Recent identification of two receptors for the adenovirus fiber protein, coxsackie B and adenovirus type 2 and 5 receptor (CAR), and the major histocompatibility complex (MHC) Class I α-2 domain allows the molecular basis of adenoviral infection to be investigated. Earlier work has shown that human airway epithelia are resistant to infection by adenovirus. Therefore, we examined the expression and localization of CAR and MHC Class I in an in vitro model of well differentiated, ciliated human airway epithelia. We found that airway epithelia express CAR and MHC Class I. However, neither receptor was present in the apical membrane; instead, both were polarized to the basolateral membrane. These findings explain the relative resistance to adenovirus infection from the apical surface. In contrast, when the virus was applied to the basolateral surface, gene transfer was much more efficient because of an interaction of adenovirus fiber with its receptors. In addition, when the integrity of the tight junctions was transiently disrupted, apically applied adenovirus gained access to the basolateral surface and enhanced gene transfer. These data suggest that the receptors required for efficient infection are not available on the apical surface, and interventions that allow access to the basolateral space where fiber receptors are located increase gene transfer efficiency.

The mechanism of infection by type 2 and type 5 adenovirus has been extensively studied. However, most of the knowledge on adenoviral infection has been obtained from studies done on immortalized cell lines. The first steps in adenovirus infection are thought to involve primarily two proteins in the capsid, fiber and penton base (1–3). The fiber protein is important for binding to a high affinity fiber receptor. In a human oral squamous carcinoma cell line (KB cells), A549 cells, and HeLa cells, this receptor is thought to be present in the range of 3,000–10,000 receptors/cell (4–6). NIH 3T3 cells, which are resistant to adenovirus infection, have less than 100 receptors/cell (7). After binding to the fiber receptor, penton base interacts with αvβ3 and αvβ5 integrins facilitates internalization via receptor-mediated endocytosis (2, 8, 9). The acidic pH the virus encounters in the endosome may trigger a conformational change that releases the virus into the cytoplasm (10–12) and allows the adenovirus capsid to travel to the nucleus (2, 13). Then viral proteins and DNA bind to the nuclear pore complex, capsid disassembly continues, and DNA enters the nucleus accompanied by DNA-associated protein 7 (1, 2, 14). These studies have concluded that a high affinity fiber receptor is required for binding and infection and that an αvβ3 integrin acts as a co-receptor.

We and others (15–22) have found infection of ciliated airway epithelia by adenovirus to be inefficient. In an in vitro model of human airway epithelia, we found that unlike infections of HeLa cells adenovirus infection of ciliated airway epithelia was quite limited and that which did occur was not blocked by co-incubation with soluble fiber protein (15). In a second set of experiments, we measured fiber-mediated binding of adenovirus and found that fiber protein had no effect on binding to differentiated airway epithelia. Based on these studies, we concluded that ciliated airway epithelia lack high affinity fiber receptor activity.

Two separate groups have recently cloned receptors that bind adenovirus fiber and may be involved in the pathogenesis of adenovirus infection. Bergelson et al. (23) identified a fiber receptor, the coxsackie B and adenovirus type 2 and 5 receptor (CAR).1 When transfected with CAR cDNA, non-permissive Chinese hamster cells became susceptible to adenovirus attachment and infection. The results from Bergelson et al. (23) are in concordance with Mayr and Freimuth (24) who found that human chromosome 21 DNA-containing CAR sequence allows Chinese hamster cells to bind fiber. Hong et al. (25) used a phage display hexapeptide library to identify mimotopes of the fiber protein receptor. One of these mimotopes was homologous to the core motif of the MHC-I α-2 consensus region (25). In a lymphoblastoid cell line they found that expression of MHC-I resulted in increased fiber protein binding and adenovirus-mediated gene transfer as compared with cells that lack expression of MHC-I. The discovery of CAR and MHC-I α-2 domain allowed us to investigate the molecular basis for our earlier finding of a lack of fiber receptor activity on the apical membrane of human airway epithelia and the limited efficiency of adenovirus infection.

