Terminal sialylation is altered in airway cells with impaired CFTR-mediated chloride transport

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Received 2 February 2000; accepted in final form 6 October 2000

Kube, Diannne, Lynn Adams, Aura Perez, and Pamela B. Davis. Terminal sialylation is altered in airway cells with impaired CFTR-mediated chloride transport. Am J Physiol Lung Cell Mol Physiol 280: L482–L492, 2001.—Reduced terminal sialylation at the surface of airway epithelial cells from patients with cystic fibrosis may predispose them to bacterial infection. To determine whether a lack of chloride transport or misprocessing of mutant cystic fibrosis transmembrane conductance regulator (CFTR) is critical for the alterations in glycosylation, we studied a normal human tracheal epithelial cell line (9/HTEo-) transfected with the regulatory (R) domain of CFTR, which blocks CFTR-mediated chloride transport; ΔF508 CFTR, which is misprocessed, wild-type CFTR; or empty vector. Reduced cAMP-stimulated chloride transport is seen in the R domain and ΔF508 transfectants. These two cell lines had consistently significantly reduced binding of elderberry bark lectin, which recognizes terminal sialic acid in the α-2,6 configuration. Binding of other lectins, including Maakia amurensis lectin, which recognizes sialic acid in the α-2,3 configuration, was comparable in all cell lines. Because the cell surface change occurred in R domain-transfected cells, which continue to express wild-type CFTR, it cannot be related entirely to misprocessed or overexpressed CFTR. It is associated most closely with reduced CFTR activity.

Cystic fibrosis; cystic fibrosis transmembrane conductance regulator; ΔF508; regulatory domain; lectins; fluorescent imaging; immunogold labeling; 3-(2,4-dinitroanilino)-3′-amino-N-methyldipropylamine

Cystic fibrosis (CF) is caused by mutations in the CF transmembrane conductance regulator (CFTR), a cAMP-responsive chloride channel. However, not all the abnormalities associated with the CF genotype can yet be directly related to CFTR. Recent studies show that CFTR itself regulates other ion channels in the plasma membrane, including the epithelial sodium channel (7) and the outward rectifying chloride channel (11, 24). Some consistent abnormalities such as the abnormal glycosylation of mucins and proteins and lipids at the cell surface (18, 19) have not yet found ready explanations, although they may be clinically important. For example, abnormal surface sialylation is associated with increased adherence to CF cells of Pseudomonas aeruginosa (21, 31), an organism with a predilection for the CF airway, acquisition of which is associated with a poorer prognosis.

The abnormal glycosylation patterns in CF may be explained, in part, by the failure of activity of CFTR at intracellular sites, which could lead to abnormal acidification of particular intracellular compartments, notably the trans-Golgi network (TGN) (2, 4, 5). Failure to acidify this compartment normally might lead to reduced activity of glycosyltransferases such as α-2,6-sialyltransferase with narrow, acidic pH optima and favor activity of enzymes such as sulfotransferases with broader pH optima. Barasch et al. (5) presented several lines of evidence in support of this hypothesis, including electron microscopy data showing increased accumulation of the weak base 3-(2,4-dinitroanilino)-3′-amino-N-methyldipropylamine (DAMP), in the TGN of SV40-transformed airway epithelial cells compared with SV40-transformed CF cell lines. However, a study using ratio-imaging confocal microscopy on various cell lines that do and do not express CFTR suggested that the pH in all intracellular compartments was normal in CF cells, even with cAMP stimulation (25). An alternative suggestion is that the abnormal processing of some mutant forms of CFTR, including the most common mutant ΔF508, interferes with processing, trafficking, and routing of other glycoconjugates including sialyltransferases (4, 29). Because most CF cell lines now in use contain at least one ΔF508 allele, it is difficult to distinguish the effects of processing mutants from those of reduced CFTR activity.

