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Cell surface and endosomal targeting of the cystic fibrosis transmembrane conductance regulator.

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CELL SURFACE AND ENDOSOMAL TARGETING OF THE CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR

by

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A DISSERTATION

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ABSTRACT OF DISSERTATION
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Defects in the CFTR molecule cause the disease cystic fibrosis. CFTR has been shown to behave as a cAMP-dependent chloride channel in the apical membranes of exocrine epithelia. Most cases of cystic fibrosis are caused by defective trafficking of the CFTR protein, but normal cell biology and trafficking of CFTR is still not understood. This work is an attempt at better understanding the subcellular localization of CFTR within the cell, the mechanisms by which CFTR can be targeted to various organelles, and how these targeting steps may affect cellular function. Because of the low expression level of CFTR even in transfected cell lines and the transmembranous/cytoplasmic orientation of the CFTR molecule, it was necessary to develop a novel biotinylation technique that allowed domain-specific biotinylation of cell surface glycoproteins and chromatographic separation of biotinylated glycoproteins from intracellular molecules. This technique proved very sensitive and efficient at measuring the relative amounts of intracellular and cell surface CFTR even at very low expression levels.

We were able to show that CFTR constitutively resides both at the cell surface and in an intracellular compartment in both epithelial cells and transfected nonepithelial cells. Using a modification of our biotinylation technique, we were able to measure the kinetics of efficient CFTR internalization from the cell surface to the endocytic pathway. Elevation of cAMP levels was capable of inhibiting the rate of CFTR endocytosis, however, did not cause a large increase in the amount of CFTR at the

plasma membrane. The inhibition of CFTR by cAMP appears to be due the CFTR ion channel activity, as cAMP had no effect in cells depleted of cellular chloride and did not affect the rate of endocytosis of G551D CFTR, which does not possess normal chloride channel activity. We next examined the possibility that CFTR may contain peptide signals important for endocytic targeting. We found that the last 40 amino acids of CFTR (aa 1440-1480) are not required for normal endocytosis, but tyrosine 1424 is required for efficient internalization, as mutation of Y1424 to alanine caused a 56% decrease in the endocytic rate of CFTR from the cell surface. All mutations that were made in the N-terminal domain disrupted normal trafficking out of the endoplasmic reticulum. Pulse chase studies on CFTR also showed that maturely glycosylated wild type CFTR is slowly trafficked to the cell surface, as the steady state value for percent CFTR on the cell surface was not reached until 4 hours after pulse. Most cell surface glycoproteins reached the cell surface within 30 minutes after pulse. Based on observations of other intracellular molecules, this finding suggests that CFTR may be targeted or sequestered to some intracellular compartment before reaching the cell surface. Y1424A CFTR was still found predominantly in an intracellular location, and the amount of protein found at the cell surface was not affected by inhibition of protein synthesis. In summary, the targeting of CFTR appears to be a complex process tightly regulated by potential signals in the CFTR protein structure. The role of intracellular CFTR, either endocytic or in some other compartment, may be a key in the understanding of epithelial biology and cystic fibrosis.

Abstract Approved by: Committee Chairman Richard B. Marchase

Program Director

Date 4/19/96 Dean of Graduate School

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INTRODUCTION TO THE SUBCELLULAR TARGETING OF CFTR

A deadly autosomal genetic disorder, cystic fibrosis is the most common cause of progressive pulmonary disease and pancreatic insufficiency in children and young adults (1). The major cause of morbidity and mortality in CF patients is pulmonary obstruction concomitant with chronic bacterial infection. The airways of a CF patient become filled with abnormally thick mucus secretions, preventing normal mucocilliary clearance and obstructing airflow (2). For reasons not completely understood, the CF lung frequently becomes colonized with *Haemophilus Influenza*, *Staphylococcus Aureus*, and *Pseudomonas Aeruginosa* (3). *P. Aeruginosa* often converts to its mucoid form in CF patients, further complicating the disease (4,5). The repetitive cycles of obstruction and infection in the CF lung are accompanied by severe inflammation and result in a complex pathology of pulmonary diseases including bronchiolectasis, intersitial fibrosis, destructive emphysema, and pulmonary hypertension (2).

While respiratory disease is the major cause of mortality in CF patients, the earliest symptoms often occur in the gastrointestinal tract. Meconium ileus, or intestinal obstruction due to fecal material, occurs in 10-20% of CF newborns, and pancreatic insufficiency begins in utero, resulting in maldigestion/malabsorption conditions (6). As in the lung, the secretory ducts of the exocrine pancreas become filled with inspisated secretions and fibrosis results. The lack of pancreatic digestive enzymes typically manifests itself initially as steatorea and failure to thrive in infants. Complete destruction of the exocrine pancreas often occurs in the first 10 years, with endocrine function being compromised later in life (7,8).

Concentrations of sodium and chloride are abnormally high in the sweat of CF patients. Still used as a common diagnostic test, CF patients often have sweat chloride levels well above 60 meq/L, with normal levels being 25-35 meq/L (1). The loss of salt through sweating can cause increases in mineralocorticoid release, which does not cause compensatory changes in electrolyte levels in CF patients (9). Mild alkalosis and hypochloremia are common, especially among children in warmer climates, and are associated with a less severe hyponatremia. While the eccrine sweat glands show little or no pathology, apocrine glands are often dilated and filled with secretory material (10,11).

The elevated levels of sodium and chloride in the sweat of CF patients first prompted researchers to compare the electrophysiological properties of normal and CF sweat glands. Normally, isotonic fluid is secreted from the secretory coil of the sweat gland. This fluid flows throught the sweat duct, where active Na^+ absorption drives Cl^- transport from the luminal sweat back into the bloodstream (12). Himself a CF patient, Paul Quinton along with his colleagues found that the resting membrane potential of CF sweat ducts was hyperpolarized compared to that in normals (-70 mV in CF vs. -7 mV in normal; ref. 13). Altering the levels of Cl^- in the lumen of the sweat duct changed the membrane potential, suggesting the lack of chloride permeability in the luminal membranes of CF sweat ducts. In normal cells, luminal chloride permeability can be increase by elevated levels of cAMP, which in vivo results from ß-adrenergic stimulation. Basal levels of stimulation causes enough cAMP to be produced to provide constitutive chloride conductance in normal sweat ducts, but not in CF sweat ducts, as increasing cAMP in permeablized CF sweat ducts had no affect on chloride permeability (14,15).

The mechanisms of electrolyte secretion and/or absorption differ in various exocrine cells. While the absence of cAMP-dependent chloride permeability is consistently observed in all of the CF cells diagrammed, other tissue-specific transport abnormalities are also observed in CF, most notably the increase in Na^+ absorption in the CF airway (16). Elevated levels of cAMP decrease Na^+ absorption in normal airway

epithelia, but not in CF airway cells. Increased Cl^- secretion and decreased Na^+ absorption in epithelia in response to cAMP have been tied to transepithelial fluid secretion, a process that is thought to be defective in CF (17). While the physiological differences between normal and CF cells became well characterized in the mid-1980's, the molecular nature of this difference was not proposed until the CF gene was cloned in 1989.

The laboratories of Jack Riordan, Lap-Chee Tsui, and Francis Collins succeeded in mapping the defect in a large number of CF patients to a previously undiscovered gene on chromosome 7 (18). The gene was named the cystic fibrosis transmembrane conductance regulator, or CFTR. It was widely speculated that the CF gene would either be a chloride channel or some regulator of channel activity. However, the sequence of CFTR was homologous to members of the ABC (ATP binding cassette) transporter family (19). ABC transporters are known to pump small, typically hydrophobic molecules outside the cell, but none had been reported to function as ion channels (20). In addition, the presence of ATP binding folds and the inferred requirement for ATP binding and hydrolysis was a mechanism thought to be reserved for pumps and other proteins mediating active transport processes, not channels, which simply allow selective diffusion. Even restoration of cAMP-mediated chloride channel activity in CF cells by transfection of wild type CFTR cDNA did not exclude the possibility that CFTR was activating endogenous chloride channels by pumping some substrate that could act as an activating ligand only at the extracellular surface or that CFTR was associating with some other protein that was actually the chloride channel (21,22).

The picture became somewhat clearer when Anderson et al. showed that CFTR itself was a chloride channel by mutating specific amino acids that were predicted to reside in transmembrane domains of CFTR. These mutants produced chloride channels with altered permeabilities when expressed in transfected cells. The changes in ion permeability were consistent with CFTR selectively passing chloride ions (23). The R

domain of CFTR, which has no relative in the ABC transporter family, was shown to regulate channel activity in a series of studies that showed PKA phosphorylation of the R domain concomitant with chloride channel activity (24). In another study, deletion of the R domain resulted in a chloride channel with constitutive activity, even in the absence of phosphorylation by PKA. These studies, however, did not assess the mechanism by which the most common mutations in CFTR cause cystic fibrosis.

Cheng et al. (25) showed that, when CFTR with the most common cystic fibrosis mutation, a deletion of phenylalanine 508 (ΔF 508), was expressed in cells not normally expressing CFTR, the translated CFTR protein did not appear to exit the endoplasmic reticulum, but was degraded before it could acquire glycosylation providing resistance to endoglycosidase H. Immunofluorescence did not detect ΔF 508 CFTR at the plasma membrane, and no chloride channel activity could be detected in the plamsa membrane of these transfected cells (25). Upon testing other, less common CF mutations, it became apparent the some mutations cause defective trafficking of CFTR out the ER, while others affected the ion channel activity of a seemingly appropriately trafficked protein (26). However, as before cloning of CFTR, the connections between CFTR and the pathology observed in cystic fibrosis were not completely obvious.

If the role of CFTR was solely as a plasma membrane chloride channel, then how did this cause the mucus secretion abnormalities seen in CF? A variety of possible roles for CFTR other than plasma membrane chloride conductance have since been proposed with the goal of explaining not only the subtleties of exocrine biology, but also the spectrum of complications and physiological irregularities in CF. CFTR has been associated with vesicle trafficking (27), Na^+ channel regulation (17), endosomal and biosynthetic acidification (28), sialylation of glycoconjugates (29), and ATP transport (30), among others. Further clouding the picture was the dispute over the exact location of CFTR in the cell. Immunofluorescent data placed CFTR at the plasma membrane, in subapical vesicles, and in other unidentified intracellular structures (31,32). The studies

described in this dissertation were begun in 1991 with the purpose of better understanding the subcellular localization and targeting of the CFTR protein to the plasma membrane and other organelles and the mechanisms regulating CFTR trafficking, with the hope that this information would be useful in elucidating the role of CFTR in epithelial biology.

CELL SURFACE LABELING OF CFTR IN T84 CELLS

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ABSTRACT

To distinguish cystic fibrosis transmembrane conductance regulator protein (CFTR) at the surface of epithelial cells from that present in intracellular membranes, intact T84 cells were treated with periodate and biotin-LC-hydrazide in order to derivatize exposed glycoconjugates. Cell lysates were then passed over a monomeric avidin column, which allows reversible avidin-biotin binding. After washing, biotinylated molecules were eluted with 2 mM biotin. CFTR was then immunoprecipitated with a mouse monoclonal antibody from both the unbound and biotin eluent fractions, radioactively labelled by in vitro phosphorylation, and analyzed by SDS-PAGE and autoradiography. Nonimmune mouse IgG failed to precipitate any CFTR, and CFTR was detectable only in the wash fractions when cells were periodate-treated but not labelled with biotin-hydrazide. In biotinylated cells, CFTR levels were approximately equal in the unbound fraction and the biotin eluent. The proportion of biotinylated CFTR did not significantly increase when cells were labelled following treatment with 10 μM forskolin. These data demonstrate that in T84 cells CFTR is constitutively expressed on the cell surface and that activation of CFTR does not primarily depend upon the cAMP-dependent trafficking of CFTR to the plasma membrane. The large unbiotinylated pool of maturely glycosylated CFTR suggests that CFTR resides in an intracellular compartment as well as being present at the cell surface.

KEY WORDS: biotin-hydrazide, chloride channel, endosomes

INTRODUCTION

Cystic fibrosis (CF) is the most common lethal genetic defect amongst Caucasians (3). CF is caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) protein (25), which possesses an adenosine 3',5'-cyclic monophosphate (cAMP)-dependent Cl^- conductance (1, 2). The CFTR protein has been shown to reside in or near the apical membrane of various epithelial cells by indirect immunofluorescence (9, 11). Its presence in the plasma membrane in many of these cells

following cAMP stimulation has been clearly established by electrophysiological techniques monitoring Cl^- channel activity (1). However, no technique has been reported that quantitatively distinguishes CFTR at the cell surface from that inside the cell. This capability would be of interest for three reasons.

First, mutations in the CFTR gene appear to result in CF either by causing defective trafficking of CFTR to the cell surface of affected cells or by disrupting its function as an ion channel directly (7). The most common mutation found among CF patients is the deletion of Phe 508 (ΔF 508) (25). CFTR with this mutation is not normally trafficked out of the endoplasmic reticulum, as assessed by its high mannose-type oligosaccharides (7). ΔF 508 CFTR, therefore, does not appear to reach the cell surface and does not result in a functional apical Cl^- channel. However, when this protein is expressed at reduced temperatures (10,12), ΔF 508 CFTR is more completely processed and results in a functional cAMP-dependent chloride channel at the plasma membrane, albeit with different characteristics than wild type (10). As other mutations of CFTR are examined, it will be important to determine if these mutations affect trafficking or disrupt the ion channel function of CFTR properly trafficked to the cell surface.

Second, CFTR has been shown to be both present and functional in endocytic vesicles (22). Even more intriguing, elevated levels of cAMP have been shown to influence membrane recycling in cells expressing CFTR but not in similar cells that lack CFTR (4). In determining if CFTR also has an intracellular function, it will be useful to quantitate the amount of CFTR that is associated with intracellular membranes as opposed to that which is present in the plasma membrane.

Lastly, a model has been proposed suggesting that the primary mechanism for CFTR activation involves the translocation of CFTR from an intracellular, subapical membraneous compartment to the plasma membrane upon stimulation (22), similar to the mechanisms of activation for the water channel and the proton pump in epithelial cells (6). If this model were correct, one would expect little or no CFTR at the cell surface in the

resting state. Then, upon stimulation, CFTR would be translocated to the cell surface, allowing Cl^- conductance across the plasma membrane. Removal of the stimulus would presumably result in the retrieval of CFTR from the cell surface. An assay for cell-surface expression could assess the validity of such a model. Denning et al. (11), utilizing an antibody directed against an extracellular epitope, demonstrated the presence of CFTR at the surface of nonpermeabilized T84 cells by indirect immunofluorescence, even in the absence of cAMP stimulation. This method of detection, however, could not assess CFTR in intracellular compartments.

One difficulty in developing a quantitative assay for cell-surface expression of CFTR stems from the apparent topology of the CFTR protein in the plasma membrane. The deduced transmembrane orientation of CFTR suggests that relatively few amino acid residues are extracellular (25). However, CFTR undergoes extensive N-linked glycosylation in most cells, even though there are only two consensus sites for such modification among the extracellular domains of the protein (7, 16, 25). This allowed the use of a labeling reagent directed toward the oligosaccharides of cell-surface glycoproteins. A second difficulty is presented by the low levels of expression of CFTR, even in cells and tissues that possess a large Cl^- current in response to cAMP (13, 16). This was overcome by utilizing a chromatographic separation of intracellular and extracellular proteins followed by a sensitive in vitro phosphorylation detection assay for CFTR. Here, this technique was used to determine if cAMP stimulation affects the cell-surface expression of CFTR.

MATERIALS AND METHODS

Cell culture. T84 cells were obtained from the American Type Culture Collection and were grown in Dulbecco's modified Eagle's medium (DMEM)/F12 media (Gibco Laboratories) supplemented with 5% fetal bovine serum (Hyclone). Cells were maintained on plastic at 37°C in a humidified atmosphere of 95% air and 5% CO_2 and passaged weekly when the cells neared confluency. For labeling experiments, cells were

seeded onto 35-mm cell culture-treated plastic dishes or permeable, collagen-coated cell culture inserts (Becton-Dickinson). Cells typically reached confluency at *day 4* of culture and were used between *days 6* and *10*.

Labeling of cell-surface proteins. Cell-surface proteins were labeled with ^{125}I using a slight modification of the lactoperoxidase method described by Pearse and Gallagher (23). Cells were washed twice with ice-cold phosphate-buffered saline containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBS c/m). To 0.5 ml of PBS c/m covering the cell monolayer were added 0.05 U lactoperoxidase (from bovine milk; Calbiochem, La Jolla, CA) and 200 µCi $Na^{125}I$ (17 Ci/mg, DuPont). The labeling reaction was started with the addition of 5 µl of H_2O_2 (1:5000 from a 30% stock into PBS c/m), with an additional 5 µl being added each minute for 5 min. Cells were washed extensively with PBS c/m to stop the labeling.

Cell-surface biotinylation of glycoproteins was performed as described by Lisanti et al. (21) for biotin hydrazide labeling, except 2 mM biotin-LC-hydrazide (Pierce Chemical, Rockford, IL) was utilized instead of 2 mM biotin hydrazide. The biotin-LC-hydrazide conjugate contains a spacer arm for increased avidin-biotin binding. Briefly, cells were cooled to 4°C, washed with PBS c/m, and incubated for 30 min with 10 mM $NaIO_4$ in the dark. The cells were again washed with PBS c/m and labeled with 2 mM biotin-LC-hydrazide in 100 mM sodium acetate (pH 5.5) for 30 min. Following labeling, cells were extensively washed and lysed in 250 µl of lysis buffer (150 mM NaCl, 20 mM Hepes, 1 mM EDTA, 1% NP40, 100 µg/ml aprotinin, 100 µg/ml leupeptin, and 2 mM phenylmethyl sulfonyl fluoride). All labeling procedures were done at 4°C with gentle agitation. One group of cells was incubated with 10 µM forskolin (Calbiochem) for 10 min at 37°C prior to labeling. Another group of cells was stimulated with forskolin for 10 min and then allowed to recover in the absence of forskolin for an additional 10 min prior to labeling.