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1 The abbreviations used are: CAR, coxsackie B and adenovirus type 2 and 5 receptor; MHC, major histocompatibility complex; GFP, green fluorescent protein; m.o.i., multiplicity of infection; PBS, phosphate-buffered saline; Rt, resistance; EMEM, Eagle's modified essential media; FITC, fluorescein isothiocyanate; CF, cystic fibrosis; FACS, fluorescence-activated cell scanning; CFTR, cystic fibrosis conductance transmembrane regulator.
Expression of Fiber Receptors by Airway Epithelia

MATERIALS AND METHODS

Human Airway Epithelia—Airway epithelial cells were obtained from surgical polyectomies or from trachea and bronchi of lungs removed for organ donation from non-CF and CF patients as indicated. Cells were isolated by enzyme digestion as described previously (26). Freshly isolated cells were seeded at a density of 5 × 10^4 cells/cm^2 onto collagen-coated, 0.6-cm^2 diameter millipore polycarbonate filters (Millipore Corp., Bedford, MA). The cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and air. Twenty four hours after plating, the mucosal layer was removed, and the cells were allowed to grow at the air-liquid interface (27, 28). The culture media consisted of a 1:1 mix of Dulbecco’s modified Eagle’s medium/Ham’s F12, 5% Ultraser G (Biosepa SA, Cedex, France), 100 units/ml penicillin, 100 μg/ml streptomycin, 1% nonessential amino acids, and 0.12 units/ml insulin. Epithelia were used for transepithelial resistance and for morphology by scanning electron microscopy.

Recombinant Adenoviruses—Recombinant adenovirus vectors expressing β-galactosidase, Ad2/Gal-2, and CFTR, Ad2/CFTR-16, were prepared as described previously (29) by the University of Iowa Gene Transfer Vector Core at titers of ~10^10 infectious units/ml. Recombinant adenovirus vectors expressing green fluorescent protein (GFP) (30, 31), Ad2/GFP, and the purified fiber knob protein used for competition experiments (32) were a gift of Dr. Sam Wadsworth (Genzyme, Framingham, MA).

Viral Infection and Binding Assays—Epithelia were allowed to reach confluence and develop a transepithelial electrical resistance (RT), indicating the development of tight junctions and an intact barrier. All epithelia had values of RT~500 Ω cm^2. Fourteen days after seeding, 50 μl of either the recombinant viruses in phosphate-buffered saline (PBS) were added to the apical surface. The particle to infectious unit ratio ranged between 24 and 34, and the cell number in a 0.6-cm diameter well differentiated airway epithelium is approximately 750,000. Following the indicated incubation time, the viral suspension was removed, and the monolayers were rinsed twice with PBS. After infection, the epithelia were incubated at 37 °C for an additional 24 h. Epithelial resistance (RT) was measured by an ohm meter (EVOM™, World Precision Instrument Inc., Sarasota, FL) before infection, and RT was not altered by application of virus. To disrupt the tight junctions, we pretreated the apical surface of the epithelia for 30 min with 400 μl of either Eagle’s modified essential media (EMEM), deionized H_2O, or 8 mM EGTA in EMEM (EGTA) at 37 °C. To evaluate virus association with cells, adenovirus was covalently labeled with the carboxyamine dye, Cy3 (Amersham Pharmacia Biotech) (13). The labeling procedure decreased the infectious units/particle ratio by 5–35%.

Ribonuclease Protection Assay—To obtain a quantitative assessment of CAR message in human airway epithelia, we used the ribonuclease protection assay. Total RNA was isolated from primary airway epithelia using RNA-STAT 60 (Tel-Test Corp., Friendswood, TX) according to the manufacturer’s instructions. [β-35S]UTP-labeled cDNA probes for CAR and β-actin were transcribed from linearized plasmid DNA using T3 polymerase for CAR and Sp6 polymerase for β-actin. Antisense probes were hybridized with 100 μg of total RNA from either CAR-protected fragment. A 216-nucleotide CAR message was normalized to β-actin message and expressed as a ratio of CAR/β-actin.