To investigate the link between the CF phenotype and abnormal glycosylation, we used 9/HTEo− cells (normal human tracheal epithelial cells known to express CFTR) stably transfected with an episomal expression vector (pCEP) that contains either the regulatory (R) domain of CFTR (pCEP-R), wild-type (WT) CFTR (pCEP-WTCFTR), ΔF508 CFTR (pCEP-ΔF508), or no expressed gene except that for hygromycin resistance (pCEP). These cell lines were isogenic except for the transfected gene. Overexpression of the R domain of CFTR (amino acids 588–857) in 9/HTEo− cells inhibits CFTR function as previously described (17). When the R domain is overexpressed in these cells,
transcription of CFTR as assessed by RT-PCR continues. Although levels of CFTR protein are too low to be detected in 9/HTEo− cells either by Western blot or by immunofluorescence assay, after R domain transfection, antibody to the R domain shows an increase in specific fluorescence in a cytoplasmic staining pattern, suggesting that the overexpressed R domain is not retained in the endoplasmic reticulum and thus probably does not retard CFTR maturation either. We demonstrate in this paper that in a heterologous expression system, coexpression of the R domain with CFTR doesn’t interfere with CFTR processing. R domain expression is associated with a reduction in basal and cAMP-stimulated chloride efflux as measured by 6-methoxy-N-(3-sulfopropyl)quinolinium (SPQ) fluorescence in a pattern similar to that produced by incubation of 9/HTEo− cells with antisense oligonucleotides to CFTR, indicating that it is due to CFTR. Calcium-stimulated chloride efflux is not reduced in R domain-transfected (or antisense-treated) cells, indicating the specificity of the R domain effect. R domain transfection does not alter cAMP levels or protein kinase (PK) A activity so its effect is not at that level. In planar lipid bilayers, unphosphorylated R domain on the intracellular side of the CFTR channel results in channel closure (15, 16), and it is likely that this is the mechanism of reduction of CFTR function in the pCEP-R cells. Besides the R domain cells, we developed and tested 9/HTEo− cells transfected with ΔF508 CFTR as well as with WT CFTR to assess the effect of the misprocessing known to occur with ΔF508-CFTR. We studied the binding of various fluorescent lectins to these cell lines using computer-assisted quantitative fluorescence microscopy. We tested these hypotheses: first, that human airway epithelial cells that express only normal CFTR but have no CFTR activity (pCEP-R cells lines) have abnormal glycosylation as detected by lectin binding due to the alkalized pH of the TGN relative to the that in the control line (pCEP) and second, that overexpression of ΔF508 CFTR in a human airway epithelial cell line that normally expresses CFTR is sufficient to produce abnormal glycosylation.

**MATERIALS AND METHODS**

**Cell lines.** Human tracheal epithelial cells (9/HTEo−) were transfected with LIPOFECTIN reagent (GIBCO BRL, Life Technologies, Gaithersburg, MD) with either empty vector (pCEP4, Invitrogen, San Diego, CA) to give the control cell line, pCEP-R (vector cloned with the R domain of CFTR) (17), pCEP-WT-CFTR (vector with WT CFTR cDNA), or pCEP-ΔF508 (vector with ΔF508 CFTR cDNA). Cells were grown in Dulbecco’s minimal Eagle’s medium supplemented with 10% fetal bovine serum and 2.5 mM l-glutamine, and all cell lines used in this study were maintained under selection with 80 μg/ml of hygromycin at 37°C in an atmosphere of 95% air/5% CO2.

**Western blot of CFTR-containing vesicles.** HEK293 cells were transfected with LIPOFECTAMINE Plus (GIBCO BRL) with either pCEP4, pCEP-CFTR, or an equal molar ratio of pCEP-CFTR and pCEP-R. Forty-eight hours after transfection, the cell vesicles were harvested following the procedure of Ma et al. (16) and duplicate 7.5% SDS-PAGE was performed for Western blot analysis with either a R domain antibody (monoclonal antibody 13-1, Genzyme, Cambridge, MA) or a mouse anti-human monoclonal CFTR COOH terminal-specific antibody (amino acids 1377–1480; Genzyme). Quantitation of blots was done with SigmaGel (Jandel Scientific, San Rafael, CA).

