Hyperacidification of Cellubrevin Endocytic Compartments and Defective Endosomal Recycling in Cystic Fibrosis Respiratory Epithelial Cells*

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The cystic fibrosis transmembrane conductance regulator (CFTR), which is aberrant in patients with cystic fibrosis, normally functions both as a chloride channel and as a pleiotropic regulator of other ion transporters. Here we show, by ratioimetric imaging with luminally exposed pH-sensitive green fluorescent protein, that CFTR affects the pH of cellubrevin-labeled endosomal organelles resulting in hyperacidification of these compartments in cystic fibrosis lung epithelial cells. The excessive acidification of intracellular organelles was corrected with low concentrations of weak base. Studies with proton ATPase and sodium channel inhibitors showed that the increased acidification was dependent on proton pump activity and sodium transport. These observations implicate sodium efflux in the pH homeostasis of a subset of endocytic organelles and indicate that a dysfunctional CFTR in cystic fibrosis leads to organelar hyperacidification in lung epithelial cells because of a loss of CFTR inhibitory effects on sodium transport. Furthermore, recycling of transferrin receptor was altered in CFTR mutant cells, suggesting a previously unrecognized cellular defect in cystic fibrosis, which may have functional consequences for the receptors on the plasma membrane or within endosomal compartments.

The cystic fibrosis transmembrane conductance regulator (CFTR) functions as an apical membrane chloride channel (1). Different CFTR mutations causing cystic fibrosis (CF) affect the processing, intracellular localization, and function of the corresponding protein (2, 3). The most common mutant form of CFTR in CF, ΔF508 CFTR, does not enter the organelles of the secretory pathway and is not delivered to the plasma membrane as it is not properly folded and remains trapped in the endoplasmic reticulum. Mutations in CFTR result in reduced apical chloride transport but also have pleiotropic effects on the function of other ion transporters including the amiloride-sensitive epithelial sodium channel (ENaC) (4, 5), outwardly rectifying chloride channels (6, 7), the Na+/H+ exchanger via EBP50 (ezrin-binding protein), Na+/H+ exchanger regulatory factor (8), bicarbonate conductance (9, 10), and aquaporin 3 (5).

It has been proposed that CFTR also plays a role in facilitating acidification of intracellular compartments, such as endosomes, by providing anions (Cl−) and maintaining charge neutrality as protons are pumped into the lumen of these organelles (11). According to this proposal, a loss of CFTR and chloride conductance would result in increased pH (11, 12). However, repeated studies have failed to detect alkalinization of intracellular compartments in CF (13–17).

It has been shown that CFTR is present in endosomes of stably transfected Swiss 3T3 and T84 cells, which normally express CFTR (15). The absence of CFTR on the plasma membrane and organelles of the secretory pathway, which communicate with the endocytic pathway, prompted us to re-examine potential consequences in CF on the pH of endocytic organelles by specific targeting of pH-sensitive GFP (18) to a defined endocytic compartment. Here we show that cellubrevin-labeled endosomes are hyperacidified in CF lung epithelial cells and that the pH of the recycling endosome depends on CFTR and its effects on sodium transport. In addition, we show physiological defects in the function of the endocytic pathway in CF, as recycling of receptor-mediated endocytic tracers (transferrin) is affected in CF lung epithelial cells.

EXPERIMENTAL PROCEDURES

Cells and Tissue Culture—CFT1 (19, 20) is a cell line derived from the tracheal epithelium of a CF patient homozygous for the most common CFTR ΔF508 mutation. Stably transfected derivatives of CFT1 were the following: CFT1-LCF5N, expressing the wild-type CFTR gene; CFT1-Δ508, transfected with ΔF508 mutant CFTR gene; and CFT1-LC3, the vector-transfected control cells. CFT1 and derivative cells were grown in F12 media (Invitrogen) supplemented with 10 µg/ml insulin, 1 µM hydrocortisone, 1 nM triiodothyronine, 10 ng/ml cholera toxin (Sigma), 3.75 µg/ml endothelial cell growth supplement, 25 ng/ml epidermal growth factor, and 5 µg/ml transferrin (Collaborative Research Inc., Bedford, MA) (19). IB3-1 is a human bronchial epithelial cell line derived from a CF patient with a ΔF508/W1282X CFTR mutant genotype (21). C38 and S9 are derivatives of IB3-1 cells and are stably transfected with a functional CFTR corrected for chloride conductance (22). The physiological levels of expression of CFTR and its functionality have been established previously for C38 cells (22). The cells were