Analysis of CAR and MHC-I by Immunocytochemistry—To evaluate CAR expression at the apical and basolateral membrane of differentiated airway epithelia, unpermeabilized ciliated airway epithelia were incubated for 3 h with 1:60 dilution of an anti-CAR monoclonal antibody (Rm6B) applied either to the apical or to the basolateral side at 4 °C. After 20 min, the epithelia were rinsed with PBS, fixed with 1.8% formaldehyde and permeabilized with Triton X-100 at 20 °C for 10 min and then quenched with 4% bovine serum albumin. The epithelia were incubated with a 1:200 dilution of RNase A/T1 provided to yield a 216-nucleotide CAR message in human airway epithelia, we used the ribonuclease protection assay (13). The labeling procedure decreased the infectious units/particle ratio by 5–35%.

Measurement of β-Galactosidase Activity—We measured total β-galactosidase activity using a commercially available method (GalactoLight™, Tropix, Inc., Bedford, MA). Briefly, after rinsing with PBS, cells were removed from filters by incubation with 120 μl of lysis buffer (25 mM Tris phosphate, pH 7.8, 2 mM dithiothreitol; 2 mM 1,2-diamino- cyclohexane-N,N,N′,N′-tetraacetic acid; 10% glycerol; and 1% Triton X-100) for 15 min. Light emission was quantified in a luminometer (Aloka Luminator, San Diego, CA).

Measurement of GFP Expression—To detect GFP mRNA, we dissociated the epithelia with 0.05% trypsin and 0.53 mM EDTA. Fluorescence from 50,000 individual cells was analyzed using fluorescence-activated cell analysis (FACScan, Lysys II software, Becton Dickinson, San Jose, CA). The percentage of cells positive for GFP was assessed by determining the percent of highly fluorescent cells in each group and subtracting the fluorescence of the control cells. To assess the GFP expression of immature, basal non-ciliated epithelial cells, aliquots of the cell suspension were used for fluorescence-activated cell analysis (16). To assess the level of CAR expression by GFP-positive cells, aliquots of the cell suspension were incubated with Rmcb antibody (1:60 dilution) for 2 h. The cells were centrifuged and resuspended with a 1:200 dilution Texas Red anti-mouse IgG (Jackson Immunoresearch) for 1 h. GFP and Texas Red (CAR) fluorescence from 10,000 individual cells were analyzed using fluorescence-activated cell analysis.

Measurement of Transepithelial Electrical Properties—Epithelia were mounted in modified Ussing chambers (Jim’s Instruments, Iowa City, IA) as described previously (33). Epithelia were bathed on the mucosal surface with a solution containing 135 mM NaCl, 2.4 mM PO_4, 0.6 mM PO_4, 1.2 CaCl_2, 1.2 MgCl_2, 10 Hepes (titrated to pH 7.4 with NaOH), and 10 dextrose. The mucosal solution was identical with the exception that NaCl was replaced with 135 mM sodium gluconate. Amiloride (10 μM) was added to the mucosal solution to inhibit Na^+ channels and transmembrane Na^+ transport. The CAMP agonists, 10 μM forskolin and 100 μM isobutylmethylxanthine, were added to the serosal and mucosal solutions, respectively, to increase transepithelial Cl^- current through CFTR Cl^- channels. To assess total Cl^- current, we then added 100 μM bumetanide to the submucosal solution and measured the change in current.

RESULTS

Expression of CAR Message by Ciliated Airway Epithelia—We investigated the abundance of CAR mRNA in airways by using primary cultures of human airway epithelia grown on permeable filter supports. We previously showed that 3 days after seeding, the airway epithelia showed a fiber-dependent expression of CAR message and measured the change in current.

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Ciliated airway epithelia expressed similar levels of CAR mRNA. These data indicate that ciliated airway epithelia express CAR message at similar levels to HeLa cells and poorly differentiated airway epithelia. There are at least two possible explanations as follows: first, CAR is expressed in differentiated epithelia, but the protein is present only on the basolateral membrane; second, only a few cells express CAR, and vectors applied to the lumen do not have access to those cells.

