Directed evolution of adeno-associated virus to an infectious respiratory virus

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Respiratory viruses evolve to maintain infectivity levels that permit spread yet prevent host and virus extinction, resulting in surprisingly low infection rates. Respiratory viruses harnessed as gene therapy vectors have illustrated this limitation. We used directed evolution in an organotypic human airway model to generate a highly infectious adeno-associated virus. This virus mediated gene transfer more than 100-fold better than parental strains and corrected the cystic fibrosis epithelial Cl⁻ transport defect. Thus, under appropriate selective pressures, viruses can evolve to be more infectious than observed in nature, a finding that holds significant implications for designing vectors for gene therapy and for understanding emerging pathogens.

The complexity of evolutionary forces that drive the adaptation of pathogens to host systems has become progressively more evident as human, agriculture, and wildlife systems have come into increasingly close proximity, resulting in emerging infectious diseases. Several theories have been developed to define relevant interactions and consequences (1, 2). The classic theory behind the evolution of viral transmission is summed up in the basic reproductive number $R_0$: the number of secondary infections resulting from one infected host in a naïve host population. $R_0$ depends on a delicate balance of viral transmission and virulence: increased $R_0$ due to enhanced transmission or infectivity (the chance a susceptible host will be infected) is counteracted by greater virulence which reduces $R_0$ by limiting the infectious period and opportunity for transmission. On the extremes of this balance, it is predicted that viruses with both high infectivity and virulence will lead to extinction of both host and virus, whereas high infectivity and low or no virulence will exhaust the reservoir of naïve hosts and lead to broad host population immunity, again resulting in viral extinction. Thus, a low infectivity for pathogenic and potentially nonpathogenic viruses may impart an evolutionary advantage by permitting the existence of a persistent naïve host population, while still facilitating viral survival within the remaining population (1, 2).

At the molecular level, the infectiousness of a virus involves several key steps: cell surface binding, entry into the target cell, and replication. To achieve evolutionary success, viruses may alter or compromise each of these steps to reduce the efficiency of infection and achieve a balance between the production of viruses that spread, but do not cause, host (and hence virus) extinction. This concept is strikingly illustrated by the inefficiency, and consequent lack of therapeutic end points, of gene transfer vectors based on respiratory viruses for respiratory diseases (3–6). A better understanding of evolutionary principles governing natural viral evolution will drive more advanced methods to successfully engineer novel virus-based gene therapeutics.

Directed evolution strategies have demonstrated the power of mutagenesis and DNA shuffling methods to investigate and enhance preexisting functions of or generate novel functions in a protein without underlying mechanistic knowledge (7, 8). Recent efforts have increasingly demonstrated the impact of these methods to address novel and high impact problems in protein engineering (9–11); however, few studies have focused efforts on engineering structurally complex protein assemblies (10) or on direct clinical application (11). We hypothesized that directed evolution in an organotypic human airway model employing recombination and mutagenesis, akin to natural evolutionary mechanisms but under selective pressures not constrained by nature, could greatly enhance the respiratory infectivity of a virus.

Adeno-associated viruses (AAV) are members of the parvovirus family and share a similar size, structure, and dependence on a helper virus for replication and gene expression. Wild-type AAV is a 4.7 kb single-stranded DNA virus that contains 2 ORFs: rep, which encodes 4 proteins necessary for genome replication; and cap, which expresses 3 proteins (VP1–3) that assemble to form the viral capsid (12). Although not necessarily considered a respiratory virus, AAV is a promising candidate to explore viral evolution for multiple reasons. First, the capsid—which determines viral infectivity and tropism (13–16)—is encoded by a single gene, and the existence of multiple serotypes with distinct tropisms indicates that the capsid is highly evolvable (17). Second, AAV evolves naturally via mutagenesis and recombination similar to other viruses (18) and, in stark contrast to pathogenic viruses, is a helper-dependent virus with a remarkable safety profile in humans (19–21). Finally, the creation of a more infectious variant will have therapeutic implications for gene therapy for airway diseases and may improve our understanding of viral evolutionary selective pressures (12, 22).

