

Abnormal Localization of Cystic Fibrosis Transmembrane Conductance Regulator in Primary Cultures of Cystic Fibrosis Airway Epithelia

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Abstract. Cystic fibrosis (CF) is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR), a membrane glycoprotein that forms Cl⁻ channels. Previous work has shown that when some CF-associated mutants of CFTR are expressed in heterologous cells, their glycosylation is incomplete. That observation led to the hypothesis that such mutants are not delivered to the plasma membrane where they can mediate Cl⁻ transport. Testing this hypothesis requires localization of CFTR in nonrecombinant cells and a specific determination of whether CFTR is in the apical membrane of normal and CF epithelia. To test the hypothesis, we used primary cultures of airway epithelia grown on permeable supports because they polarize and express the CF defect in apical Cl⁻ permeability. Moreover, their dysfunction contributes to disease. We developed a semiquantitative assay,

using nonpermeabilized epithelia, an antibody directed against an extracellular epitope of CFTR, and large (1 μm) fluorescent beads which bound to secondary antibodies. We observed specific binding to airway epithelia from non-CF subjects, indicating that CFTR is located in the apical membrane. In contrast, there was no specific binding to the apical membrane of CF airway epithelia. These data were supported by qualitative studies using confocal microscopy: the most prominent immunostaining was in the apical region of non-CF cells and in cytoplasmic regions of CF cells. The results indicate that CFTR is either missing from the apical membrane of these CF cells or it is present at a much reduced level. The data support the proposed defective delivery of some CF-associated mutants to the plasma membrane and explain the lack of apical Cl⁻ permeability in most CF airway epithelia.

CYSTIC fibrosis (CF)¹ is an autosomal recessive disease caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) (8, 15, 22, 28, 30). Recent studies have shown that CFTR is a Cl⁻ channel (2, 3, 6, 21) which is regulated by phosphorylation with cAMP-dependent protein kinase (7, 10, 29, 32) and by nucleoside triphosphates (1). The properties of recombinant CFTR Cl⁻ channels and those of the cAMP-regulated apical membrane Cl⁻ permeability are similar (5) and CFTR has been immunocytochemically localized in the apical region of several epithelia and in the apical membrane of Cl⁻ secreting, intestinal epithelial cell lines (12, 14, 26). These observations indicate that CFTR generates the cAMP-regulated apical membrane Cl⁻ permeability of normal epithelia. They are also consistent with the observation that CF epithelia lack such a Cl⁻ permeability (27).

There is, however, considerable uncertainty about how CF-associated mutations lead to Cl⁻ impermeability. Mutations

in CFTR could result in a loss of Cl⁻ permeability in one of two general ways: the mutated CFTR channel might not be delivered to the apical membrane where it normally mediates Cl⁻ permeability, or the mutated channel might reach the plasma membrane, but have little or no function. It is also possible that some mutations could lead to a defect in both delivery and function.

Recent studies (9, 19) indicate that several CF-associated mutations, including the most common (deletion of phenylalanine at position 508, CFTRΔF508) (22, 24), lead to incomplete protein processing. These observations suggest that the mutant proteins are retained in the ER rather than being transported to the plasma membrane. This inference was based on the finding that wild-type CFTR expressed in heterologous cells underwent two stages of glycosylation, a core glycosylation (endoglycosidase H sensitive) characteristic of processing in the ER, and more extensive glycosylation characteristic of processing in the Golgi complex. In contrast, CFTRΔF508 only underwent core glycosylation. This result suggested that the mutant protein did not reach the Golgi complex and was not delivered to the plasma membrane. Of nine CF-associated mutants that were studied, six

1. *Abbreviations used in this paper:* CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator.

were incompletely glycosylated, and all six failed to generate cAMP-stimulated halide efflux as assessed using the SPQ assay (19). Incomplete glycosylation does not in itself cause Cl⁻ impermeability, because a protein that lacked glycosylation sites (constructed by site-directed mutagenesis) was present in the plasma membrane and had normal Cl⁻ channel activity (9). Three of the nine CF-associated mutants were processed appropriately, but still failed to generate cAMP-regulated halide efflux, suggesting that in those cases the protein was transported normally but had little or no channel function. Because the incompletely glycosylated mutants represent well over 70% of all CF chromosomes, it was proposed that defective trafficking of CFTR is the molecular basis for most of CF.

Several other studies have suggested the alternative: that mutant CFTR is processed appropriately and delivered to the plasma membrane. But none of these studies directly tested the hypothesis. In one study (16), CFTR Δ F508 was expressed in *Xenopus* oocytes. A cAMP-regulated current was generated that was 62% of that observed with wild-type CFTR. It was suggested that CFTR Δ F508 may be appropriately located in CF epithelia, but that cAMP levels may not have been elevated sufficiently in previous studies to detect the current. Another study of CFTR overexpressed in heterologous cells (13) indicated that CFTR Δ F508 retained some Cl⁻ channel activity, although the number of functional channels in the plasma membrane appeared to be decreased. Neither of these studies could directly test the hypothesis, because they used heterologous overexpression systems and the cAMP-regulated current in *Xenopus* oocytes may reflect differences in protein processing in amphibian oocytes as compared to mammalian cells. A study using membrane fractionation of cultured cells suggested that mutant CFTR is located in the plasma membrane (31). However that technique is probably not sufficiently specific to be certain that CFTR Δ F508 is in the apical membrane. Finally, a study using Western blots of proteins from CF airway epithelia, suggested that mutant protein is processed and glycosylated correctly, although no assessment of its location could be obtained (35).