**RT-PCR.** pCEP-R cells were previously characterized and shown to overexpress R domain while retaining expression of CFTR (17). Coupled RT-PCR (1) was used to show expression of ΔF508 CFTR in the 9/HTEo−-transfected cells. The primers chosen amplified a 359-bp fragment of cDNA. The 5’ primer was in exon 9 of CFTR (5’-GAAAAGGAGCACTGTT-GTGGCGGTTCG-3’), and the 3’ primer crossed the boundary of exon 11/12 (5’-CAATGAGCATCTTGTATACGTGC-3’). Primers (100 ng each) were incubated with 40 U of RNase inhibitor and 4 μg of total RNA at 65°C for 15 min. To obtain a total reaction volume of 100 μl, 95 μl of the reaction mixture were added for a final concentration of 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl2, 200 nM each deoxynucleotide triphosphate, and 2.5 U of Taq polymerase (Boehringer Mannheim, Indianapolis, IN) with and without 200 U of SuperScript reverse transcriptase (GIBCO BRL). Reagents were mixed and overlaid with mineral oil. The thermocycler protocol was 37°C for 0.5 h and 42°C for 0.5 h for RT followed by PCR that included denaturation at 95°C for 3 min and 30 cycles consisting of 92°C for 1 min, 59°C for 1 min, and 72°C for 1.5 min and then a 10-min extension at 72°C and a 4°C hold. Ten microliters of reaction products were size fractionated on a 2.5% Tris-acetate-EDTA agarose gel and visualized with ethidium bromide. For Southern hybridization, the gel was denatured (0.5 N NaOH and 1.5 M NaCl) for 20 min and transferred by downward capillary action onto Immobilon-N transfer membranes (Millipore, Bedford, MA) in 10× saline-sodium citrate (0.3 M NaCl and 30 mM trisodium citrate). The filter was prehybridized in Church buffer (1% BSA, 1 mM EDTA, 0.5 M NaHPO4, pH 7.5, and 7% SDS) at 55°C for 1 h and hybridized overnight in fresh Church buffer with [32P]dCTP-tailed antisense probes corresponding to the region of sequence divergence from the WT, covering the ΔF508 (5’-AACACATGTATTTTCTTTCT-3’) mutation. RT-PCR was also used to confirm expression of transfected WT CFTR cDNA and endogenous CFTR in pCEP-WT-CFTR-transfected cells (data not shown). WT CFTR 5’ primer (5’-GTATATGCTACCGTACGTGAC-3’) in the 5’-untranslated region of pCEP vector and 3’ primer (5’-ATCCTGATTCAGAGAGGG-3’) in exon 3 of CFTR amplified a 586-bp product. Endogenous CFTR expression was confirmed by amplification of a 673-bp product with primer pairs with the plus primer (5’-TGGAAGATTGAGCTGGG-3’) in exon 3/4 of CFTR and the minus primer (5’-GAGAGGCGCTTTATCAAGACCC-3’) in the 3’-untranslated region of CFTR that is not present in the pCEP-WT-CFTR construct.

**Chloride efflux assays.** The presence of a cAMP-stimulated chloride efflux in the pCEP and pCEP-WT-CFTR clones was assayed by [36Cl] efflux (28). Cells were plated on vitrogen-coated 35-mm dishes, grown to confluence, and then loaded with 5 μCi of [36Cl] for 1 h. The cells were washed, and 1 ml of HEPES-buffered Ringer solution was added to the dishes. The buffer was sampled and replaced every 30 s during the 10-min assay for scintillation counting. Theophylline (1 mM) and forskolin (10 μM) were added at 240 s. The cells were lysed with 0.1 N HCl and 1 N NaOH at the end of the experiment to determine the [36Cl] remaining in the cells. The apparent rate constant (r; in min−1) was calculated at each efflux interval with the equation 

\[ r = \frac{\ln(C_i) - \ln(C_f)}{t_2 - t_1} \]
terminal sialic acid residues in an α-2,3 linkage to galactose[β1→4]N-acetyl-d-glucosamine (GlcNac) of glyco-
proteins (14, 22). ECL binds terminal galactose[β1→4]GlcNac, a substrate for both α-2,6- and α-2,3-linked sialic acid, but sialic acid substitution on this structure interferes with lectin binding (27). Because WGA is able to bind N-acetylneuraminic acid as well as cell surface glycoconjugates containing GlcNac and its β1→4 oligomers and SWGA binds only glycoconjugates containing GlcNac and does not bind to sialic acid, using a combination of WGA and SWGA allows an estimation of total membrane sialic acid (6). PNA binds preferentially to galactosyl[β1→3]N-acetyl-d-galactosamine, common to O-linked oligosaccharides and glycosphingolipids including asialo-GM1 (12). PNA does not bind to its recognition sugar sequence if it is substituted for by sialic acid, and even sialic acid not bound to the receptor sequence itself may inhibit lectin binding. The B subunit of cholera toxin recognizes the glycosphingolipid GM1 (23).

Cells were plated on vitrogen-coated Permanox four-well tissue culture chamber slides (Lab-Tek, Naperville, IL) and used within 2–4 days. At near confluence, the cells were rinsed two times in cold phosphate-buffered saline (PBS; 2 mM KH2HPO4, 6 mM Na2HPO4, 2 mM KCl, and 136 mM NaCl) and fixed with 4% paraformaldehyde, 1% t4), where C1 and C2 are the percentages of counts remaining in the cell layer at times t1 and t2, respectively.

SPQ assays. SPQ assays were performed following the protocol of Perez et al. (17). Briefly, cells on aclar-coated coverslips were loaded with 5 mM SPQ and studied cell by cell with an upright Zeiss epifluorescence microscope. The images were quantified with Image-1 F1 Software (Universal Imaging, West Chester, PA). Successive images were taken at a rate of ~1 every 5 s. The cells were continually perfused at 37°C with either chloride-containing or chloride-free solution in the presence and absence of 10 µM isoproterenol. The results are expressed as rates (relative fluorescence units per minute).