Separation and isolation of biotinylated proteins. A 250-µl column of immobilized monomeric avidin (Pierce Chemical) was prepared according to the manufacturers instructions and then equilibrated with PBS containing 1% NP40 (PBS-N). Cell lysates were applied to the column, which was then washed with 2 ml of PBS-N to remove nonbiotinylated proteins from the column. The first five wash volumes (total of 1.25 ml) were collected and pooled. For elution of biotinylated proteins, 1.25 ml of 2 mM biotin (Sigma) in PBS-N was added, and the eluent fractions (total of 1.25 ml) were collected and pooled. The column was then regenerated with 100 mM glycine, pH 2.8 and reused up to six times with no detectable loss of binding capacity. The pooled wash fractions are referred to in this paper as the unbound fraction, and the pooled biotin-containing fractions as the biotin eluent. For analysis of iodinated proteins, 50 µl each of the total cell lysate, the pooled unbound fractions, and the pooled biotin eluent fractions were reduced with 2-mercaptoethanol-containing sample buffer and analyzed by SDS-PAGE (7.5% polyacrylamide) (19). After fixation, the gels were dried and processed for autoradiography at -80°C for 10 h.

Detection of biotinylated proteins . T84 cells were biotinylated, lysed, separated as described above, and were subjected to SDS-PAGE followed by electrophoretic transfer onto a PVDF membrane (Immobilon-P; Millipore). The membrane was blocked overnight with 5% nonfat dry milk in PBS containing 0.05% Tween-20. Following washing, the membrane was incubated with streptavidin-conjugated horseradish peroxidase (DuPont;1:1000 in PBS containing 0.05% Tween-20, 5% polyethylene glycol, and 1% bovine serum albumin) for 1 h. The bound horseradish peroxidase was visualized with diaminobenzidine/$NiCl_2$ in the presence of 0.03% H_2O_2.

Immunoprecipitation and detection of CFTR. CFTR was immunoprecipitated from the pooled fractions as previously described (16), using a monoclonal antibody to the C-terminus of CFTR generously supplied by Dr. Seng Cheng (Genzyme Corporation). The immunoprecipitated CFTR was phosphorylated with cAMP-dependent

protein kinase (Promega) and 10 μCi [^{32}P]ATP (3000 Ci/mmol; DuPont) (16), followed by analysis by SDS-PAGE on 6% polyacrylamide gels. After fixation, the gels were dried and processed for autoradiography and phosphorimaging. Exposure times ranged from 2 to 10 h.

Fluorescence microscopy. T84 cells were seeded on permeable supports and allowed to grow to confluency. At 2 days post-confluency, the cells were biotinylated on the apical side only using the biotin-LC-hydrazide technique described above. The filters were then fixed in 3% formaldehyde, permeabilized with 0.5% Triton X-100, and treated with Texas Red-conjugated avidin (1:50) and Hoescht's reagent. Cross-sectional views were obtained by folding the filter prior to visualization. Control monolayers were processed and visualized as just described, omitting the biotinylation step.

Quantitation of cell-surface CFTR. Gels were exposed to a Molecular Dynamics phosphorimaging cassette and anaylzed using a Molecular Dynamics phosphorimager. Percentages of cell-surface CFTR were calculated by dividing the number of counts found in the CFTR band in the biotin eluent fraction by the total amount of CFTR found in both the unbound and biotin eluent fractions. Values are expressed +/- the standard error of the mean of three independent experiments. Significance values were determined using a Student's *t* test.

Measurement of halide permeability. Confluent T84 cell monolayers were assayed for halide permeability in response to forskolin, using the ^{125}I-efflux method described by Venglarik et al. (29).

RESULTS

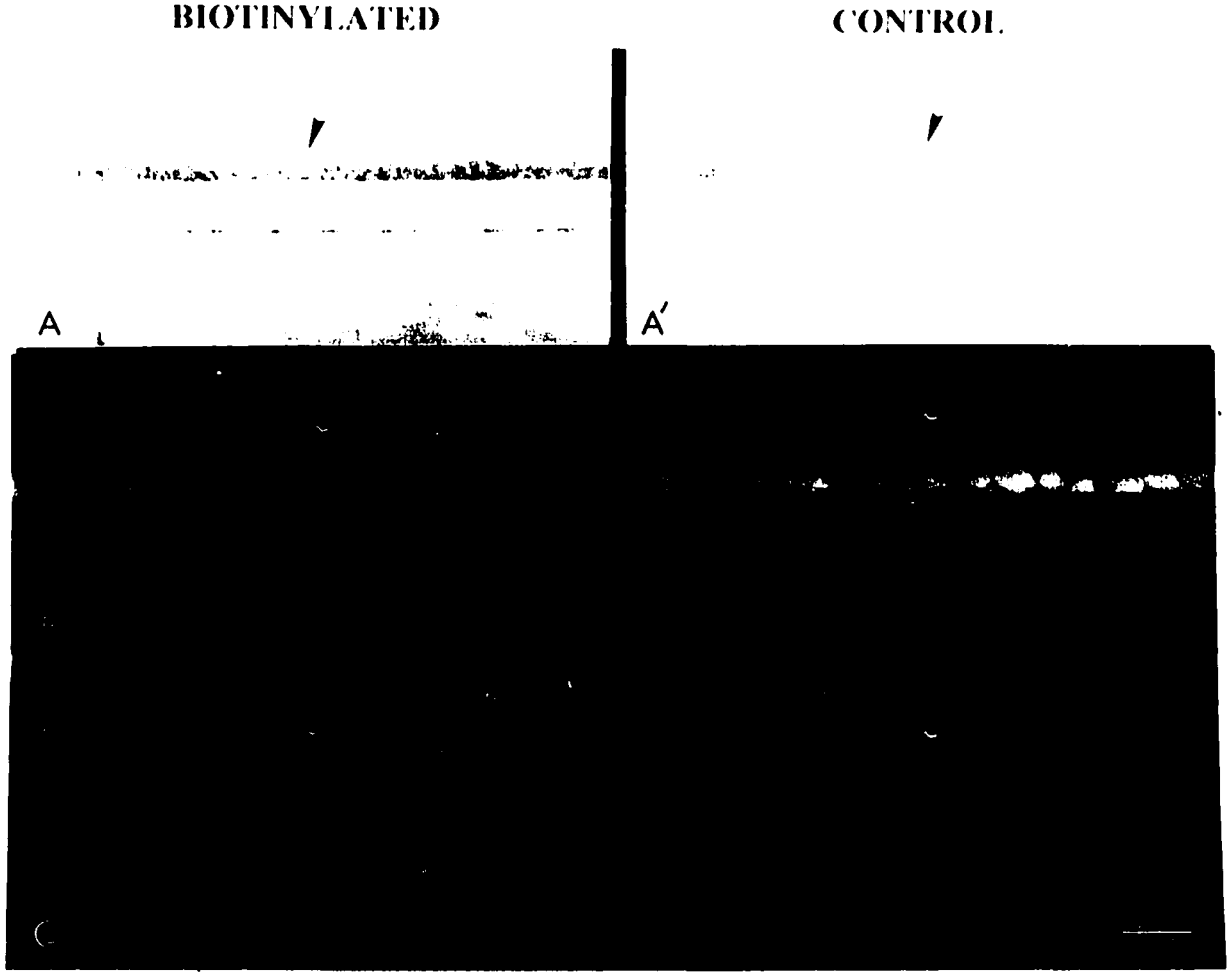
To be useful in an assay that quantitatively distinguishes between proteins present at the cell surface and those present in intracellular compartments, an extracellularly applied labeling reagent must be both nonpermeable, so as to not label intracellular proteins, and efficient in its capacity to label proteins on the cell surface. As has already been noted, the vast majority of the CFTR peptide is either cytoplasmic or

transmembranous (25) and, therefore, not accessible to labeling probes specific for amino acid side chains. However, other cell-surface labelling techniques have made use of nonpermeable reagents that derivatize carbohydrate residues of cell surface glycoproteins (21, 26, 27). The observation that CFTR undergoes extensive glycosylation (16) made these probes attractive for use in a CFTR detection assay.

Several studies have utilized nonpermeable biotin hydrazide conjugates that, following periodate oxidation of the cell surface, form covalent attatchments exclusively to extracellular glycoproteins (21, 26). To examine the feasablility of using such a technique on T84 cell monolayers, fluorescence microscopy was utilized to visually assess the labeling procedure. Filter-grown T84 monolayers were biotinylated with biotin-LC-hydrazide on their apical surfaces only. The cells were then fixed, permeabilized, and incubated with Texas Red-avidin. As visualized by fluorescence microscopy (Fig. 1*C*), Texas Red-avidin staining is restricted to the apical surface of the monolayer. Because the cells were permeabilized after labeling and before treatment with Texas Red-avidin, the lack of staining of intracellular glycoprotein-containing organelles (Golgi, endoplasmic reticulum, etc.) suggests that the biotin-LC-hydrazide was in fact nonpermeable. Control cells (Fig. 1*C*") that were not labeled with biotin-LC-hydrazide did not show staining when incubated with Texas Red-avidin. Figure 1*D* shows a higher magnification of a monolayer that has been photographed to reveal both Texas Red-avidin staining of the cell surface and Hoescht's staining of the nuclei. Further support for this selectivity was found from experiments in which extracellular avidin and anti-biotin antibody were shown to completely block subsequent accessibility of the biotinylated glycoproteins to the conjugated avidin (data not shown).

Attempts at using cell-surface labeling with biotin-LC-hydrazide and then directly detecting CFTR on blots using conventional avidin-biotin visualization techniques lacked the sensitivity necessary to visualize CFTR, even after immunoprecipitation of

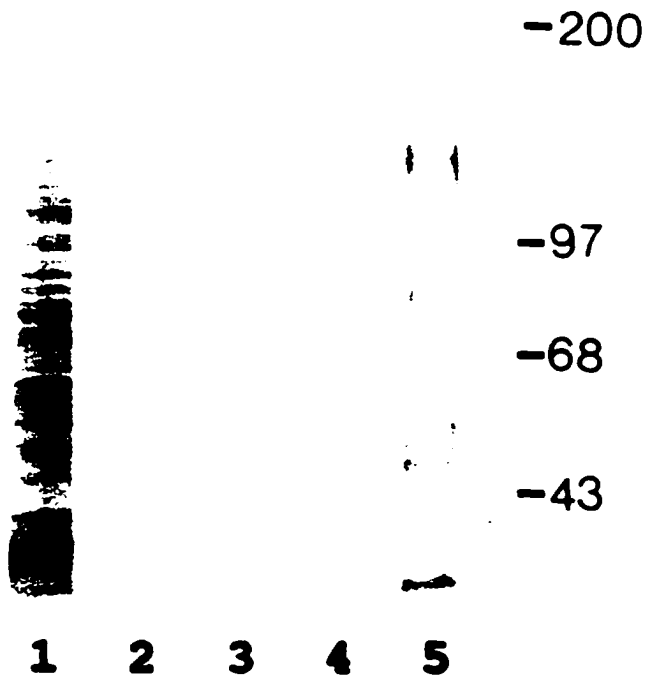
Fig. 1. Cross-sectional views of T84 cell monolayers with (left hand panels) and without (right hand panels) treatment with 2 mM biotin-LC-Hydrazide. Both monolayers were subsequently formaldehyde fixed, permeabilized with Triton X-100, and incubated with Texas Red-avidin (1:50) and Hoescht's reagent. Cross-sectional views were obtained by folding the filter in half and then examining the folded edge. Panels *A* and *A'* show phase contrast images of the cell monolayers. Panels *B* and *B'* show nuclei visualized fluorescently with Hoescht's reagent. Panels *C* and *C'* show Texas Red-avidin fluorescence. Panels *D* and *D'* show higher magnification views of the monolayers doubly exposed to reveal both Texas Red-avidin and Hoescht's fluorescence. Panels *C* and *D* show sharp delineation of the apical surface, specific for biotinylated cell-surface glycoproteins, with no intracellular staining. Arrowheads show the apical surface of the cell monolayer. Bar = 20 μm.



protein from 10^7 cells (data not shown). This and all subsequent immunoprecipitations were carried out with a monoclonal antibody against the C-terminus of CFTR found to be >95% efficient in immunoprecipitating CFTR (S. H. Cheng, personal communication). In order to use the more sensitive *in vitro* phosphorylation method for CFTR detection (16), it was necessary to utilize a technique capable of separating biotinylated from nonbiotinylated molecules.

A monomeric avidin column has been developed that allows for separation and recovery of biotinylated proteins through reversible avidin-biotin interaction (17). Intact T84 cells were treated as controls or subjected to the biotin-LC-hydrazide labelling protocol. Cell lysates were then passed over the monomeric avidin column. Unbound proteins were collected, as were proteins that were retained by the column and eluted with 2 mM biotin. *Lane 1* of Fig. 2 shows a Coomassie stain of the major proteins from T84 cells following transfer to a PVDF membrane. Coomassie stains of the biotin eluent fractions from both the control and the biotinylated cells contained insufficient levels of protein to allow detection of stained bands. However, aliquots of these samples were also visualized using streptavidin-conjugated horseradish peroxidase (Fig. 2, *lanes 2-5*). No biotinylated proteins were detectable in either the unbound or biotin eluent fractions from the control, unlabeled cells. The biotinylated cells showed five major biotinylated proteins. These proteins were distinct from the major Coomassie staining bands, consistent with previous reports demonstrating the specificity of this technique for labeling cell-surface glycoproteins (21). In addition, proteins that were biotinylated were found exclusively in the biotin eluent fraction and not in the unbound fraction, demonstrating the efficiency of the monomeric avidin column. None of the major biotinylated glycoproteins was immunoprecipitated with a monoclonal anti-CFTR antibody.

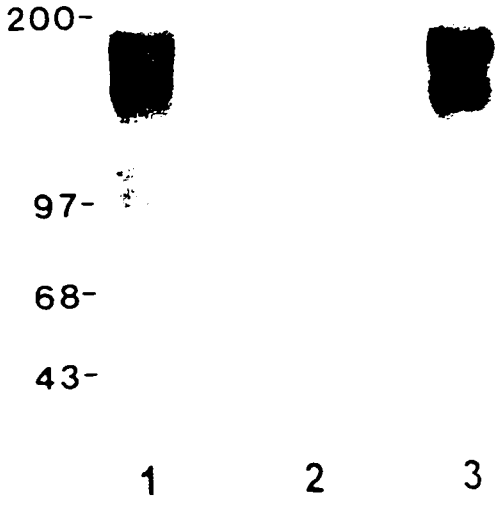
Fig. 2. Biotinylation of cell-surface glycoproteins from T84 cells. T84 cell monolayers were biotinylated with 2 mM biotin-LC-hydrazide, lysed, and applied to a monomeric avidin column. *Lane 1* shows Coomassie staining of a total T84 cell lysate following electrophoretic transfer to a PVDF membrane. *Lanes 2-5* show visualization of biotinylated proteins with streptavidin-horseradish peroxidase. *Lanes 2* and *3* are the unbound and biotin eluent fractions, respectively, from control T84 cells that were not treated with biotin-LC-hydrazide. *Lanes 4* and *5* are the unbound and biotin eluents from cells treated with 2 mM biotin-LC-hydrazide.



While the previous experiment demonstrates that biotinylated glycoproteins are efficiently retained by the monomeric avidin column, it does not assess the completeness of the biotinylation of glycoproteins present on the cell surface. To do this, T84 cells were labeled with ^{125}I using a lactoperoxidase method, which results in the iodination of only extracellular tyrosine residues on cell-surface proteins (23) and would not prejudice the subsequent biotinylation of oligosaccharides on cell-surface glycoproteins. The distribution of these arbitrary, labeled proteins following separation by the monomeric avidin column would provide a measure of the completeness of the biotinylation. Before iodination, the cells were cooled to 4°C and kept at that temperature for the remainder of the experiment so that internalization of labeled proteins was inhibited (18). Following iodination the cells were derivatized with biotin-LC-hydrazide, and cell lysates were then applied to the monomeric avidin column. As seen in Fig. 3, the two major iodinated, high molecular weight proteins present in the cell lysates (*lane 1*) were found exclusively in the biotin eluent fraction (*lane 3*), suggesting that both the biotinylation and the separation of these major cell-surface proteins were very efficient. Darker exposures revealed several bands of lower molecular weight that also were detected only in the biotin eluent fraction. No detectable iodinated protein was immunoprecipitated with the anti-CFTR antibody, consistent with the finding (16, Fig. 2) that CFTR is relatively rare, even in comparison to other cell-surface proteins.

For the detection of cell-surface CFTR, nonradioactive T84 cells were labeled with biotin-LC-hydrazide, and the biotinylated, cell-surface proteins were separated from intracellular proteins using the monomeric avidin column. CFTR was immunoprecipitated from both the unbound and biotin eluent fractions and then radioactively labeled by phosphorylation with [^{32}P]ATP and the catalytic subunit of cAMP-dependent protein kinase (16). The immunoprecipitates were then analyzed by SDS-PAGE and autoradiography. CFTR initially present on the cell surface would be expected to be found in the biotin eluent, whereas that which was not present on the cell

Fig. 3. Biotinylation and separation of ^{125}I-labelled surface glycoproteins. T84 cells were labelled with ^{125}I using the lactoperoxidase method (23). Cells were then biotinylated with 2 mM biotin-LC-hydrazide, lysed, and applied to a monomeric avidin column. Shown is an autoradiograph following SDS-PAGE of the total cell lysate (*lane 1*), the unbound fraction (*lane 2*), and the biotin eluent (*lane 3*).



surface would not bind to the column and would therefore be found in the unbound fraction.

In order to determine if this technique could define the extent of surface expression of CFTR, a series of control experiments were performed (Fig. 4). As noted above, a monoclonal antibody against the C-terminus of CFTR was shown to successfully immunoprecipitate from T84 cell lysates a 170-kDa protein that was a substrate for in vitro phosphorylation (*lane 1*). This band was not detected when nonimmune mouse IgG was used in place of the antibody (*lane 2*). Secondly, it has been shown that stimulation of cells with forskolin, an adenylate cyclase agonist that results in CFTR-mediated increases in Cl^- conductance, causes CFTR to become phosphorylated in vivo by cellular protein kinases (8). To determine if this in vivo phosphorylation would interfere with the detection method, total cellular CFTR was immunoprecipitated from both control cells and cells that had been stimulated with 10 μM forskolin for 10 min and then subjected to in vitro phosphorylation. In experiments not shown, T84 cell monolayers were found to exhibit a marked increase in Cl^- conductance in response to 10 μM forskolin. As seen in *lanes 3* and *4*, the amount of radioactivity associated with CFTR was not changed by prior stimulation with forskolin. This is likely due in part to the presence of cellular phosphatases during the immunoprecipitation. Therefore, CFTR detected by in vitro phosphorylation appeared to be representative of protein levels and not cAMP-dependent changes in phosphorylation state. Lastly, when T84 lysates from cells that had not been exposed to biotin-LC-hydrazide were passed over the monomeric avidin column, all detectable CFTR was found, as expected, in the unbound fraction (*lane 5*), suggesting that nonspecific binding of CFTR to the column was negligible.