Nevertheless, if CFTR Δ F508 retains some Cl⁻ channel activity, it is imperative that its location be determined. If CFTR is not at the apical membrane, then therapeutic interventions designed to increase the function of mutant CFTR without affecting its localization are unlikely to appreciably alter transepithelial Cl⁻ transport.

There are several requirements for testing the hypothesis that CFTR Δ F508 is not in the apical membrane of CF epithelia. First, it requires nonrecombinant normal and CF epithelia. Because the protein processing studies were done with recombinant cells, there has been concern that defective trafficking could be an artifact of overexpressing CFTR or of expressing it in nonpolarized cell lines. For our study, we used airway epithelial cells because numerous studies have shown that they express the CF defect in Cl⁻ permeability in their apical membrane and because their dysfunction contributes to disease (8, 27). We used primary cultures rather than cell lines, because transformation could conceivably alter protein expression or processing. Finally, we cultured the cells on filter supports so that they formed polarized epithelia with a distinct apical membrane that expresses the CF defect. Second, a method is required that determines if mutant

CFTR is in the apical membrane, because that is where the CF defect in Cl⁻ permeability resides. Direct evidence that CFTR is in, rather than just near the membrane, is also required to avoid ambiguity in the interpretation. Third, well characterized antibodies are required. We used mAbs that were previously shown to recognize CFTR (14, 18). These antibodies also recognize CFTR Δ F508 (18 and see Materials and Methods). The fourth requirement is a detection method that is sensitive and which clearly distinguishes signal from background. This is essential because CFTR is often present at low levels in nonrecombinant cells: mRNA levels are very low in airway epithelia (a few copies/cell) (30, 33) and the protein has been difficult to detect by immunoprecipitation or immunoblotting. Sensitivity and specificity are also required so that epithelia from different subjects can be compared.

Materials and Methods

Materials

Biotinylated sheep anti-mouse IgG, FITC-conjugated streptavidin, and fish gelatin were purchased from Amersham Corp. (Arlington Heights, IL). Normal goat serum, mouse IgG, DL-DTT, BSA (fraction V), and neuraminidase (Type VIII from *Clostridium perfringens*) were obtained from Sigma Chemical Co. (St. Louis, MO).

Cell Culture

We cultured NIH 3T3 cells that were stably expressing CFTR or CFTR Δ F508 (via recombinant retroviral infection) or were mock transfected (2), T84 cells (25), or human airway epithelial cells (4) as previously described. The airway epithelial cells were obtained from nine normal patients (six from nasal polyps and three from nasal turbinates) and six CF patients (nasal polyps). The genotype of the CF cells was Δ F508/N1303K for CF1 and CF4, Δ F508/G542X for CF2, Δ F508/other for CF3, other/other for CF5, and Δ F508/ Δ F508 for CF6. Data include all specimens processed by the lab during the course of the study. One CF culture (CF6) was not studied with the antibody/bead complex technique, because it was received before the method was developed; these cells were used for immunostaining. For localization experiments, 3T3 and T84 cells were seeded on Millicell-HA filters (12 mm, 0.45 μ m, Millipore Corp., Bedford, MA) at 5×10^5 cells/cm². Airway cells were seeded at the same density on Millicells or on Living Tissue Substrate filters (Organogenesis Inc., Cambridge, MA) using seeding techniques recommended by the manufacturer. Studies were done when the airway epithelia had developed a maximal transepithelial resistance (200–800 $\Omega \cdot$ cm²), between 5 and 12 d after seeding.

Monoclonal Antibodies

mAbs against the R domain (M13-1), the carboxyl terminus (M1-4), and the first extracellular domain (M6-4) of CFTR all recognize wild-type CFTR as previously described (14, 18). Fig. 1 shows that they also recognize CFTR Δ F508. All three antibodies stained NIH 3T3 fibroblasts expressing either wild-type CFTR (Fig. 1, *d-f*) or CFTR Δ F508 (Fig. 1, *g-i*). In contrast, staining of mock-transfected cells (Fig. 1, *a-c*), which lack CFTR (2) was similar to staining with a nonspecific mouse IgG (not shown). Similar results were obtained with HeLa cells expressing recombinant CFTR, using a hybrid T7/vaccinia virus expression system (17, 28) (not shown). In addition, the antibodies stained CF airway epithelia expressing CFTR Δ F508 (see Fig. 6). Thus, the antibodies recognize both wild-type CFTR and CFTR Δ F508.

Immunofluorescence Staining

All cells were stained as previously described for T84 cells (14). Briefly, mucus was removed from epithelial cells by incubating with 5 mM DTT and 0.2 U/ml neuraminidase for 30 min at 37°C. Cells were washed with PBS and fixed for 30 min with 4% paraformaldehyde in PBS. Residual aldehydes