Fluorescent lectin binding. All fluorescent lectins (elderberry bark lectin (EBL), Erythrina cristagalli lectin (ECL), Maackia amurensis lectin I (MAL I), peanut agglutinin (PNA), succinyllated wheat germ agglutinin (SWGA), and wheat germ agglutinin (WGA)) were purchased from Vector Laboratories (Burlingame, CA). FITC-conjugated cholera toxin B subunit was purchased from List Biochemicals (Campbell, CA).

EBL binds specifically to sialic acid in an α-2,6 linkage to galactose (Gal) or N-acetyl-d-galactosamine present in N- or O-linked glycoproteins and glycolipids (26). MAL I binds to

![Fig. 1](http://ajplung.physiology.org/) Expression of regulatory (R) domain does not interfere with cystic fibrosis transmembrane conductance regulator (CFTR) processing. Shown are Western blots of membrane vesicles isolated from HEK293 cells transfected with equal molar amounts of plasmids expressing either empty vector (pCEP) plus CFTR (vector:CFTR; lanes 1 and 4), R domain (pCEP-R) plus CFTR (R:CFTR; lanes 2 and 5); or pCEP-CFTR (CFTR; lanes 3 and 6). Left, blot probed with a COOH-terminal antibody (C-term Ab) for CFTR; right, blot probed with an R domain Ab. Quantitation of CFTR by densitometry showed that band C constitutes 79.08% of total CFTR. Band B was negligible by densitometry and so percentage of band C is not meaningful. The appearance of fully glycosylated CFTR in lanes 2 and 5 in quantities not reduced compared with control level demonstrates that coexpression of the R domain does not inhibit processing. Nos. at right, molecular mass in kDa.

![Fig. 2](http://ajplung.physiology.org/) A: 2.5% Tris-acetate-EDTA agarose gel stained with ethidium bromide (ETBr). B: results of Southern hybridization of filter from same gel as A probed with an antisense oligo corresponding to the pCEP-ΔF508 mutation of CFTR. Lane 1, 2 µg of total RNA from pCEP-ΔF508 clone 1 cells with RT-Taq polymerase; lane 2, 2 µg of total RNA with Taq and without RT; lane 3, 2 µg of total RNA treated with RNase 1 before RT-PCR, with both RT and Taq present; lane 4, same as lane 3 but without RT; lane 5, all reagents but no RNA; lane 6, molecular weight marker; lane 7, pCEP-CFTR plasmid DNA [wild-type CFTR (WTCFTR)] with RT-Taq.
glutaraldehyde, and 3% sucrose in PBS for 2 h. Fixed cells were rinsed two times with PBS and incubated with 20–100 μg of FITC-conjugated lectin in 300 μl of PBS for 0.5 h except for control wells that were incubated only with 300 μl of PBS. The wells were rinsed three times with PBS and air-dried. The slides were then fixed in methanol for 10 min, air-dried, and mounted for fluorescent microscopy with Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL) and coverslips. Neuraminidase treatment incubation with EBL to demonstrate lectin specificity with Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL) and coverslips. Neuraminidase treatment incubation with EBL to demonstrate lectin specificity with Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL) and coverslips. Neuraminidase treatment incubation with EBL to demonstrate lectin specificity with Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL) and coverslips. Neuraminidase treatment incubation with EBL to demonstrate lectin specificity with Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL) and coverslips. Neuraminidase treatment incubation with EBL to demonstrate lectin specificity with Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL) and coverslips. Neuraminidase treatment incubation with EBL to demonstrate lectin specificity with Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL) and coverslips. Neuraminidase treatment incubation with EBL to demonstrate lectin specificity with Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL) and coverslips.
and applied Student’s *t*-test for significance. 2) We assessed the distribution of fluorescence by using the Kolmogorov-Smirnov (K-S) nonparametric statistical test for analysis of the histograms (30).

The fluorescence distribution of the images was assessed on the original images that were contrast compressed without any contrast enhancement to make use of the full range of values within the image. For K-S analysis, these histograms were converted to a cumulative frequency or probability distribution curve. The maximum vertical displacement (D) or difference between sample and control curves was then computed. From this “D value,” a K-S statistic was derived, and significance was tested by comparison of D against critical table values. The lower threshold was set at the intensity value below which 97% of the pixels fell in control wells, and an upper threshold was set at the intensity value below which 97% statistic was derived, and significance was tested by comparing the distribution of fluorescence by using the Kolmogorov-Smirnov (K-S) nonparametric statistical test for analysis of the histograms (30).