This approach was then utilized to determine whether CFTR is expressed at the cell surface in the resting state prior to stimulation and whether stimulation with forskolin increases the amount of CFTR found at the cell surface (Fig. 5*A*). In untreated cells

Fig. 4. Immunoprecipitation of CFTR from T84 cells. *Lane 1* shows that a 170-kDa protein is specifically immunoprecipitated from a T84 cell lysate with an anti-CFTR antibody. *Lane 2* shows a nonimmune control. The immunoprecipitates were subjected to in vitro phosphorylation with [^{32}P]ATP. Samples were then analyzed by SDS/PAGE and autoradiography. In *lanes 3* and *4*, forskolin stimulation had no effect on the total amount of cellular CFTR that can be immunoprecipitated and visualized by in vitro phosphorylation. Cells were either left unstimulated (*lane 3*), or stimulated with 10 μM forskolin for 10 min. (*lane 4*). In *lanes 5* and *6*, cell lysates from T84 monolayers that were not biotinylated were passed over a monomeric avidin column, and all detectable CFTR was found in the unbound fraction (*lane 5*). No CFTR was seen in the biotin eluent (*lane 6*).

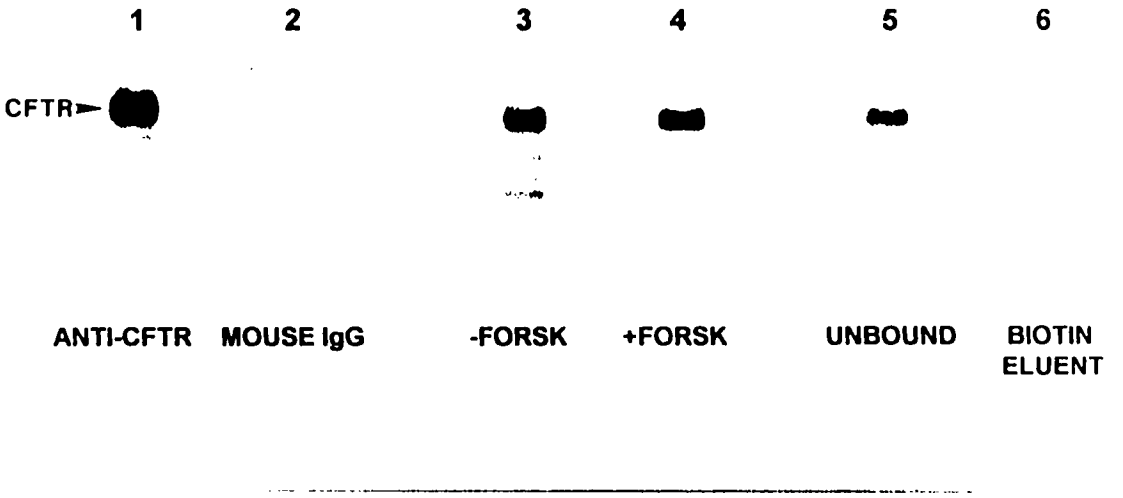
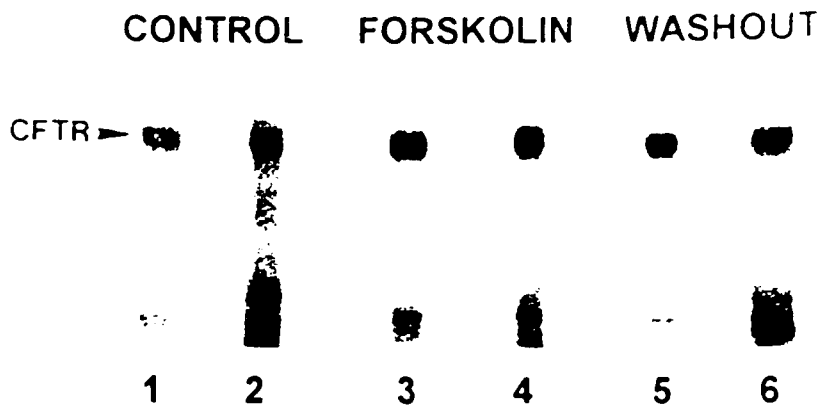
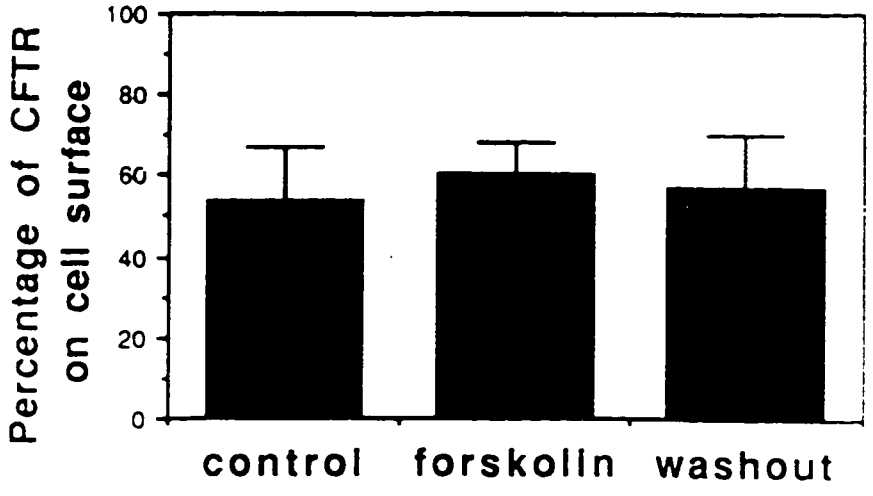


Fig. 5. Effect of forskolin on cell-surface expression of CFTR in T84 cells. Panel *A* shows autoradiographs following in vitro phosphorylation with [^{32}P]ATP and SDS-PAGE. Cells were either left untreated (*lanes 1* and *2*), stimulated with 10 μM forskolin for 10 min.(*lanes 3* and *4*), or stimulated with 10 μM forskolin for 10 min and allowed to recover in the absence of forskolin for 10 min (*lanes 5* and *6*). *Lanes 1*, *3*, and *5* show CFTR from unbound fractions; *lanes 2*, *4* , and *6* show CFTR from biotin eluents. Panel *B* shows quantitation of a series of independent experiments ($n = 3$). Values are expressed as the percentage of total detected CFTR that was in the biotin eluent lane for each of the above described conditions.

A



B



These data corroborate a previous study by Denning et al. (11), who, using a monoclonal antibody to an extracellular epitope of CFTR, determined that the pattern of CFTR immunofluorescence in nonpermeabilized cells did not change upon elevation of cAMP levels. In addition, this study showed that cooling the cells to a temperature that prevents membrane trafficking (18), and therefore vesicle insertion, does not prevent a cAMP-dependent increase in transepithelial Cl^- current. While other studies have shown membrane trafficking to be influenced by cAMP in cells expressing CFTR (4), the data presented here suggest that CFTR activation is not primarily dependent upon this trafficking.

A second finding of our study is that approximately 50% of maturely glycosylated CFTR appears to be intracellular. This distribution is analogous to other cell-surface proteins that actively recycle from the cell surface to endocytic compartments. The intracellular portion of the transferrin and LDL receptors at any given time range from 45% to 70%, and 15% to 50%, respectively, depending on the type of cell studied (5,30). In contrast, only 5% of the MHC class I and II molecules are normally found in intracellular compartments (24), and GPI-anchored proteins are found almost exclusively on the cell surface (20). This is consistent with CFTR being present in endosomal membranes, as suggested by Lukacs et al. (22). These authors examined an endosomal preparation isolated by differential centrifugation and with physiological techniques detected functional CFTR in the endosomes. Bradbury et al. (personal communication) have recently detected CFTR in clathrin-associated vesicles, and in studies utilizing a modification of the technique described here we have determined that CFTR actively undergoes endocytosis in T84 cells (Prince and Marchase; manuscript in preparation).

cAMP decreases endocytosis and increases exocytosis in cells expressing CFTR (4). If CFTR is present in vesicles participating in membrane recycling, then activation of the CFTR Cl^- channel could cause ion flux-related swelling of these vesicles. Such swelling has been implicated in the mechanism underlying fusion of exocytic vesicles

with target membranes (15). The effects of cAMP on membrane recycling could therefore be due solely to the well-described ion channel activity of CFTR. This explanation is supported by data from Bradbury et al. (4) that show stimulation of exocytosis with ionomycin treatment, which is known to stimulate a different Cl^- channel than CFTR and which occurs in cells completely lacking CFTR (14). Similarly, the reduced endocytosis observed could be due to an inhibition in the pinching off of incipient vesicles, again due to ion fluxes and the accompanying flow of water. Previous studies have shown that cytoplasmic acidification also causes a decrease in budding of vesicles in the recycling pathway, presumably due to H^+ transport into the abortive vesicles (28). In addition, amiloride has been shown to inhibit EGF-stimulated pinocytosis (30), again suggesting a role for ion transport in membrane recycling.

Since cAMP has been shown to cause an increase in exocytosis of vesicles (4) that would presumably contain CFTR, cAMP might be expected to cause an increase in the insertion of CFTR into the plasma membrane. However, if at the time of stimulation 50% of the cellular CFTR is already present at the cell surface, an increase of exocytic insertion might result in only a small increase in the total amount of CFTR at the cell surface. The physiologic relevance of these findings and their possible relation to the pathogenesis of CF have yet to be determined.

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RAPID ENDOCYTOSIS OF CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR CHLORIDE CHANNEL

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ABSTRACT

The cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel is found at the apical region of exocrine epithelial cells, both at the cell surface and in an apically localized intracellular compartment. In order to determine if this internal pool was due to endocytosis, a technique was developed that allows the rate of CFTR internalization from the cell surface to be monitored. A two-step periodate/hydrazide biotinylation procedure was used to derivatize cell surface glycoconjugates. Because both of these steps are required for derivitization and are conducted at 4°C, the inclusion of a 37°C incubation between the treatments resulted in an assay for the internalization of cell-surface glycoconjugates. CFTR was found to be targeted to a rapidly recycling endocytic pathway, as approximately 50% of cell surface CFTR was internalized within minutes and unavailable for biotinylation. In contrast, the major glycoproteins of the apical surface were not significantly endocytosed during even longer incubations at 37°C. Elevating cAMP levels either by forskolin or cAMP analogs, which has been shown to activate CFTR chloride channel activity, inhibited CFTR internalization. cAMP, however, did not affect the internalization of G551D CFTR, a naturally occurring mutant that is expressed at the cell surface but lacks normal ion channel function. In addition, the inhibition by cAMP of CFTR was not observed when cells were depleted of cellular chloride. The presence of CFTR in epithelial cells had previously been shown to confer a cAMP-mediated inhibition on the rate of fluid phase endocytosis. This effect was not seen in chloride-depleted cells, suggesting that CFTR's ion channel function and localization to incipient endosomes may be responsible for the observed inhibition. The finding that CFTR is targeted to the endocytic pathway may provide insight into the role of CFTR in normal exocrine function. In addition, these findings suggest that the expression of a regulated ion channel in a membranous subcellular compartment provides a mechanism by which a cell can regulate vesicular trafficking through that compartment.

INTRODUCTION

Cystic fibrosis (CF) is a lethal, autosomal recessive defect that results in abnormally viscous mucus secretions in the exocrine ducts of the airway and gastrointestinal tract (1). CF is caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) (2), which normally functions as a regulated chloride channel at the apical surface of exocrine epithelial cells (3, 4). In CF cells, CFTR is either not trafficked out of the endoplasmic reticulum (5) or displays abnormal channel regulation and/or activity at the cell surface (6), depending upon the particular mutation. While it is clear that CFTR functions as a cAMP-dependent chloride channel, it is not apparent how defective chloride channel activity results in the pathology observed in CF. Recent studies have shown that wild type CFTR resides in an intracellular compartment as well as at the cell surface (7-9), raising further questions as to the role of CFTR in normal exocrine function.

In two of the above mentioned studies, vesicle fractions enriched in endosomes were shown to contain a cAMP-dependent chloride permeability (8, 9). From these findings, it was suggested that regulation of CFTR chloride channel activity might occur through insertion of channels into the plasma membrane from a latent intracellular pool, much like the regulation of the glucose transporter GLUT4 (10) or water transport in the kidney via CHIP28 (11). However, these studies made no quantitative comparisons between cell surface and intracellular pools of CFTR molecules. In our previous study, a cell surface labeling assay was developed that allowed such a comparison (7). Since the deduced transmembrane orientation of CFTR suggests that relatively few amino acid residues are extracellular (2), standard probes for labeling cell surface proteins were not employed. However, CFTR undergoes extensive N-linked glycosylation (12), allowing labeling of cell surface glycoconjugates through periodate oxidation followed by hydrazide-mediated biotinylation. Biotinylated molecules were then separated from remaining cellular material using immobilized monomeric avidin. Because of its

extrememly low copy number (12), it was necessary to detect CFTR by immunoprecipitation followed by *in vitro* phosphorylation with cAMP-dependent protein kinase and [^{32}P]-ATP (12). Using this approach, we determined that approximately half of maturely glycosylated CFTR resides in an intracellular location. More importantly, we determined that CFTR is constitutively expressed at the cell surface in the absence of stimulus, and that a 10-min stimulation with cAMP did not cause a large increase in the relative amount of CFTR at the cell surface (7). This provided evidence against the latency model of CFTR regulation, but did not provide insight into the nature of the intracellular pool of CFTR.

Because both the periodate oxidation of cell surface glycoconjugates and the subsequent reaction with biotin-LC-hydrazide are absolutely required for labeling (13), we have been able to examine the dynamics of CFTR trafficking from the cell surface by including 37°C incubations between these two treatments, both of which are conducted at 4°C. The studies presented here show that cell-surface CFTR is rapidly and selectively internalized, suggesting that it is efficiently targeted to a constitutively recycling endocytic compartment. In addition, these studies suggest an explanation for the previous finding that CFTR confers a cAMP-dependency to fluid-phase endocytosis in cells that express it (14).

MATERIALS AND METHODS

Cell Culture. T84 cells were obtained from the American Type Culture Collection and were grown in Dulbecco's modified Eagle's medium with Ham's nutrient mixture F12 (Gibco Laboratories) supplemented with 5% fetal bovine serum (Hyclone). Cystic fibrosis pancreatic adenocarcinoma cells (CFPAC-1; ref. 21) and CFPAC-1 cells transfected (22) with the PLJ retrovirus alone (CFPAC-1 PLJ), PLJ-CFTR (wild type) (CFPAC-1 PLJ-CFTR), or with PLJ-G551D CFTR (CFPAC-1 PLJ-G551D CFTR) were obtained from Dr. Raymond A. Frizzell (University of Alabama at Birmingham) and were cultured in Iscove's Modification of Dulbecco's Medium supplemented with 10%

fetal bovine serum. All cells were maintained on plastic at 37°C in a humidified atmosphere of 95% air and 5% CO_2 and passaged weekly when the cells neared confluency. For labeling experiments, cells were seeded on 35-mm cell culture-treated plastic dishes or Cyclopore permeable cell culture inserts (Falcon). Cells typically reached confluency at day 4 of culture and were used between days 6 and 10. Chloride depletion studies (15) were conducted following a 1-hr incubation in gluconate buffer containing 135 mM Na gluconate, 2.4 mM K_2HPO_4, 0.6 mM KH_2PO_4, 10 mM HEPES, 25 mM glucose, 0.1 mM calcium gluconate, 1.0 mM magnesium gluconate (pH 7.0).

Labeling and Internalization of Cell Surface Proteins. Cell-surface biotinylation of glycoproteins was performed as described by Lisanti *et al.* (16) for biotin hydrazide labeling, except 2 mM biotin-LC-hydrazide (Pierce Chemical) was utilized instead of biotin hydrazide. The biotin-LC-hydrazide conjugate contains a spacer arm for increased avidin-biotin binding efficiency. Briefly, cells were cooled to 4°C, washed with phosphate buffered saline containing 1.0 mM $MgCl_2$ and 0.1 mM $CaCl_2$ (PBS c/m), and incubated for 30 min with 10 mM $NaIO_4$ in the dark. The cells were again washed with PBS c/m and labeled with 2 mM biotin-LC-hydrazide in 100 mM sodium acetate (pH 5.5) for 30 min. Following labeling, cells were extensively washed and lysed in 250 µl of lysis buffer (150 mM NaCl, 20 mM Hepes, 1 mM EDTA, 1% nonidet P-40 (NP40), 100 g/ml aprotinin, 100 g/ml leupeptin, and 2 mM phenylmethyl sulfonyl fluoride). To measure internalization of glycoproteins, the above labeling protocol was conducted with the addition of a 37°C incubation in prewarmed media following periodate oxidation and prior to biotinylation, both of which were conducted at 4°C. Cells were then lysed, and proteins were isolated as below.

Separation and Isolation of Biotinylated Proteins. A 250-µl column of immobilized monomeric avidin (Pierce Chemical) was prepared according to the manufacturer's instructions and then equilibrated with PBS containing 1% NP40 (PBS-

N). Cell lysates were applied to the column, which was then washed with 2 ml of PBS-N to remove nonbiotinylated proteins. The wash volumes were collected and pooled. For elution of biotinylated proteins, 1.25 ml of 2 mM biotin in PBS-N was added, and the fractions were collected and pooled. The column was then regenerated with 100 mM glycine (pH 2.8) and reused up to six times with no detectable loss of binding capacity. The pooled wash fractions are referred to in this paper as the unbound fraction (U), and the biotin-containing fractions, as the biotin eluent (E).

Immunoprecipitation and Detection of CFTR. CFTR was immunoprecipitated from the pooled fractions as previously described (12), using a monoclonal antibody to the C-terminus of CFTR generously supplied by Dr. Seng Cheng (Genzyme Corporation). The immunoprecipitated CFTR was phosphorylated with cAMP-dependent protein kinase (Promega) and 10 μCi [^{32}P]-ATP (3000 Ci/mmol; DuPont, NEN), followed by analysis by SDS-PAGE on 6% polyacrylamide gels (12). After fixation, the gels were dried and processed for autoradiography and phosphorimaging. Exposure times ranged from 2 to 10 hr.

Quantitation of Cell-Surface CFTR. Gels were exposed in a Molecular Dynamics phosphorimaging cassette and anaylzed using a Molecular Dynamics phosphorimager. Percentages of cell-surface CFTR were calculated by dividing the number of counts detected in the CFTR C-band (5) of the biotin eluent fraction by the total amount of CFTR C-band found in both the unbound and biotin eluent fractions.