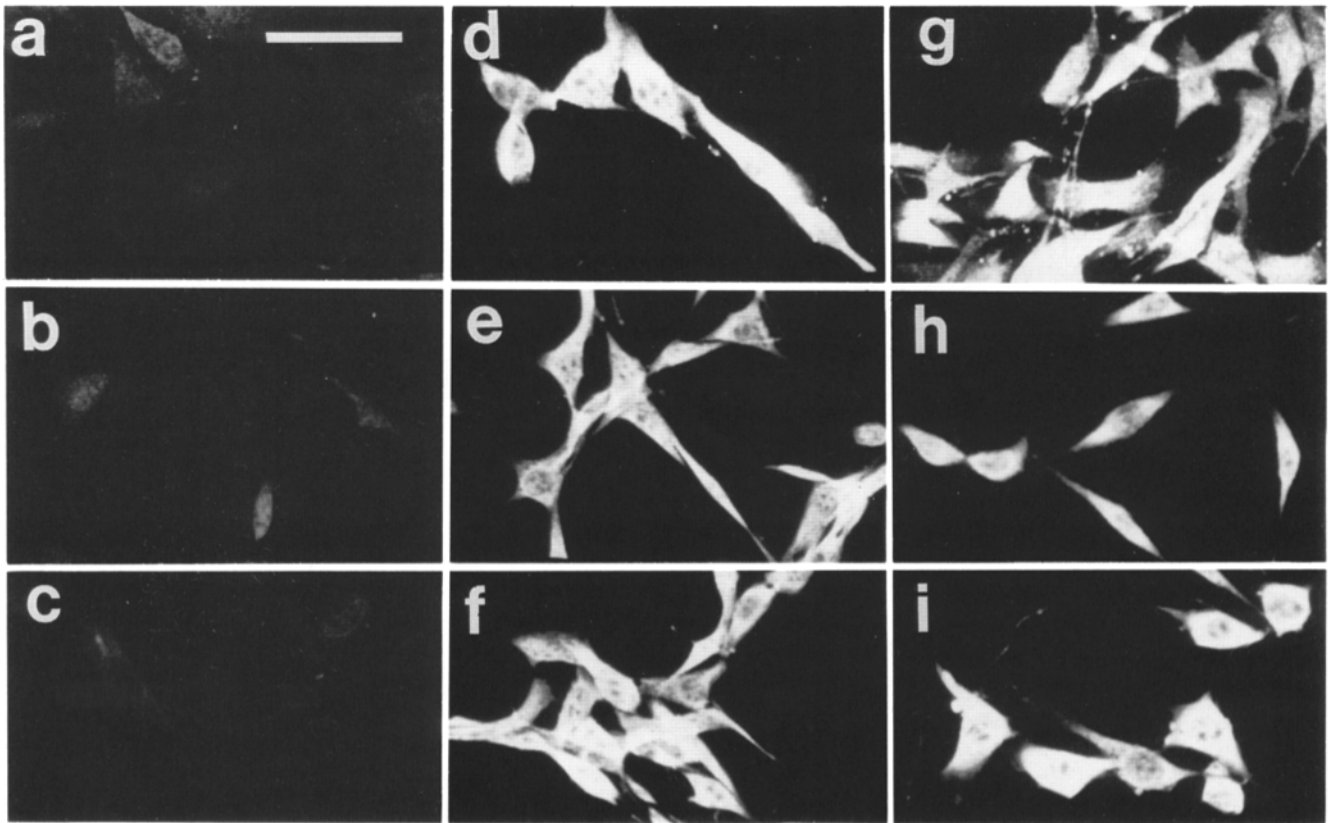


Figure 1. Immunofluorescence staining of stably transfected 3T3 cell lines using antibodies against CFTR. Mock transfected cells (*a-c*) and cells expressing CFTR (*d-f*) or CFTR Δ F508 (*g-i*) were stained with mAbs against the R domain, M13-1 (*a, d, and g*); the carboxyl terminus, M1-4 (*b, e, and h*); or an extracellular domain, M6-4 (*c, f, and i*). All cells were permeabilized before incubation with antibody. Bar, 50 μ m.

were inactivated by incubation with 50 mM glycine in PBS for 30 min. For some studies, cells were permeabilized by incubating 10–20 min with 0.2% Triton X-100 in PBS. Nonspecific staining was blocked by incubating cells for 30–60 min with incubation buffer (PBS containing 1% BSA and 0.1% fish gelatin) supplemented with 5% normal goat serum. Single filters were cut into pieces and the pieces were incubated with the different antibodies. Incubation with primary antibody in incubation buffer with goat serum (M13-1, 5 μ g/ml; M1-4, 25–50 μ g/ml; M6-4, 25–40 μ g/ml) was for 2 h at room temperature or overnight at 4°C. Between antibody incubations, cells were washed three times with ice-cold incubation buffer for 5 min each. Incubation with secondary antibody was for 1 h at room temperature and with FITC-streptavidin for 30 min. Biotinylated fluorescent beads (≥ 1 - μ m diam) (Cell Bright Beads, Diversified Biotech, Newton Centre, MA) were suspended in incubation buffer with 5% goat serum and briefly sonicated just before use. Filters were inverted over a drop of bead suspension and cells were incubated for 1–2 h. Filters were washed by gently immersing in 3–5 changes of PBS. Cells were covered with mounting medium (Gelmount, Biomedica Corp., Foster City, CA) and a glass coverslip was placed on the cells.

Confocal Microscopy and Bead Quantitation

Confocal images were obtained using the Bio-Rad MRC-600 confocal imaging system with an argon ion laser (Bio-Rad Microsciences Division, Cambridge, MA). To quantitate binding of beads, images of several randomly selected fields (630 \times 420 μ m) were collected and the beads were counted. We routinely counted 10 fields, although occasionally up to 20 fields were counted. Values are expressed as beads/field \pm SEM.

The use of bead antibody complexes may prove to be of value for the study of other membrane proteins. Although the quantitation is not absolute, it is similar in principle to that obtained with immunogold electron microscopic techniques.