We therefore used directed evolution of the AAV capsid to select viral variants with enhanced infection of human airway epithelium, PCR-based mutagenesis coupled with high-throughput in vitro recombination generated a diverse library of chimeric cap genes with components from 2 divergent serotypes that use distinct receptors, AAV2 (heparan sulfate) and AAV5 (sialic acid) (13–16). Subsequent selection of this library for enhanced infection of organotypic human airway epithelial cultures identified a single novel AAV chimera with a unique point mutation that exhibits enhanced binding to the apical surface of airway epithelia as well as improved gene transfer. Furthermore, the novel AAV virus mediates successful cystic fibrosis transmembrane conductance regulator (CFTR) cDNA-
Evolution of highly efficient AAV vector for pulmonary gene delivery. Directed evolution platform in organotypic polarized, well-differentiated human airway epithelia (A). AAV library was applied apically for decreasing times and multiplicity of infection (MOI = i (vg/cell)) over 5 rounds. Additional diversification (A) was performed after round 3. Recovered virus per epithelium (vg/ep) was approximately 550-fold greater than wtAAV2 after 5 rounds (B). *P < 0.01.

gene transfer to correct the chloride transport defect in human cystic fibrosis (CF) epithelia.

Results

Library Construction and Selection Results in a Novel AAV with Improved Airway Infection. We combined 2 divergent serotypes that use distinct receptors, AAV2 and AAV5, by subjecting the cap genes encoding the viral capsomeres to DNA shuffling and error-prone PCR. This strategy produced a highly diverse library of approximately 10⁶ unique chimeric viral cap genes that were used to generate replication competent viruses, each carrying its own chimeric viral capsid sequence (10, 23). Extensive selections by apical inoculation of the AAV viral library were performed on organotypic human airway epithelial cultures from 3 different donors during each round (15 donors total)(Fig. 1). AAV infection was allowed to proceed for 3 days, to allow for entry and trafficking and followed by basolateral inoculation with helper wild-type adenovirus to drive the amplification of AAV variants that successfully reached the nucleus. The stringency of selection was gradually increased during subsequent rounds by decreasing the dose and incubation period. After round 3, the successful cap genes were subjected to additional diversification via recombinant and mutagenesis. With every round of selection, we recovered increasing numbers of viral progeny relative to wild-type AAV2. By round 5, recovery of the evolved progeny was approximately 550-fold higher than AAV2 (Fig. 1B). Surprisingly, sequencing 8 random clones from the selected pool revealed a single AAV variant, AAV2.5T, which is a chimera between AAV2 (aa1–128) and AAV5 (aa129–725) with one point mutation (A581T) (Fig. S1).

Improvement of Apical Airway Transduction. Apical transduction (50,000 vg/cell) by the novel AAV chimera; several corresponding variants; and serotypes 2, 5, and 9—each harboring luciferase—was quantified over a 28 day time course (Fig. 2A). To improve the time course of gene expression, these cultures were treated basolaterally with Hoechst 33342 at the time of apical infection. Hoechst treatment of AAV-infected airway only affects gene expression from the CMV promoter and does not affect other transduction steps. Strikingly, 21 days posttransduction, AAV2.5T outperformed all other AAV serotypes evaluated: AAV2-Luc (100-fold), AAV5-Luc (10-fold), and AAV9-Luc (20-fold). The decrease in expression at day 28 may affect other transduction steps. No significant difference in basolateral transduction between AAV2.5T and other wild-type serotypes was observed (Fig. 2B), indicating that the advantage of AAV2.5T was specific for the apical surface.

