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Incorporation of Adeno-Associated Virus in a Calcium Phosphate Coprecipitate Improves Gene Transfer to Airway Epithelia In Vitro and In Vivo

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Adeno-associated virus (AAV) is inefficient at infecting differentiated airway epithelia because of a lack of receptors at the apical surface. We hypothesized that incorporation of AAV in a calcium phosphate coprecipitate would circumvent this barrier. Interestingly, coprecipitation of AAV type 2 improved gene transfer to differentiated human airway epithelia in vitro and to the mouse lung in vivo. These results suggest that delivery of AAV as a CaP_i coprecipitate may significantly enhance its utility for gene transfer to the airway epithelia in vivo.

Adeno-associated viruses (AAVs) show promise as gene transfer vectors because they are capable of long-term gene expression in vivo, and since they do not encode viral genes, they do not elicit a cell-mediated immune response (4–7, 12, 17). Thus, AAV has some advantages over first-generation adenovirus vectors. However, AAV infection from the apical surfaces of well-differentiated airway epithelia is inefficient (2, 20). This inefficiency stems in large part from the lack of AAV receptors and coreceptors in the apical membrane. AAV is thought to infect cells by binding heparan sulfate proteoglycan (19). The virus may then interact with fibroblast growth factor receptor 1 and $\alpha_v\beta_5$ integrin to further stabilize binding and mediate endocytosis (14, 18). However, in human airway epithelia, these receptors and coreceptors are located on the basolateral membrane, where they are not accessible from the airway lumen (2).

It has recently been reported that a lack of receptors for adenovirus on the apical surfaces of well-differentiated human airway epithelia limits adenovirus-mediated gene transfer (13, 21, 24). In attempting to circumvent this barrier, it has been found that delivery of adenovirus as a calcium phosphate (CaP_i) coprecipitate improves gene transfer to the airway epithelia in vitro and in vivo (3, 9, 10). Furthermore, members of our group observed that delivery of adenovirus as a CaP_i coprecipitate improves gene transfer by increasing binding to the apical surface and that infection occurs independently of fiber knob-coxsackie adenovirus receptor and penton base-integrin interactions (22). Since AAV infection of human airway epithelia may also be limited by a lack of apical receptors, we hypothesized that delivery of AAV in a CaP_i coprecipitate would increase gene transfer to well-differentiated airway epithelia.

We tested our hypothesis in vitro by infecting well-differentiated human airway epithelia grown at the air-liquid interface. Airway epithelia cells were obtained from tracheae and bronchi of lungs removed for organ donation. Cells were cultured and maintained as previously described (8, 16, 23, 25). The culture medium consisted of a 1:1 mixture of Dulbecco's modified Eagle medium and Ham's F-12 medium, 5% Ultraser G (Biosepra SA, Villeneuve-la-Garenne, France), 100 U of penicillin per ml, 100 μg of streptomycin per ml, 2.5 μg of am-

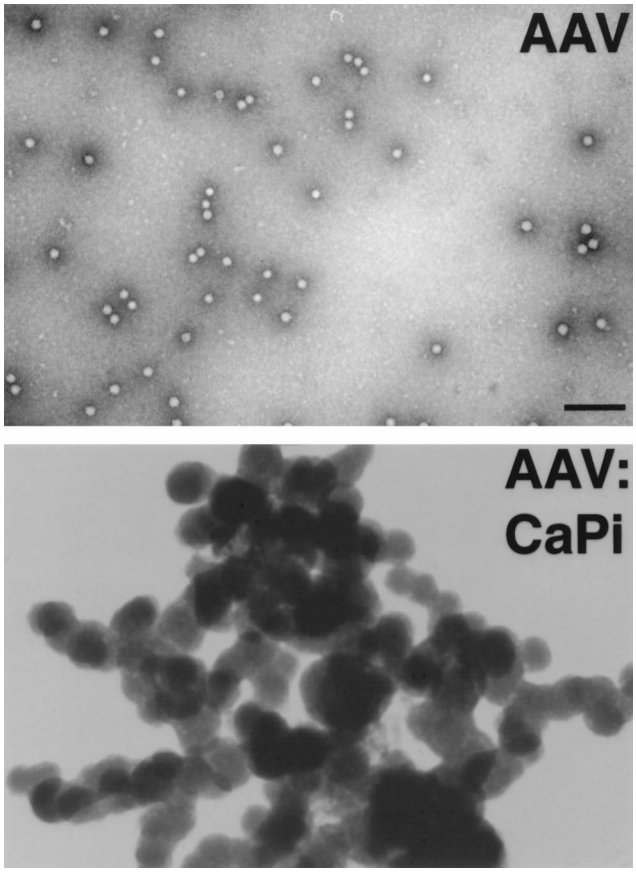

FIG. 1. Electron micrographs of AAV and AAV-CaP_i coprecipitates. Bar = 100 nm.