Fluid Phase Endocytosis. Filter-grown T84 cells were incubated with 2 mg/ml fluorescein-dextran (10 kDa) or Texas Red-dextran (10 kDa), applied apically for 5 min at 37°C in the presence or absence of 10 μM forskolin (forsk). In addition, some monolayers (gluc, gluc + forsk) were chloride depleted in gluconate buffer as described above. The same buffer was used for all subsequent labeling and internalization steps. Following uptake, the cell monolayers were washed extensively at 4°C, and label was extracted with cold 100% ethanol (17). Fluorescence was measured using a fluorimeter

with excitation/emmission set at 480/520 nm for fluorescein and 580/612 nm for Texas Red. Experimental values ($n = 7$ for each condition) were subtracted from background values, obtained by incubating cells for 5 min at 4°C with 2 mg/ml labeled dextran. Control means were set equal to 100%.

RESULTS

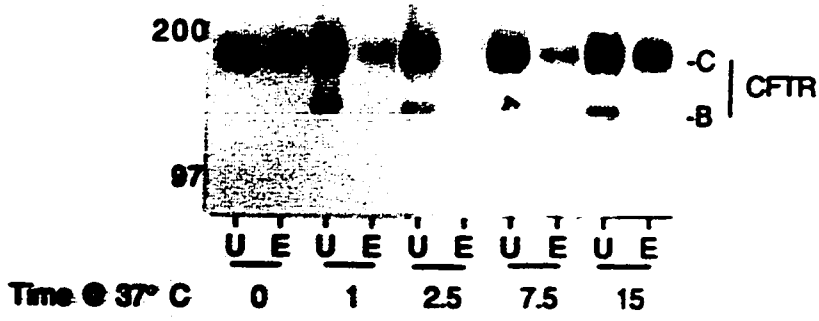
The labeling of glycoconjugates requires both a periodate oxidation step and exposure to biotin-LC-hydrazide, and both of these steps appear to be limited to cell surface glycoconjugates during incubations at 4°C (13, 16). Therefore endocytosis of cell-surface CFTR could be studied by including a 37°C incubation following periodate oxidation but prior to biotinylation. Any CFTR present at the cell surface at the time of periodate treatment but then internalized during the 37°C incubation would not be available for biotinylation, while CFTR not on the cell surface at the time of periodate treatment would not be biotinylated, regardless of its subsequent movement.

When periodate-treated T84 cell monolayers were kept at 4°C prior to biotinylation, approximately 50% of the maturely glycosylated CFTR was detected at the cell surface (Fig. 1*A*), as previously reported (7). However, when membrane recycling at 37°C was allowed to occur between the treatments, a decrease in biotinylated CFTR was seen. On the average ($n = 16$), half of the total CFTR on the cell surface at the time of periodate treatment was internalized in the first few minutes of 37°C incubation. The rapid kinetics of internalization observed for CFTR are thus comparable to those of proteins that recycle through a clathrin-mediated endocytic pathway, such as the LDL (18) and mannose-6-phosphate receptors (19). Longer 37°C incubations resulted in return of periodate-treated CFTR to the cell surface, as seen by the increase in biotinylated CFTR at 7.5 and 15 min compared to the 1- and 2.5- min time points.

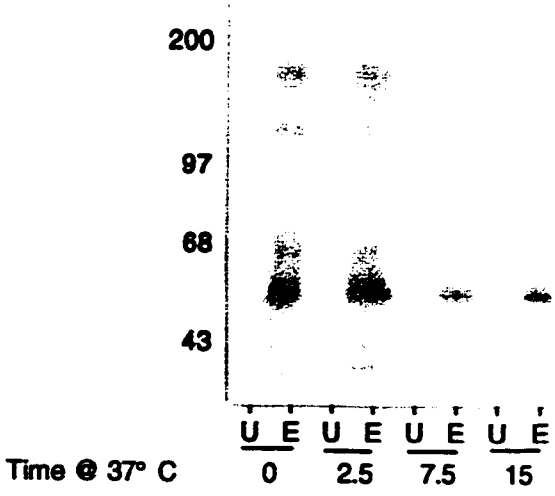
To assess the specificity of apical endocytosis for CFTR, internalization of the major apical glycoproteins of T84 cells was examined using a parallel protocol (Fig. 1*B*).

FIG. 1. (*A*) CFTR is internalized from the cell surface of T84 cells. Cell surface labeling of CFTR from confluent monolayers of T84 human colon carcinoma cells was conducted as previously described (7), except that individual monolayers were incubated at 37°C for the indicated time periods (in minutes) following periodate treatment and prior to biotinylation. The remainder of the labeling protocol was conducted at 4°C. Shown is a sample autoradiograph of a typical experiment in which CFTR was immunoprecipitated from the unbound (U) and the biotin eluent (E) proteins following fractionation of the cell lysates over a monomeric avidin column. The core glycosylated B form of CFTR was found in the unbound fraction only. (*B*). The predominant glycoproteins on the apical surface of T84 cells are not rapidly endocytosed. Total protein from the unbound and biotin eluent fractions as generated in Fig. 1*A* were separated by SDS/PAGE, electrophoretically transferred to a PVDF membrane, and visualized by incubation with streptavidin-conjugated horseradish peroxidase. In addition to the lack of any apparent decrease in biotinylated proteins, no increases in periodate-oxidized proteins could be detected by streptavidin-horseradish peroxidase following incubation of PVDF blots of the unbound fractions with 2 mM biotin-LC-hydrazide, even though this procedure was found to be as effective as biotinylation prior to SDS/PAGE in derivatizing control glycoproteins (data not shown).

A



B



Cell monolayers were warmed to 37°C between periodate oxidation and exposure to biotin-LC-hydrazide, and biotinylated proteins were again separated with immobilized monomeric avidin. Following SDS/PAGE total proteins from the unbound and biotin eluent fractions were electrophoretically transferred to PVDF, where the predominant biotinylated proteins were visualized with streptavidin-conjugated horseradish peroxidase. As seen in Fig. 1*B*, biotinylation of the major apical glycoproteins did not decrease following warming, although other aliquots of these biotinylated fractions exhibited a marked decrease in CFTR (data not shown). In addition, an increase in glycoproteins that were periodate oxidized but then sequestered from biotinylation could not be detected in the unbound fractions. Taken together, these data suggest that the major glycoproteins on the apical surface of T84 cells do not undergo significant internalization. These findings demonstrate that, in contrast to the major apical glycoproteins of epithelial cells, CFTR is specifically targeted to a rapidly recycling endocytic pathway. The kinetics and efficiency of internalization, when compared to those of other well-studied recycling proteins (18, 19), suggest that a discrete structural motif directs CFTR to clathrin-coated pits and endocytic vesicles (20).

In cells expressing CFTR, increasing cAMP has been shown to decrease fluid phase endocytosis (14, 17), prompting us to examine the effects of agents that raise intracellular cAMP, and therefore activate CFTR chloride channel activity, on the endocytosis of CFTR itself. We had previously shown that *in situ* phosphorylation of CFTR following exposure of intact T84 cells to forskolin does not change the total amount of CFTR detectable by immunoprecipitation and subsequent *in vitro* phosphorylation (7), demonstrating that radioactivity detected in this assay reflects protein levels and is not influenced by the preexisting state of CFTR phosphorylation. This allows the use of our internalization assay in studying the effect of cAMP on CFTR internalization. Including 10 μM forskolin in the media during the 37°C incubation following periodate treatment caused a marked inhibition of CFTR internalization at early

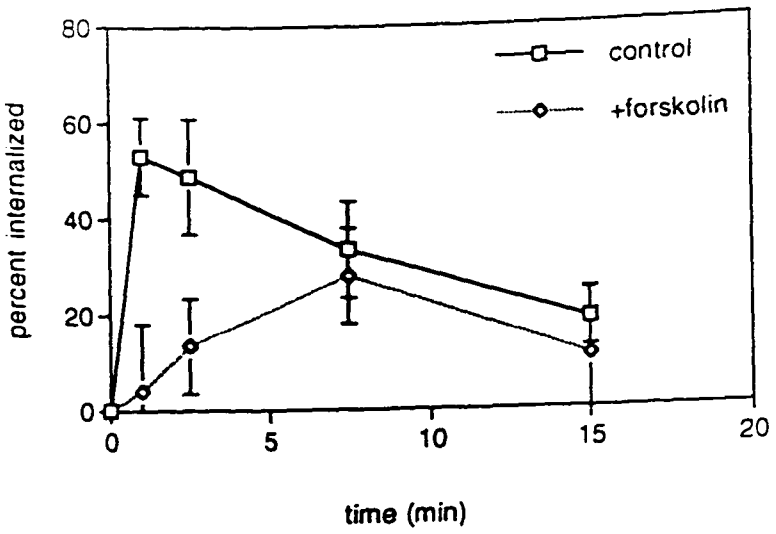
time points (Fig. 2*A*), demonstrating that conditions that lead to opening of the CFTR chloride channel also inhibit internalization of the protein. As seen in Figure 2*B*, this effect appeared to be due to elevated levels of cAMP, since dideoxy-forskolin did not slow CFTR endocytosis while both dibutyryl cAMP and 8-chlorphenythio-cAMP (8-CPT cAMP) inhibited its internalization.

Our previous studies (7) demonstrated only a slight increase in the amount of CFTR on the cell surface following 10 min of forskolin stimulation. After having established the rapidity of CFTR internalization, the steady state measurements were repeated with T84 cells following a 1-min incubation at 37°C in the presence of 10 μM forskolin. This resulted in a 12% increase in the steady state levels of CFTR on the cell surface (data not shown), still less than would be predicted without compensatory changes in other, yet undefined processes.

To assess the possibility that CFTR's ion channel function might be involved in the regulation of its endocytosis by cAMP, experiments were carried out with CFPAC-1 cells (21) that had been stably transfected with either the PLJ vector alone or with the vector containing normal or mutated CFTR (22). The mutation examined was the naturally occurring substitution of aspartate for glycine at amino acid position 551 (G551D) (24). This protein shows defective ion channel activity in response to cAMP (6), but displays a mature pattern of glycosylation (23). Wild-type CFTR and G551D CFTR were expressed at comparable levels in the transfected CFPAC-1 cells (Fig. 3*A*). As seen in Fig. 3*B*, when expressed in CFPAC-1 cells, both wild-type and G551D CFTR were detected at the cell surface and internalized with high efficiency. However, while endocytosis of wild-type CFTR was inhibited by cAMP, endocytosis of G551D CFTR was not affected, suggesting that normal chloride channel function in response to cAMP is required for the inhibition of CFTR endocytosis.

FIG. 2. Forskolin inhibits internalization of CFTR. (*A*). Quantitation of CFTR recycling. Multiple experiments were conducted as described in Fig. 1, either with (squares; $n = 9$) or without (circles; $n = 16$) 10 μM forskolin being present during the 37°C incubation. Data represent the percentage of total mature CFTR detected in the biotin eluent fraction by phosphorimaging. The mean values +/- standard errors are given. The values at 1 and 2.5 min in the absence of forskolin are significantly different from the 0 min time point ($P < 0.005$). These are also significantly different from the respective values in the presence of forskolin at 1 min ($P < 0.005$) and 2.5 min ($P < 0.05$). (*B*). Amount of periodate-treated CFTR remaining on the cell surface following a 1-min incubation at 37°C as detected by biotinylation with biotin-LC-hydrazide and quantitated by phosphorimaging (see above). T84 cell monolayers were periodate treated at 4°C and warmed to 37°C for 1 min in the presence of 10 μM forskolin (forsk; $n = 9$), 10 μM 1,9-dideoxyforskolin (ddforsk; $n = 11$), 500 μM 8-cpt-cAMP (8-cpt-cAMP; $n = 16$), or 500 μM dibutyryl cAMP (dibu-cAMP; $n = 9$). The values with forskolin ($P < 0.005$), 8-cpt-cAMP ($P < 0.05$), and dibutyryl cAMP ($P < 0.05$) were significantly different from the 37°C value.

A



B

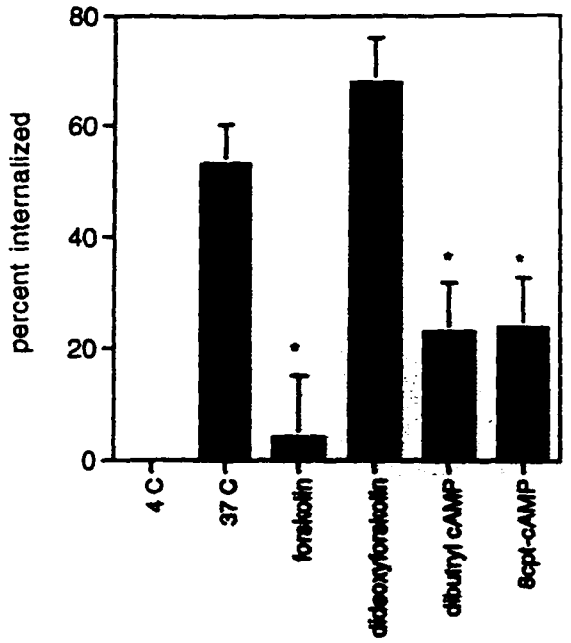
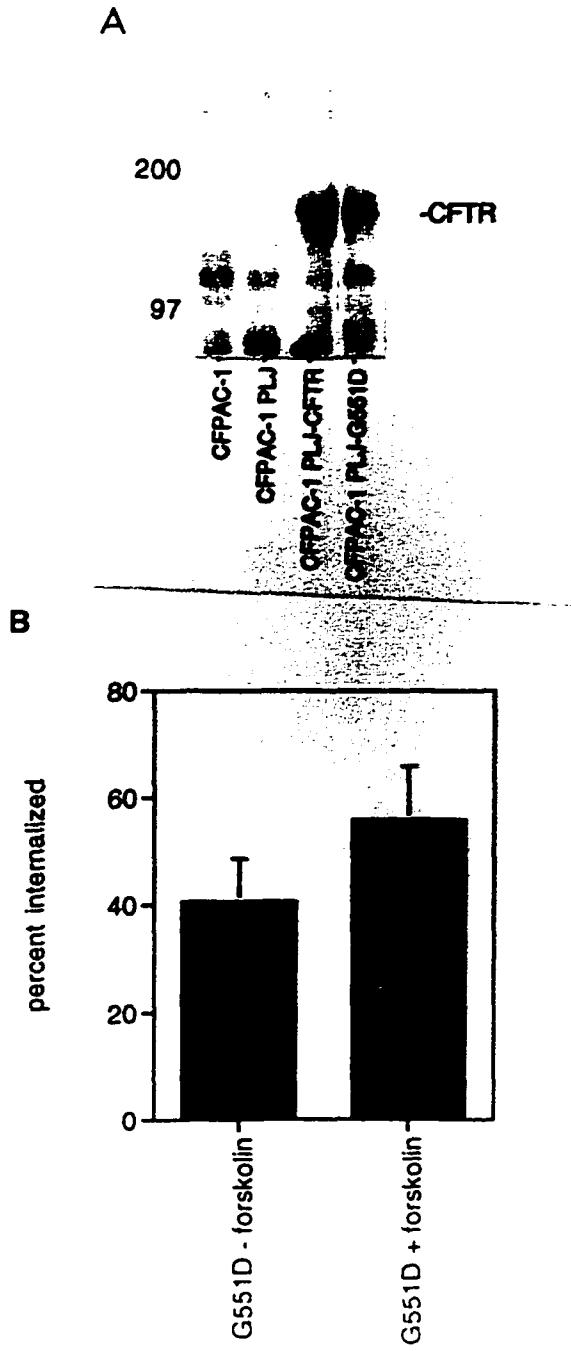


FIG. 3. (*A*). Immunoprecipitation of CFTR from stably transfected CFPAC-1 cells. CFPAC-1 (lane1), CFPAC-1 PLJ (lane 2), CFPAC-1 PLJ-CFTR (lane 3), and CFPAC-1 PLJ-G551D CFTR (lane 4) cells were lysed, immunoprecipitated with a monoclonal antibody against the C-terminus of CFTR, and phosphorylated with [32P] ATP and cAMP-dependent protein kinase as described. Expression of CFTR in CFPAC-1 and CFPAC-1 PLJ could not be detected. Wild type and G551D cell lines expressed CFTR at comparable levels, approximately 25% of that detected in T84 cells. (*B*). Elevated levels of cAMP do not affect the internalization of G551D CFTR. The relative amount of periodate-treated CFTR that was removed from the cell surface during 1-min at 37°C was measured as in Fig. 2. Both wild type and G551D CFTR were expressed on the cell surface of transfected CFPAC-1 cells and underwent rapid internalization. Addition of 10 μM forskolin during the 37°C incubation inhibited wild type CFTR internalization ($P < 0.05$; $n = 6$), but did not affect internalization of G551D CFTR ($n = 6$).



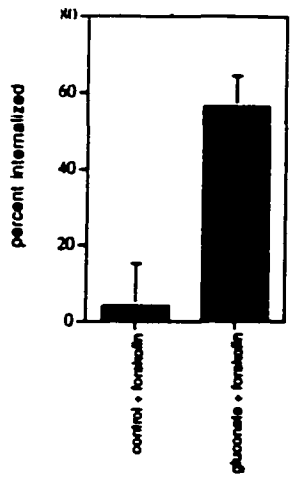
To further assess the possible role of chloride conductance on vesicle trafficking, CFTR endocytosis was also examined under conditions in which cellular chloride was depleted by incubation with gluconate (15). Under these conditions, cAMP would not be expected to elicit chloride flux through CFTR. In chloride-depleted T84 cells, CFTR endocytosis was comparable to that seen in control cells, but forskolin was found to no longer inhibit internalization (Fig. 4*A*).

