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Enhanced Sialic Acid-Dependent Endocytosis Explains the Increased Efficiency of Infection of Airway Epithelia by a Novel Adeno-Associated Virus▽

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We previously used directed evolution in human airway epithelia to create adeno-associated virus 2.5T (AAV2.5T), a highly infectious chimera of AAV2 and AAV5 with one point mutation (A581T). We hypothesized that the mechanism for its increased infection may be a higher binding affinity to the surface of airway epithelia than its parent AAV5. Here, we show that, like AAV5, AAV2.5T, uses 2,3N-linked sialic acid as its primary receptor; however, AAV2.5T binds to the apical surface of human airway epithelia at higher levels and has more receptors than AAV5. Furthermore, its binding affinity is similar to that of AAV5. An alternative hypothesis is that AAV2.5T interaction with 2,3N-linked sialic acid may instead be required for cellular internalization. Consistent with this, AAV2.5T binds but fails to be internalized by CHO cells that lack surface expression of sialic acid. Moreover, whereas AAV2.5T binds similarly to human (rich in 2,3N-linked sialic acid) and pig airway epithelia (2,6N-linked sialic acid), significantly more virus was internalized by human airway. Subsequent transduction correlated with the level of internalized rather than surface-bound virus. We also found that human airway epithelia internalized significantly more AAV2.5T than AAV5. These data suggest that AAV2.5T has evolved to utilize specific 2,3N-linked sialic acid residues on the surface of airway epithelia that mediate rapid internalization and subsequent infection. Thus, sialic acid serves as not just an attachment factor but is also required for AAV2.5T internalization, possibly representing an important rate-limiting step for other viruses that use sialic acids.

Adeno-associated viruses (AAVs) are dependoviruses within the parvovirus family which require helper viruses (such as adenovirus or herpesvirus) in order to replicate (1, 2). Because of their nonpathogenic nature and ability to mediate long-term transgene expression, AAVs hold promise as gene therapy vectors. Directed evolution strategies have proven to be a powerful tool for gaining insight into the biology of AAV and for generating novel AAV capsids with enhanced infectivity and tissue and cell-specific targeting (8, 17, 19).

The AAV life cycle begins with the virus binding its receptor on the cell surface. Currently, over 100 AAV variants have been described, many of which demonstrate distinct receptor binding characteristics (34). In the case of the best-studied serotype, AAV serotype 2 (AAV2), the primary receptor is heparan sulfate proteoglycan (29). After binding to this primary receptor, AAV2 binds a coreceptor, $\alpha_v\beta^5$ integrin or fibroblast growth factor receptor 1 (23, 28). In contrast, AAV5 uses 2,3N-linked sialic acid as its primary receptor and platelet-derived growth factor receptors α and β as its coreceptors (5, 33). Once bound to the coreceptor(s), AAV undergoes receptor-mediated endocytosis, trafficking through the late endosome, endosomal escape, and nuclear transport (4, 6, 9, 12, 25). In the nucleus, the single-stranded viral DNA undergoes second-strand synthesis to yield a variety of genomic forms, including concatemers, episomes, or integrants into the host cell genome (4). The virus likely remains latent until coinfection with a helper virus triggers expression of viral genes and subsequent production of viral progeny (1, 2).

In human airway epithelia, it is thought that viral binding to the apical surface is the rate-limiting step in AAV infection (8, 14, 32, 36). Accordingly, incorporation of AAV in a calcium phosphate coprecipitate improves gene transfer to differentiated human airway epithelia *in vitro* and to the mouse lung *in vivo* (31). Increased infection of human airway epithelia by serotypes analyzed to date has generally correlated with increased binding efficiency. For instance, AAV5 binds the apical surface of human airway epithelia more effectively than AAV2, which correlates with increased transduction. One exception is AAV4, whose primary receptor is 2,6O-linked sialic acid, which is abundant on airway mucins. Despite its higher levels of apical surface binding compared to AAV2, AAV4 demonstrates decreased infection. Thus, binding alone is not sufficient to ensure infection.

Once a virus binds to the cell surface, subsequent steps can become rate-limiting. Duan et al. (7) showed that AAV2 internalization is lower from the apical side compared to the basolateral side of human airway epithelia. Interestingly, these authors also found that virus entering via the apical route subsequently suffered from impaired nuclear trafficking compared to the basolateral route. Furthermore, proteasome inhibitors increase AAV2 transduction of airway epithelia, sug-

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gesting that, under some conditions, viral capsid ubiquitination and targeted degradation may also be limiting factors (7). Finally, within the nucleus second strand synthesis of the viral genome is rate-limiting in mouse airway epithelia (11), although Ding et al. (4) found in human airway epithelia that this single- to double-strand DNA conversion is not rate-limiting for either AAV2 or AAV5.