**CAR and MHC Immunocytochemistry**—We first evaluated the possibility that CAR is not expressed at the apical membrane of differentiated airway epithelia by immunocytochemistry. No staining was observed when the antibody was added to the apical side (Fig. 2A). However, CAR staining was detected on the surface of most cells when the antibody was added to the basolateral side. Furthermore, a small population of cells showed very high levels of CAR staining (Fig. 2A). When no primary or non-immune ascites fluid was used, no stain was visible (data not shown). To more quantitatively analyze CAR expression, we used fluorescence-activated cell scanning (FACS) to determine the number of cells expressing CAR and the level of CAR expression by each cell. Fig. 2B shows examples of histograms obtained from cells stained with anti-CAR antibody. The shift in fluorescence suggests that most cells express CAR. In addition, the histogram shows that a subpopulation of the epithelial cells was highly fluorescent. These highly fluorescent cells may represent basal cells, since we found a similar number of cells were positive for cytokeratin 14 (CK-14) expression, a basal cell marker (16) (data not shown). Because both anti-CAR and anti-CK-14 were mouse monoclonals, studies to evaluate co-localization of CK-14 and CAR by FACS were not possible.

We also studied the cellular distribution of MHC-I α-2 domain on well differentiated human airway epithelia. Epithelia were incubated with an FITC-labeled anti-human MHC-I antibody applied either to the apical or basolateral surface. As with anti-CAR antibodies, no staining was observed when the antibody was added to the apical side (Fig. 3). However, membrane staining was observed throughout the epithelium when the antibody was added to the basolateral side (Fig. 3). No staining was seen with the FITC-labeled secondary antibody alone or when mouse cells were used (data not shown). These results suggest that the adenovirus fiber receptors, CAR and MHC-I α-2 domain receptor, are polarized to the basolateral plasma membrane of the airway epithelia. In addition, they suggest that a small population of cells, probably basal cells, express higher levels of CAR. This polarity may account for the relative resistance to adenovirus infection when vector is applied to the apical membrane. Furthermore, the results predict that infection from the basolateral side should be more efficient and fiber-dependent.

**Adenovirus Infection of Airway Epithelia Is Polar**—We investigated the ability of adenovirus to infect human airway epithelia through the basolateral side by a fiber-dependent
mechanism. Since the adenovirus diameter is only 70–130 nm (3), it should be able to pass freely through the Millipore filters (0.4 μm pores) used to grow the airway epithelia. To test this hypothesis, we turned the epithelia upside down and carefully applied 50 m.o.i. of Ad2/βGal-2 in a volume of 25 μl to the bottom of the Millipore filter. After 30 min, the epithelia were rinsed thoroughly. Fig. 4 shows that airway epithelia expressed β-galactosidase activity at levels 2 logs greater when the virus was applied from the basolateral side than when it was placed on the apical surface. In contrast to apical infection, infection from the basolateral surface was blocked by addition of 70 μg/ml fiber knob.

To estimate the number of cells achieving gene transfer via the basolateral side, we applied Ad2/GFP to the basolateral surface for 30 min, and after 48 h we assayed GFP expression by FACS. Fig. 5A shows that 41.3 ± 4.4% of the cells were positive for GFP expression when the virus was applied via the basolateral side compared with 1.3 ± 0.2% when it was applied via the apical side. Fig. 5B shows a fluorescence photomicrograph of epithelia infected with Ad2/GFP via the apical and basolateral side. Arrows indicate a subpopulation of GFP-positive cells that are higher in fluorescence. The bar represents 50 μm.

Fig. 4. Effect of polarity of infection on adenovirus-mediated transgene expression by human airway epithelia. Well differentiated airway epithelia were exposed to 50 m.o.i. Ad2/βGal-2 for 30 min either from the apical or basolateral side (BL). Vector was then removed, and the epithelia were cultured for 2 additional days before analysis. Control epithelia received no virus. Data are β-galactosidase activity/mg of protein under control conditions (open bars) or in the presence of 70 μg/ml fiber knob (shaded bars). Data are mean ± S.E., n = 14. Asterisk indicates p < 0.01 between basolateral and apical viral application. ¶ indicates p < 0.01 between basolateral expression and fiber knob competition.