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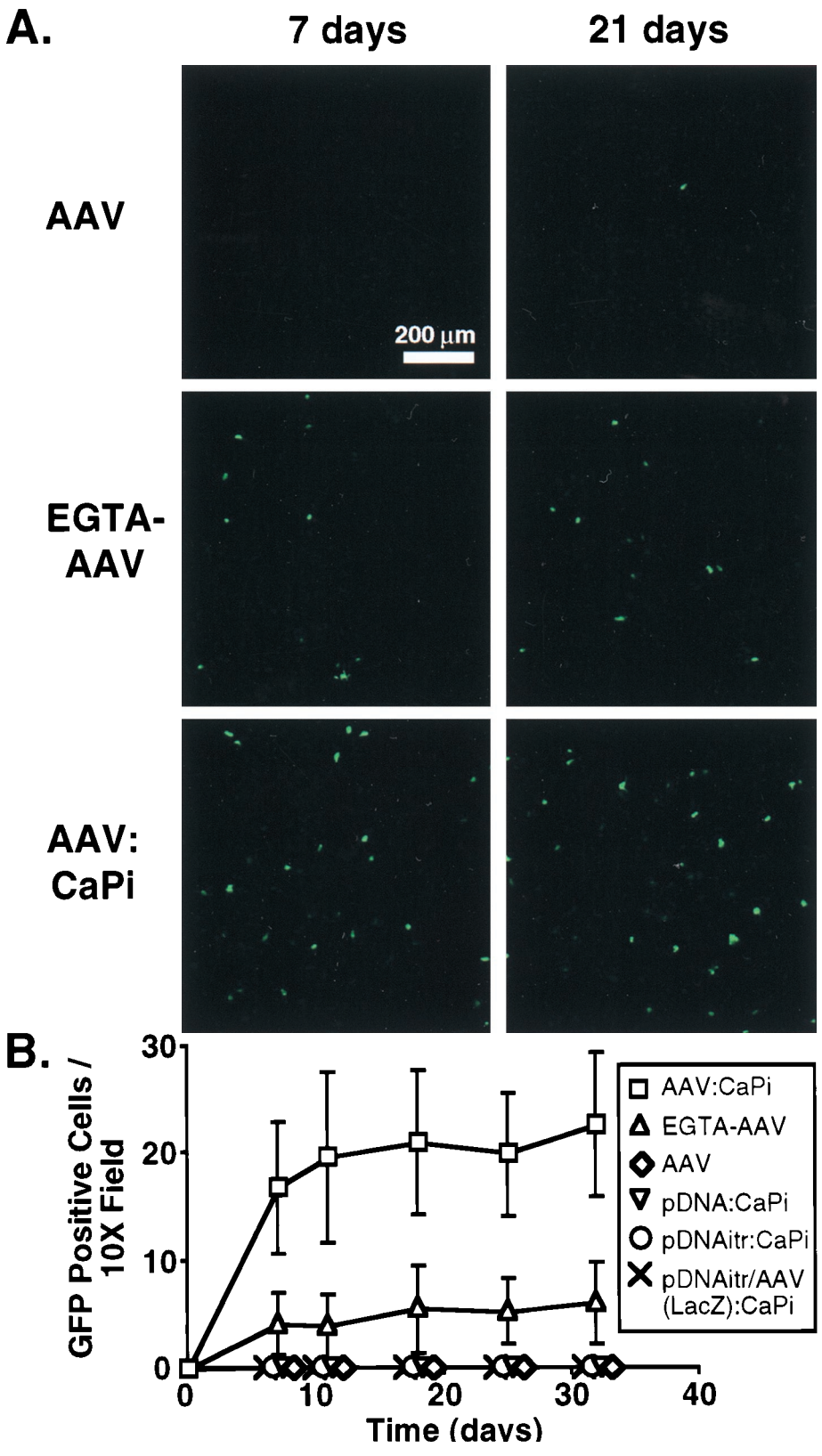

FIG. 2. Gene transfer to human airway epithelia with AAV-CaP_i. Epithelia were incubated with AAV alone, plasmid DNA-CaP_i both with and without inverted terminal repeats (itr), plasmid DNA/AAV(LacZ)-CaP_i, AAV alone following EGTA pretreatment, or AAV-CaP_i. All were then assayed for GFP expression. (A) Confocal photomicrographs of epithelia at 7 and 21 days after infection; (B) transgene expression as indicated by the number of GFP-positive cells at the indicated time after infection. Data are means ± standard deviations; $n = 9$.