In theory, one virus is sufficient to infect a cell. Selecting viruses that bind with high affinity to their receptors has advantages over selecting viruses that bind with low affinity to increased numbers of receptors/cell. We hypothesized that cell surface receptor binding is the rate-limiting step for AAV infection of human airway epithelia. We have previously described a chimeric AAV capsid mutant, derived from the AAV2 and AAV5 capsids together with a single point mutation (A581T), named AAV2.5T (8). AAV2.5T contains the VP1 specific region of the AAV2 capsid and the VP2 and VP3 regions of the AAV5 capsid. We generated AAV2.5T from a highly diverse viral library by directed evolution for substantially improved apical infection of human airway epithelia, although the mechanism responsible for this improved infectivity was unknown. AAV2.5T demonstrated roughly 500-fold improvement over AAV2 and 10-fold improvement over AAV5 in infection of human airway epithelia. In the present study, we investigate whether, in accordance with our initial hypothesis, the improved transduction efficiency is due to increased binding affinity. Interestingly, the results showed conclusively that the mechanism for improved transduction was due to increased sialic acid-dependent internalization.

MATERIALS AND METHODS

Primary human and pig airway epithelia. Primary airway epithelia from humans and from pigs were isolated from the trachea and bronchus. Cells were seeded onto collagen-coated, semipermeable membranes (Millipore) and grown at the air-liquid interface as previously described (15, 37). Approximately 2 weeks after seeding, cultures were well differentiated and attained a measurable transepithelial resistance.

Lectins. Fluorescein-conjugated lectins were purchased from Vector Laboratories (Burlingame, CA). The lectins were bound to cells on ice for 15 min at the following concentrations: *Maakia amurensis* lectin I, 100 μg/ml; *Sambucus nigra*, 100 μg/ml; and wheat germ agglutinin, 30 μg/ml. Next, the cells were washed three times with ice-cold phosphate-buffered saline (PBS). The cells were then fixed with 4% paraformaldehyde in PBS at room temperature for 25 min, followed by another PBS wash. Samples were then visualized using an Olympus IX71 fluorescence microscope.

Cell lines and viral production. CHO-Pro5, and -Lec2 cells (American Type Culture Collection) were cultured in αMEM (Sigma-Aldrich). Cos7 cells were cultured in Dulbecco modified Eagle medium (Invitrogen). All media were supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin-streptomycin (Invitrogen). The recombinant AAV vectors were packaged and purified with iodixanol gradient centrifugation as previously described (17–19). DNase-resistant genomic titers were determined by using quantitative PCR. Electron microscopy of the preparations routinely showed less than 10% empty vectors. Flow cytometry was used to obtain transduction titers as previously described (17–19).

AAV2.5T transduction. AAV2.5T was diluted in EMEM (Invitrogen) and added to either CHO cells, the apical side of human airway epithelia, or pig airway epithelia at the doses indicated in the figures and text. Samples were incubated for 4 h at 37°C, followed by two washes with PBS. Green fluorescent protein (GFP)-positive cells were manually counted by viewing the cells with an Olympus IX71 fluorescence microscope.

AAV binding and internalization. Virions of AAV2.5T-CMV-eGFP or AAV5-CMV-eGFP were allowed to bind to CHO-Pro5 cells, CHO-Lec2 cells, the apical surface of human airway epithelia, or the apical surface of pig airway epithelia at the doses indicated in the figures for 1 h on ice, followed by two washes with 250 μl of ice-cold PBS. For internalization studies, AAV binding was followed by shifting the cells to 37°C for the times shown. The cells were then treated with 200 μl of 0.25% trypsin at 37°C for 20 min to remove the virus bound on the cell surface. Human and pig airway epithelia were treated with trypsin on the apical surface only. After trypsin treatment, 1 ml of growth media was added to the CHO-Pro5 and -Lec2 cells, and the cells were centrifuged at 600 × *g* for 8 min at 4°C to pellet the cells, followed by the removal of the supernatant and DNA extraction. Apical trypsin treatment did not disrupt the airway epithelia, so the trypsin was removed, followed by one wash with 250 μl of ice-cold PBS, and the DNA was then extracted. For all cell types, the DNA was extracted, and quantitative PCR was performed with SYBR green Extract-N-Amp (Sigma-Aldrich) according to the manufacturer's instructions. Quantitative PCR primers were designed for the cytomegalovirus (CMV) promoter of the virus (5′-AAATCAA CGGGACTTTCCAA-3′ and 5′-GGTTCACTAAACGAGCTC-3′) and compared to known amounts of viral DNA standards to determine the unknown amounts of viral DNA. Electron microscopy of viral preparations routinely showed that <10% of the capsids are empty (do not contain DNA). Thus, it is possible that we are underestimating capsid binding by <10%.

Sialylation of CHO cells. 2,3*N*-sialyltransferase, 2,3*O*-sialyltransferase, 2,6*N*-sialyltransferase, and CMP-sialic acid were obtained from Calbiochem. CHO-Lec2 cells were seeded onto 48-well plates and sialylated by incubating with 100 μl of 15 mU of each sialyltransferase/ml individually with 0.1 mM CMP-sialic acid at 37°C for 150 min. Cells were washed one time with 250 μl of EMEM and either stained with lectins or inoculated with AAV2.5T as indicated.

