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Rapid endocytosis of the 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. 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 ≈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. However, cAMP did not affect the internalization of G551D CFTR, a naturally occurring Gly-551 → Asp 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.**

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 either is 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 (wt) 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 used. 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 by using immobilized monomeric avidin. Because of its extremely low copy number (12), it was necessary to detect CFTR by immunoprecipitation followed by *in vitro* phosphorylation with cAMP-dependent protein kinase and [γ^{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

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Abbreviations: CF: cystic fibrosis; CFTR: cystic fibrosis transmembrane conductance regulator; 8-CPT-cAMP, 8-(4-chlorophenylthio)-cAMP; wt, wild type.

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modified Eagle's medium with Ham's nutrient mixture F-12 (GIBCO) supplemented with 5% (vol/vol) fetal bovine serum (Hyclone). Cystic fibrosis pancreatic adenocarcinoma cells (CFPAC-1; ref. 15) and CFPAC-1 cells transfected with the PLJ retrovirus alone (CFPAC-1 PLJ), PLJ carrying wt CFTR (CFPAC-1 PLJ-CFTR), or with PLJ carrying mutant G551D CFTR (CFPAC-1 PLJ-G551D CFTR) were obtained from Raymond A. Frizzell (University of Alabama at Birmingham; ref. 16) 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 were 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 on day 4 of culture and were used between days 6 and 10. Chloride depletion studies (17) were conducted after a 1-hr incubation in gluconate buffer containing 135 mM sodium gluconate, 2.4 mM K_2HPO_4, 0.6 mM KH_2PO_4, 10 mM Hepes, 25 mM glucose, 0.1 mM calcium gluconate, and 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.* (18) for biotin hydrazide labeling except that 2 mM biotin-LC-hydrazide (Pierce) 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. These labeled cells were extensively washed with PBS c/m and lysed in 250 μl of lysis buffer (150 mM NaCl/20 mM Hepes/1 mM EDTA/1% Nonidet P-40/100 μg each of aprotinin and leupeptin per ml/2 mM phenylmethylsulfonyl fluoride). To measure internalization of glycoproteins, the above labeling protocol was conducted with the addition of a 37°C incubation in prewarmed medium after periodate oxidation and before biotinylation, both of which were conducted at 4°C. Cells were then lysed, and proteins were isolated as described below.

Separation and Isolation of Biotinylated Proteins. A 250-μl column of immobilized monomeric avidin (Pierce) was prepared according to the manufacturer's instructions and then equilibrated with PBS containing 1% Nonidet P-40 (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 described (12) by using a monoclonal antibody to the C terminus of CFTR generously supplied by Seng Cheng (Genzyme). The immunoprecipitated CFTR was phosphorylated with cAMP-dependent protein kinase (Promega) and 10 μCi (370 kBq) of [γ^{32}P]ATP (3000 Ci/mmol; DuPont/NEN), followed by SDS/PAGE analysis 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 analyzed with a Molecular Dynamics PhosphorImager. Percentages of cell surface CFTR were calculated by dividing the number of cpm 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 of fluorescein-dextran (10 kDa) or Texas Red-dextran (10 kDa) per ml applied apically for 5 min at 37°C in the presence or absence of 10 μM forskolin. In addition, some monolayers were chloride-depleted in gluconate buffer as described above. The same buffer was used for all subsequent labeling and internalization steps. After uptake, the cell monolayers were washed extensively at 4°C, and label was extracted with cold 100% ethanol (19). Fluorescence was measured by using a fluorimeter with excitation/emission 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 of labeled dextran per ml. 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, 18). Therefore, endocytosis of cell surface CFTR could be studied by including a 37°C incubation after periodate oxidation but before 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, whereas 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, ≈50% of the maturely glycosylated CFTR was detected at the cell surface (Fig. 1*A*), as 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 is thus comparable to those of proteins that recycle through a clathrin-mediated endocytic pathway, such as the low density lipoprotein (20) and mannose 6-phosphate receptors (21). 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 with 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 by using a parallel protocol (Fig. 1*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. After SDS/PAGE, total proteins from the unbound and biotin eluent fractions were electrophoretically transferred to polyvinylidene difluoride, 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 after 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