Binding Is Significantly Improved and Requires Sialic Acid. To investigate the mechanism of improved transduction, apical binding was analyzed. Recombinant AAV2.5T bound to the apical surface significantly better than AAV5 (100-fold; Fig. 3A), and in contrast to AAV5, binding of AAV2.5T did not saturate at doses ranging from 10 to 1000 genome copies/cell. This suggested a dramatic increase in the number of viral receptors and possibly in binding affinity. Studies on cell lines with specific deficiencies in glycosylation revealed that, similar to AAV5 and in contrast to AAV2, AAV2.5T requires sialic acid (Lec2 cells) but not heparan sulfate (pgsA or pgsD cells) for efficient transduction (Fig. 3B). Furthermore, apical neuraminidase pretreatment of airway epithelia significantly decreases AAV2.5T binding, indicating that sialic acid binding is required for efficient transduction (Fig. 3C). Additional transduction studies on other cell types indicate the advantage of AAV2.5T is cell-type specific (Fig. 3D).

Interestingly, when the point mutant (AAV5-A581T) or chimera (AAV2.5) was studied independently, neither was better than AAV5 at binding or transduction (Fig. 2A and B). The A581T mutation occurs in a region critical to AAV5 sialic acid binding; therefore, a mutation in this region may influence the binding affinity for sialic acid and/or the type of linkages recognized. For example, a study of a related parvovirus demonstrated that point mutations adjacent to the sialic acid binding pocket conferred recognition of additional sialic acid linkages (24). However, the AAV5-A581T virus produced low genomic titers and failed to bind or transduce airway epithelia (Fig. 2A and B).
AAV2.5T binding does not saturate at doses between 300 and 1,000 viral genomes per cell (vg/cell). AAV2.5T mirrors parental AAV5 sensitivity for sialic and does not efficiently transduce sialic acid deficient Lec2 cells whereas transduction is not altered on heparan sulfate deficient mutants pgsA or pgsD (B). TU, transducing unit. AAV2.5T binding to the apical surface of human airway epithelia is significantly reduced by pretreatment with neuraminidase (C) (P < 0.001). In contrast to airway epithelia, similar transduction was observed between AAV2.5T and AAV5 in several cell lines and primary human astrocytes (D).

Enhanced binding and cell specificity of evolved AAV2.5T. In contrast to the saturation of AAV5 binding to the apical surface of human airway epithelia, AAV2.5T binding does not saturate at doses between 300 and 1,000 viral genomes per cell (vg/cell) (A). AAV2.5T mirrors parental AAV5 sensitivity for sialic acid and does not efficiently transduce sialic acid deficient Lec2 cells whereas transduction is not altered on heparan sulfate deficient mutants pgsA or pgsD (B). TU, transducing unit. AAV2.5T binding to the apical surface of human airway epithelia is significantly reduced by pretreatment with neuraminidase (C) (P < 0.001). In contrast to airway epithelia, similar transduction was observed between AAV2.5T and AAV5 in several cell lines and primary human astrocytes (D).

and B). Likewise, AAV2.5 offered no advantage over AAV5 in airway epithelia, even though aa1–128 from AAV2 may potentially alter intracellular trafficking (25). Collectively, our data suggest that the recombination event rescues a structurally deleterious yet functionally advantageous mutation (A581T). Furthermore, the data demonstrate that under artificial pressures that select for enhanced infectivity and do not require a balance between transmission and virulence within a population, AAV can evolve to be significantly more infectious than naturally occurring serotypes.