Neuraminidase treatment. CHO-Pro5 cells were pretreated with 100 μl of a 20-mU/ml concentration of neuraminidase (*Vibrio cholerae*; Sigma-Aldrich) for 2 h at 37°C and then washed. Treatment of CHO cells resulted in the lack of binding of wheat germ agglutinin. The cells were then inoculated with 10^5 viral genomes (vg) of AAV2.5T-CMV-eGFP/cell diluted in EMEM in a volume of 150 μl for 4 h at 37°C and then washed. At 48 h after AAV transduction, cells were imaged with fluorescence microscopy (Olympus IX71 microscope).

RESULTS

AAV2.5T exhibits higher binding to airway epithelia than AAV5, but with similar affinity. Increased viral binding can result either from an increased number of receptors on the cell surface or from higher binding affinity of the virus to these receptors. The aim of our prior work was to generate a gene therapy vector highly specific for human airway epithelia (8). To achieve specificity, the selection process included sequential infection of airway epithelia from multiple donors with increasing stringency (i.e., decreasing the incubation time and the multiplicity of infection [MOI] with each round). The first round of selection consisted of apical infection of airway epithelia with 1,000 vg of library virus/cell, while the final round of selection utilized an MOI of 0.01 vg/cell. Moreover, the library for each round was constructed from the lowest inoculation condition that resulted in AAV *cap* gene recovery. Because our final MOI was 0.01 vg/cell, a condition that would be predicted to favor viruses with high binding affinities, we anticipated that AAV2.5T would have higher binding affinity than AAV5. To measure the binding properties of AAV2.5T, different doses of virions were bound to the apical surface of human airway epithelia for 1 h at 4°C. Total binding (Fig. 1A) was separated into nonspecific and specific components by fitting the binding curve to a single-site binding isotherm based on the assumption that nonspecific binding is directly proportional to the ligand or virus concentration. Scatchard plot analysis of the specific binding component showed that AAV2.5T had 7.70×10^3 receptor sites per cell (Fig. 1B), within an order of magnitude of the 2.59×10^3 receptor sites per cell measured for AAV5 and the $\sim 10^3$ receptor sites per cell previously measured for AAV5 (33). Its increased number of receptors/cell may explain the total increased binding of AAV2.5T compared to prior studies with AAV2 and AAV5. Surprisingly, nonlinear

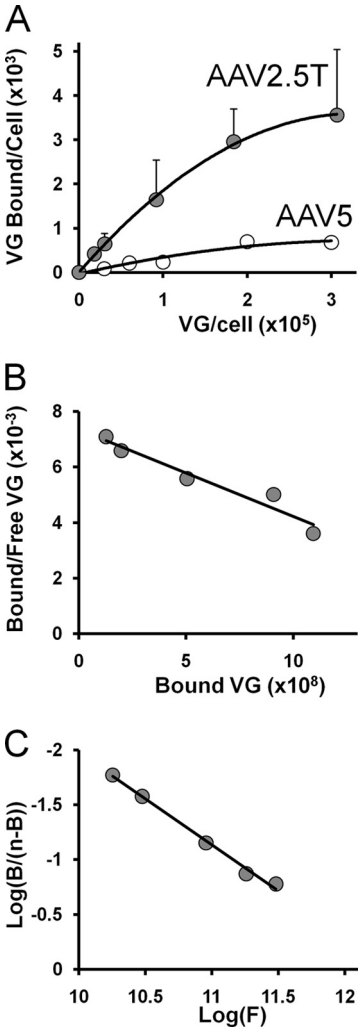

FIG. 1. AAV2.5T has increased binding on the apical surfaces of human airway epithelia. (A) AAV2.5T or AAV5 virions were allowed to bind the apical surfaces of human airway epithelia at the indicated doses for 1 h at 4°C. A Scatchard analysis (B) and a Hill plot (C) were performed to measure the total number of receptors and the cooperativity coefficients of AAV2.5T on human airway epithelia. Nonlinear regression analysis was performed to determine the binding affinities of the viruses. $n = 3$ different donors in four different experiments.

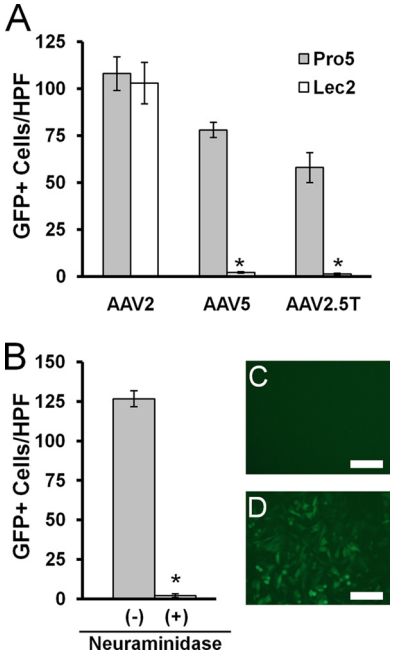

FIG. 2. AAV transduction of CHO-Pro5 and CHO-Lec2 cells. AAV2, AAV5, and AAV2.5T, all carrying the CMV promoter driving eGFP expression, were allowed to transduce CHO-Pro5 and CHO-Lec2 cells at 37°C for 4 h. (A) At 48 h posttransduction, fluorescence microscopy was performed, and the numbers of GFP-positive cells per high-power field (HPF) were counted. $n = 5$ in three different experiments ($P < 0.01$). CHO-Pro5 cells were treated with 150 μl of 20 mU of neuraminidase/ml from *Vibrio cholerae* to remove the sialic acid from the surface, followed by transduction with AAV2.5T-CMV-eGFP at an MOI of 10^5 vg/cell. (B) GFP-positive cells were quantified by using fluorescence microscopy. $n = 3$ in four different experiments ($P < 0.01$). (C and D) Representative images of CHO-Lec2, (C) and CHO-Pros (D) cells after transduction with AAV2.ST-CMV-eGFP. Scale bar, 100 μM.