Results and Discussion

Use of Fluorescent Bead/Antibody Complexes to Localize CFTR

We developed a new technique designed to localize CFTR that is in the apical membrane and provide a readily quantifiable method for assessing antibody binding. We used mAbs that we have previously described: M13-1, raised against the R domain; M1-4, against the carboxyl terminus; and M6-4, against an extracellular domain (14, 18). Primary cultures of airway epithelial cells were grown on permeable filter supports, so that they formed polarized epithelia with a distinct apical membrane. We incubated the apical surface of nonpermeabilized airway epithelia with antibody. Then, we incubated with biotinylated anti-mouse IgG followed by streptavidin. Finally, we incubated the cells with a suspension of biotinylated fluorescent beads. The beads were easy to count because of their large size (≥ 1 μ m), uniform shape, and high fluorescence intensity. To detect CFTR in the apical membrane, we used antibody M6-4, directed against an extracellular epitope. Our earlier studies had shown that M6-4 detects CFTR in the membrane of nonpermeabilized cells (14). To define nonspecific binding, we used antibodies against intracellular domains (M13-1 or M1-4), a nonspecific mouse IgG, or omitted primary antibody altogether. Our

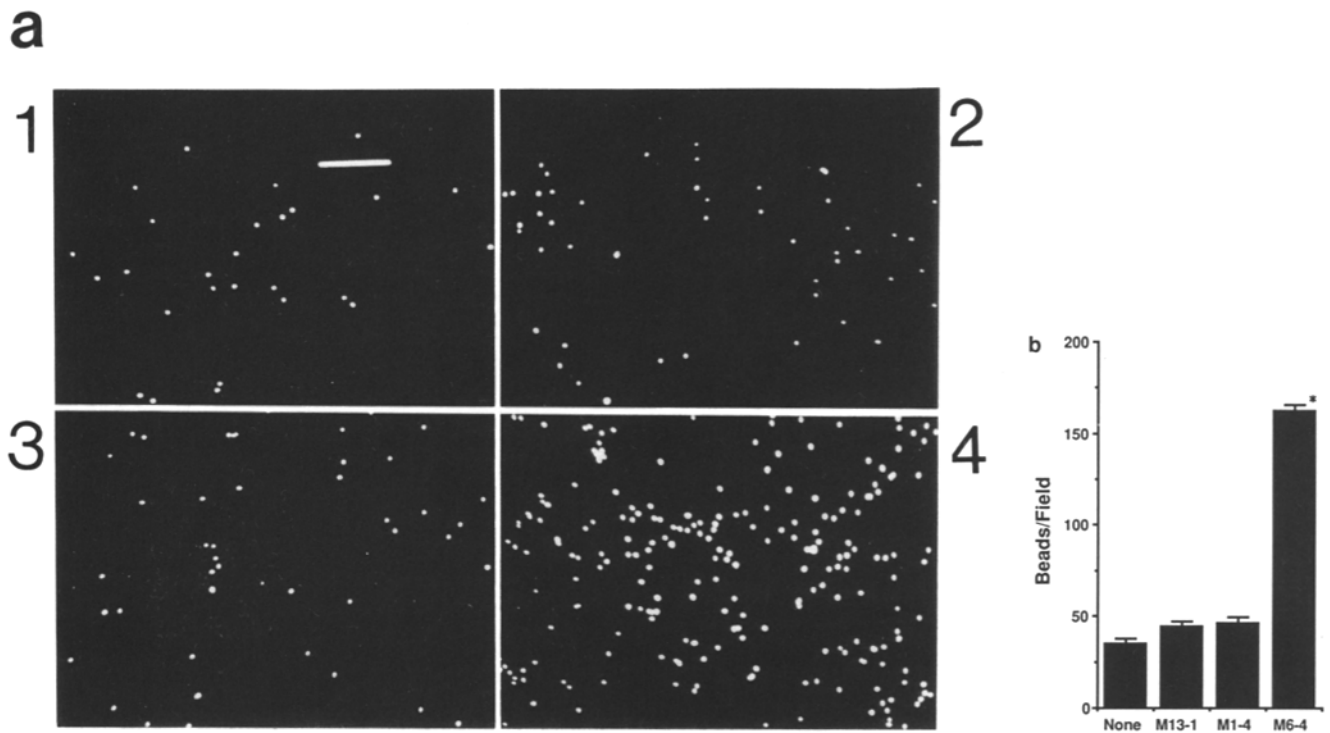


Figure 2. Binding of antibody/bead complexes to the apical membrane of nonpermeabilized T84 cells. Confocal images (*a*) were collected at the apical surface of T84 cells incubated without primary antibody (1); with antibodies against intracellular epitopes, M13-1 (2) and M1-4 (3); or with an antibody against an extracellular epitope, M6-4 (4). Cells were then incubated with biotinylated secondary antibody, streptavidin, and biotinylated fluorescent beads. These images show the fluorescent beads against the negligible background staining of the cell monolayer. The number of beads in each of 10 random fields/sample was determined and the data (*b*) are expressed as mean number of beads/field \pm SEM. The number of beads/field was significantly different with M6-4 (*asterisk*) than with the other conditions ($P < 0.001$, analysis of variance). Bar, 100 μ m.

previous work had shown that antibodies M13-1 and M1-4 do not have access to their epitopes in nonpermeabilized cells; i.e., they only stain permeabilized cells (14). We reasoned that if CFTR is in the apical membrane, more beads would bind to the surface of cells incubated with M6-4 than with the other antibodies. Although this procedure does not allow an absolute quantitation of CFTR, it provided an effective means for defining nonspecific binding and for detecting CFTR in the apical membrane.