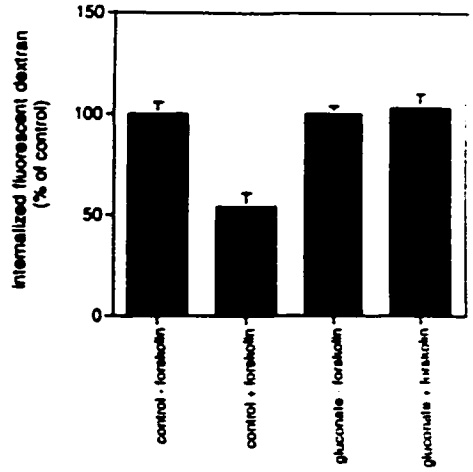
Previous findings have shown that elevated levels of cAMP inhibit fluid phase endocytosis in cells expressing CFTR (14, 17). Therefore, to assess more generally the possible inhibitory effects of ion flux on endocytosis, apical uptake of fluorescent dextrans by filter-grown T84 cells was measured in control and chloride-depeleted cells (Fig. 4*B*). As seen previously (14, 17), addition of 10 μM forskolin caused a 46% inhibition of uptake of this fluid phase marker over 5 min. However, as with internalization of the CFTR protein, forskolin again had no effect in chloride-depleted cells on endocytosis of fluid-phase markers. Taken together with the results reported above, these data suggest that the inclusion of CFTR in incipient endocytic vesicles and its ion channel function are both critical to the observation that endocytosis is inhibited by cAMP in cells expressing CFTR.

DISCUSSION

The results presented here suggest that CFTR is targeted to a rapidly recycling endocytic compartment. This is not due to a general turnover of apical membrane proteins, as the major apical glycoproteins do not undergo significant internalization on the same time scale as CFTR. The kinetics and selectivity of CFTR internalization and its subsequent return to the cell surface are comparable to proteins that are known to recycle via a clathrin-mediated mechanism (18,19). Increasing cellular cAMP levels, which activates CFTR chloride channel activity, inhibits the internalization of wild-type CFTR, but does not affect the rapid endocytosis of G551D CFTR, suggesting that this inhibition

FIG. 4. (*A*). Internalization of CFTR is not inhibited by forskolin when cells are chloride depleted. T84 cells were chloride depleted as described in *Materials and Methods*. Internalization of periodate-treated CFTR following 1-min incubation at 37°C prior to biotinylation was determined as described in Fig. 2*B*. Forskolin significantly decreased internalization of CFTR in control cells ($P < 0.005$; $n = 9$), but not in cells that were chloride depleted ($n = 12$). (*B*). Forskolin inhibits fluorescent dextran uptake from the apical surface of T84 cells only in the presence of chloride. Uptake in the presence of forskolin was significantly inhibited for control ($P < 0.005$) but not gluconate-treated cells.





of endocytosis of CFTR by cAMP requires normal chloride channel function. In addition, depletion of cellular chloride, which would reduce the flux of chloride through open CFTR channels, also removes the effect of cAMP on endocytosis of not only CFTR but also fluorescently labeled dextrans, a marker of fluid phase uptake.

Our findings suggest that activation of CFTR chloride channel activity may regulate trafficking of incipient vesicles containing CFTR. While increasing intracellular cAMP will cause multiple changes in cell behavior, apical endocytosis appears to be inhibitable by cAMP only under conditions allowing for chloride efflux through CFTR. It has been previously reported that CFTR expression correlates with the inhibition of fluid phase endocytosis by cAMP (14), but no information regarding the mechanism of this inhibition had been provided. Our findings suggest that this effect on vesicle trafficking by CFTR might be related to its chloride channel activity.

In polarized epithelial cells, most proteins that recycle via a clathrin-mediated mechanism reside on the basolateral surface and are receptors for nutrients or signal-transducing ligands (25,26). In contrast, CFTR is an apically expressed ion channel, but it too is selectively targeted to a rapidly recyling compartment. While targeting receptors to a recycling pathway provides cells with the ability to efficiently internalize nutrients and appropriately respond to various stimuli, the purpose of targeting CFTR to an endocytic compartment is not readily obvious. However, it is clear that functional CFTR is crucial for the proper hydration of glandular secretions and mucins, as shown in both CF patients (1) and transgenic mice lacking a functional CFTR gene (27). We suggest that CFTR's inclusion in intracellular vesicles is primarily to position an ion channel within the final stages of the biosynthetic pathway. The presence there of CFTR may confer a cAMP-dependence to this pathway that synchronizes macromolecular release with the efflux of chloride and water. As a corollary, we suggest that an influx of ions and water into fusing exocytic vesicles may be required to ensure efficient exit of the relatively dehydrated complex glycoconjugates found within them. If this pool of CFTR is to

influence the biosynthetic pathway, it is necessary that endocytosed CFTR and the biosynthetic pathway intersect. Previous studies by other groups (28,29) have determined that proteins internalized from the cell surface are exposed to a sialyltransferase in the trans Golgi network (TGN) about 1 out of every 10 internalization cycles. This provides a minimum estimate for the intersection of these pathways. It is possible that even higher levels of mixing occur, but in a more distal compartment that lacks sialyltransferase. The cAMP inhibition of endocytosis that is attributable to CFTR is viewed as a secondary consequence of the need to target CFTR to this rapidly recycling pathway.

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THE ROLE OF THE CYTOPLASMIC DOMAINS OF CFTR IN INTRACELLULAR TRAFFICKING

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Abstract

Proteins that are found in the endocytic pathway are specifically targeted to endosomes via discrete structural motifs found in their cytoplasmic domains. To date, these motifs have only been characterized in type I and type II transmembrane receptors. However, preliminary work involving the GLUT4 glucose transporter suggests that intracellularly targeted type III membrane proteins may also use specific targeting sequences to mediate organelle-specific localization. We have studied the endocytic trafficking of the CFTR chloride channel and here present our findings implicating the cytoplasmic tails of CFTR in the trafficking of CFTR to the cell surface and from the cell surface into the endocytic pathway. In contrast to previous findings using adenovirus-expressed CFTR mutants, the N-terminal cytoplasmic region of CFTR was critical for the normal trafficking of CFTR through the biosynthetic pathway, as even small deletions in the first 80 amino acids caused the retention of CFTR in the endoplasmic reticulum, possibly in association with calnexin. Deletion of the last 40 amino acids of CFTR do not appear to be essential for CFTR endocytosis, as CFTR with a stop codon inserted after amino acid 1440 (CFTR-1440X) was endocytosed from the cell surface as efficiently as wild type. However, mutation of tyrosine 1424 to alanine (CFTR-Y1424A) was not internalized from the cell surface as efficiently as wild type, implicating tyrosine 1424 in a possible cytoplasmic internalization signal. Further investigation of both wild type and Y1424A CFTR showed that a large portion of maturely glycosylated CFTR is targeted to some yet-unidentified intracellular compartment from the golgi apparatus before reaching the cell surface. This existence compartment may help explain the large pools of intracellular CFTR found in both epithelial and nonepithelial cells.

Introduction

Previous work by our group and others has dealt with the surface expression and rapid endocytosis of CFTR from the apical membrane of epithelial cells (5, 18). Relevant to this work, it has been reported by others that CFTR copurifies with adaptin molecules,

which are implicated in the formation of clathrin-coated invaginations and vesicles (5). In addition, the kinetics of endocytosis we have observed for CFTR are consistent with internalization via clathrin-coated pits (18). However, no additional information is available regarding the mechanisms by which CFTR is targeted to endosomes. The objective of this study was to determine if CFTR contains peptide signals that mediate its incorporation into endocytic vesicles. It was hoped that, by mutating any such regions of CFTR, we could decrease the rate of endocytosis of CFTR and then reduce the intracellular pool of CFTR. Not only would this be the first such investigation into the endocytic targeting of an apically expressed protein, but the construction of an endocytosis-defective CFTR may provide a model system for investigating the role of intracellular CFTR in epithelial biology

Cell surface receptors that internalize via clathrin-coated pits appear to possess one of two structural motifs in their cytoplasmic domains that mediate their efficient endocytosis. The first of these motifs consists of a tyrosine based 4-6 amino acid region with tyrosine at position 1 and a hydrophobic residue at position 4. The second class of signal is a dileucine/leucine-isoleucine motif with the first leucine being critical for endocytosis (24). The GLUT4 glucose transporter is the only type III transmembrane protein that has been shown to contain one of these motifs. GLUT4 is normally located in intracellular vesicles but is inserted into the plasma membrane upon insulin stimulation (22). Upon removal of stimulation, GLUT4 is retrieved from the cell surface via a clathrin-mediated endocytic mechanism. The dileucine sequence found in the C-terminal region of GLUT4 appears to be required for endocytosis and possibly intracellular sequestration, as mutation of LL to either AA or AS causes GLUT4 to be located at the plasma membrane in the absence of insulin (7,26).

Unlike GLUT4, CFTR does not undergo stimulus-dependent translocation to the plasma membrane (17). However, the observation that CFTR does undergo rapid internalization from the cell surface prompted us to consider the possibility that CFTR

contains an internalization signal like those described above. Because CFTR contains numerous predicted intracellular tyrosines and leucine-leucine/leucine-isoleucine repeats (26 to be exact), we decided to concentrate our studies on the N-terminal and C-terminal tails, for which no function had yet been described. The C-terminal cytoplasmic tail of GLUT4 contains the LL critical for endocytosis. The N-terminal tail of CFTR (methionine to the first transmembrane residue) consists of 80 amino acids, while the C-terminal tail (from the end of nucleotide binding fold #2 to aa 1480) consists of 89 amino acids. Fig. 1 shows the potential internalization motifs found in the primary sequences of the N-terminal and C-terminal tails. We conducted mutagenesis studies on the N-terminal and C-terminal tails of CFTR to examine these potential internalization signals in CFTR.

Materials and Methods

Surface Labeling and Internalization of CFTR

Cell-surface biotinylation of CFTR was performed as described previously. Briefly, cells were washed in PBS at 4°C and then incubated in 10 mM NaIO4 for 30 min to oxidize vicinal hydroxyl-containing carbohydrates to reactive aldehydes. Cells were then treated with 2 mM biotin-LC-hydrazide (Pierce Chemical) followed by lysis in 1% NP40. The cell lysates were then passed over a monomeric avidin column (Pierce) which allowed for the reversible binding of biotinylated molecules. Unbound fractions were pooled, as were fractions eluted from the column using excess free biotin.

CFTR was immunoprecipitated from the pooled fractions with either anti-C-terminal or anti-R domain monoclonal antibodies generously supplied by Dr. Seng Cheng (Genzyme). The resulting immunoprecipitates were phosphorylated with [γ-32P] ATP and cAMP-dependent protein kinase. The radiolabeled protein was then analyzed by SDS-PAGE and autoradiography. Quantitation was performed using a Molecular Dynamics phosphorimager.

Figure 1. Potential internalization signals found in the N-terminal and C-terminal cytoplasmic domains of CFTR.

N-term.	C-term.
^{38}YQIP	^{1424}YDSI
^{65}KNPKLI	^{1427}IQKLL

To examine the internalization rates of CFTR, the above protocol was performed with the insertion of 37° C incubations following NaIO4 treatment and before reaction with biotin-LC-hydrazide, both of which are performed at 4° C, thereby inhibiting membrane trafficking.

Construction and Expression of CFTR Mutants

pMT-CFTR (wild-type) and pMT-CFTR-1440X were obtained from Dr. Seng Cheng (Genzyme). In addition, wild-type and mutant CFTR cDNA were expressed using the SV40-based vector pGT1 (Dr. Eric Sorsher, University of Alabama at Birmingham). For mutagenesis of the N-terminal region of CFTR, the Xma I-XbaI fragment of pGT1 was subcloned into pSK-Bluescript (Stratagene). Single-stranded DNA was then used as a template for oligonucleotide-directed mutagenesis. The resulting mutant cDNA was then put back into pGT1 for expression in COS-7 cells. COS-7 cells were transfected with 1.0 mg/ml DEAE-dextran and 10 μg of the appropriate DNA according to established protocols. 100 μM chloroquine was added to the media for 3 h following transfection. Cells were assayed 48 h later.

Immunofluorescence of CFTR in COS-7 Cells

Transfected COS-7 cells were trypsinized 12 h after transfection and plated onto glass coverslips. 48 h later, the coverslips were fixed in methanol: acetic acid (3:1), blocked in PBS/1% BSA, and incubated with either monoclonal anitbody 24-1 or 13-1 (diluted 1:50 in PBS/BSA). Coverslips were then incubated with Texas Red-conjugated goat anti-mouse antibody (diluted 1:1000 in PBS/BSA/5% normal goat serum), followed by mounting and visualization under a Leitz fluorescence microscope.

V8 Protease Digestion

Cleveland digestion of CFTR was conducted by excising radiolabeled CFTR from a 6% polyacrilamide gel and placing the gel slices into loading wells of a 12% polyacrilamide gel. Various concentrations of V8 protease were added along with Laemli sample buffer. The samples were electrophoresed into the stacking gel, where

electrophoresis was stopped for 1 h to allow digestion. The samples were then completely electrophoresed followed by fixation, drying, and autoradiography.

Endoglycosidase H and N-glycanase Digestion of CFTR

Immunoprecipitated and radiolabeled CFTR was excised from a polyacrilamide gel and extracted in NH_4HCO_3 /β–mercaptoethanol buffer containing 0.1% SDS. Protein was precipitated with trichloroacetic acid and washed with cold acetone. Protein pellets were resuspended in buffer containing either endoglycosidase H or N-glycanase (both from Genzyme). Digestion was carried out at 37° C for 24 h. The samples were concentrated under vacuum and analyzed by SDS-PAGE and autoradiography.

Results

Examination of CFTR Endocytosis in Transfected COS-7 Cells

To examine the regions of CFTR responsible for efficient endocytosis, we decided to transiently express CFTR constructs in COS-7 cells, which lack endogenous CFTR. As shown in Fig. 2, wild-type CFTR is expressed both at the cell surface and in an intracellular location when expressed in transfected COS-7 cells. CFTR expressed in COS-7 cells using a vector containing the mouse metallothionein promoter (pMT-CFTR) was distributed between the cell surface and intracellular compartments comparable to what had been seen previously in T84 cells, with approximately 50% of the maturely glycosylated C form of CFTR being detected at the cell surface. When the internalization kinetics of pMT-CFTR was measured, 16% of the cell surface CFTR was internalized per minute ($K_e = 0.16$; reference 29), slightly slower than the internalization rate seen for CFTR in epithelial cells, but very comparable to the rate reported for the transferrin receptor in nonpolarized cells (25).

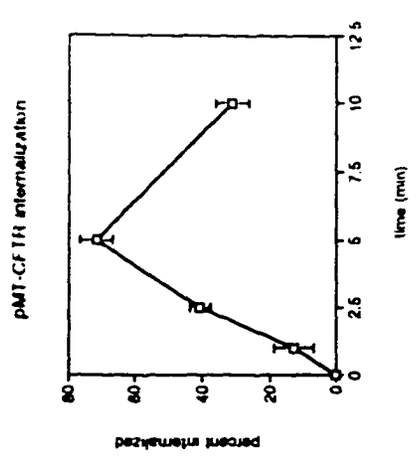
When wild-type CFTR was expressed using a vector containing the SV40 early promoter (pGT-CFTR), the total expression level of CFTR was approximately one-fifth of that seen with pMT-CFTR. The distribution of pGT-CFTR was slightly different than pMT-CFTR, as 23% of the total C-form was found at the cell surface in COS-7 cells

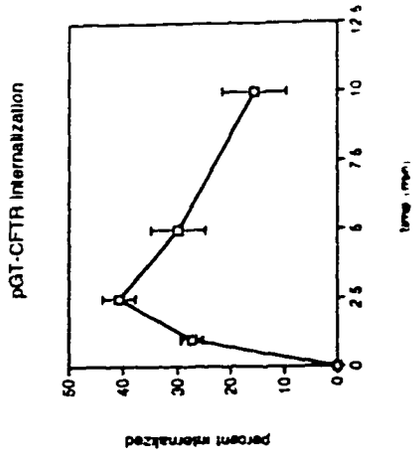
transfected with pGT-CFTR. However, the internalization rate for pGT-CFTR from the plasma membrane was 0.27, higher than that seen for pMT-CFTR. The differences between the cellular distributions of CFTR expressed from the two different vectors is discussed later. For the experiments described below, the trafficking of mutant CFTR protein is always compared to the trafficking of wild-type CFTR in the context of the same expression vector. The efficient internalization of CFTR from the cell surface in transfected COS-7 cells indicates that CFTR is capable of utilizing the endocytic machinery in unpolarized nonepithelial cells for its own internalization. In addition, the levels of CFTR expression in transfected COS-7 cells
allow trafficking consistent with that observed for CFTR in epithelial cells.

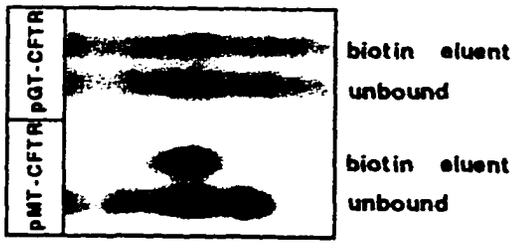
Deletion of amino-terminal regions of CFTR cause mistrafficking.

To examine the possibility that targeting information is contained in the N-terminal tail of CFTR, a series of deletion mutants were constructed (Fig. 3). Deletion of amino acids 2-79 (Δ2-79) removed most of the N-terminal tail before the first transmembrane domain, Δ2-59 removed a smaller region, and Δ35-45 and Δ60-72 removed the areas surrounding potential internalization motifs (see Fig. 1). As seen in Fig. 4, the maturely glycosylated C-form of CFTR was not produced in COS-7 cells transfected with any of the N-terminal deletion mutant constructs. The phosphoprotein observed could be immunoprecipitated with anitbodies to the C-terminus of CFTR and was presumed to represent an ER form of CFTR. In addition, the band possessed the same V8 protease digestion pattern as the B and C forms of wild-type CFTR. These proteins could not be biotinylated at the cell surface, and SPQ analysis of cells transfected with each of the N-terminal deletions failed to show normal chloride channel activity in response to elevated levels of cAMP (Fig. 5*a*). The approximately 130-kDa CFTR band immunoprecipitated from cells transfected with N-terminal deletion mutants was sensitive to both N-glycanase

Figure 2. CFTR is expressed at the cell surface and the endocytic compartment of transfected COS-7 cells. COS-7 cells were transfected with pMT-CFTR or pGT-CFTR as described in the *Materials and Methods* section. 48 h post transfection, cells were biotinylated with biotin-LC-hydrazide. The labeled cells were lysed in 1% NP-40 and passed over a monomeric avidin column. CFTR was then immunoprecipitated from both the unbound, nonbiotinylated fraction and the biotin eluent fraction, which contains all biotinylated cell surface proteins. Following in vitro phosphorylation with [γ-^{32}P] ATP and cAMP-dependent protein kinase, the immunoprecipitates were subjected to SDS-PAGE and autoradiography. Internalization of CFTR was measured as described in the previously (18).







as well as endoglycosidase H (Fig. 5*b* and *c*), the latter result confirming that the deletion mutants studied were retained in the ER/cis-Golgi and acquired only core glycosylation.