regression analysis yielded an apparent K_d for AAV2.5T of ~560 nM, representing a receptor affinity similar to that of AAV5, whose K_d is ~920 nM. This result was in contrast to our initial hypothesis that AAV2.5T would have much higher binding affinity and suggested that, while surface binding is rate-limiting for AAV2, AAV5 may encounter a downstream rate-limiting step that AAV2.5T is able to overcome. Finally, the Hill plot coefficient was nearly 1 (1.04), indicating that AAV2.5T binds with no cooperativity (Fig. 1C), a finding consistent with the binding of adenovirus at 4°C (22).

We were surprised to find that AAV2.5T has evolved to bind a receptor or receptors present in higher abundance on the surface of human airway epithelia and yet binds this receptor or receptors with an affinity similar to that of AAV5 for its receptor. This is surprising because our selection strategy used an MOI as low as 0.01 vg/cell, which we predicted would favor viruses with higher binding affinity. However, since our strategy selected for infection rather than binding we considered the possibility that AAV2.5T may have evolved to overcome a rate-limiting step subsequent to binding. Accordingly, we investigated the role of sialic acids in AAV2.5T binding to, internalization by, and transduction of cells.

AAV2.5T requires sialic acids to transduce CHO cells. In contrast to airway epithelia transduction, we previously found that AAV2.5T does not demonstrate an advantage over AAV5 in CHO cells. We hypothesized that, like AAV5, AAV2.5T requires sialic acid for binding. The variant CHO line Lec2 has a mutation in the CMP-sialic acid transporter that prevents the cells from transporting CMP-sialic acid into the Golgi apparatus, thus blocking the addition of sialic acid moieties to glycosylated cell surface proteins. CHO-Pro5 cells are the parental, wild-type line from which CHO-Lec2 cells were derived. Although AAV2.5T and AAV5 both transduce CHO-Pro5 cells, neither is able to transduce the CHO-Lec2 cells (Fig. 2A). In contrast, AAV2, which utilizes heparan sulfate proteoglycan as its receptor, transduces both CHO-Lec2 and CHO-Pro5 cells (Fig. 2A). Treatment of CHO-Pro5 cells with neuraminidase from *Vibrio cholerae* leads to the removal of α2,3-, α2,6-, and α2,8-sialic acid from surface glycoproteins. Consistent with