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Transfections—Cellubrevin-pHluorin GFP and glycosylphosphatidylinositol (GPI)-pHluorin GFP DNA constructs were from J. Rothman (18). IB3-1 cells and their derivatives were seeded at 1000 cells/ml on 25-mm coverslips in 6-well plates. Cells were transfected with 1 μg/ml DNA using Lipofectin (Invitrogen) for 6 h at 37 °C, 5% CO2, CPT1 cells and their derivatives were seeded at 1000 cells/ml on 25-mm coverslips in 6-well plates and grown in the medium without cholerin toxin. Cells were transfected with GenePorter (Gene Therapy Systems, San Diego, CA) with 2.5 μg/ml DNA for 4 h at 37 °C, 5% CO2. Transfected cells were mounted in a perfusion chamber after 48 h of expression (Harvard Instruments, Holliston, MA) set at 37 °C for live microscopy or otherwise processed for colocalization studies.

Fluorescence Microscopy and pH Measurements—Fluorescence microscopy was carried out using an Olympus IX-70 microscope and Olympus KAI-100 CCD camera (LSR, Olympus, Melville, NY). The ratio of emission at 508 nm upon excitation at 410 versus 470 nm was obtained using the previously described (18) filter sets (Chroma Technology Corp., Brattleboro, VT) mounted in a Sutter filter wheel (Sutter Instruments, Novato, CA) and controlled by the Merlin program (version 1.18, LSR, Olympus, Melville, NY). For the pH standard curve, two types of calibration were carried out. (i) Cells transfected with GPI-pHluorin GFP were mounted in a perfusion chamber in a perfusion chamber in a medium containing buffer A (25 mM HEPES (pH changing from 7.4 to 5.5), 119 mM NaCl, 2.5 mM KCl, 2 mM CaCl2, 2 mM MgCl2, 30 mM glucose) at 37 °C. Fluorescence images were taken upon excitation at 410 and 470 nm (six consecutive exposures). Three regions of interest were selected, and the standard curve was plotted as averaged 410/470 ratio values for a given buffer pH. (ii) At the end of experiments, the pH gradient was collapsed by incubating cells in 10% sucrose for 10 min at room temperature, mounted with PermaFluor (Shandon, Pittsburgh, PA), and examined by fluorescence microscopy using a 570/20 excitation filter and a dichroic mirror/Emitter cube set 8300 (Chroma Technology Corp.). For localization studies with co-transfected with 0.5 μg of cellubrevin-pHluorin GFP and Myc-tagged α2,6-sialyltransferase DNA using 10 μl of Lipofectin. After 48 h of expression, cells were fixed with 3.7% paraformaldehyde and permeabilized with 0.2% saponin for 5 min. Mouse monoclonal antibody (9E10) against c-myc (Santa Cruz Biotechnology, Santa Cruz, CA) was used as a secondary antibody conjugated to Alexa 568 (Molecular Probes). Glass slides were mounted using PermaFluor and analyzed by fluorescence microscopy using a 570/20 excitation filter and a dichroic mirror/Emitter cube set 8300. For localization studies with dextran-Texas Red, cellubrevin-pHluorin-transfected IB3-1 cells and IB3-1 derivatives were incubated with 10 μl of dextran-Texas Red followed by three washes. Cells were either fixed or live sequences were carried out immediately after removal of dextran-Texas Red every 10 s for 30 min using a monochromator excitation light source and emission filter sets on a microscope and camera controlled by TILLvisTRAC, version 3.3 (T.I.L.L. Vision, GMBH). For localization studies of cellubrevin-pHluorin GFP and EEA-1, IB3-1 and derivative cells were transfected with cellubrevin-pHluorin GFP. EEA1 was visualized using primary human anti-EEA1 antibody (Transduction Laboratories, Lexington, KY) and secondary Alexa 488-conjugated goat anti-mouse antibody (Molecular Probes). Glass slides were mounted using PermaFluor and analyzed by fluorescence microscopy using a 570/20 excitation filter and a dichroic mirror/Emitter cube set 8300.