Fig. 5. Effect of polarity of infection on adenovirus-mediated GFP expression by human airway epithelia. A, well differentiated airway epithelia were exposed to 50 m.o.i. Ad2/GFP for 30 min from either the apical or basolateral (BL) side. Vector was then removed, and the epithelia were cultured for 2 additional days before FACS analysis. Data are the percentage of dissociated airway epithelia cells that were positive by FACS analysis for GFP. Ad2/βGal-2 and non-infected cells were used as negative controls. Data are mean ± S.E.; n = 4. Asterisk indicates p < 0.01 between basolateral and apical viral application. B, fluorescent photomicrograph of epithelia infected with Ad2/GFP from the apical and basolateral side. Arrows indicate a subpopulation of GFP-positive cells that are higher in fluorescence. The bar represents 50 μm.
levels of CK14 and CAR suggesting that other receptors (perhaps MHC-1) may also be involved in adenovirus binding and infection. Moreover, most of the cells expressing moderate levels of GFP expressed low levels of CAR (bottom left quadrant in Fig. 6B) suggesting that low levels of CAR may limit the degree of adenovirus infection.

Adenovirus-mediated Gene Transfer of CFTR through the Basolateral Side—For CFTR to mediate transepithelial Cl⁻ transport it must be present in the apical membrane of cells that are in contact with the lumen. Thus, basal cells cannot contribute to Cl⁻ transport across the apical membrane. Therefore we asked if delivery of adenovirus expressing CFTR via the basolateral side would correct the Cl⁻ transport defect in well differentiated CF epithelia. Finally, whereas the relationship between β-galactosidase expression on measured β-galactosidase activity is linear, the relationship between expression of CFTR and transepithelial Cl⁻ transport is not (33). Fig. 7 shows that 48 h after 50 m.o.i. of Ad2/CFTR-16 were added to the basolateral side of CF epithelia for 30 min, total Cl⁻ current (ΔIscBum) increased into the range observed in normal epithelia (34). No correction was observed when the virus was added to the apical side. We cannot exclude the possibility that cell division and differentiation of basal cells resulted in adenovirus-mediated expression of CFTR in columnar cells; however, the low rate of cell division (35) and the short time between adenovirus infection and the expression assay make this less likely. These data suggest that columnar non-basal airway epithelia can be infected with adenovirus via the basolateral side and can express sufficient amounts of CFTR to correct the CF Cl⁻ transport defect.

The Basolateral Surface Is Accessible to Virus in Airway Epithelia following Disruption of the Tight Junctions—Chelation of extracellular Ca²⁺ with 8 mM EGTA can reversibly increase the permeability of tracheal epithelia (36, 37). Also, a brief apical application of H₂O has been reported to transiently increase permeability of airway epithelia to macromolecules and DNA (38). We tested the effect of exposing well differentiated human airway epithelia to EGTA or to H₂O in an attempt to disrupt the tight junctions, improve access of adenovirus to the basolateral surface of cells, and improve adenovirus-mediated gene transfer. Fig. 8 shows that both 8 mM EGTA and H₂O added to the apical surface for 30 min significantly decreased Rt, indicating disruption of the tight junctions. The effect was fully reversible with time, with Rt returning to base-line values within 12 h.

We used fluorescently labeled adenovirus to test the hypothesis that disruption of the tight junctions with H₂O or 8 mM EGTA would allow adenovirus to reach the basolateral surface of the airway epithelial cells. Fig. 9, A, C, and E, shows 180-μm thick X-Z projections of confocal images taken of control epithelia and epithelia treated with H₂O or EGTA prior to addition of the fluorescently labeled adenovirus. The location of cells is shown by staining for F-actin. The experiments were performed at 4 °C to avoid endocytosis of vector. Control epithelia showed no detectable apical surface binding or virus between the cells. In contrast, epithelia pretreated with H₂O or
8 mM EGTA showed large amounts of virus within the epithelia but not at the apical surface. Fig. 9, B, D, and F, shows a single X-Z confocal section (0.35 μm). The fluorescently labeled adenovirus can be seen in the intercellular spaces of the epithelia pretreated with H2O or 8 mM EGTA. Thus, disruption of the tight junctions increased adenovirus access to the basolateral surface of airway epithelia where CAR and MHC Class I are expressed.