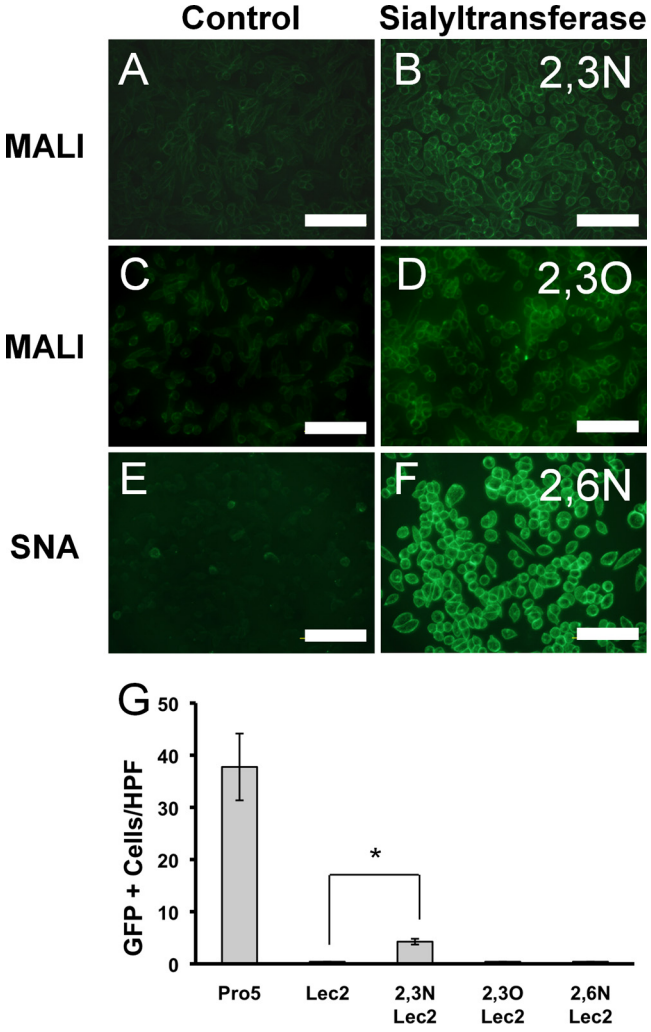

FIG. 3. Sialylation of CHO-Lec2 cells. The surface proteins of CHO-Lec2 cells were sialylated using 2,3*N*-, 2,3*O*-, or 2,6*N*-sialyltransferases (B, D, and F). Lectin staining was used to confirm the cells were sialylated by comparing to untreated controls (A, C, and E). MALI was used to stain the 2,3*N*- and 2,3*O*-sialyltransferase-treated cells (B and D) and untreated control cells (A and C). SNA was used to stain the 2,6*N*-sialyltransferase-treated cells (F) and untreated control cells (E). Scale bar, 100 μM. (G) After the surfaces of the cells were sialylated, they were transduced with AAV2.5T-CMV-eGFP at an MOI of 10^5 vg/cell for 4 h at 37°C, and GFP-positive cells were counted 48 h after transduction by fluorescence microscopy. $n = 3$ in three different experiments ($P < 0.01$).

results on CHO-Lec2 cells, AAV2.5T loses the ability to transduce neuraminidase-treated CHO-Pro5 cells (Fig. 2B). Figure 2C and D are representative images of CHO-Lec2 and CHO-Pro5, respectively, after transduction with AAV2.5T-CMV-eGFP. These data suggest that AAV2.5T requires sialic acid to transduce CHO cells.

AAV2.5T exhibits specificity for 2,3N-linked sialic acids. To address the likelihood that the poor transduction of CHO-Lec2 cells by AAV2.5T is due to the cellular sialylation defect, we used three different sialyltransferases to sialylate the surface proteins of CHO-Lec2 cells and then tested transduction by AAV2.5T. Sialylation of CHO-Lec2 cells treated with either 2,3*N*- and 2,3*O*-sialyltransferases was confirmed by the binding of *Maackie amurensis* lectin (MALI), which binds all 2,3-linked sialic acid (Fig. 3A to D). Treatment with 2,6*N*-sialyltransferase was verified by the binding of *Sambucus nigra* lectin (SNA), which binds to 2,6-linked sialic acid (Fig. 3E and F). Sialylated cells were then transduced with AAV2.5T-CMV-eGFP at an MOI of 10^5 vg/cell for 4 h at 37°C. Transduction efficiency was determined 48 h later by GFP fluorescence. Sialylation of CHO-Lec2 cells with 2,3O- or 2,6N-linked sialic acid had no effect on transduction with AAV2.5T. However, sialylation with 2,3N-linked sialic acid partially restored AAV2.5T transduction of CHO-Lec2 cells. CHO cells were not transduced when either substrate or 2,3N-linked sialyltransferase were not included. These data suggest that, similar to AAV5, AAV2.5T uses 2,3N-linked sialic acid to transduce cells. Even though these cells do not recapitulate the difference in infection between AAV2.5T and AAV5 in human airway epithelia, they are useful to investigate the mechanism of sialic acid-mediated binding and internalization.

AAV2.5T requires sialic acid for internalization, but not for binding, on CHO cells. Due to the requirement of sialic acid and the ability of 2,3N-linked sialic acid to mediate AAV2.5T transduction in CHO cells, we tested the possibility that 2,3N-linked sialic acid may be required for binding of the virus. We bound AAV2.5T virions to CHO-Pro5 and CHO-Lec2 cells at 4°C for 1 h and measured binding by quantitative PCR. Surprisingly, binding was similar on both cell types at all doses tested. In addition, virion binding did not reach saturation even at a very high MOI (Fig. 4A). These data show that AAV2.5T does not require sialic acid to bind CHO cells. Moreover, the lack of saturation suggests that binding to CHO cells is either nonspecific, or the doses tested were not high enough to achieve receptor-binding saturation. The disconnect between transduction efficiency and binding suggests that binding is not the rate-limiting step of AAV2.5T transduction. These data are consistent with the unexpectedly similar binding affinity between AAV2.5T and AAV5 on human airway epithelia. We therefore hypothesized that sialic acid is required for viral internalization. To investigate this, AAV2.5T virions (10^5 vg/cell) were bound to CHO-Pro5 and CHO-Lec2 cells at 4°C and then allowed to internalize by shifting the cells to 37°C. Virions that had not been internalized were removed from the cell surface by treatment with trypsin at various time points. After 8 h at 37°C, ~80% of the detectable virus was trypsin resistant in CHO-Pro5 cells compared to ~2% in CHO-Lec2 cells (Fig. 4B), indicating that very little of the virus bound to CHO-Lec2 cells had entered the cells. These data show that AAV2.5T requires a specific sialic acid-mediated endocytosis in order for transduction to occur in CHO cells.