RESULTS

Expression and Localization of Cellubrevin- and GPI-pHluorin GFP Chimeras in CF and CFTR-corrected Bronchial Epithelial Cells—In this study, we employed the recently developed pH-sensitive GFP (pHluorin GFP) system for ratiometric determination of the luminal pH in intracellular organelles (19). Two pHluorin GFP fusion constructs were used (Fig. 1, A and B). IB3-1 cells were co-transfected with 0.5 μg of cellubrevin-pHluorin GFP and Myc-tagged α2,6-sialyltransferase DNA using 10 μl of Lipofectin. After 48 h of expression, cells were fixed with 3.7% paraformaldehyde and permeabilized with 0.2% saponin for 5 min. Mouse monoclonal antibody (9E10) against c-myc (Santa Cruz Biotechnology, Santa Cruz, CA) was used as a secondary antibody conjugated to Alexa 568 (Molecular Probes). Glass slides were mounted using PermaFluor and analyzed by fluorescence microscopy using a 570/20 excitation filter and a dichroic mirror/Emitter cube set 8300. For localization studies with dextran-Texas Red, cellubrevin-pHluorin-transfected IB3-1 cells and IB3-1 derivatives were incubated with 10 μl of dextran-Texas Red followed by three washes. Cells were either fixed or live sequences were carried out immediately after removal of dextran-Texas Red every 10 s for 30 min using a monochromator excitation light source and emission filter sets on a microscope and camera controlled by TILLvisTRAC, version 3.3 (T.I.L.L. Vision, GMBH). For localization studies of cellubrevin-pHluorin GFP and EEA-1, IB3-1 and derivative cells were transfected with cellubrevin-pHluorin GFP. EEA1 was visualized using primary human anti-EEA1 antibody (Transduction Laboratories, Lexington, KY) and secondary Alexa 488-conjugated goat anti-mouse antibody (Molecular Probes). Glass slides were mounted using PermaFluor and analyzed by fluorescence microscopy using a 570/20 excitation filter and a dichroic mirror/Emitter cube set 8300.

Horseradish Peroxidase (HRP) Uptake and Fluid Phase Endocytosis—The assay was carried out according to Li and co-workers (26, 27). Cells were seeded in 6-well plates at 5 × 104/well at 24 h prior to assay. After being washed in serum-free DMEM, cells were incubated for 15 min at 37 °C with either DME M or 100 ng/ml wortmannin in DME M. After washing, cells were incubated with 5 mg/ml HRP in DME M, 0.2% BSA for 60 min at 37 °C. Uptake was stopped by washing with 4 °C phosphate-buffered saline, 0.2% BSA. Cells were lysed in phosphate-buffered saline, 0.1% Triton X-100. Lysate was added to O-phenylenediamine solution (HRP substrate) in a 96-well plate and incubated at room temperature for 5 min. Reaction was stopped by addition of 1 M H2SO4, and A540 was measured using a spectrophotometer (Shimadzu UV-1601, Shimadzu, Columbia, MD). Protein concentration of the lysate was determined by BCA reaction (Pierce), and uptake was expressed as A540/mg protein.

Statistics—All statistical analyses were carried out using Fisher's Protected LSD post hoc test (analysis of variance) (SuperANOVA v1.11, Abacus Concepts, Inc., Berkeley, CA).
have been used as standard cell lines to model the effects of CFTR (6, 21, 22, 24, 28).