To verify the technique, we tested it with T84 cells, which express CFTR (18) in the apical membrane (14). Fig. 2 *a* shows an example of the results; more beads were bound to cells incubated with M6-4 (*panel 4*) than with M13-1 (*panel 2*), M1-4 (*panel 3*), or no primary antibody (*panel 1*). When we counted the number of beads in multiple microscopic fields, we found significantly more beads/field with antibody M6-4 than in the other conditions (Fig. 2 *b*). These results suggest that antibody M6-4 specifically binds CFTR in the apical membrane. They also suggest that antibodies against intracellular epitopes are appropriate controls.

We also evaluated the technique using nonpermeabilized NIH 3T3 fibroblasts. In cells not expressing CFTR (mock), the number of beads/field was similar with antibodies against intracellular and extracellular epitopes (Fig. 3). This result indicates similar levels of nonspecific binding for all the antibodies. In contrast, with cells expressing wild-type CFTR, the number of beads/field was significantly greater with M6-4 than with intracellular domain antibodies. This result

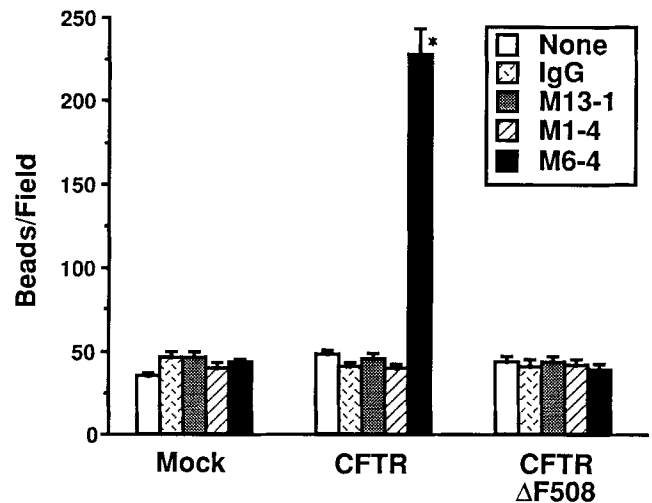


Figure 3. Binding of antibody/bead complexes to stably transfected NIH 3T3 fibroblasts. Mock transfected cells or cells expressing wild-type CFTR or CFTR Δ F508 were incubated with the indicated antibody, with nonspecific mouse IgG (*IgG*) or without primary antibody (*None*). Cells were then incubated with biotinylated secondary antibody, streptavidin, and biotinylated fluorescent beads. The number of beads in each of 10 random fields/sample was determined and the data is expressed as mean number of beads/field \pm SEM. In the CFTR-expressing cells, the number of beads/field was significantly greater for antibody M6-4 (*asterisk*) than for the other conditions ($P < 0.001$, analysis of variance).

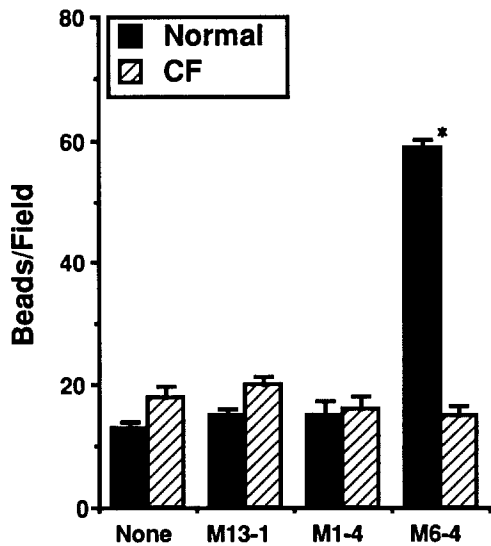


Figure 4. Binding of antibody/bead complexes to the apical membrane of a nonpermeabilized normal (N6) and CF (CF5) airway epithelium. Primary cultures of normal and CF airway cells were grown as epithelia on permeable filter supports. Nonpermeabilized monolayers were incubated with the indicated primary antibody, biotinylated secondary antibody, streptavidin, and biotinylated fluorescent beads. Confocal images were collected, the number of beads in each of 10 random fields/sample was determined, and data are expressed as mean number of beads/field \pm SEM. The number of beads/field was significantly different (*asterisk*) for antibody M6-4 in the normal cells ($P < 0.001$, analysis of variance).

indicates that CFTR is in the plasma membrane where it is accessible to M6-4, a conclusion consistent with immunocytochemical (Fig. 1) and functional (2) studies of these cells. When we did the same experiment using cells expressing CFTR Δ F508, we saw no difference in the number of beads/field with the different antibodies. The lack of specific binding with M6-4 suggests that CFTR Δ F508 is missing from or is present in markedly reduced amounts in the plasma membrane of recombinant cells.

Comparison of CF and Normal Airway Epithelia

Although our results with recombinant CFTR support the hypothesis that CFTR Δ F508 does not reach the membrane, the critical test requires nonrecombinant epithelia where the CF defect has been well characterized. Fig. 4 shows binding of antibody/bead complexes to the apical membrane of nonpermeabilized airway epithelia. The figure compares a CF and a normal epithelium processed on the same day. There are three main points. First, the number of beads/field was similar with intracellular domain antibodies (M13-1 and M1-4) and in the absence of primary antibody. This result again suggests that binding with antibodies against intracellular epitopes represents nonspecific, background binding. Second, background binding was similar for normal and CF epithelia. Third, and most importantly, antibody M6-4 bound specifically to normal, but not to CF epithelia. This result suggests that CFTR is in the apical membrane of normal epithelia, but is missing from the apical membrane of CF epithelia.