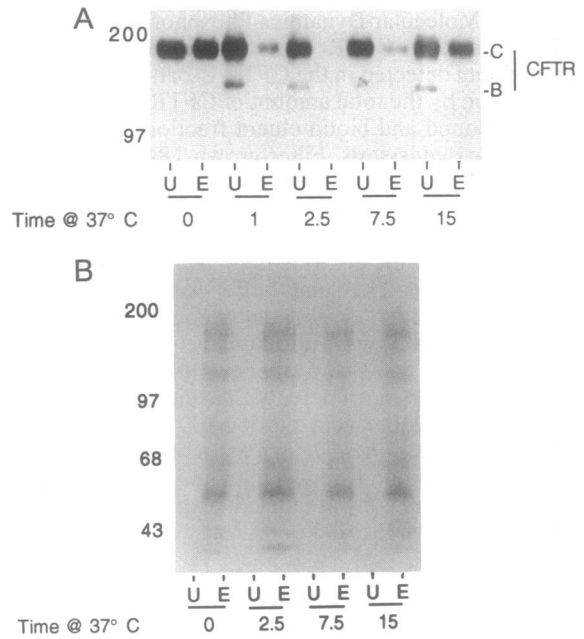

FIG. 1. (*A*) CFTR is internalized from the cell surface of T84 cells. Cell surface labeling of CFTR from confluent monolayers of T84 human colon carcinoma cells was conducted as described (7) except that individual monolayers were incubated at 37°C for the indicated time periods (in minutes) after periodate treatment and before 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 (lanes U) and the biotin eluent (lanes E) proteins after 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*) 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 *A* were separated by SDS/PAGE, electrophoretically transferred to a polyvinylidene difluoride (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 after 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).

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 (20, 21), suggest that a discrete structural motif directs CFTR to clathrin-coated pits and endocytic vesicles (22).

In cells expressing CFTR, increasing cAMP has been shown to decrease fluid-phase endocytosis (14, 19), 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 after 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 medium during the 37°C incubation after 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. This effect appeared to be due to elevated levels of cAMP, since dideoxyforskolin did not slow CFTR endocytosis, while both dibutyryl cAMP and 8-(4-chlorophenylthio)-cAMP (8-CPT-cAMP) inhibited its internalization (Fig. 2*B*).

Our previous studies (7) showed only a slight increase in the amount of CFTR on the cell surface after 10 min of forskolin stimulation. After having established the rapidity of CFTR internalization, the steady-state measurements were repeated with T84 cells after a 1-min incubation at 37°C in the

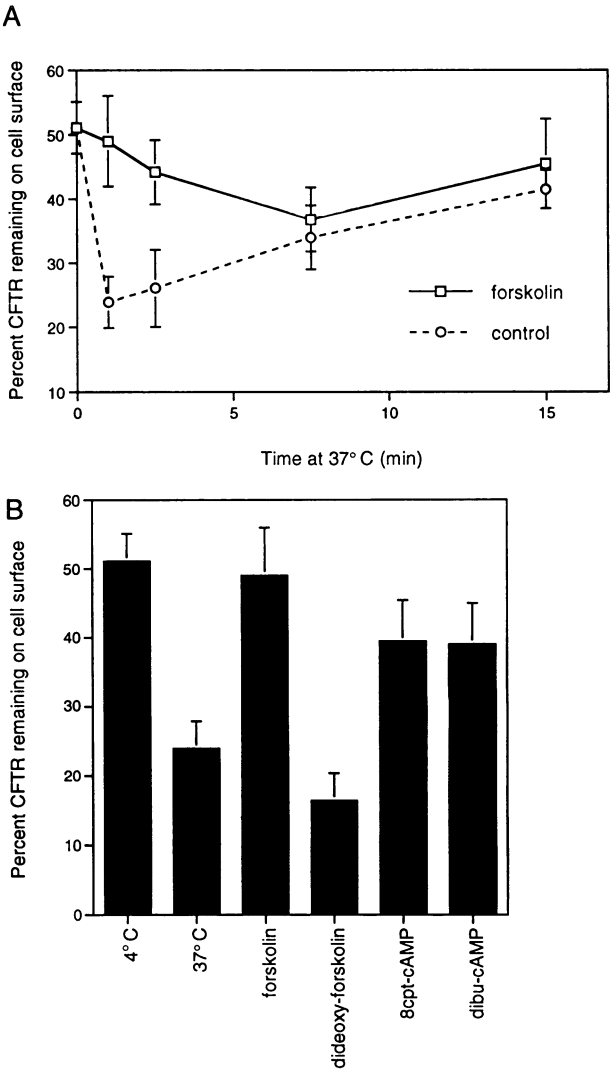

FIG. 2. Forskolin inhibits internalization of CFTR. (*A*) Quantitation of CFTR recycling. Multiple experiments were conducted as described in Fig. 1, either with (□; $n = 9$) or without (○; $n = 16$) 10 μM forskolin being present during the 37°C incubation. Data represent the percentage of total mature CFTR detected in the biotin eluent fraction by phosphorimaging. The means ± SEM 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 after 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 ($n = 9$), 10 μM 1,9-dideoxyforskolin ($n = 11$), 500 μM 8-CPT-cAMP (8cpt-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.

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 (15) that had been stably transfected with either the PLJ vector alone or with the vector containing wt or mutated CFTR (16). The mutation examined was the naturally occurring substitution of aspartate for glycine at amino acid position 551 (G551D) (23). This protein shows defective ion-channel activity in response to cAMP (6) but displays a mature pattern of glycosylation (24). wt CFTR and G551D CFTR were expressed at comparable levels in the transfected CFPAC-1 cells (Fig. 3*A*). When expressed in CFPAC-1 cells, both wt and G551D CFTR were detected at the cell surface and internalized with high efficiency (Fig. 3*B*). However, while endocytosis of wt 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.