**Confocal microscopy.** Cells prepared for fluorescence microscopy as in *Fluorescent lectin binding* were examined under a Bio-Rad MRC600 confocal microscope with a ×40, 1.3-numerical aperture objective. Z stacks of ∼20 images with 0.5-μm spacing were collected. For EBL, most of the fluorescence was at the cell surface, but for the other lectins, substantial cytoplasmic fluorescence was observed as well (confocal images not shown).

**Electron microscopy.** The DAMP method for cytochemical visualization of acidic compartments was a modification of the technique of Anderson and Pathak (3). pCEP and pCEP-R cells were plated on 60-mm vitamin-coated cell culture dishes, and at confluence, the cells were rinsed and 2 ml of fresh medium with 50 μM DAMP (Oxford Biomedical Research, Oxford, MI) were added and incubated at 37°C for 0.5 h. The cells were rinsed twice in medium and incubated for an additional 10 min. As a control for DAMP accumulation, 25 μM monensin (Oxford Biomedical Research) was added to one set of dishes. The cells were rinsed twice in PBS and fixed in 3% paraformaldehyde, 1% glutaraldehyde, and 3% sucrose for 2 h. The cells were postfixed in 1% OsO₄ and embedded in Polybed 812 (Epon). Thin sections were mounted on Formvar-coated nickel grids. For immunogold labeling, the sections were first etched by floating the grids section side down over a saturated solution of NaIO₃ for 20–40 min and then rinsing three times by floating the grids over fresh drops of 0.2-μm filtered distilled water. The grids were then floated for 1 h over 50-μl drops of buffer *B* (20 mM Tris·HCl, pH 9.0, 200 mM NaCl, and 1% BSA) followed by incubation in a humidified chamber at 4°C overnight (16–18 h) over 25-μl drops of 15 μg/ml of anti-N-dinitrophenyl (Oxford Biomedical Research) in buffer *C* (20 mM Tris·HCl, pH 9.0, 200 mM NaCl, and 0.1% BSA). The grids were subsequently incubated over 25-μl drops of 10 μg/ml of affinity-purified rabbit anti-mouse IgG (Sigma, St. Louis, MO) in buffer *C* for 2 h. Bound antibodies were visualized by floating sections for 1 h over 25-μl drops of a 1:40 dilution of goat anti-rabbit IgG gold conjugate (10 nm; Sigma). The grids were then rinsed by floating them over large drops of 0.2-μm filtered distilled water or dipping them repeatedly in water for 45 s and were air-dried. All incubations except for the primary antibody were carried out at room temperature. The grids were counterstained with uranyl acetate and lead citrate. Two separate DAMP-labeling experiments were performed, and multiple grids from two separate immunogold labeling treatments from each experiment were analyzed.

Gold-labeled grids were examined under a JEOL 100CX transmission electron microscope at 80 kV and photographed at ×20 magnification. Final magnification of the micrographs that were printed for the counting of gold particles was approximately ×60,000. A transparent grid was placed over the micrograph to determine the area of the intracellular organelles and background regions, and the number of gold particles in the region was counted. Background gold counts were obtained by evaluating areas of the cytoplasm without identifiable membrane-bound organelles. Significance was determined by *t*-test or nonparametric Mann-Whitney rank sum analysis when normality testing failed.

<table>
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<tr>
<th>Lectin</th>
<th>Carbohydrate Specificity</th>
<th>Intensity No. of experiments</th>
<th>Distribution No. of experiments</th>
<th>R Binding ΔF508</th>
<th>R Binding ΔF508</th>
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<tr>
<td>EBL</td>
<td>Neu5Acα2→6Gal/GalNAc</td>
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<td>1/5</td>
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<tr>
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<td>Neu5Acα2→3Gal/β1→4GlcNAc/Glc</td>
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<td>ECL</td>
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<td>SWGA</td>
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<td>WGA</td>
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<td>1/7</td>
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Note: ΔF508 values were obtained by evaluating areas of the cytoplasm without identifiable membrane-bound organelles. Significance was determined by *t*-test or nonparametric Mann-Whitney rank sum analysis when normality testing failed.
RESULTS

Expression of R domain. pCEP-R cells have been previously characterized (17). They express the exogenous R domain mRNA (confirmed by RT-PCR) and protein (shown by immunoprecipitation and/or phosphorylation assay as well as by immunofluorescence) as well as the mRNA for endogenous CFTR. During the course of these experiments, R domain expression was verified periodically by immunoprecipitation of the cell supernatant with monoclonal antibody 13-1 (Genzyme) directed against the R domain of CFTR, phosphorylation with PKA, and SDS-PAGE. Figure 1 demonstrates that coexpression of equal molar amounts of CFTR and R domain in a heterologous expression system (HEK293 cells) results in little change in the ratio of band C (fully processed) to band B (core-glycosylated) CFTR, indicating no interference by the R domain in CFTR processing. This suggests that the lack of cAMP-activated chloride channel activity in pCEP-R cells is not due to increased misprocessing of CFTR. Although this heterologous, high-level expression system is not identical to the low-level expression in the 9/HTEo cell line, there is no reason to suspect qualitatively different protein-protein interaction in airway epithelial cells.