**CFTR Expression and Phenotype Correction.** We next investigated potential therapeutic applications of AAV2.5T by analyzing whether it could efficiently express CFTR and correct the CF chloride transport defect. CF airway epithelia were transduced with AAV2.5T encoding a shortened CFTR expression cassette (CFTRAR, 50,000 vg/cell), then analyzed in Ussing chambers 30 days posttransduction, as previously described (26, 27). Normal epithelia demonstrated chloride transport (Fig. 4A), as shown by an increase in current, after treatment with IBMX/Forskolin (ΔIscAMP 12 ± 2 μA.cm⁻²), that is blocked with GlyH-101, a CFTR blocker (ΔIscGlyH 10 ± 2 μA.cm⁻²), CFTR is barely detectable by immunocytochemistry in normal epithelia (Fig. 4B). CF epithelia did not transport chloride (Fig. 4C), as shown by the lack of change in current, after treatment with IBMX/Forskolin (ΔIscAMP 0 ± 0 μA.cm⁻²) or with GlyH-101 (ΔIscGlyH 0 ± 0 μA.cm⁻²). CFTR was also undetectable by immunocytochemistry (Fig. 4D). In contrast, AAV2.5T-CFTRAR restored CFTR chloride current to normal levels (Fig. 4E, ΔIscAMP 12 ± 4 μA.cm⁻², ΔIscGlyH 18 ± 9 μA.cm⁻²), and CFTR protein strikingly localized at the apical membrane of the epithelial cells (Fig. 4F). This is in contrast to AAV2, which does not correct this model, and AAV5, which requires a substantially higher multiplicity of infection (MOI) (26). Furthermore, a dose-response with AAV2.5T-CFTRAR in CF epithelia showed, surprisingly, that as little as 10 vg/cell was sufficient for chloride transport correction, and a dose of 100-1000 vg/cell was equivalent to adenovirus carrying the wild-type CFTR gene (Fig. 4G dotted line, 200 pfu/cell).

**Discussion.** These data demonstrate that under the appropriate selective pressure, viruses, in particular AAV, can evolve significantly greater infectivity within the airway epithelium than generally observed for naturally occurring variants. This result supports the theory that a highly infectious phenotype may be evolutionarily deleterious, and, therefore, naturally evolved viruses likely fail to possess optimal phenotypes for highly efficient respiratory gene delivery vectors (3, 22). Our experimental system with artificial selective pressures thus enabled the evolution of a novel viral variant with a specific phenotype (i.e., enhanced infectivity) by removing natural viral evolutionary pressures and constraints, such as host survival and long transmission times.

We combined AAV2 (the best characterized serotype) with AAV5, which binds a distinct receptor, infects airway epithelia, and contains only 57% primary protein sequence homology to AAV2 (13–16). The stringency of selection was increased each round (decreased inoculation MOI and time) to drive the selection of viral variants with enhanced infectivity from the apical side of airway epithelia. Clones were isolated from the lowest MOI for which virus could subsequently be recovered by PCR, which surprisingly resulted in the enrichment of a single variant after 5 rounds of selection. The high stringency of our protocol may have prevented the identification of additional variants, as observed in a previous study (28). This novel solution is a chimera between the VP1 specific region of AAV2 and the VP2/VP3 region of AAV5, with a single mutation within loop 4 of the exposed VP3 region of AAV5 (A581T).

The AAV capsid contains approximately 3–6 VP1 molecules. The VP1 specific region of AAV2 and some other parvoviruses contains a phospholipase A2 (PLA2) domain that has been shown to be crucial for viral infection due to its role in virion endosomal escape (25, 28). No such activity has yet been ascribed to the AAV5 VP1 region, which possesses only 57.7% protein sequence homology to the AAV2 VP1 region and differs in key PLA2 consensus residues. Interestingly, evidence suggests that amino acid differences within this region among other parvoviruses confers differences in PLA2 activity of 2 to 3 orders of magnitude, along with altered specificities for various phospholipids (25, 28). Furthermore, Takeuchi et al. have recently discussed the VP1-VP2 boundary as a recombination hot spot (29). Two recent attempts to produce AAV vectors in insect cells have shown that swapping the AAV2 VP1 region with the corresponding regions of AAV5 and AAV8 can alter the viral tropism (30, 31). These studies along with our data highlight modularity of this critical capsid domain. Potentially, enhancement of the endosome escape process for AAV may avoid high levels of lysosomal or proteasomal degradation of the virus and thus increase viral transduction as seen in previous airway
studies that used proteasomal inhibitors to augment AAV transduction (32).