The plasma membrane localization of GPI-pHluorin GFP was demonstrated by responsiveness of GFP fluorescence to pH changes of the external buffer. Fig. 1, a–d, displays the fluorescence appearance of GPI-pHluorin GFP at pH 7.4 and 5.5. The cells expressing GPI-pHluorin GFP were used to generate a standard curve (Fig. 1i). All cells showed identical dependence of the GPI-pHluorin GFP fluorescence on pH of the external buffer. In addition to the plasma membrane labeling, as evidenced in Fig. 1, a–d, all cells transfected with GPI-pHluorin GFP showed a perinuclear fluorescence corresponding to a lipid raft recycling compartment, recently described by Lipincott-Schwartz and colleagues (29). Based on our observations, this compartment responds to external buffer pH (Fig. 1, a–d), most likely because of the previously described rapid cycling of these membranes in constant communication with plasma membrane (29). GPI-pHluorin GFP fluorescence was not dependent on changes in concentration of other ions in the medium (e.g., sodium; data not shown). There were no differences in fluorescence ratios obtained with GPI-pHluorin GFP in IB3-1, C38, and S9 cells.

Localization of cellubrevin-pHluorin GFP was examined in both CF and CFTR-corrected cells by fluorescence microscopy using EEA1 antibodies, Texas Red-conjugated endocytic tracers. First, the cells were allowed to endocytose fluorescent transferrin, which was followed by chasing this marker of receptor-mediated endocytosis into the pericentriolar/paranuclear recycling compartment. This resulted in a significant colocalization of transferrin with cellubrevin-pHluorin GFP fluorescence in the transfected cells as evidenced by a similar overall organelar distribution (Fig. 2, a–c, IB3-1 cells; d–f, CFTR-corrected S9 cells). Both the CF and CFTR-corrected cells showed similar overall organelar distribution. The colocalization of cellubrevin-pHluorin GFP and transferrin was not absolute in either cell line, as some of the cellubrevin- and transferrin-labeled profiles did not fully overlap, consistent with previous observations of strong but incomplete colocalization between transferrin and cellubrevin labeled vesicles (30).

The most complete overlap was seen in the pericentriolar recycling endosomal compartment, strongly labeled by fluorescent transferrin, which was also the site of the majority of cellubrevin-pHluorin GFP labeled intracellular organelles. In further support of the overlap between cellubrevin and the recycling endosomal compartment, the treatment of cells with nocodazole, which causes depolymerization of microtubules and dispersion of the recycling endosome, resulted in redistribution of both transferrin and cellubrevin-pHluorin GFP fluorescence with a preservation of the significant overlap between the two markers (Fig. 2, g–i). These observations suggest that cellubrevin-pHluorin GFP is localized in human bronchial epithelial cells with similar distribution in both CF and CFTR-corrected cells in the endosomal recycling compartment equivalent to what has been observed in several model cell lines (30–33). Importantly, CFTR partially overlapped with the recycling endosome in bronchial epithelial cells (Fig. 2, j–l). The colocalization of CFTR-GFP and transferrin was similar to the one observed with cellubrevin-pHluorin GFP and transferrin (Fig. 2, a–f).