A 220-kDa band was occasionally observed in the immunoprecipitates of N-terminal deletion mutants (Fig. 6*a*). This 220-kDa band was not seen in untransfected cells and showed the same V8 protease digestion pattern as the B forms of mutant CFTR and the B and C forms of wild-type CFTR (Fig. 6*b*). This strange band could result from either an abnormally large degree of glycosylation, causing a 90-kDa shift in apparent molecular weight, or by coimmunoprecipitation of another protein with CFTR. CFTR immunoprecipitates cannot be boiled prior to electrophoresis, increasing the possibility that a CFTR binding protein could remain associated with CFTR during electrophoresis. To test these possibilities, the 220-kDa band (or D form) was excised from a SDS-PAGE gel and digested with N-glycanase and endoglycosidase H. If the D forms were to be the result of high levels of glycosylation, then the band would be expected to be sensitive to digestion with N-glycanase but resistant to endoglycosidase H (23). However, both N-glycanase and endoglycosidase H were able to increase the mobility of D form CFTR to that of a 130-kDa protein, the approximate size of unglycosylated CFTR (Fig. 6*c*). This finding suggests that immaturely glycosylated CFTR can associate with another protein of approximately 90-kDa and that this association is at least partially mediated by Asn-linked high-mannose glycosylation.

Pind et al. have shown association of B form CFTR with the 90-kDa molecular chaperone calnexin, also called p88 (16). Calnexin has been shown to copurify with newly synthesized glycoproteins in the endoplasmic reticulum, and glycosylation appears to be important for calnexin binding (11,15). The lower expression levels we obtained with CFTR transient transfections, compared to the stable cell lines used by Pind et al., prohibited coimmunoprecipitation of our mutant CFTR proteins with anti-calnexin antibodies. However, preclearing of the cell lysate with immobilized anti-calnexin antibodies reduced the amount of D form obtained from COS-7 cells transfected with N-

Figure 3. Schematic of deletion mutants of CFTR. Deletions of the N-terminal and C-terminal tails of CFTR were constructed. The strategy for mutagenesis is described in the *Materials and Methods* section.

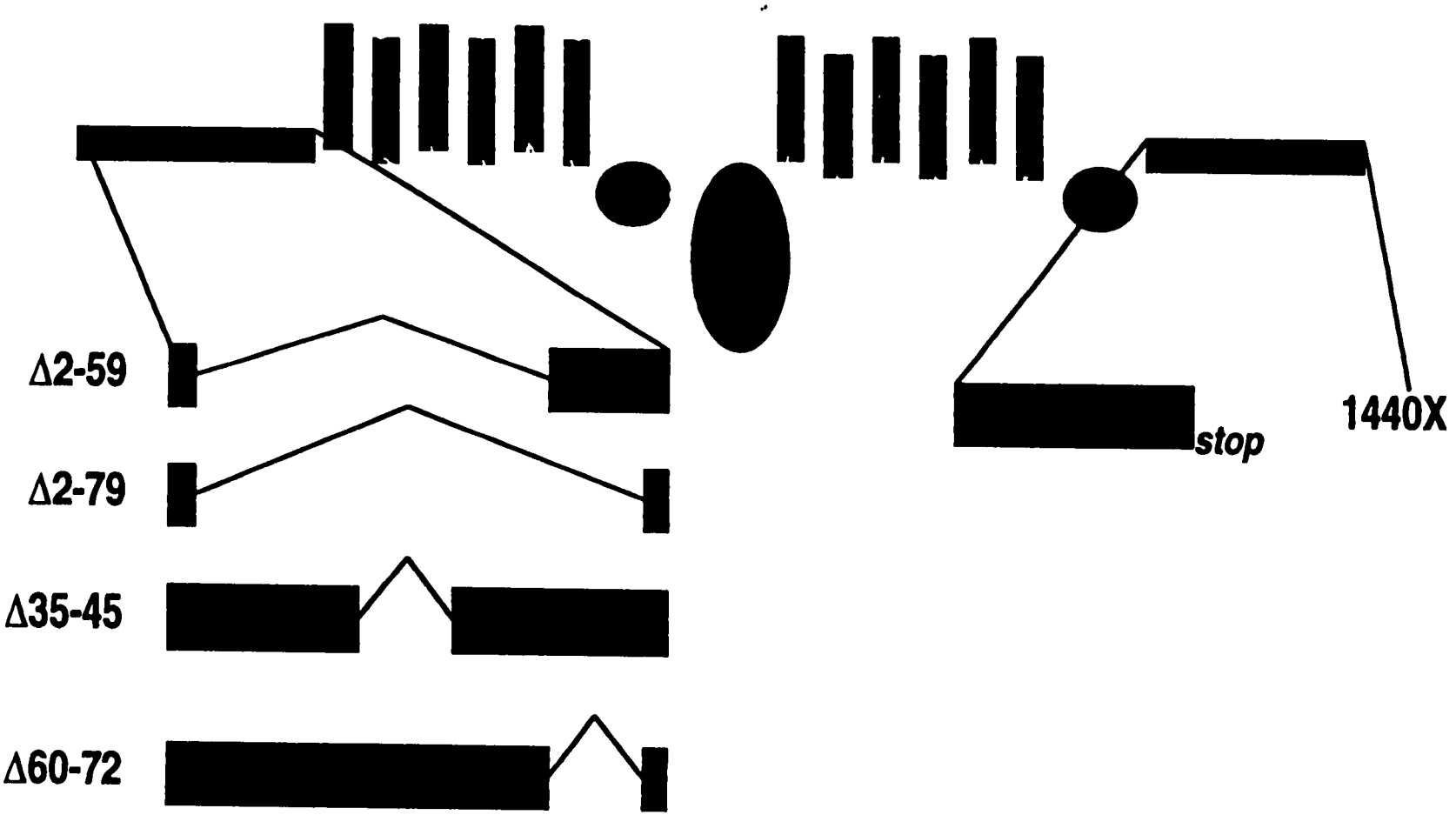
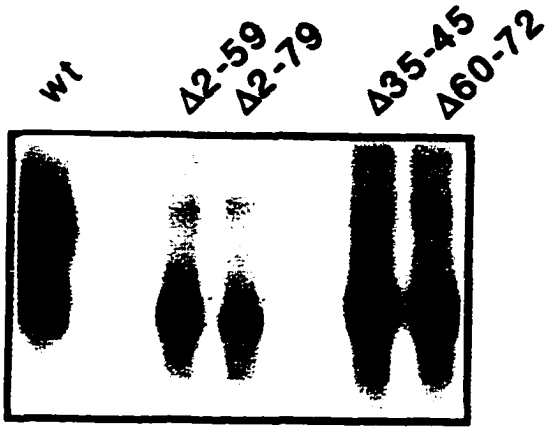
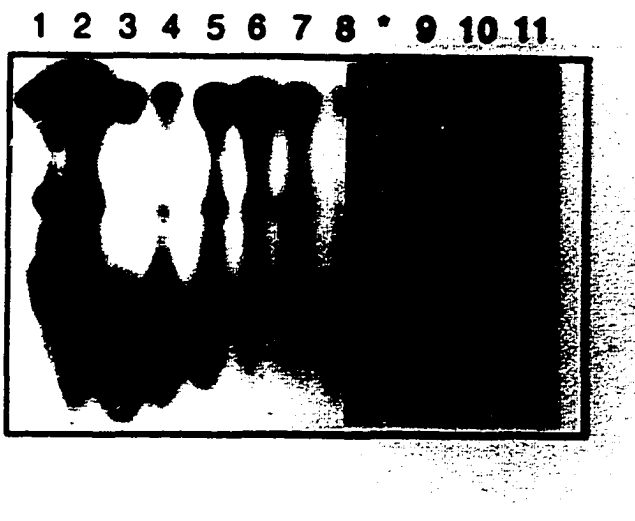


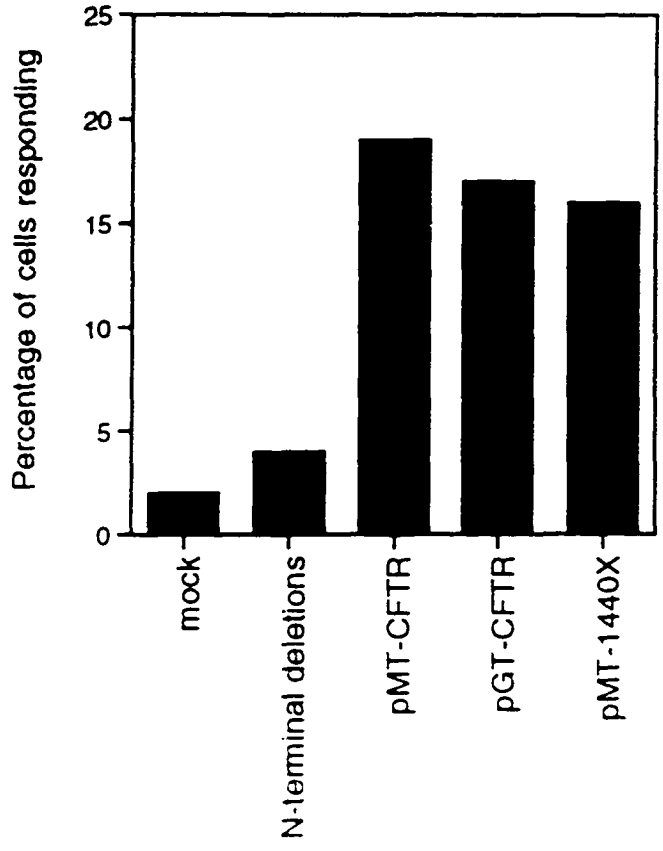
Figure 4. Immunoprecipitation of N-terminal CFTR mutants from transfected COS-7 cells. N-terminal CFTR mutant cDNAs were constructed using the PGT1 vector as a template and transfected into COS-7 cells. 48 h post-transfection, CFTR was immunoprecipitated using monoclonal antibody 24-1 or 13-1. Following in vitro labeling with [γ-^{32}P] ATP and cAMP-dependent protein kinase, the immunoprecipitates were analyzed by SDS-PAGE and autoradiography, as shown in the top panel *a*. In the lower panel *b*, immunoprecipitated protein as described above was excised from the polyacrilamide gel, digested with V8 protease, and electrophoresed a second time. Note that all of the immunoprecipitated proteins show the same V8 digestion pattern.

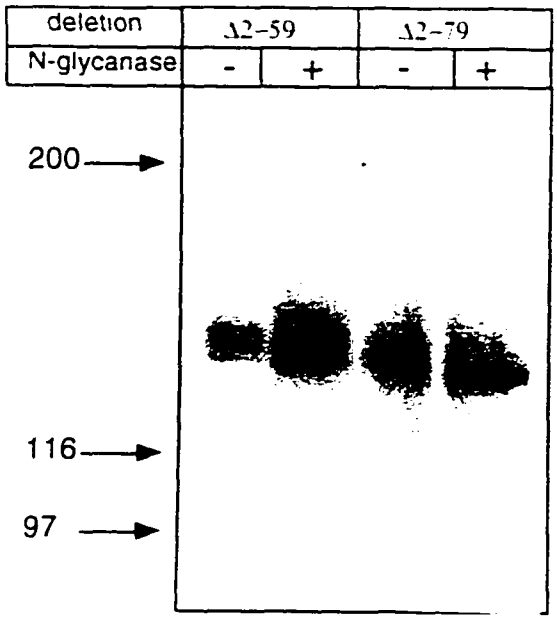




1 wild-type C-form
2 wild-type C-form
3 wild-type C-form
4 Δ2-59 B-form
5 Δ2-59 B-form
6 Δ2-59 B-form
7 Δ2-79 B-form
8 Δ2-79 B-form
9 Δ2-79 D-form
10 Δ2-79 D-form
11 Δ2-79 D-form

Figure 5. Expression N-terminal mutant CFTR molecules does not result in plasma membrane CFTR chloride channels. (*a.*) COS-7 cells transfected with mutant and wild-type CFTR constructs were analysed by SPQ analysis. Percent of cells responding refers to the percentage of cells visualized that display a cAMP-dependent halide permeability and is also a measure of transfection efficiency. (*b.*,*c.*) Immunoprecipitated CFTR from COS-7 cells expressing N-terminal mutant CFTR was digested with either N-glycanase or endoglycosidase H and analyzed by SDS-PAGE.





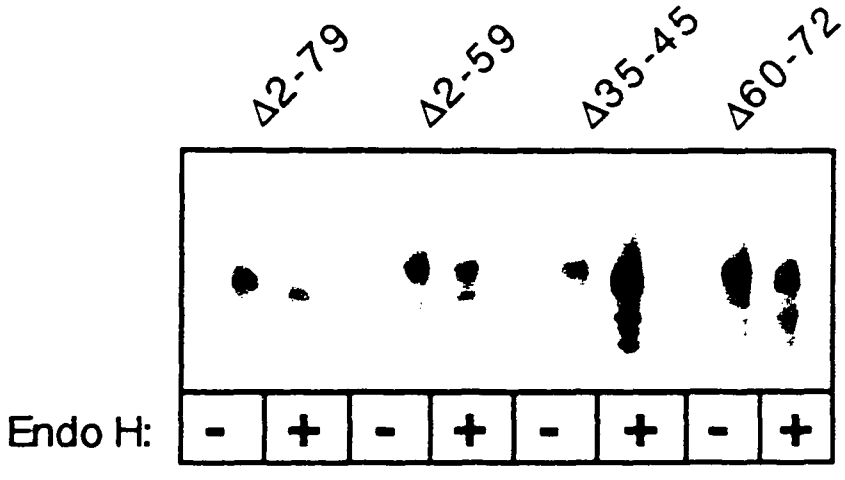
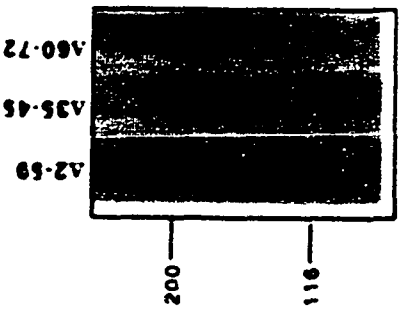
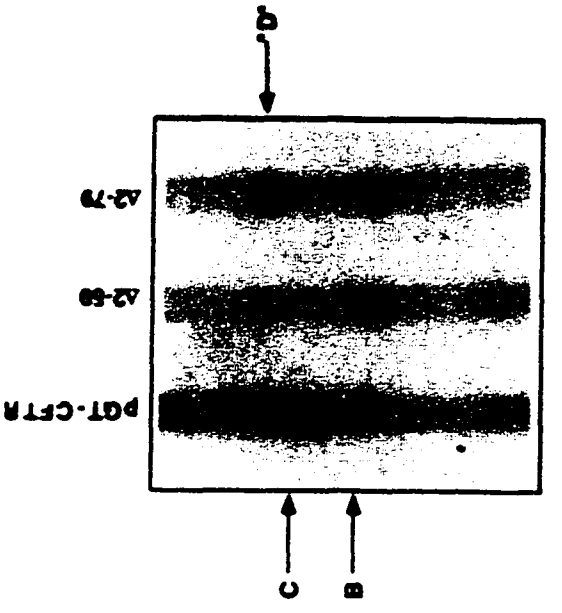


Figure 6. Characterization of an abnormal "D" form of CFTR. As shown in the left panel, a phosphoprotein of aproximately 220 kDa was occasionally detected in immunoprecipitates of N-terminal CFTR deletion mutants. This protein was confirmed to be CFTR in Fig. 4 by V8 protease digestion. The right panel of this figure shows that, upon digestion with endoglycosidase H, the 130-kDa A form of CFTR is regenerated, suggesting that the D form is stabilized by the presence of endo H sensitive glycosylation.





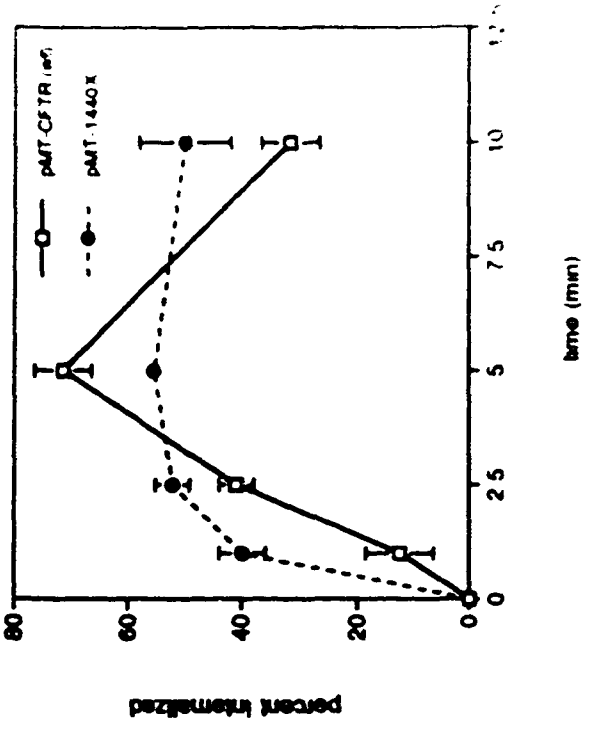
terminal deletion CFTR mutants (data not shown), suggesting that the D form of CFTR may result from interaction of immaturely glycosylated CFTR with calnexin in the endoplasmic reticulum.

Flotte et al. have reported chloride channel activity in cells transfected with CFTR-AAV chimeras lacking the N-terminus and first transmembrane domain of CFTR (9). However, their studies lacked a comprehensive biochemical analysis of the resulting mutant protein. Our data suggest that deletion of even small regions of the N-terminal tail of CFTR results in protein mistrafficking. This mistrafficking is not completely surprising, as point mutations in the N-terminal tail have been found in genotyped CF patients, suggesting that integrity of the N-terminal cytoplasmic domain is critical for normal CFTR expression/function.

The role of the carboxy-terminal region of CFTR in endocytosis in endocytic targeting.

To examine the role of the C-terminal 40 amino acids in endocytic targeting, CFTR containing a premature stop codon placed after amino acid 1440 was expressed in COS-7 cells using a vector containing the metallothionein promoter (pMT-1440X; reference 19). The 1440X truncated protein could be immunoprecipitated with an anti-R domain antibody but not by an anti-C-terminal antibody (Fig. 7), providing evidence that the protein was indeed truncated. SDS-PAGE of CFTR 1440X did show the truncated protein to have a slightly lower apparent molecular weight. Unlike the N-terminal deletion constructs, CFTR missing the last 40 amino acids was maturely glycosylated and expressed at the cell surface with similar distribution between the cell surface and intracellular compartments as wild-type pMT-CFTR. CFTR 1440X was internalized from the cell surface with similar kinetics as wild type, suggesting that any peptide signals necessary for endocytic targeting were not contained after amino acid 1440 (see Fig. 1). In addition, SPQ analysis of CFTR-1440X (Fig. 7) showed functional chloride channel response after stimulation with cAMP.