The A581T mutation is at the mouth of a region we have recently identified as the sialic acid-binding pocket of AAV5 (Fig. 5). Many other viruses, including influenza, polyomaviruses, coronaviruses and paramyxoviruses, have been shown to use sialic acid as a receptor (24, 33, 34), and alterations of only a few amino acids have been shown to significantly alter sialic acid binding of some viruses. For example, the type of sialic acid linkage is critical for the species specificity of influenza A. Avian influenza binds \( \text{2,3-} \) linked sialic acid while human viruses prefer \( \text{2,6-} \) linked sialic acid, and mutations within the influenza hemagglutinin protein can shift this preference between \( \text{2,3-} \) to \( \text{2,6-} \) linked sialic acid (35). Mutation at a critical receptor-binding site could thus significantly alter tissue specificity, especially for human respiratory tissues that possess a remarkably diverse array of glycan structures (36). In our case, modifying a small hydrophobic residue to a large polar group could alter the binding affinity and specificity by modulating viral capsid interactions with the terminal sialic acid moieties, adjacent sugar residues, or the underlying glycoprotein. In contrast to AAV5, AAV2.5T does not readily saturate its binding to airway epithelia, suggesting it accesses abundant receptor sites on the apical membrane (Fig. 3A). Dose-response related correction of CF epithelia by AAV2.5T-CFTR\( \Delta R \) relative to adenovirus-CFTR correction \((n = 3–6 \text{ CF epithelia from 2 different donors})\) (G). Representative Ussing chamber tracing: (1) Inhibition of Na\(^+\) current with amiloride (\(10^{-4} \text{ M}\)) hyperpolarized apical membrane voltage and increased the driving force for Cl\(^-\) secretion; (2) Inhibition of non-CFTR Cl\(^-\) channels with DIDS (\(10^{-4} \text{ M}\)); (3) CFTR activity stimulation (\(\Delta \text{IsccAMP}\)) by cAMP levels elevated via forskolin (\(10^{-5} \text{ M}\)) and IBMX (\(10^{-4} \text{ M}\)); (4) Reduction of transepithelial Cl\(^-\) current (\(\Delta \text{IscGlyH}\)) with the CFTR-specific blocker GlyH-101 (\(10^{-4} \text{ M}\)). ISC, short-circuit current. 60× oil immersion confocal microscopy.

The AS81T mutation is at the mouth of a region we have recently identified as the sialic acid-binding pocket of AAV5 (Fig. 5). Many other viruses, including influenza, polyomaviruses, coronaviruses and paramyxoviruses, have been shown to use sialic acid as a receptor (24, 33, 34), and alterations of only a few amino acids have been shown to significantly alter sialic acid binding of some viruses. For example, the type of sialic acid linkage is critical for the species specificity of influenza A. Avian influenza binds \( \text{2,3-} \) linked sialic acid while human viruses prefer \( \text{2,6-} \) linked sialic acid, and mutations within the influenza hemagglutinin protein can shift this preference between \( \text{2,3-} \) to \( \text{2,6-} \) linked sialic acid (35). Mutation at a critical receptor-binding site could thus significantly alter tissue specificity, especially for human respiratory tissues that possess a remarkably diverse array of glycan structures (36). In our case, modifying a small hydrophobic residue to a large polar group could alter the binding affinity and specificity by modulating viral capsid interactions with the terminal sialic acid moieties, adjacent sugar residues, or the underlying glycoprotein. In contrast to AAV5, AAV2.5T does not readily saturate its binding to airway epithelia, suggesting it accesses abundant receptor sites on the apical surface (Fig. 3A). Interestingly, the improvement in transduction appears to be airway-specific since transduction is not significantly improved on other cell types (Fig. 3B and D). Future studies will address the underlying basis for the airway-specificity, which may be a function of airway polarization or glycosylation profile. It is exceedingly difficult to predict which capsid regions should be reengineered to form novel viral-cell interactions that