Transient Disruption of Tight Junctions Enhances Gene Transfer by Apical Adenovirus—We tested the hypothesis that disrupting the tight junctions of differentiated epithelia would increase adenovirus-mediated gene transfer. The apical surfaces of airway epithelia were pretreated for 30 min with either H2O or 8 mM EGTA followed by a 30-min incubation with 50 m.o.i. of Ad2/βGal-2. Fig. 10 shows that these treatments increased β-galactosidase expression 8–9-fold as compared with epithelia pretreated with EMEM. Furthermore, the increase in β-galactosidase expression could be blocked by excess fiber knob protein. Moreover, if Ad2/βGal-2 was applied 12 h after treatment at a time when R0 had returned to basal values, β-galactosidase expression was not significantly different from that in control epithelia (data not shown).

We also tested the hypothesis that disrupting the tight junctions of well differentiated epithelia would increase adenovirus-mediated CFTR gene transfer to CF airway epithelia. We observed a significant correction of total Cl− current (ΔIsc bum) in CF airway epithelia transfected with 50 m.o.i. of Ad2/CFTR-16 after treatment with H2O or 8 mM EGTA (Fig. 11). No correction in ΔIsc bum was seen when the virus was added to the apical surface of epithelia for 30 min. We had previously shown that adenovirus-mediated gene transfer to airway epithelia can correct the CF defect if the incubation time with the virus was increased (16); therefore, we also applied Ad2/CFTR-16 to the apical surface for 24 h. We also found that treatment of airway epithelia with H2O or 8 mM EGTA alone (without adding Ad2/CFTR-16) did not affect the ΔIsc bum defect of CF airway epithelia (n = 3, data not shown). Fig. 6 shows that there was more correction of Cl− transport in epithelia treated with H2O or 8 mM EGTA for 30 min than with a 24-h exposure to virus alone. These data indicate that disruption of the tight junctions before apical application of the virus significantly improved adenovirus infection and adenovirus-mediated expression of CFTR. However, the efficiency of adenovirus-mediated gene transfer was slightly lower with H2O or 8 mM EGTA pretreatment than with application of virus to the basolateral side.

DISCUSSION

Our earlier work showed that ciliated airway epithelia are relatively resistant to adenovirus infection and adenovirus-mediated gene transfer because they lack high affinity fiber receptor activity on the apical surface (15, 16). Our current work confirms those findings and provides a molecular explanation, the two cellular receptors for fiber, CAR and MHC Class I, were not present on the apical membrane of well differentiated ciliated airway epithelia. Lack of fiber binding appears to be the rate-limiting step to explain the inefficient adenovirus-mediated gene transfer to the airway epithelia seen in vitro (16, 17, 21) and in vivo (19, 39, 40). Our results are also in agreement with a recent report by Pickles et al. (41) suggesting that adenovirus binding and CAR expression are limited to the basolateral surface of human airway epithelia. However, Pickles et al. (41) found that in contrast to human, adenovirus internalization was rate-limiting for adenovirus infection to the apical membrane of rat airway epithelia.

Despite their absence on the apical membrane, both receptors were expressed on the basolateral membrane. Our data showing that excess fiber knob inhibits basolateral gene transfer suggests that fiber receptor is responsible, at least in part,
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for the efficiency of gene transfer from the basolateral surface. However, it is still not clear if one or both of the two fiber receptors are required for adenovirus binding and infection of airway epithelia. Our data cannot rule out the requirement for MHC Class I (25) since we found it uniformly expressed in the basolateral membrane of most cells. However, our data also suggest that basal cells may express higher levels of CAR and MHC Class I expression on the apical surface of airway cells. Experiments using recombinant expression and knock-out or antisense down-regulation of these receptors will be required to assess their relative contribution to fiber binding and adenovirus internalization.

Some viruses infect epithelia via the basolateral side (35, 42, 43), the apical side (44–48), or both sides (49, 50). The sidedness of infection may even vary with maturation of the epithelia. Rossen et al. (51) found that porcine-transmissible gastroenteritis virus infected equally from the basolateral side and the apical side of a porcine epithelial cell line (LLC-PK1) infected with 50 m.o.i. of Ad2/CFTR-16 applied to the apical surface for 30 min. Forty eight hours later the epithelia were studied in Ussing chambers. Data are mean ± S.E. of β-galactosidase activity/mg of protein. Asterisk indicates p < 0.01 compared with EMEM control, n = 6.

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