Figure 7. CFTR-1440X is expressed at the cell surface of COS-7 cells and is internalized with rapid kinetics. Cell surface labeling of CFTR and internalization assays were described in chapters 1 and 2. COS-7 cells were transfected with pMT-CFTR-1440X and assayed 48 h later. Immunoprecipitations were conducted using monoclonal antibody 13-1 (Genzyme), raised against the R-domain of CFTR, since the C-terminal epitope recognized by the 24-1 monoclonal is not produced due to the premature stop codon inserted after amino acid 1440 in the pMT-CFTR-1440X construct.



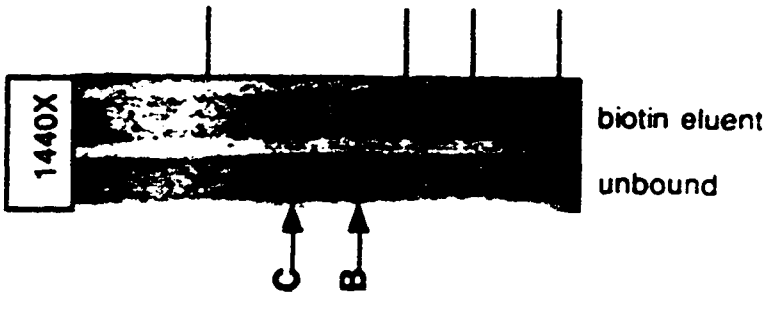
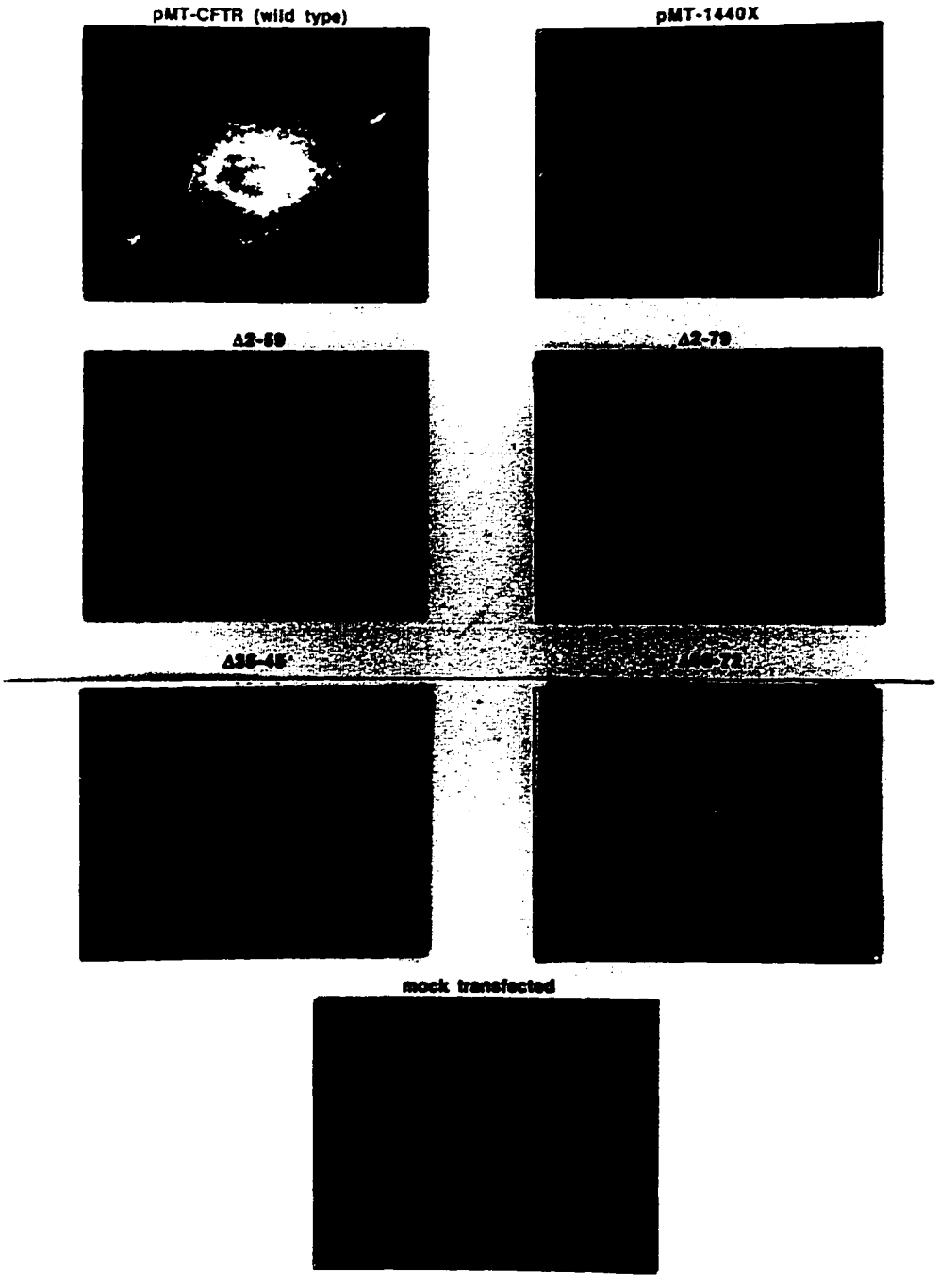


Figure 8. Immunofluorescence of COS-7 cells transfected with wt and mutant CFTR constructs. COS-7 cells were transfected with CFTR constructs as described earlier in this chapter and assayed 48 h later. Cells were plated onto glass coverslips, fixed with methanol:acetic acid (3:1), incubated with anti-CFTR antibodies (13-1 in the case of pMT-1440X and 24-1 for the other constructs), and then incubated with Texas Red-conjugated goat anti-mouse antibodies. Mounted cells were visualized under a fluorescent microscope and photographed.



Anti-CFTR immunofluorescence showed a staining pattern consistent with a cell-surface protein being targeted to endosomes in COS-7 cells transfected with either wild-type or 1440X CFTR. Fig. 8 shows the presence of both wild-type and 1440X CFTR at the plasma membrane and in a punctate distribution resembling coated pits/endocytic vesicles. There is also a considerable amount of perinuclear staining, probably representing newly synthesized CFTR in the biosynthetic pathway. Consistent with the biochemical and physiological data presented earlier, COS-7 cells expressing N-terminal deletion mutants show only perinuclear staining, suggesting retention within the biosynthetic pathway. This staining closely resembles the pattern seen in cells transfected with ΔF508 CFTR, which is retained in the endoplasmic reticulum and degraded (27).Untransfected cells show little staining, as seen in panel G. To avoid potential problems of mistrafficking like those we had observed with N-terminal deletion mutants, we continued our mutagenesis studies by utilizing mutagenesis of specific amino acids in the C-terminal tail. We focused our attention on amino acids 1424YDSIQKLL1431, a sequence that resembles the internalization signals in other proteins (24). Since most internalization signals described to date contain either a critical tyrosine or a dileucine/leucine-isoleucine, we decided to change tyrosine 1424 to alanine and examine its endocytic targeting. As seen in Fig. 9, CFTR Y1424A is expressed in comparable levels to wild type CFTR. However, a larger percentage of maturely glycosylated CFTR Y1424A is expressed at the cell surface compared to wild type (36.1% of total for Y1424A vs. 23.1% for wild-type; $p<0.01$; Fig. 10). In addition, the internalization rate of the Y1424A mutant is lower (0.12 vs. 0.27; $p<0.01$) than wild-type CFTR. These data suggest that tyrosine 1424 is critical for the proper internalization of CFTR from the cell surface.

The effect of a decrease in the internalization rate of CFTR would be to cause a large increase in the percentage of the total maturely glycosylated (C-form) CFTR biotinylated at the cell surface. We have in fact observed a substantial increase in the

Figure 9. Immunoprecipitation of tyrosine mutant CFTR proteins expressed in COS-7 cells. CFTR was immunoprecipitated from T84 cells and from COS-7 cells transfected with wild-type pGT-CFTR or the indicated tyrosine mutant CFTR cDNAs. Cells were assayed 48 h after transfection. pGT-CFTR (wt) and pGT-Y1424A are expressed at comparable levels. Mutation of Y38 to A seems to decrease the efficiency by which A/B form CFTR is converted to band C.

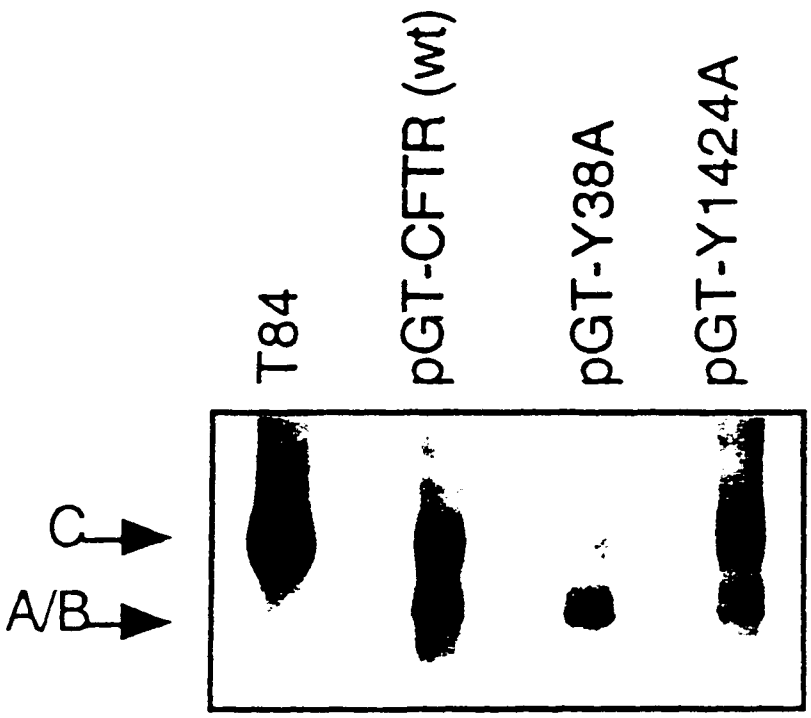


Figure 10. Kinetics of internalization for Y1424A CFTR are defective. pGT-CFTR (wt) and pGT-Y1424A CFTR were expressed in COS-7 cells and assayed as described above. The time course of internalization is shown in *a*. The steady state surface expression and internalization rate (fraction of cell surface CFTR removed in the first minute of 37°C incubation) for each construct is listed in *b*.

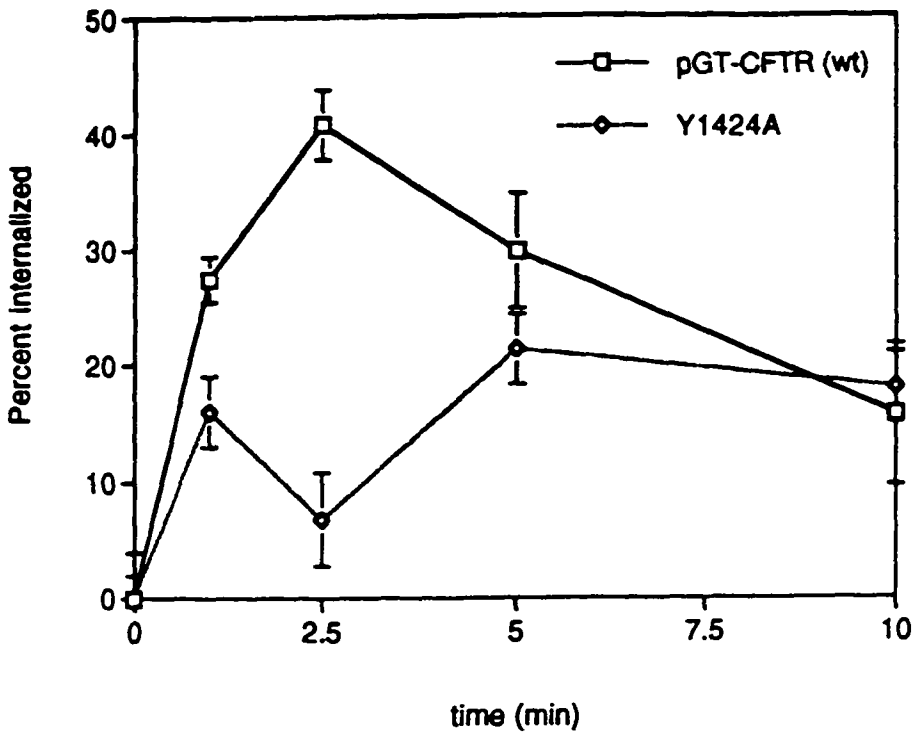
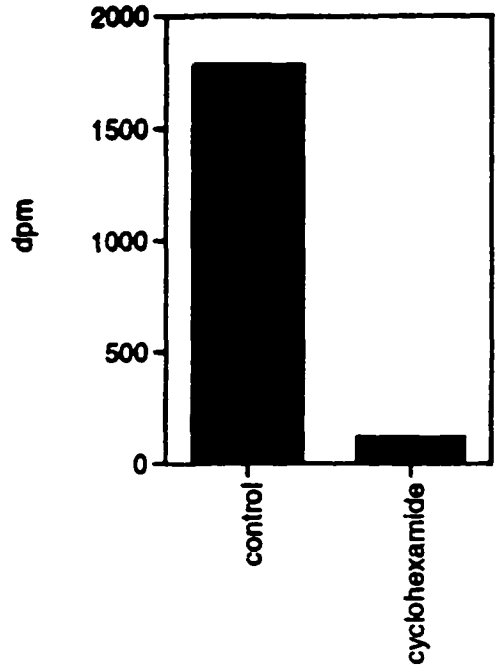
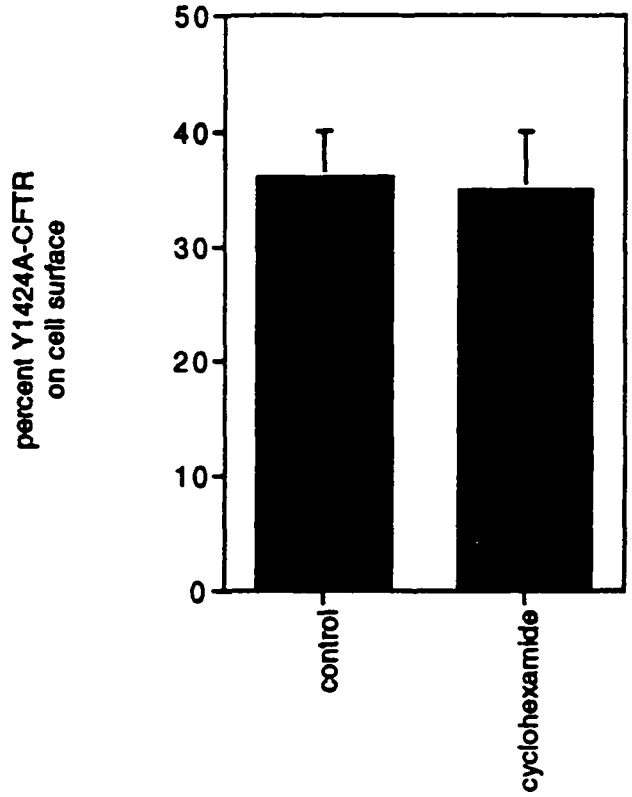


Figure 11. Inhibition of protein synthesis by cyclohexamide does not affect the amount of Y1424A-CFTR found at the cell surface in transfected COS-7 cells. (*A*) COS-7 cellswere incubated for 2 h in the presence of 15 mg/ml cyclohexamide, after which 15 μCi of ^{35}S-methionine was added to the media for 15 min. Cells were lysed, and 25 ml (5%) of the lysate was counted by liquid scintillation. Background dpm's were subtracted from each value. Incorporation of radiolabeled methionine is drastically inhibited by cyclohexamide. (*B*) COS-7 cells transfected with pGT-Y1424A-CFTR were incubated with 15 mg/ml cyclohexamide for 2 h. Cell surface labeling of Y1424A-CFTR was conducted as described in this chapter and previous chapters. Cyclohexamide had little effect on the relative amount of CFTR at the cell surface.

A



B



surface expression of Y1424A CFTR compared to wild type, but not the extent of change in distribution that was anticipated. Mutation of tyrosine 1424 increases surface expression of CFTR by 56% and decreases internalization rate by 54%. However, the majority (63%) of C-form CFTR still resides in an intracellular compartment. This could result from a population of cell surface CFTR not being labeled with biotin hydrazide or from the contribution of newly synthesized CFTR in the biosynthetic pathway on its way to the cell surface (see immunofluorescence). Alternatively, intracellular CFTR could reside in an intracellular compartment other than the rapidly recycling endocytic compartment. Biotinylation of radioiodinated cell surface proteins is nearly 100% complete (17), so the likelihood of the first possibility, cell surface CFTR not being biotinylated, although possible, seems unlikely.

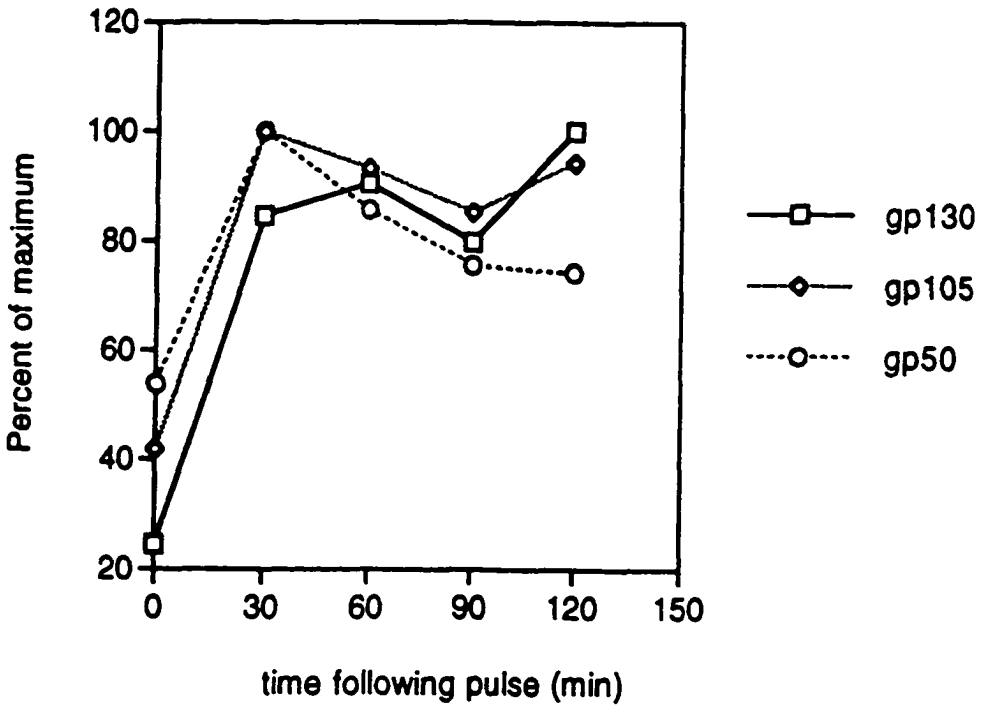
Biosynthetic trafficking of CFTR.