Expression of ΔF508 and WT CFTR. Figure 2A shows the results of a coupled RT-PCR demonstrating the presence of mRNA for ΔF508 (Fig. 2A, lane 1) in pCEP-ΔF508 cells, which was probed with the appropriate 32P-tailed antisense oligo for the ΔF508 muta-
tion (Fig. 2B, lane 1) to confirm that the message detected was not endogenous CFTR. WT CFTR does not bind the allele-specific probe (Fig. 2B, lane 6). Total RNA was DNase treated, and the negative controls were samples that were further treated with RNase I before RT-PCR to confirm that the reaction product originated from mRNA, not from transfected cDNA. RT-PCR was also used to confirm the expression of transfected WT CFTR cDNA (data not shown).

Chloride efflux assays. The cAMP-mediated stimulation of the rate of chloride efflux, characteristic of functioning CFTR chloride channels, has previously been shown to be markedly reduced in pCEP-R cells as measured by SPQ fluorescence (17). As reported previously (17), the two pCEP-R clones used in this study, clones F and E, failed to respond to 10 \( \mu \)M isoproterenol with an increased rate of chloride efflux. The control cell line transfected with the empty vector pCEP responded to the cAMP agonist with an approximately twofold increase in rate of chloride efflux. The basal rate of chloride efflux (without isoproterenol stimulation) was also significantly reduced in the pCEP-R clones. Responses of these clones were checked periodically and new vials were opened if the chloride transport response changed. Figure 3 shows the results of the SPQ assays with two clones (clones 1 and 9) of pCEP-\( \Delta F508 \) cells. They also failed to respond to isoproterenol with an increased rate of chloride efflux. Basal chloride efflux rate was reduced in pCEP-\( \Delta F508 \) clone 9 but not in clone 1 compared with pCEP control clone 3. Assays of \( ^{36} \text{Cl} \) efflux in pCEP-WTCFTR cells showed that this cell line, like cells transfected with empty vector (pCEP), could be stimulated by a combination of the cAMP agonists theophylline and forskolin (Fig. 4A). Thus the control cell line transfected with empty vector (pCEP) and the line transfected with WT CFTR both express functioning CFTR channels, whereas pCEP-R- and pCEP-\( \Delta F508 \)-transfected cell lines lack cAMP-stimulated chloride effluxes. Because the responses of pCEP and pCEP-WTCFTR were so similar, pCEP was the standard control cell line.

Quantitative fluorescence microscopy. Using two statistical methods of quantitation, we found that sialic acid in an \( \alpha-2,6 \) linkage (as assessed by EBL binding) showed no difference between the control cell lines pCEP-WTCFTR and pCEP (Fig. 4B). Thus 9/HTEo- cells transfected with the empty vector or WT CFTR cDNA retain cAMP-stimulated chloride efflux and have similar cell surface lectin binding properties. Simultaneous studies of the control pCEP line and two clones of both R domain- and \( \Delta F508 \)-transfected 9/HTEo- cells were performed for six FITC-conjugated lectins and the B subunit of choler toxin at concentrations that minimize low-affinity binding.

Based on previous reports (5, 9), we expected that on CF-phenotypic cells, sialic acid in the \( \alpha-2,6 \) linkage that could be measured with EBL binding would be reduced and that there would be either a commensurate reduction in total sialic acid or a compensatory increase in sialic acid in the \( \alpha-2,3 \) configuration and in asialo-GM\(_1\). The most consistent difference in fluorescent lectin binding between the control and experimental cell lines was observed with EBL (Table 1). A typical experiment (Fig. 5) shows a significant reduction (59\%) in the mean pixel intensity of EBL binding to pCEP-R cells and a 45\% reduction in mean pixel intensity of the pCEP-\( \Delta F508 \) images compared with that in control cells. EBL specifically recognizes \( \alpha-2,6 \)-sialic acid linkages. Clostridium perfringens neuraminidase, specific for \( \alpha-2,6 \)-sialic acid, significantly reduced EBL binding almost to background values (Fig. 5), whereas incubation with a sialidase with preference to the \( \alpha-2,3 \) linkage from Salmonella typhi- murium did not significantly alter the binding of EBL (data not shown).