Lectin binding on human airway epithelia and pig airway epithelia. Human airway epithelia express abundant 2,3N-linked sialic acid in the lower airways and 2,6N-linked sialic acid in the upper airways (27). In contrast, pig airway epithelia express both 2,3N- and 2,6N-linked sialic acid in the upper and lower airways (21). Thus, AAV2.5T could conceivably bind to, be internalized by, and transduce pig airway epithelia similarly to human airway epithelia. To confirm the reported sialylation patterns of cultured primary pig (21) and human (27) airway epithelia, lectins were used to probe their glycosylation profiles. Wheat germ agglutinin (WGA), which binds to all sialic

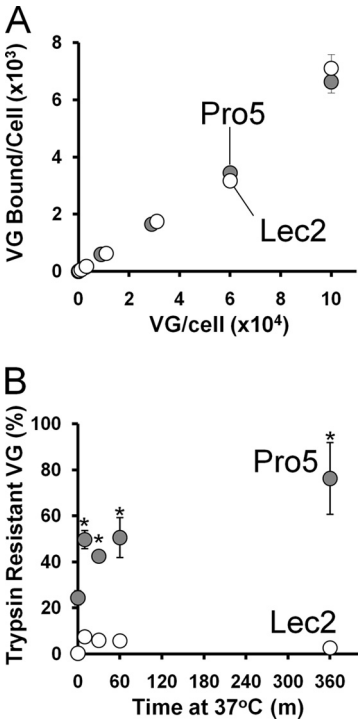

FIG. 4. AAV2.5T binding and internalization in CHO-Pro5 and CHO-Lec2 cells. AAV2.5T virions were allowed to bind to CHO-Pro5 and CHO-Lec2 cells at the doses shown at 4°C for 1 h (A). $n = 4$ in three different experiments. To allow endocytosis of the virus, after binding 10^5 vg/cell for 1 h at 4°C, the cells were shifted to 37°C for the times shown. After the specified times, the cells were treated with trypsin to remove the virus that had not been internalized (B). $n = 4$ in three different experiments ($P < 0.01$). Binding and internalization of the virus were both measured by using quantitative PCR to determine the number of viral genomes.

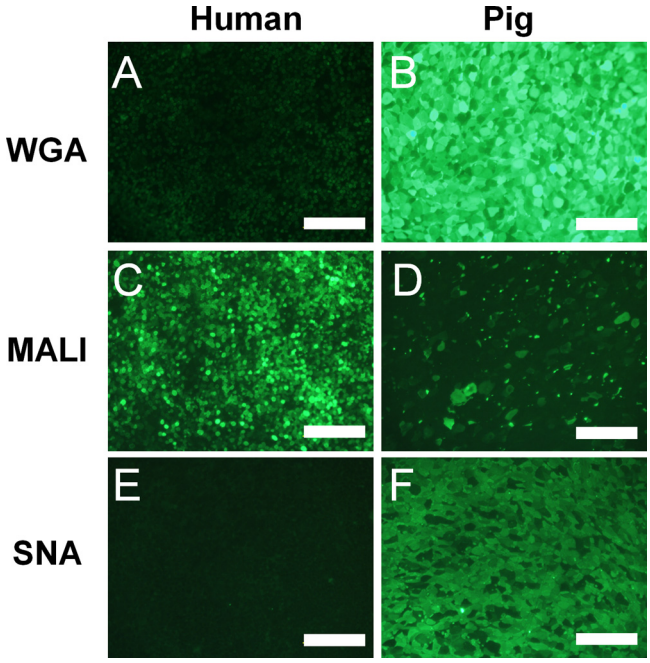

FIG. 5. Lectin binding to human and pig airway epithelia. The apical surfaces of airway epithelial cultures from humans (A, C, and E) and pigs (B, D, and F) were stained using fluorescein isothiocyanate -conjugated wheat germ agglutinin (WGA; A and B), MALI (C and D), or SNA (E and F) to determine the levels of the different sialic acids present. Scalebar, 100 μM.

acids, bound to human and to a much greater extent pig airway epithelia (Fig. 5A and B). On the other hand, MALI bound to the apical surface of pig and to a much greater degree human airway epithelia (Fig. 5C and D). Finally, SNA bound to pig but not human airway epithelia (Fig. 5E and F). These data indicate that although both human and pig airway epithelial cultures present sialic acid on their surfaces, consistent with the published literature (21, 27), cultured human airway epithelia have more α2,3-linked sialic acid than those of pigs, whereas pig airway epithelia have more α2,6-linked sialic acid than those of humans. These data are consistent with other studies (13) that have shown that the viral infection profile of airway epithelia derived from humans may be distinct from pig.

AAV2.5T binds human and pig airway epithelia but is only internalized by and transduces human airway epithelia. To determine whether AAV2.5T shows differential binding, internalization, or transduction in primary human versus pig airway epithelial cultures, AAV2.5T virions were incubated on the apical side of differentiated airway epithelial cultures at 4°C at doses ranging from 1.8×10^4 to 3.0×10^5 vg/cell, and quantitative PCR was conducted to determine the numbers of bound virions. Binding assays demonstrated that viral binding to pig airway epithelia was slightly lower compared to human airway epithelia. In addition, binding was linear and failed to reach saturation (Fig. 6A). To determine the amounts of internalized virus, 10^5 AAV2.5T virions per cell were bound to the apical surface airway cultures for 1 h at 4°C, followed by 1 h at 37°C to allow viral endocytosis. Epithelia were then apically treated with trypsin for 20 min at 37°C to remove uninternalized surface-bound virions, followed by quantitative PCR. Although approximately 68% of the virus was internalized by human airway epithelia, only 2.2% was internalized by pig airway epithelia (Fig. 6B). These data suggest that 2,3N-linked sialic acid is required for AAV2.5T internalization in this *in vitro* model system. Importantly, we also found differences in the transduction efficiency of airway epithelia from different donors. Based on AAV2.5T's ability to bind sialic acid-deficient CHO-Lec2 cells, along with its inability to be internalized by and to transduce these cells, we hypothesized that transduction efficiency would correlate with endocytosis rather than cell surface binding on airway epithelia. We compared the levels of binding, internalization, and transduction by AAV2.5T using different donors of human and pig airway epithelia at 10^5 vg/cell. As anticipated, transduction correlates with internalization of the virus but not with viral binding (Fig. 6C).