Fig. 4. AAV2.5T-CFTR\( \Delta R \)-mediated correction of CF epithelia. Normal epithelia exhibit cAMP-regulated chloride transport that is sensitive to the CFTR blocker, GlyH-101 (A) and CFTR (green) is barely detectable by immunocytochemistry (B) (representative X-Y and X-Z plane shown). Tight junction protein ZO-1 (red), nuclei (blue). In contrast, cystic fibrosis (CF) epithelia lack cAMP-regulated chloride transport (C), and CFTR is not detectable by immunocytochemistry (D). AAV2.5T-CFTR\( \Delta R \) (MOI 50,000) corrected cAMP-regulated chloride current (E) and CFTR (green) is detectable by immunocytochemistry and appropriately localized to the apical membrane (F). Dose-response related correction of CF epithelia by AAV2.5T-CFTR\( \Delta R \) relative to adenovirus-CFTR correction \((n = 3–6 \text{ CF epithelia from 2 different donors})\) (G). Representative Ussing chamber tracing: (1) Inhibition of Na\(^+\) current with amiloride (\(10^{-4} \text{ M}\)) hyperpolarized apical membrane voltage and increased the driving force for Cl\(^-\) secretion; (2) Inhibition of non-CFTR Cl\(^-\) channels with DIDS (\(10^{-4} \text{ M}\)); (3) CFTR activity stimulation (\(\Delta \text{IsccAMP}\)) by cAMP levels elevated via forskolin (\(10^{-5} \text{ M}\)) and IBMX (\(10^{-4} \text{ M}\)); (4) Reduction of transepithelial Cl\(^-\) current (\(\Delta \text{IscGlyH}\)) with the CFTR-specific blocker GlyH-101 (\(10^{-4} \text{ M}\)). ISC, short-circuit current. 60× oil immersion confocal microscopy.
Library Construction. For the initial plasmid library, the AAV2 cap gene was amplified via PCR using 5’-CATGCGGAAATGGCAGCG-3’ and 5’-CGCA-GAGACCAAAGTTCAACTGA-3’ as forward and reverse primers, respectively and AAV5 cap gene was amplified via PCR using 5’-CATGCGGAAATGGCAGCG-3’ and 5’-AAAGGCCGCGAATGGTTAAGGGG-3’ as forward and reverse primers, respectively. DNA shuffling was performed as previously described (7, 8), and chimeric cap genes were cloned into p5B2 for rcAAV production (10). For subsequent evolution rounds, error-prone PCR was performed as previously described (10).

Cell Lines and Viral Production. Unless otherwise mentioned, cell lines were obtained from the ATCC and cultured at 37 °C and 5% CO2. HEK293T, HeLa, CHO K1, CHO pgsA, and CHO pgsD were cultured in Iscove’s modified Dulbecco’s medium (IMDM) (Mediatech). Pro-5, and LeC2 were cultured in minimal essential medium, alpha modification (mEM) (Sigma–Aldrich). AAV293 cells (Stratyne, Teneo) and Cos7 were cultured in DMEM of of Ad5 at 10 pfu/cell (we provided the manufacturer’s instructions (ScienCell)). All media were supplemented with 10% FBS (Invitrogen) and 1% penicillin/streptomycin (Invitrogen).

The rcAAV library and rAAV vectors were packaged and purified via iodixanol gradient centrifugation as previously described (10, 23). Viral vectors were harvested and titered via quantitative PCR to obtain DNase-resistant genomic titers or flow cytometry to obtain transduction titers as previously described (10, 23).

CFTR transgene in AAV2.5T contains a deleted portion of the R domain (CFTRΔR, 708–759), a shortened CMV immediate/early (173CMV/ie) enhancer/promoter, and a minimal polya (signal) as described by Ostedgaard et al. (26, 27). Ad-CFTR contained the full length CMV promoter and wild-type CFTR.