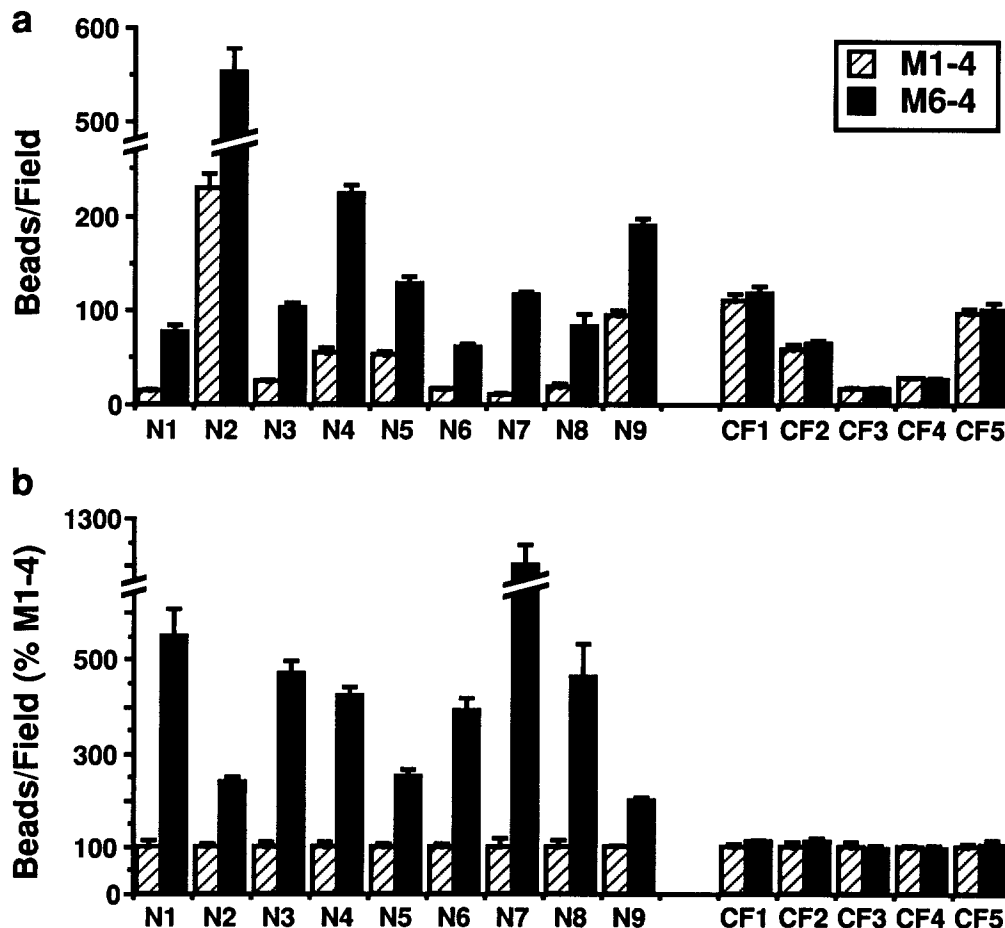


Figure 5. Binding of antibody/bead complexes to the apical membrane of nonpermeabilized normal and CF airway epithelia. Data are from nine normal (N1-9) and five CF (CF1-5) cultures (each from a different subject) grown as epithelia on permeable filter supports. Nonpermeabilized monolayers were incubated with antibodies against an intracellular, M1-4 (▨), or an extracellular, M6-4 (■), domain of CFTR. *a* shows the number of beads/field mean \pm SEM, $n =$ at least 10 random fields/experiment. Similar results were obtained with antibody M13-1 or no antibody; (data not shown because each was not used in every culture). *b* shows the data from *a* normalized to the mean value of beads/field for antibody M1-4. The number of beads/field is significantly greater with M6-4 than with M1-4 in every normal culture ($P < 0.01$, unpaired *t* test), but in none of the CF cultures.

Fig. 5 *a* summarizes results from nine normal and five CF airway epithelia; each came from a different subject. The absolute level of binding varied from culture to culture, but similar variability between experiments was observed with T84 and 3T3 cells (not shown). In every normal culture, there was a significantly greater number of beads/field with antibody M6-4 than with the intracellular antibody M1-4, indicating that CFTR is in the apical membrane.

In sharp contrast, there was no significant difference between the two antibodies in any of the CF epithelia. The genotype of the CF cells was $\Delta F508/N1303K$ for CF1 and CF4, $\Delta F508/G542X$ for CF2, $\Delta F508/other$ for CF3, and *other/other* for CF5. The $\Delta F508$ mutation accounts for approximately 76% of the mutations in the American Caucasian population (24). The N1303K and G542X mutations are two of the more common non- $\Delta F508$ mutations (see ref. 34 for a review). As with CFTR $\Delta F508$, CFTR-N1303K is not appropriately glycosylated in a recombinant system (9, 19). We expect that CFTR-G542X is a truncated protein or, if the levels of mRNA are decreased (20), that there is no G542X protein at all.

The difference between normal and CF epithelia is illustrated more clearly in Fig. 5 *b*, which shows the data from Fig. 5 *a* normalized to the average number of beads/field observed with antibody M1-4. In normal epithelia, binding with antibody M6-4 averaged 4.65 ± 1.00 times the binding with antibody M1-4. Whereas in CF epithelia, binding with antibody M6-4 was 1.02 ± 0.03 times the binding with antibody M1-4. Thus, we conclude that some mutant CFTR proteins, including CFTR $\Delta F508$, CFTR-N1303K, CFTR-G542X, and at least one other mutant CFTR, are not present or are present at greatly reduced amounts in the apical membrane of these CF epithelia.