AAV2.5T is internalized by human airway epithelia more efficiently than AAV5. We have previously shown that AAV2.5T binds and infects human airway epithelia more efficiently than AAV5. Based on the data presented above, we speculated that AAV2.5T's improved binding ability revealed a new rate-limiting step at the level of virus internalization in the transduction of human airway epithelia. Thus, we predicted that more of the bound AAV2.5T would be internalized than that of AAV5. AAV2.5T and AAV5 virions (10^5 vg/cell) were

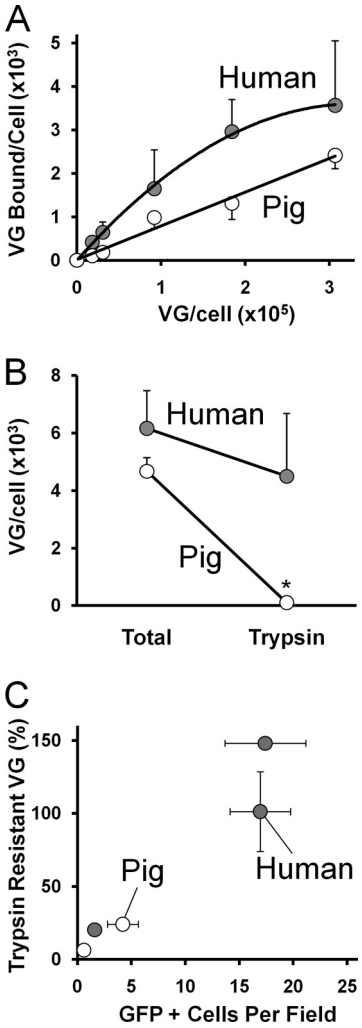

FIG. 6. AAV2.5T binding, internalization, and transduction of human and pig airway epithelia. (A) AAV2.5T virions were allowed to bind the apical surfaces of human and pig airway epithelial cultures for 1 h at 4°C at the doses shown. (B) After 10^5 vg/cell were bound to the apical surfaces of human and pig airway epithelia, the bound AAV2.5T virions were allowed to undergo endocytosis by shifting the cells to 37°C for 1 h, followed by trypsin treatment to remove the virus that was not internalized by the cells. (C) Viral internalization and transduction efficiencies (10^5 vg/cell) of different donors of human and pig airway epithelia were compared. AAV2.5T-CMV-eGFP was inoculated on the apical side of airway epithelia for 4 h at 37°C and, 33 days later, GFP-positive cells were counted by using fluorescence microscopy. Viral internalization and binding were measured by using quantitative PCR. $n = 3$ in three different donors ($P < 0.05$).

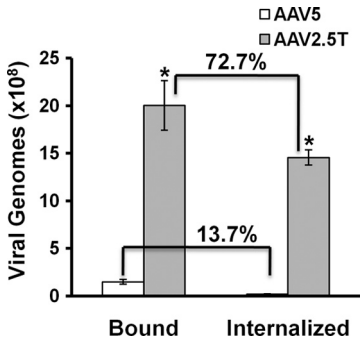

FIG. 7. AAV2.5T is internalized by human airway epithelia better than AAV5. AAV2.5T and AAV5 virions were allowed to bind to the apical surfaces of human and pig airway epithelial cultures for 1 h at 4°C at an MOI of 10^5 vg/cell. After 1 h, the temperature was shifted to 37°C for 1 h to allow endocytosis of the virus to occur. Cells were treated with trypsin to remove the virus that was not internalized by the cells. The bound virus was measured on cells that were not treated with trypsin, and the internalized virus was measured in cells that were treated with trypsin. A total of 72.7% of the bound AAV2.5T virions were internalized, while only 13.7% of the bound AAV5 virions were internalized. $n = 3$ in three different donors ($P < 0.01$).

incubated for 1 h on the apical side of differentiated airway epithelial cultures at 4°C. The cultures were shifted to 37°C to allow viral endocytosis for 1 h, followed by trypsinization to remove uninternalized virions. As previously shown, AAV2.5T demonstrated increased binding to the apical surface of airway epithelia over AAV5 (13.5-fold) (Fig. 7). However, the difference in internalized virus was 70-fold. Whereas 13.7% of AAV5 bound was internalized, 72.7% of AAV2.5T bound was internalized. Therefore, although both AAV5 and AAV2.5T require sialic acid for transduction of cells, these data are consistent with AAV2.5T requiring sialic acid for a distinct step during the infection process after the initial binding.