The cellubrevin-pHluorin GFP probe did not colocalize with the early endosomal marker EEA1, although the large EEA1-positive profiles and the cellubrevin recycling endosome appeared to be closely apposed (Fig. 3, a–c). Treatment of cells with nocodazole confirmed that cellubrevin-pHluorin GFP and EEA1 were in distinct compartments (Fig. 3, d–f). The organelar distribution of EEA1 and cellubrevin compartments was
similar in CFTR-corrected (Fig. 3, a–c) and CF cells (Fig. 3, insets in a–c). Cellubrevin-pHluorin GFP did not colocalize with peripheral endocytic organelles labeled with the fluid phase tracer dextran-Texas Red in fixed cells (data not shown) and in live cells monitored by time lapse microscopy (Fig. 3, g–l). Cellubrevin-pHluorin GFP was also tightly apposed to the a2,6-sialyltransferase, as revealed by immunofluorescence (Fig. 4), but remained localized distinctly from the TGN marker. There were no differences in localization of cellubrevin-pHluorin GFP and EEA1 visualization of Myc-tagged cellubrevin-GFP (18) were used to determine the pH of cellubrevin-containing endosomal compartments. Fig. 1, e–h, illustrates the difference in fluorescence between cellubrevin-pHluorin GFP-transfected IB3-1 and C38 cells upon illumination at 410 versus 470 nm. The apparent pH of cellubrevin-containing endosomes was 6.7 ± 0.1 (mean ± S.E., n = 15) for the CFTR-corrected C38 and 6.7 ± 0.1 (mean ± S.E., n = 32) for S9 cells compared with the apparent pH of IB3-1 CFTR mutant cells, which was 6.2 ± 0.1 (mean ±

**FIG. 3.** Fluorescence microscopy analysis of cellubrevin-pHluorin GFP and EEA1 localization. IB3-1 and derivative cells were transfected with cellubrevin-pHluorin GFP. EEA1 was visualized using primary human anti-EEA1 antibody and secondary Alexa 568 conjugated antibody. a–c, CFTR-corrected C38 cell (insets, CF cells IB3-1): a, GFP fluorescence; b, EEA1 immunofluorescence; e, merged images a and b, d–f, C38 cells as described in a–c upon treatment with nocodazole; d, GFP fluorescence; e, EEA1 visualization; f, merged images a and b. g–l, time lapse recording of cellubrevin-pHluorin GFP labeled and dextran-Texas Red-containing vesicles. Shown is a portion of a live IB3-1 cell transfected with cellubrevin GFP (green) following endocytosis of the fluid phase endocytic tracer dextran-Texas Red. f, dextran-Texas Red-labeled vesicle; 2, cellubrevin-pHluorin GFP vesicle.

**FIG. 4.** Close apposition of cellubrevin-pHluorin GFP recycling endosomes and TGN in human bronchial epithelial cells. TGN was revealed by Myc-tagged α2,6-sialyltransferase Stα, IB3-1 and S9 cells were co-transfected with cellubrevin-pHluorin GFP and myc tagged α2,6-sialyltransferase Stα, expressing constructs. a–d, main panels, S9 (CFTR-corrected cell). b–d, insets, IB3-1 (mutant CFTR cell); a, phase contrast; b, GFP fluorescence; c, immunofluorescent visualization of Myc-tagged α2,6-sialyltransferase, Stα, using anti-Myc antibody and secondary Alexa 568-conjugated antibody (red fluorescence); d, merged images b and c.

S.E., n = 19) (Table I). Thus, cellubrevin-labeled compartments in CF mutant cells show hyperacidification of 0.5 pH unit (p = 0.0001). The pH of the cellubrevin-labeled compartments remained unaltered regardless of whether the cells were subconfluent or confluent, retaining the difference in pH between CF and CFTR-corrected cells (n = 66).

The observation that cellubrevin-labeled endosomes are hyperacidified in CF cells was confirmed using another well characterized CF cell line, CFT1 (19, 20), derived from the tracheal epithelium of a CF patient homozygous for the ΔF508 CFTR mutation. CFT1 and its stably transfected derivatives, CFT1-LCFSN (expressing the wild-type CFTR gene), CFT1-ΔF508 (expressing the ΔF508 mutant CFTR gene), and CFT1-LC3 (vector control), were transiently transfected with cellubrevin-pHluorin GFP constructs. The cellubrevin-pHluorin GFP-labeled compartment in the CFTR-corrected variant CFT1-LCFSN had an apparent pH of 6.6 ± 0.3 compared with pH 6.1 ± 0.1 in CFT1, pH 6.2 ± 0.1 in CFT1-ΔF508, and pH 6.0 ± 0.1 in CFT1-LC3 cells (Table I). Thus CF tracheal epithelial cells, similar to bronchial epithelial cells, had hyperacidified cellubrevin endosomal compartments. As in the case of IB3-1, C38, and S9 cells, the differences in cellubrevin endosomal pH between CFT1, CFT1-ΔF508, CFT1-LC3, and CFT1-LCFSN (CFTR-corrected) cells remained unaltered whether cells were confluent or not (n = 63).