However, there was no significant change in overall total sialic acid (as assessed by ECL binding as well as by WGA and SWGA) or in \( \alpha-2,3 \)-linked sialic acid (as assessed by MAL I binding). Moreover, we detected no difference in asialo-GM\(_1\) using the binding of PNA, although both the pCEP-R and pCEP-\( \Delta F508 \) cells used in this study, when assessed by specific antibody binding, showed increases in asialo-GM\(_1\) and also increased binding of Pseudomonas aeruginosa compared with those in the control pCEP cell line (8). However, when the cells were incubated with an \( \alpha-2,3 \)-specific siali-
dase, the pCEP-R and pCEP-ΔF508 cells showed significant increases in binding PNA, whereas pCEP cells did not (K-S test results comparing untreated vs. neuraminidase treated: pCEP, $P > 0.1$; pCEP-R, $P < 0.05$; pCEP-ΔF508, $P < 0.001$; data not shown). Thus asialo-GM₁ sites may be inaccessible to lectin unless the surrounding molecules are modified to better expose the receptor similar to the increased binding of *Pseudomonas* observed after incubation with PAO1 supernatant (20, 21).

Table 1 summarizes the results of comparisons of the total intensity of fluorescent lectin binding ($t$-test) to pCEP-R or pCEP-ΔF508 cells compared with that in pCEP control cells. Separate analysis of individual experiments allows both internal experimental comparisons and assessment of experimental variability as well as quantitative determination of significance. All of the EBL binding experiments showed a reduction in mean pixel intensity or a reduction in the intensity of the overall fluorescence in the experimental cell lines, and most reached significance ($P < 0.05$). Four of the experiments comparing pCEP-R cells with pCEP cells showed a significant decrease in mean pixel intensity ranging from 14 to 60% of control values, and 4 of 6 experiments showed a significant reduction in EBL binding to pCEP-ΔF508 cells of 16–61% of the quantitated mean pixel intensities of the control cells. For the other lectins, there were no consistent changes in the intensity of overall fluorescence in experimental compared with control lines.

We also compared the distribution of fluorescence intensity in the various cell lines using the K-S test. For this analysis, the absolute intensity values are ignored, and the distribution of bright fluorescence within an image is considered. Figure 6 illustrates one experiment of EBL binding. Figure 6A, inset, shows the average pixel intensities of the original individual images from which the mean pixel intensities of the
experiment were obtained. Figure 6A shows these data combined. The average pixel intensity obtained from the histogram is the same value as the mean pixel intensity shown in Fig. 6A, inset. Figure 6B represents the same images as in Fig. 6A converted for analysis of distribution of fluorescence by the K-S nonparametric test for analysis of histograms. Figure 6B, inset, shows the first step in the statistical analysis, conversion of the histogram to probability distribution functions from which the K-S values are derived. Figure 6 shows that there are both more pixels at lower intensity values and fewer pixels at higher intensity values for both the pCEP-R cell line and the ΔF508 cell line compared with those in the control line. Thus in the region that represents specific EBL binding, there is less EBL bound to pCEP-ΔF508 and pCEP-R cells than to the control line pCEP (Table 1). Figure 7 shows a representative experiment.

To examine the subcellular accumulation of the fluorescent labeling by lectins in these permeabilized cells, confocal microscopy was performed. More than 80% of the EBL labeling was at the cell surface, but for the other lectins, fluorescence was distributed throughout the cytoplasmic compartment as well (data not shown).

**DAMP accumulation in intracellular organelles.** To determine whether the sialylation changes in pCEP-R cells were associated with the higher pH in the TGN, we used DAMP and subsequent immunogold labeling to assess the pH change across the membranes delimiting intracellular compartments. Because prior measurements with 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, a proton-sensitive fluorescent dye, showed no difference in the cytoplasmic pH between pCEP and pCEP-R cells (13), DAMP labeling directly compared the pH in the organelles in pCEP and pCEP-R cells. Figure 8 illustrates that DAMP accumulated in acidic vesicles, shown in pCEP-R (A) and pCEP (B) cells as the clustering of many gold particles in the electron-dense or gray organelles and few particles in the cytoplasm or background label. We observed no apparent differences in cellular morphology, including size and structure of intracellular organelles, between pCEP-R (Fig. 8C) and pCEP-R (Fig. 8D) cells. We identified intracellular organelles by their distinctive morphology and ability to accumulate DAMP. Gold granules were counted in mitochondria, identified by a round to elongated shape and lamellar folds of inner membrane; nuclei; multivesicular bodies, identified as membrane-bound organelles with internal membrane structures resembling vesicles; and uniformly gray or electron-dense organelles that may be late endosomes or lysosomes. Although the electron-dense vesicles shown in Fig. 8, A (pCEP-R) and B (pCEP), are of different sizes, there was no overall difference in size of this lysosomal or endosomal compartment when these organelles from many cells were quantitated. Gold particles in the TGN were identified in vesicles near the classically defined Golgi stacks (at least three visible organized stacks) and the area of the TGN included the region of cytoplasm encompassing those vesicles. In two separate DAMP experiments, each with two separate immunolabeling procedures of multiple grids, we found no significant difference in the pH of intracellular organelles in pCEP and pCEP-R cells (Fig. 9). Grids from monensin-treated samples showed no accumulation of gold particles in intracellular vesicles, and grids immunolabeled without primary antibody were void of gold (data not shown).