Wild-type adenovirus serotype 5 (Ad5) and Ad-CFTR were produced by the University of Iowa Gene Transfer Vector Core.

In Vitro Selection and Characterization. To select the viral library, AAV was diluted in EMEM and added to the apical side of primary human airway epithelium from 3 donors in a final volume of 25 µl or 50 µl at 50,000 viral genomes per cell or at the indicated dose and incubated at 37 °C for 1, 4, or 16 h as indicated. Unbound virus was removed by washing 2 times with 250 µl of PBS. After 3 days, AAV was amplified by infection with Ad5, as described (10). For human airway epithelium were infected and 25 µl of Ad5 at 10 pfu/cell was allowed to adsorb from the basolateral side. Epithelia were then placed in fresh media. Three days later, AAV viral genomic DNA was recovered by PCR amplification and cloned into p5B2 for additional rcAAV production and selection or pX2 NotI for rAAV production (23). One round of evolution consisted of capsid recombination and mutagenesis followed by 3 selection steps of infection of human airway epithelia. A second round of recombination and mutagenesis was performed followed by another 2 rounds of selection for a total of 5 rounds of selection.

To determine the relative transduction efficiencies of various clones in cell lines, 1–2.5 × 104 cells were infected with rAAV vectors carrying cDNA encoding green fluorescent protein at a genomic MOI of 10 or 5 × 104. Forty-eight hours postinfection, the percentage of GFP+ cells was quantified by flow cytometry.

For human airway epithelium, apical inoculation was performed at 37 °C for 4 h and basolateral was performed by inverting the microliter to allow adsorption, as described above. For apical inoculations only, Hoescht 33342 (5 µM in USG medium, Invitrogen) was applied basolaterally at the time of infection and incubated for 4 h at 37 °C, after which the basolateral media was aspirated and replaced with fresh USG media containing no Hoescht 33342.

Human airway epithelia infected with eGFP carrying virus were visualized by confocal microscopy. Luciferase gene expression was determined by adding 50 µl of 150 µg/ml D-Luciferin Potassium Salt (PN 122769, Xenogen Corp.) dissolved in EMEM to the apical surface of human airway epithelia. Epithelia were then imaged using the IVIS Imaging System 200 and quantitated using Living Image 2.50.1 software.

Neuraminidase Treatment and Binding Assays. Human airway epithelia were pretreated apically with 10 milliliters of neuraminidase (Vibrio cholerae, Sigma–Aldrich) for 2 h at 37 °C. Cells were then put on ice, neuraminidase removed, and the apical surface washed 3 times with cold EMEM. For all binding assays, epithelia were incubated on ice for 10 min before virus adsorption. Virus was diluted in EMEM and applied to the apical side at the indicated doses for 30 min on ice. Unbound virus was removed by washing 2 times with 250 µl of ice cold EMEM. DNA was isolated with the QIAamp DNA Blood Mini Kit (Qiagen) according to the manufacturer’s instructions. Quantitative PCR was performed using the Power SYBR Green PCR Master Mix (Applied Biosystems) with primers designed to the CMV promoter (5'-
Epithelia were treated with forskolin (10^{-5} M) in modified Ussing chambers (Jim's Instruments) as previously described. Current was measured.

**Immunostaining.** Human airway epithelia were washed once with PBS, fixed with ice cold methanol containing 1% paraformaldehyde for 20 min at −20 °C, and blocked with 2% BSA in SuperBlock (Pierce). Cells were incubated with primary Ab, washed extensively, and incubated with goat anti-mouse or -rabbit secondary Ab. After washing, epithelia were coverslipped with Vectashield mounting media (Vector Laboratories, Inc.). Images were acquired with an Olympus Fluoview FV1000 Laser Scanning Confocal Microscope using a 60X oil immersion lens and analysis was performed with Olympus Fluoview software version 1.4a and Image J.

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