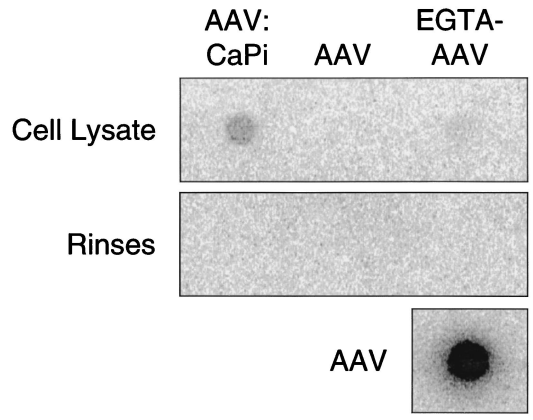

FIG. 3. Effect of CaP_i coprecipitation of AAV on virus association with human airway epithelia. Epithelia were treated with AAV alone, AAV following pretreatment with 6 mM EGTA to disrupt tight junctions, or AAV-CaP_i. Samples were applied to a nylon membrane, and AAV was detected with a ^{32}P-labeled probe. Also shown are rinses and application of 5×10^9 particles of AAV to the membrane.

photericin B per ml, 1% nonessential amino acids, and 0.12 U of insulin per ml. When the epithelia are grown at the air-liquid interface, they differentiate and develop a ciliated surface that is resistant to gene transfer with AAV (2). Differentiated epithelia were infected with recombinant AAV type 2 (rAAV2) which was produced by a CaP_i cotransfection protocol and was purified through three rounds of isopycnic cesium chloride ultracentrifugation, as previously described (2). The proviral plasmid pCisAv.GFP3ori was used to generate rAAV2 (AV.GFP3ori) encoding the enhanced green fluorescent protein (eGFP) under the transcriptional control of the cytomegalovirus enhancer/promoter and the simian virus 40 polyadenylation signal. Recombinant viral stocks were heated at 58°C for 60 min to inactivate contaminating helper adenovirus. Typical yields were 10^9 particles/μl, determined on the basis of DNA slot blot hybridization assays against plasmid standards, and 10^6 transducing units/μl, determined by infection of 293 cells assayed for eGFP expression. The level of adenoviral contamination as based on a second reporter assay for the recombinant adenovirus used for propagation (Ad.CMV Alkphos) was less than 1 functional particle per 10^{10} DNA particles of rAAV2. Viral preparations were evaluated for contamination of wild-type AAV by immunocytochemical staining of AV.GFP3ori- and Ad.CMVLacZ-coinfected 293 cells with anti-Rep antibodies (American Research Products, Inc., Belmont, Mass.). All rAAV2 stocks demonstrated an absence of Rep immunoreactivity when 10^{10} rAAV2 particles were used for infection. Transfection with Rep- or Cap-encoding plasmids served as controls for antibody staining of Rep protein. The virus was dialyzed in phosphate-buffered saline (PBS) prior to use. CaP_i coprecipitates were then formed by placing 1.5×10^{10} particles (1.5×10^7 IU) of AAV2 or 1.5×10^{10} copies of plasmid DNA in 1 ml of Eagle's modified essential medium, which contains 1.8 mM Ca^{2+} and 0.86 mM P_i. An aliquot of 2 M $CaCl_2$ (Promega, Madison, Wis.) solution was then added to achieve a final Ca^{2+} concentration of 12 mM (3). The solutions were mixed by gentle vortexing and incubated at room temperature for 30 min prior to infection. The dose response for the incubation time was similar to that seen with adenovirus coprecipitates, with an optimum formation time of 30 min (data not shown) (3). Figure 1 shows representative electron micrographs of AAV alone and in a CaP_i coprecipitate formed for 30 min. Samples were processed for transmission electron microscopy as previously described (3).