**DISCUSSION**

Our results show that reduced function of CFTR in a human airway epithelial cell line, whether achieved by overexpression of the R domain in the face of persistent CFTR expression or by overexpression of ΔF508 CFTR, is associated with altered sialylation in the cell. Binding of EBL, which recognizes sialic acid in the α-2,6 linkage, is reduced in two separate clones of each transfectant in multiple experiments, and the results were quantitative, consistent, and highly significant. These cell lines were transfected with an episomal vector (pCEP) so that they could not be subject to insertional mutagenesis or unusual phenomena from the chromosomal placement of transfected DNA and could be closely matched in properties except for the genes added via the vector. Both the total amount and the distribution of EBL fluorescence were altered in the...
pCEP-R and pCEP-ΔF508 lines compared with both the pCEP and pCEP-WTCFTR lines.

9/HTEo− cells transfected with the R domain continued to express the mRNA for endogenous WT CFTR but lacked cAMP-stimulated chloride conductance. As discussed above, this impairment is most likely due to direct interaction of the R domain with CFTR (15, 16), which continues to be transcribed (17) and processed normally. This is demonstrated by our results in HEK293 cells where transfection of both the R domain and CFTR results in no change in CFTR maturation compared with cells transfected with CFTR only. The abnormal sialylation in these cells must thus be attributed to reduced CFTR function, not to misprocessed mutant CFTR. We also tested whether cells transfected with the R domain have a higher pH in intracellular compartments compared with that in cells transfected with empty vector but could not demonstrate significant differences in pH in any organelle. Our experiments achieved an intensity of DAMP label in intracellular vesicles, after background subtraction, comparable to that reported by Barasch et al. (5) for SV40-transformed airway epithelial cells, who concluded from distribution of DAMP in these cells that TGN pH is increased in CF cells. Thus differences between our findings and theirs are probably not due to inefficient DAMP label penetration in our cells. Our results are in agreement with those of Seksek et al. (25), who found a normal pH in the intracellular compartments in CF cells using fluorescent labels.

In addition, we found that transfecting ΔF508 CFTR into 9/HTEo− cells resulted in a marked decrement of endogenous CFTR activity, whereas transfection of WT CFTR had little effect on chloride transport. We had expected that endogenous WT CFTR would continue to be produced and processed and function at the cell surface, but this was not the case. It is possible that transfection of a misprocessed CFTR allele increased the proportion of WT CFTR that is retained and degraded in the endoplasmic reticulum, but we speculate that it is more likely that the reduction in activity comes from dimerization of WT with mutant CFTR. Recent structural studies (10, 32) indicated that CFTR may exist and function in membranes as two closely associated molecules, including sialyltransferases, this is unlikely to account for the results in the R domain cells where the only CFTR is WT. Alterations in overall sialic acid uptake should affect sialic acid available for addition in an α-2,3 as well as in an α-2,6 linkage, and this is not the case. It is possible that the sialyltransferases are mislocalized in CF (29) for unknown reasons. It is also possible that sialyltransferase function somehow requires functional CFTR. Alternatively, transcriptional regulation of these enzymes may be abnormal in CF. Further experiments are required to allow us to choose among these possibilities.

We thank Cathy Silski for making the pCEP-ΔF508 and pCEP-wild-type cystic fibrosis transmembrane conductance regulator cell lines, Yoshiie Hervey for excellent cell culture care, and Dave Fletcher for the 36Cl efflux assays. Also, this work has benefited from the helpful discussions and critical reviews of Ulrich Hopfer, Mitch Drum, Tom Ferkol, and Alice Prince.

This work is supported by National Heart, Lung, and Blood Institute Grants RO1-HL/MD-4903 and T32-HL-07415; National Institute of Diabetes and Digestive and Kidney Diseases Grant P30-DK-27651; and a Research Development Program grant from the Cystic Fibrosis Foundation.

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