DISCUSSION

Here we show that internalization rather than binding of AAV5 is the rate-limiting step for gene transfer to airway epithelia. AAV2.5T has solved this hurdle. Interestingly, this step requires a sialic acid in a specific linkage (2,3N-linkage). Sialic acids are thought to function as viral receptors in three ways. One model suggests that these sugars may behave as abundant, low-affinity attachment factors similar to heparan sulfate proteoglycan, bringing the virus closer in proximity to a secondary receptor that is likely present in lower abundance on the cell surface. Once bound to its secondary receptor, viral endocytosis can occur. Thus, by enabling viral binding to its secondary receptor, sialic acid facilitates internalization. A second, related model suggests that sialic acid is required for the initial viral attachment and that this binding event induces a conformational change in the virus that allows access to a coreceptor. In a third model, sialic acid is attached to a specific glycoprotein or glycolipid and acts as the primary receptor for virus binding, which then allows endocytosis of the virus (3, 10, 30). Our data suggest that sialic acid is required for internalization by, but only partially for attachment to, both CHO cells and airway epithelia. Thus, different AAV variants can (i) bind to sialic acid and not infect (AAV4) (14), (ii) bind to sialic acid and then to a coreceptor in order to infect (AAV5) (33), or (iii) bind to sialic acid on the glycocalyx and infect without the need of a coreceptor (bovine AAV) (24). AAV2.5T illustrates a novel interaction. A total of 90% of surface binding is attributed to sialic acid on human airway epithelia (8), although sialic acid is not required for binding to CHO cells. However, under all conditions, 2,3N-linked sialic acid is required for internalization of AAV2.5T.

Our data suggest that sialic acid is involved in AAV2.5T transduction in an unknown manner, independent of initial

viral attachment. One possibility is that sialic acid may function by allowing multimerization of coreceptor proteins after AAV2.5T binds. Sialic acid interactions between the cellular proteins may be required for subsequent signaling and induction of endocytosis of the coreceptor proteins and the bound AAV2.5T. For example, Kitazume et al. (16) found that the platelet endothelial cell adhesion molecule requires α2,6-sialylation to interact with itself, thereby enabling homodimerization to initiate its intracellular antiapoptotic signaling. Another possibility is that AAV2.5T binds to cells in the absence of sialic acid, but internalization only occurs when sialic acid is present and interacting with the virus. This interaction may cause the virus to undergo structural changes, allowing it to interact with a nonsialylated coreceptor protein and signal endocytosis (3, 10). A third possibility is that AAV2.5T binds to an unknown attachment factor (or it binds nonspecifically) and, subsequent to its initial sialic acid-independent attachment to the cell surface, its ability to interact with its coreceptor proteins is dependent on the coreceptors being sialylated.

By evolving a virus to efficiently replicate in human airway epithelia, we anticipated that one or more rate-limiting steps could be overcome and subsequently identified. In particular, we assumed our strategy of progressively lowering the virus/cell ratio may have selected for virus with increased binding and higher affinity. However, AAV2.5T surprisingly does not have increased affinity for its receptor, indicating an advantage beyond initial receptor binding. For AAV to replicate, multiple steps have to be overcome in its life cycle. We previously found that increased binding leads to increased infection (36). AAV2.5T not only has increased binding due to increased receptor number, but it also has increased and rapid internalization and perhaps overcomes subsequent rate-limiting steps as well.

It is interesting that this virus binds to pig airway epithelia but does not transduce or get internalized by these cells. These data suggest that species-specific differences in airway sialic acids may result in different efficiency of infection. However, there are so many differences between the human and porcine cells that it is not possible to conclude that it is all a difference between sialic acids. We speculate that directed evolution of an AAV library in pig airway epithelia will result in a different capsid that binds to a different sialic acid moiety or to other receptors.

AAV infection and gene transfer to human airway epithelia are inefficient. Our previous work has shown that AAV5 has increased binding to the apical surface of human airway epithelia compared to AAV2, suggesting that binding is a rate-limiting step. We have also shown that infection is increased when AAV entry is aided by calcium phosphate precipitation, presumably by increasing the close association between the virus and target cells (31). Increased infection has also been accomplished by increasing viral binding by mutagenesis of the binding loops in the capsid (20, 26). However, none of these modifications have resulted in a virus that is good enough for gene therapy in the human lung. Others have suggested that different steps in the AAV life cycle are rate-limiting steps, including endosomal escape and intracellular trafficking (7, 35). Double-stranded DNA conversion is not a rate-limiting step, at least in airway epithelia (4).

Using our directed evolution strategy, we hypothesized that we would generate viruses that overcome rate-limiting steps in a sequential manner. Here, we show that we selected a virus that has increased infection and that the mechanism involves a novel sialic acid-mediated internalization step. This suggests that, for viruses requiring sialic acid for infection, the requirement for sialic acid may be at the point of entry into the cell and not at the initial attachment site on the cell. This novel solution for enhanced infection highlights the power of directed evolution to reveal entirely new and unexpected capsid protein functions. Thus, further improvements in the specificity and efficacy of AAV through directed evolution will lead to better AAV vectors (with increased transduction in addition to binding) with real promise for gene therapy.

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