Human airway epithelia were incubated with 5×10^9 particles of AAV in a CaP_i coprecipitate (AAV-CaP_i) and compared to epithelia incubated with AAV alone. As additional controls, we applied 5×10^9 copies of plasmid DNA (both with and without inverted terminal repeats) in a CaP_i coprecipitate and plasmid DNA with AAV in a DNA/AAV(LacZ)-CaP_i coprecipitate. Following a 2-h incubation at 37°C, epithelia were washed twice with Eagle's modified essential medium to remove unbound virus and DNA. We then evaluated transgene expression by counting fluorescent green cells per 10× microscopic field using indirect fluorescence microscopy (2). Fluorescent photomicrographs were obtained with a Bio-Rad MRC-1024 confocal microscope equipped with a Kr-Ar laser at a ×10 magnification. Figure 2 shows that gene transfer with AAV alone was minimal. In addition, no eGFP expression was observed with CaP_i coprecipitates of eGFP plasmid DNA either with (pCisAv.GFP3ori) or without (pCMVeGFP [Clontech, Palo Alto, Calif.]) inverted terminal repeats. Moreover, we did not see eGFP expression with eGFP plasmid DNA delivered in a CaP_i coprecipitate along with recombinant AAV expressing an irrelevant gene, *lacZ*. As a positive control, tight junctions were disrupted by incubating the epithelia with 6 mM EGTA for 20 min prior to vector application (2, 21). As previously reported (2), following pretreatment with EGTA, gene transfer with AAV alone increased 10-fold compared to that with AAV alone on intact epithelia. More importantly, when we infected intact epithelia with AAV-CaP_i, gene transfer increased 100-fold. Thus, delivering AAV as a CaP_i coprecipitate markedly enhanced AAV-mediated gene transfer to human airway epithelia in vitro.

Because CaP_i coprecipitation enhances adenovirus-mediated gene transfer by binding virus to the cell surface, we hypothesized that AAV-mediated gene transfer is enhanced by a similar mechanism. To test this hypothesis, we used a dot blot assay to probe for AAV viral DNA in epithelia treated with AAV alone, AAV alone following EGTA pretreatment, and AAV-CaP_i. Epithelia were studied 24 h after infection. Prior to lysis, epithelia were rinsed six times with PBS at pH 5.0 in order to remove any free virus (a pH of 5 dissociates the CaP_i coprecipitates [3]). Cells were then lysed with RNase-free H_2O. Samples were prepared and applied to a nylon membrane (Ambion, Austin, Tex.). As a positive control, we blotted 5×10^9 particles of AAV (i.e., the same amount of virus used to infect the epithelia). Dot blots were probed with ^{32}P-labeled pCisAv.GFP3ori and developed with a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.). As shown in Fig. 3, we did not detect AAV viral DNA in epithelia which were infected with AAV alone. When we infected EGTA-pretreated epithelia with AAV, a faint dot was present. However, when we infected epithelia with AAV-CaP_i, we observed a 15-fold-greater hybridization than with AAV applied to EGTA-pretreated epithelia. These data suggest that enhanced gene transfer with AAV-CaP_i is due to an increase in the amount of virus that infects cells.

Since CaP_i coprecipitation enhances infection from the apical surfaces of human airway epithelia and receptors for AAV are not present on the apical surfaces of these epithelia, we predicted that AAV-CaP_i would not require an interaction with heparan sulfate proteoglycan for gene transfer. To test this prediction, we preincubated AAV or AAV-CaP_i with 20 μg of soluble heparin sulfate (Sigma, St. Louis, Mo.) per ml for 30 min at room temperature and then infected the epithelia for 2 h at 37°C (1, 19). Figure 4 shows minimal gene transfer with AAV alone in the absence and presence of soluble heparin