We considered alternative explanations for the lack of specific binding in CF epithelia. First, an equal number of beads could bind to CF cells incubated with intracellular and extracellular domain antibodies, if all three epitopes were equally accessible at the surface. Such a scenario could result from misfolding of mutant CFTR, so that the R domain and carboxyl terminus were located extracellularly. This alternative is unlikely because the number of beads/field was similar to that in the absence of primary antibody or with nonspecific mouse IgG (not shown), i.e., background levels. Second, the number of beads bound to CF cells incubated with M1-4 and M6-4 could be equal if the CF, but not normal cells, were somehow permeabilized or lysed. In this scenario, antibodies against intra and extracellular epitopes would have equal access to CFTR. This interpretation is unlikely because the epithelia were treated identically, because there was no greater binding with, than without antibody, and because antibodies M1-4 or M13-1 were not able to immunostain CFTR unless we first permeabilized the epithelia (see below).

Localization of CFTR in Airway Epithelia

If mutant CFTR is not in the apical membrane, where is it? To begin to address this question, we used the three antibodies to stain permeabilized epithelia and localized staining with a laser scanning confocal microscope.

Fig. 6 *a* shows a series of confocal images of a normal airway epithelium stained with mAb M13-1. We saw the same pattern with antibodies directed against the other epitopes of

CFTR. The first image (Fig. 6 *a*, row 1) is above the plane of the apical membrane; subsequent images are taken as the plane of focus moves down into the cell monolayer (3- μm increments). The most intense staining was encountered at the apical membrane (Fig. 6 *a*, row 2). Then, as the focal plane moved down beneath the apical surface, the staining intensity decreased. There was, however, detectable intracellular staining in some of the cells (Fig. 6 *a*, rows 3 and 4). At the base of the cells, we saw bright spots of fluorescence (Fig. 6 *a*, row 7). These most likely represent autofluorescent collagen fibers in the supporting matrix, because they were also observed in cells stained with nonspecific mouse IgG (Fig. 6 *b*, row 7) and in the absence of antibody staining (not shown). This pattern of staining in normal epithelia suggests that most CFTR is in or near the apical membrane. The results also suggest that there is some intracellular CFTR.

We saw a different staining pattern in airway epithelia from a patient homozygous for the $\Delta F508$ mutation (Fig. 6 *c*). As the plane of focus moved down into the cells, the brightest staining was encountered beneath the apical membrane, in a punctate pattern throughout the cytoplasm (Fig. 6 *c*, rows 3-6). Again, we saw the same pattern with antibodies directed against the other epitopes of CFTR. In this and other studies, there was no significant difference in the size or morphology of normal and CF cells.

The difference in staining patterns between normal and CF cells was a consistent finding. We never saw a predominantly intracellular pattern in normal epithelia or a predominantly apical pattern in these CF cells. Thus, these results support the more quantitative data presented above. They indicate that there is defective trafficking of some mutant forms of CFTR in CF epithelia. Because of the low level of immunostaining, we have not been able to localize mutant CFTR to a specific intracellular organelle. However, in some CF epithelia the pattern appeared to be perinuclear.

These data allow us to consider additional alternatives for the lack of specific antibody binding in CF epithelia (Fig. 5). First, binding with M6-4 might be reduced if mutant CFTR was appropriately located, but the total amount of CFTR was markedly reduced. The observation that the overall intensity of immunostaining was approximately similar in normal and CF epithelia suggests that this is unlikely in every case. However, even if the absolute levels of mutant CFTR are reduced (as a result of defective processing or mutations causing premature termination of CFTR synthesis), our conclusion would be the same: there is much less mutant CFTR in the apical membrane. Second, although we know M6-4 recognizes CFTR $\Delta F508$ in permeabilized cells (Figs. 1 and 6), it is possible that CFTR $\Delta F508$ is present in the apical membrane, but the epitope for M6-4 (the first extracellular loop) is no longer located extracellularly. A normal apical membrane distribution of mutant CFTR with an unexposed epitope seems unlikely, because we see very different patterns of immunostaining in permeabilized normal and CF epithelia (Fig. 6).

Conclusions

These data indicate that CFTR is located in the apical membrane of normal airway epithelia. This location places CFTR in a position where it can mediate transepithelial Cl^- transport; for a Cl^- channel to govern Cl^- secretion it must be