To estimate the contribution of CFTR within the biosynthetic pathway to the pool of maturely glycosylated intracellular CFTR, we first wanted to measure the kinetics of other proteins travelling through the biosynthetic pathway of COS-7 cells. Using a pulse chase procedure, we observed that proteins travel through the biosynthetic pathway rapidly. Following a 15 min pulse with ^{35}S-methionine, radiolabeled proteins appear at the cell surface within 30 min and reach a maximum within 60 min. Fig. 12*b* shows the kinetics of surface expression of several major glycoproteins. TCA precipitable counts in the media (Fig. 12*c*) also rise quickly following the pulse, again providing evidence that newly synthesized proteins destined for the cell surface or for constitutive secretion into the media move through the biosynthetic pathway quite rapidly.

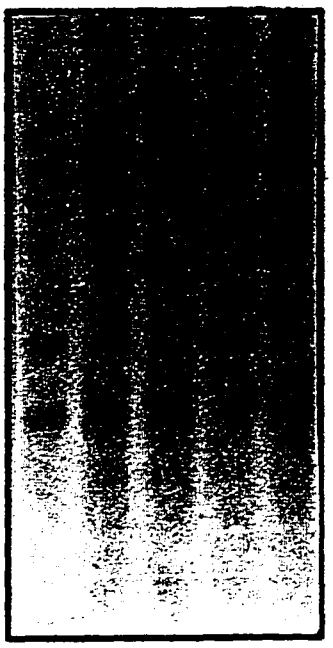
Our first approach was to inhibit protein synthesis in CFTR expressing COS-7 cells for a duration long enough to allow proteins to exit the biosynthetic pathway and then measure the size of the intracellular pool of CFTR. A 2-h pretreatment of COS-7 cells with 15 μg/ml cyclohexamide, a potent inhibitor of protein synthesis, can drastically inhibit the incorporation of radiolabeled methionine into cellular protein (Fig. 11) without

Figure 12. Appearance of labeled glycoproteins at the cell surface of COS-7 cells. COS-7 cells were labeled with ^{35}S-methionine for 15 min and chased for the indicated amounts of time. After chase, the cells were biotinylated with biotin hydrazide as described in chapter 1. Biotinylated proteins were isolated by incubation of cell lysates with immobilized streptavidin. Bound glycoproteins were eluted by boiling the beads in SDS-PAGE sample buffer. *(a).* Eluted samples were separated by SDS-PAGE, processed, dried, and exposed to film for three days. Phosphorimages were obtained for quantitation purposes. *(b).* The appearance at the cell surface of three selected glycoproteins is shown. The maximum value during the chase for each protein was set equal to 100%. Note that all proteins appear at the cell surface soon (within 30 min) after labeling.

b



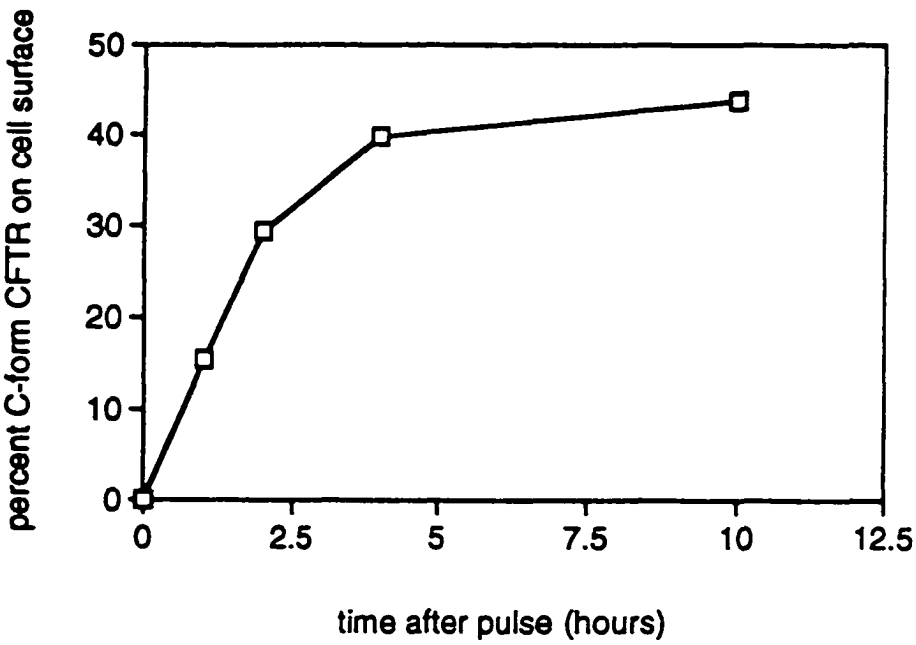
Time following pulse: 0 .5 1 1.5 2



causing detectable cell death or changes in morphology (data not shown). We therefore arrested protein synthesis with cyclohexamide in cells expressing CFTR to allow newly synthesized CFTR molecules to reach the cell surface, therefore depleting the biosynthetic pool of CFTR. As seen in Fig. 11*b*, pretreatment of cells for 2 h with 15 μg/ml cyclohexamide did not increase the amount of Y1424A CFTR C-form expressed at the cell surface. This finding initially implied that biosynthetic CFTR contributes little to the pool of intracellular C-form CFTR.

However, to more accurately assess the identity of the intracellular pool of CFTR, we needed to directly examine the movement of CFTR to the cell surface. COS-7 cells expressing pMT-CFTR (wild-type) were pulsed with 150 μCi of ^{35}S-methionine and chased for up to 10 h in the presence of excess unlabeled methionine. The cells were biotinylated at the end of the chase and CFTR was immunoprecipitated from the unbiotinylated and biotinylated fractions (Fig. 13). As has been reported previously (27), the translation rate of CFTR is slow, and the conversion of B-form CFTR to C-form is inefficient, as seen in the 0- and 1-h time points. C-form CFTR was detected 1 h following pulse, but most C-form CFTR at this time point was found in the unbound fraction, not at the cell surface. The percentage of biotinylated C-form did not approach the steady state value untill after 4 h following pulse, suggesting that CFTR is either trafficked from the trans-Golgi to the cell surface very slowly or that CFTR is targeted to some intracellular compartment after traversing the trans-Golgi and retained there before reaching the cell surface. Our data, as well as previous work (13) have suggsted that most glycoproteins travel through the biosynthetic pathway quickly (within 30 min) and do not show the same retention-like behavior as CFTR. Therefore, the intracellular pool of C-form CFTR probably results from the additional processes of targeting CFTR from the cell surface to endosomes in addition to a sequestration mechanism for intracellular CFTR somewhere between the Golgi apparatus and the plasma membrane.

Figure 13. Appearance of CFTR at the cell surface. COS-7 cells transfected with pMT-CFTR 48 h earlier were labeled with ^{35}S methionine for 30 min and then chased in the presence of excess methionine for 1, 2, 4, and 8 h. After chase, the cells were biotinylated with biotin-LC-hydrazide. Biotinylated proteins were separated using a monomeric avidin column, and CFTR was immunoprecipitated from the unbound and eluent fractions. Percent of total C-form CFTR found in the eluent fraction was calculated as described earlier following SDS-PAGE and phosphorimaging of the immunoprecipitates. CFTR appears at the cell surface more slowly than the proteins examined in Fig. 12.



Discussion

Utilizing a transient expression system in nonepithelial cells, we have been able to show that CFTR can be targeted to the endocytic pathway of nonpolarized cells in a fashion that is similar to its targeting in epithelial cell lines. The kinetics of internalization for CFTR in transfected COS-7 cells is comparable to the internalization rates of other rapidly endocytosed proteins. We have detected a slight difference in the internalization capacity of CFTR dependent upon expression level. Using a metallothinein promoter, the pMT vector can produce approximately five times more CFTR protein than the SV-40 based pGT1. We have also determined that cells transfected with pMT-CFTR have 45-50% of their CFTR on the cell surface, whereas cells transfected with pGT1-CFTR have only 25% of their CFTR on the cell surface. 16% ($K_e = 0.16$) of the pMT-CFTR can be internalized in the first minute, whereas 27% ($K_e = 0.27$) of the cell surface pGT-CFTR is internalized in 1 min. The higher internalization rate seen for pGT-CFTR actually represents the endocytosis of fewer CFTR molecules than that seen for pMT-CFTR due to the differences in expression level. The expression level of pMT-CFTR therefore may saturate the mechanisms for endocytosis/intracellular retention of the CFTR protein, although no data have been obtained regarding the number of CFTR molecules that can be included in a clathrin-coated endosome or pertaining to the nature of CFTR retention. Therefore, to avoid misinterpretations of data, all mutants have been compared to wild type in the context of the same expression vector and expression level.

The mutations that we have made in the N-terminal tail lead us to conclude that the trafficking and folding requirements of the N-terminal tail of CFTR are very sensitive to changes in structure. Deletion of even 11 amino acids from the N-terminal tail caused the mutant CFTR molecules to remain sensitive to endoglycosidase H and be retained in a perinuclear compartment. This finding has been confirmed by immunofluorescence, cell surface biotinylation, SPQ anaylsis, and carbohydrate digestion. Even the substitution of alanine for tyrosine 38 causes a decrease in the efficiency of maturation of CFTR to the

endo H resistant C form. Several missense mutations in the N-terminal tail of the CFTR have been reported in CF patients (28), but the function of the N-terminal tail of CFTR is still not known.

The role of the C-terminal tail of CFTR in endocytic targeting was studied by mutagenesis of regions containing putative internalization signals. Truncation of CFTR after amino acid 1440 caused no detectable decrease in internalization rate, suggesting that the last 40 amino acids of CFTR do not play an active role in the endocytic targeting of CFTR. This was not suprising, as the last 40 amino acids of CFTR do not contain tyrosine- or dileucine-based sequences. However, amino acids 1424-1431 (YDSIQKLL) resemble internalization signals found in other proteins. By mutating tyrosine 1424 to alanine, we were able to decrease the rate of CFTR endocytosis from the cell surface by 56%. This suggests that tyrosine 1424 is critical for the targeting of CFTR to endocytic vesicles.

GLUT4 is the only other type III membrane protein whose endocytosis has been studied in such a manner. Mutation of a carboxy terminal LL sequence in GLUT4 to either AA or AS causes a decrease in internalization rate of GLUT4 from the plasma membrane (7,25). The studies on CFTR reported here describe the first essential tyrosine involved in the endocytic targeting of a type III membrane protein. Further research will be necessary to determine if CFTR contains additional internalization signals.

In addition, the endocytic signal described here for CFTR is the first such signal in a protein expressed at the apical membrane of polarized epithelial cells. Little is known about the structural requirements for proteins entering apical coated pits versus basolateral coated pits. Gottleib et al. (10) have reported that apical endosomes may require a different cytoskeletal machinery than basolateral endosomes. Continuing studies of CFTR internalization mutants in polarized epithelial cells will reveal if the observations seen in COS-7 also hold true at the apical membrane of polarized epithelial cells.

While mutation of tyrosine 1424 caused a large decrease in the endocytic rate of CFTR, internalization was not completely inhibited. Several possibilities would explain this. If the entire sequence YDSIQKLL is necessary for internalization of CFTR, complete removal of endocytic activity may require the mutagenesis of other amino acids, potentially isoleucine 1427 and leucine 1430. Mutation of leucine 1430 alone does not appear to affect endocytosis, but might when combined with a mutation of tyrosine 1424. Alternatively, mutation of both Y1424 and I1427 (or some other amino acid) may be necessary to completely inhibit CFTR endocytosis. This is suggested by studies on the human transferrin receptor, in which mutation of both Y20 and F23 in the YTRF internalization signal is necessary to decrease endocytosis to the level of a "tailless" mutant (6). Lastly, there could be other internalization signals elsewhere in the CFTR molecule. The N-terminal tail may be a potential region for either internalization signals or sorting signals that may act to route CFTR to various intracellular compartments, a process suggested by the apparent retention of CFTR within some compartment before reaching the cell surface.

This mutagenesis approach should prove effective in producing a CFTR molecule found mostly at the cell surface, with little protein detected in intracellular organelles. In order to accurately assess the function of intracellular CFTR and its role in epithelial biology, it will be important to compare the physiology of cells expressing wild-type CFTR compared to cells expressing CFTR that is located only or predominantly at the cell surface.

Based on the work to date regarding CFTR function and epithelial biology, predictions can be made regarding the effects of reduced intracellular CFTR. The overall transepithelial chloride current will probably not change, since plasma membrane channel number does not appear to be limiting in cells expressing wild type CFTR (20). Likewise, effects of CFTR on Na^+ absorption and HCO_3^- secretion would probably not be effected, as these effects are proposed to be due to the action of CFTR at the plasma

membrane. The data regarding intraorganellar pH have not been able to provide any conclusive evidence for the role of CFTR in regulating vesicular acidification (2,4). This is not suprising, since all cells acidify intracellular organelles, such as endosomes, lysosomes, and TGN (8), and epithelial cells expressing CFTR to do not have lower pH values than nonepithelial cells (2,12). Sulfate incorporation into proteoglycans is regulated by the activity of the SO_4^-/Cl^- exchanger at the cell surface (14), and there is no proposed model for the contribution of intracellular CFTR to this mechanism.

What then is the role of intracellular CFTR? Recent work has implicated ATP transport by CFTR in the regulation of chloride channels in secretory epithelial cells (21). They propose that ATP secreted by CFTR can activate ORCCs by binding to an extracellular ATP receptor. However, it could be difficult for CFTR at the plasma membrane to raise the concentration of ATP in the extracellular space very efficiently due the vast extracellular volume. ATP secreted by CFTR into the lumen of a vesicle, whether it be an endosome or exocytic vesicle, however, could increase the ATP concentration of the luminal material very rapidly, considering the measured ATP current of 5.7 pS through CFTR (Cl- current through CFTR has been measured as 7-9 pS; reference 3).

We believe that mucin hydration and swelling is tightly regulated to electrolyte and water secretions by epithelial glands. Work on the heparan sulfate granules found in mast cells of the beige mouse has shown that changes in voltage across the proteoglycan granule can cause dramatic, reversible swelling of the heparan matrix, presumably by altering the counterion hydration radii of the cations found in the gel (1). Proper hydration of mucous granules is essential for the proper secretion of mucus from epithelial glands. If this hydration is a result of altered ion concentrations or osmotic gradients, the ideal site for these events would be in the secretory vesicle of gland cells. A small ion or water conductance in a secretory vesicle could cause a giant change in concentration of that ion or a change in osmotic volume. This may be the driving force

behind not only hydration and expansion of the mucus granule, but also swelling of the secretory vesicle and/or fusion with the target membrane.

The functional presence of CFTR in intracellular vesicles, therefore, may provide the cell with a mechanism for the rapid concentration of either chloride or other molecules within small, finite volumes. Such a rapid intracvesicular concentration could have hydrodynamic consequences in addition to biochemical or physiological effects. This function could be very distinct from the actions of CFTR at the plasma membrane and its roles in chloride and bicarbonate secretion and sodium absorption.

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SUMMARY

The transmembraneous topology, biochemical characteristics, and low expression levels of CFTR have necessitated novel approaches to understanding the subcellular localization and trafficking of the CFTR protein. Using a carbohydrate-specific biotinylation protocol, we have been able to compare the relative amount of CFTR at the plasma membrane compared to intracellular organelles. Using a modification of this protocol, we were then able to ask questions regarding the dynamics of CFTR targeting to the cell surface and from the cell surface to endocytic compartments. The work presented here is the first description of a quantitative measurement of the amount of CFTR in these various compartment.

We first discovered that CFTR is present both at the cell surface and in intracellular compartments, a finding that was intriguing, but not completely unexpected. What was somewhat unexpected, however, was that stimulation of at least T84 cells with agonists that raise cAMP levels did not cause a large movement of CFTR from intracellular locations to the cell surface. It was observed that the intracellular pool of CFTR was not a latent population for stimulus-dependent insertion into the plasma membrane, but the result of rapid internalization of CFTR from the cell surface into the endocytic pathway.

Activation of CFTR chloride channel activity was capable of transiently inhibiting the endocytosis of CFTR, and this inhibition required the chloride channel function of CFTR, as inhibition was not observed for G551D-CFTR or when chloride ions were depleted. The physiological significance of such a regulation of endocytosis has not been determined, but it is our hypothesis that CFTR may regulate vesicle trafficking in more

than one intracellular compartment, and that its function in endosomes may be an example as to how CFTR might regulate the membrane trafficking of other vesicles in the regulated secretory pathway involved in mucus secretion. This hypothesis is supported by the observation in transfected COS-7 cells that CFTR appears to be routed to some retention or sequestration compartment before it reaches the cell surface. CFTR endocytosed from the plasma membrane may well reach that same compartment. A molecular approach has begun with the goal of dissecting the trafficking mechanisms related to CFTR distribution.

This molecular approach has proven initially successful in finding a tyrosine residue in the C-terminal tail of CFTR that appears to be critical for the efficient endocytosis of CFTR from the plasma membrane. This is the first such description of a tyrosine crucial for endocytosis in a type III membrane protein in addition to being the first study finding specific amino acids required for the efficient internalization of an apically expressed glycoprotein. The finding that an ion channel can contain similar sorting motifs as type I and type II receptors may prove valuable in the future. It is our hope that this continuing examination of the targeting of CFTR will further our knowledge of secretory epithelial biology in addition to accelerating our understanding of the pathogenesis of cystic fibrosis.

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