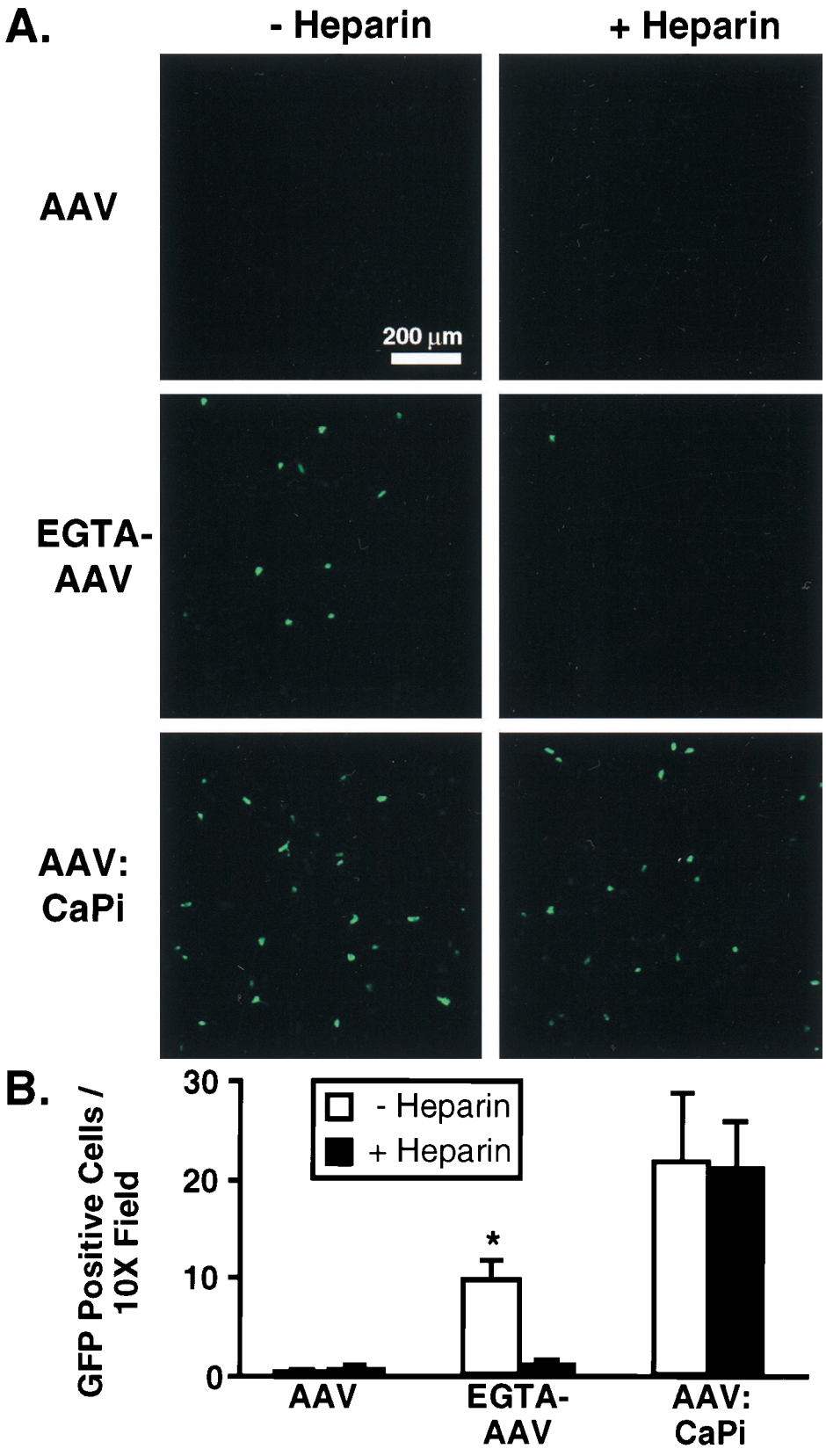

FIG. 4. Effect of heparin sulfate on AAV-CaP_i gene transfer. Prior to infection, virus was incubated in the absence or presence of soluble heparin sulfate. Then human airway epithelia were treated with AAV alone, AAV following pretreatment with 6 mM EGTA to disrupt tight junctions, or AAV-CaP_i. (A) Confocal photomicrographs of epithelia at 14 days after infection; (B) transgene expression as indicated by the number of GFP-positive cells at 14 days after infection. Data are means ± standard deviations; $n = 5$. The asterisk indicates a P value of <0.005.