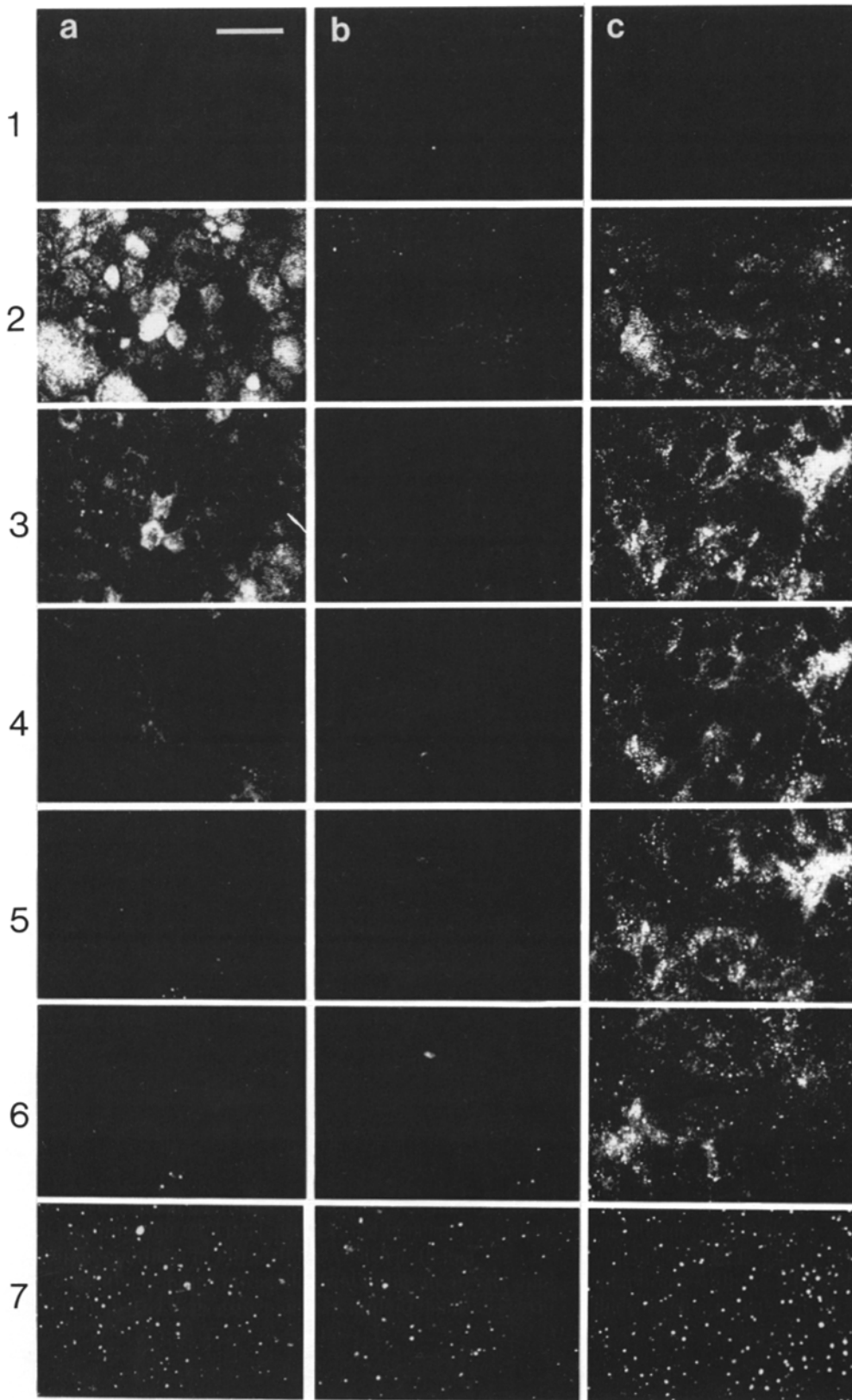


Figure 6. Series of confocal images of a normal (N5) and a CF (CF6) airway epithelium. Normal (a, rows 1-7) and CF (c, rows 1-7) epithelia were stained with monoclonal antibody M13-1. Also shown is a normal epithelium stained with nonspecific mouse IgG (b, rows 1-7). Images were collected with the focal plane above the cell monolayer (row 1) and at 3- μ m increments moving toward the matrix support (row 7). Epithelia were permeabilized before incubation with antibody. Bar, 25 μ m.

located in the apical membrane. Of course we cannot exclude the possibility that CFTR is also located beneath the apical membrane, perhaps in intracellular vesicles. In fact, we observed relatively more intracellular staining in airway epithelia than in T84 cells grown under similar conditions (14).

In contrast, we could not detect mutant CFTR in the apical membrane of CF airway epithelia. Thus we conclude that mutant CFTR is either entirely missing from the apical membrane of these CF epithelia or the levels are much reduced. This conclusion is valid for CFTR Δ F508, CFTR-

N1303K, CFTR-G542X, and at least one other undetermined mutation. These results support the hypothesis that cellular processing of CFTR Δ F508 is defective in native epithelia (9, 19). Because some recombinant CF-associated mutant proteins are appropriately glycosylated (9, 19), we predict that we would detect surface CFTR in CF epithelia bearing such mutations.

Defective intracellular trafficking of mutated proteins has also been observed in other genetic diseases. In familial hypercholesterolemia, class 2 mutations of the low density lipoprotein (LDL) receptor (representing 50% of all mutants) cause intracellular trafficking defects that result in an abnormally glycosylated and abnormally located receptor (23). The absence of surface receptor leads to a reduced clearance of circulating LDL and eventually to disease. α_1 -antitrypsin deficiency has a similar molecular basis (11).

Several studies have shown that the apical membrane of CF airway epithelia lacks a cAMP-stimulated Cl⁻ conductance (27). Although it was clear that this abnormality resulted from mutations in the CFTR Cl⁻ channel, it was unclear how mutations lead to a defective Cl⁻ permeability. An understanding of why CF epithelia lack Cl⁻ permeability became even more pressing after the reports that CFTR Δ F508 might retain some Cl⁻ channel activity (13, 16). Our data indicate that CFTR Δ F508 and several other mutants are not efficiently delivered to the apical membrane. This result can explain the Cl⁻ impermeability in most of CF.

These results have several implications. First, as previously suggested (9), it is possible that variations in disease severity might be explained by the location of different CFTR mutants. For example, it is possible that some mutations code for proteins that reach the apical membrane and those might confer a milder phenotype. Second, the data suggest that therapeutic attempts to open mutant CFTR Cl⁻ channels are unlikely to successfully increase apical Cl⁻ permeability, when CFTR is not there. Interestingly, if a pharmacologic method could be discovered for inducing function in mutant CFTR, the assay we have developed might identify which patients (or CFTR mutations) were amenable to such treatment.

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