Additional experiments were also carried out to confirm these findings. Hyperacidification of cellubrevin containing endosomes in CF cells was corrected by the addition of low concentrations of the weak base NH4Cl (0.1 mM) bringing pH to the values matching those observed in CFTR-corrected cells (Fig. 5a). Based on these experiments, we conclude that cellubrevin-labeled compartments are hyperacidified by 0.5 pH units in CF lung epithelial cells. This phenomenon was due to defective CFTR function and trafficking, as expression of a functional CFTR, but not that of ΔF508 CFTR, restored the normal pH in CFT1 cells (Table I). To confirm this notion, we treated mutant CFTR IB3-1 cells by growing them at a permissive temperature (26 °C), which allows CFTR folding and trafficking (23), or by adding the chemical chaperone 4-PBA, which restores trafficking and CFTR function (24, 25), followed by pH determination using cellubrevin-pHluorin GFP-transfected cells. The results of these experiments are shown in Fig. 6. As both treatments (low temperature and chemical chaperone) restored normal pH of the cellubrevin-endosome in CFTR-mutant cells, it is possible to conclude that defective CFTR causes aberrantly low pH in this organelle.

To examine the pH of other parts of the endocytic pathway, IB3-1, C38, and S9 cells were allowed to endocytose 5 μM ZD, a fluid phase pH-sensitive ratiometric dye, for 5 min. The ratio of fluorescence was measured after 10 and 60 min...
amiloride for the indicated period of time: 60 min, however, after 60 min there was no significant difference in the pH range from 7.4 to 5.5. These results indicate that an early versus late inhibition of amiloride, a sodium channel inhibitor, led to an increase in Na\(^+\) efflux from the organelles could play a role in determining luminal pH. It is known that in CF bronchial epithelial cells, the epithelial sodium channel, ENaC, is under negative regulation by CFTR (3–5, 35–37). In the absence of CFTR, as is the case in CF, ENaC is relieved from CFTR inhibition in lung epithelial cells, leading to an increase in Na\(^+\) transport. To test for the possibility that altered sodium transport could play a role in affecting organellar acidification in CF, we first established whether H\(^+\)-ATPase played a role in hyperacidification of the cellubrevin endosomes in CF cells. Treatment with baflomycin A\(_1\) abrogated hyperacidification of cellubrevin-labeled compartments in CF cells (Fig. 5b). Next, a role for sodium transport in hyperacidification was tested. The addition of amiloride, a sodium channel inhibitor, led to an increase in pH of 1 unit in cellubrevin labeled endosomes of IB3-1 CFTR mutant cells after 2 h of incubation (Fig. 5c). This observation is consistent with sodium transport, (i.e. sodium efflux from the organelles) playing a role in determining the pH of the cellubrevin endosomal compartment. Additional experiments (Fig. 5d) with acetylstrophanthin, an Na\(^+\)/K\(^+\)-ATPase inhibitor, and ion substitution studies (data not shown) confirmed the role of Na\(^+\) conductance in organellar hyperacidification in CF.