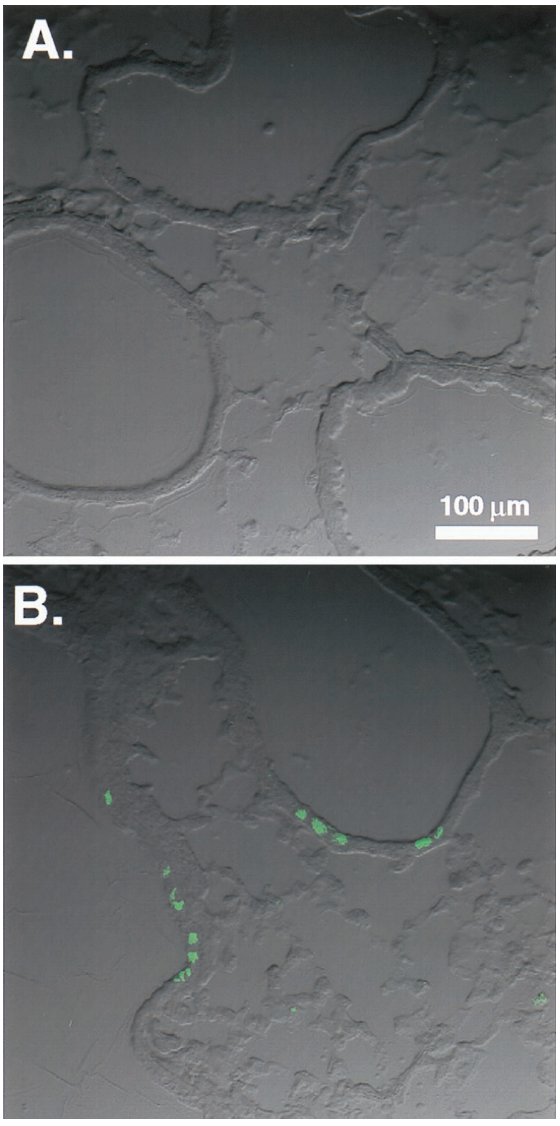

FIG. 5. AAV-CaP_i gene transfer to mouse lung. Recombinant AAV was administered alone or as AAV-CaP_i. Twenty-eight days later, lungs were fixed and cryosectioned. Confocal photomicrographs are from animals infected with AAV alone (A) or AAV-CaP_i (B). (C) Transgene expression as indicated by the number of GFP-positive cells per centimeter of airway as a function of airway diameter. Data are means ± standard deviations; $n = 15$. The asterisk indicates a P value of <0.005.

sulfate. However, when we delivered AAV alone to EGTA-pretreated epithelia, gene transfer was increased. This increase was inhibited by preincubation of AAV with soluble heparin sulfate, suggesting a receptor-dependent infection. In contrast, when we infected cells with AAV-CaP_i, the increase in gene transfer was not blocked by soluble heparin sulfate. Thus, infection with AAV-CaP_i did not require an interaction between AAV capsid proteins and cell surface heparan sulfate proteoglycan in order to enhance gene transfer.

To determine whether CaP_i coprecipitation would improve AAV-mediated gene transfer to airway epithelia in vivo, we infected six 6- to 8-week-old C57BL/6 mice (Jackson Laboratory, Bar Harbor, Maine) with 1.2×10^{10} particles of either AAV alone or AAV-CaP_i by intranasal instillation of two 62.5-μl doses delivered under methoxyflurane anesthesia. Twenty-eight days after infection, animals were sacrificed. PBS (10 ml) was instilled into the right ventricle, and then the lungs and heart were removed intact. The trachea was intubated and inflated at 10 cm of H_2O pressure with PBS, 4% paraformaldehyde, and then PBS again. Lungs were cryosectioned. Figure 5A and B show representative photomicrographs from lung sections of AAV- and AAV-CaP_i-infected mice, respectively. Sections were analyzed by measuring the airway diameter and counting the number of eGFP-expressing cells per airway (Fig. 5C). We observed that gene transfer with AAV alone was minimal. In contrast, delivery of AAV as a CaP_i coprecipitate significantly increased gene transfer to small, medium, and large airways, with a tendency toward infecting small airways. Based on an average width of airway cells of 4.9 μm, we calculated the percentage of cells in the small airways which expressed the transgene (3, 11). In small airways of AAV-infected mice, 0.02% ± 0.02% of cells expressed the transgene, whereas 0.25% ± 0.20% of cells expressed the transgene in AAV:CaP_i-infected mice. Although these percentages are low, it is important to note that the dose of vector delivered was also low. Thus, delivery of AAV as a CaP_i coprecipitate markedly enhanced AAV-mediated gene transfer in vivo, with particular propensity for the small airways.

Our data are consistent with earlier observations that apical delivery of AAV is inefficient at gene transfer (2, 7, 20). Our results with EGTA and heparin sulfate are also in agreement with a basolateral localization of AAV receptors. Recent reports have questioned the role of heparan sulfate proteoglycan and α_v integrins as receptors for AAV (15). However, whatever the receptor is, its activity appears to be localized to the basolateral side. Our data show that by increasing binding to the apical surface, CaP_i coprecipitation helps overcome this barrier. The enhancement in gene transfer is similar to that seen with adenovirus-CaP_i and is more efficient than gene transfer with plasmid DNA-CaP_i (9). Unlike plasmid DNA, AAV has viral proteins which may facilitate steps subsequent to binding. The 100-fold improvement in gene transfer when AAV was delivered as a CaP_i coprecipitate supports this conclusion.

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