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 (17). 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, 19). 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-depleted cells (Fig. 4*B*). As seen previously (14, 19), 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.

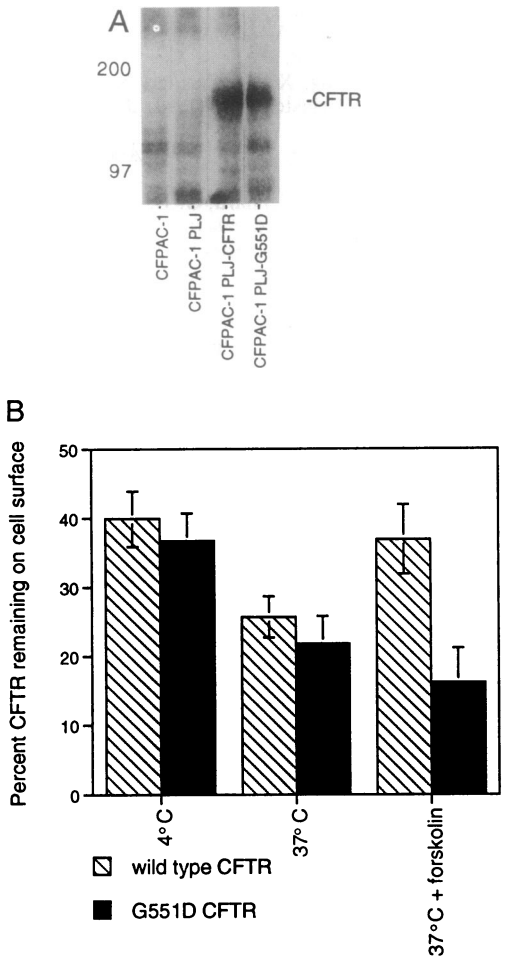

FIG. 3. (*A*) Immunoprecipitation of CFTR from stably transfected CFPAC-1 cells. CFPAC-1 (lane 1), CFPAC-1 PLJ (lane 2), CFPAC-1 PLJ-CFTR (lane 3), and CFPAC-1 PLJ-G551D CFTR (lane 4) cells were lysed, immunoprecipitated with a monoclonal antibody against the C terminus of CFTR, and phosphorylated with [γ-^{32}P]ATP and cAMP-dependent protein kinase as described. Expression of CFTR in CFPAC-1 and CFPAC-1 PLJ could not be detected. wt and G551D mutant cell lines expressed CFTR at comparable levels, ≈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 wt 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 wt CFTR internalization ($P < 0.05$; $n = 6$) but did not affect internalization of G551D CFTR ($n = 6$).

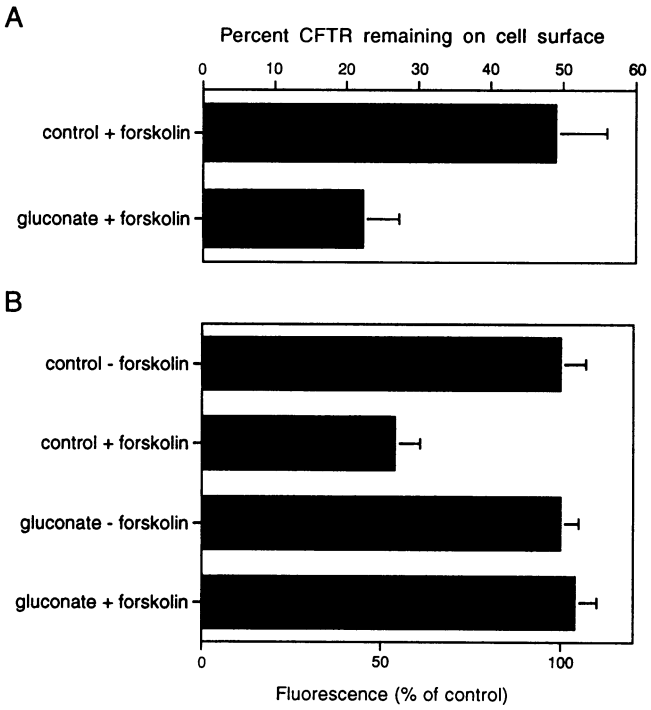

FIG. 4. (*A*) Internalization of CFTR is not inhibited by forskolin when cells are depleted of chloride. T84 cells were chloride-depleted by incubation with gluconate as described in text. Internalization of periodate-treated CFTR after a 1-min incubation at 37°C before biotinylation was determined as 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.

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 (20, 21). Increasing cellular cAMP levels, which activates CFTR chloride channel activity, inhibits the internalization of wt CFTR, but does not affect the rapid endocytosis of G551D CFTR, suggesting that this inhibition 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 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 recycling 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.

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 Snider and Rogers (28) and Duncan and Kornfeld (29) have determined that proteins internalized from the cell surface are exposed to a sialytransferase in the trans-Golgi network (TGN) in about 1 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 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. 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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