### Table I

| Cell line\(^a\) | Apparent pH\(^b\) |
|-----------------|-----------------|
| IB3-1 (mutant)  | pH 6.2 ± 0.1 (n = 19) |
| C38 (corrected) | pH 6.7 ± 0.1 (n = 15) |
| S9 (corrected)  | pH 6.7 ± 0.1 (n = 32) |
| CFT1 (mutant)   | pH 6.0 ± 0.1 (n = 19) |
| CFT1-LCFSN (corrected) | pH 6.6 ± 0.03 (n = 14) |
| CFT1-LCFSN (mutant) | pH 6.0 ± 0.1 (n = 15) |
| CFT1-LCFSN (mutant) | pH 6.2 ± 0.1 (n = 19) |

\(^a\) IB3-1 is a human bronchial cell line derived from a CF patient with a ΔF508/W1282X CFTR mutant genotype (21). C38 cells express a functional CFTR with a fortuitous N-terminal in frame deletion. S9 cells express a functional full size CFTR (22). CFT1 (19) is a tracheal cell line derived from a CF patient homozygous for mutant ΔF508/ΔF508 CFTR. CFT1-LCFSN (wild-type CFTR), CFT1-L508 (Δ508 CFTR), and CFT1-LCS (vector control) are stably transfected derivatives of CFT1.

\(^b\) P < 0.0001 for IB3-1 versus C38; IB3-1 versus S9; CFT1-LCFSN versus CFT1-LCS; CFT1-LCFSN versus CFT1-L508; CFT1-LCFSN versus CFT1.

### Fig. 5

**Analysis of the mechanism of hyperacidification of cellubrevin endosomes in CF.** Changes in pH (ΔpH) in IB3-1 (○), C38 (△), and S9 (□) cells expressing cellubrevin-pHuroin GFP were determined in five distinct cells for each treatment as indicated (bar, mean value). a, IB3-1, C38, and S9 were either not treated (Control, open symbols) or treated with 0.1 mM NH\(_4\)Cl (filled symbols) for 48 h. IB3-1, p = 0.0057; C38, p = 0.8805; S9, p = 0.2982. b, untreated cells (Control, open symbols) and baflomycin A\(_1\)-treated cells (filled symbols). IB3-1, p < 0.0001; C38, p = 0.0028; S9, p = 0.0500. c, IB3-1 cells treated with amiloride for the indicated period of time: 60 min, p = 0.0085; 120 min, p < 0.0001. d, untreated cells (Control, open symbols) and 10 μM acetylstrophanthin-treated cells (filled symbols) for 60 min. IB3-1, p = 0.4528; C38, p < 0.0001.

(n = 5). After 10 min IB3-3 CFTR mutant cells (ratio 3.26 ± S.E. 0.42) were significantly more acidic than C38 (ratio 2.40 ± S.E. 0.21) and S9 (ratio 2.54 ± S.E. 0.18) (both corrected cells) (p = 0.0149 IB3-1 versus C38, p = 0.038, IB3-1 versus S9). However, after 60 min there was no significant difference in the ratios between IB3-3 CFTR mutant cells (ratio 3.02 ± S.E. 0.159) and C38 (ratio 2.88 ± S.E. 0.15) or S9 (ratio 2.84 ± S.E. 0.14) cells (p = 0.6732 IB3-1 versus C38, p = 0.5881, IB3-1 versus S9). HTPS probe responsiveness was linear over a pH range from 7.4 to 5.5. These results indicate that an early endosomal compartment accessible to the exogenously added fluid phase probe is hyperacidified in CF bronchial epithelial cells but that the late, degradative endocytic organelles are not affected.

**Hyperacidification of Cellubrevin Endosomal Compartments in CF Epithelial Cells Is a Sodium-dependent Process—**How might the absence of CFTR affect endosomal pH? It has previously been suggested that chloride channel activity of CFTR may affect organellar acidification in nasal polyp epithelial cells from CF (11). Such proposals are consistent with the role that Cl\(^–\) anions are believed to play in dissipating membrane potential generated by proton pumping into the lumen, which otherwise inhibits H\(^+\)-ATPase activity (34). However, this model would predict organellar alkalinization in CF and could not explain hyperacidification observed in our experiments. Instead, we considered an alternative hypothesis, in which Na\(^+\) efflux from the organelles could play a role in determining luminal pH. It is known that in CF bronchial epithelial cells, the epithelial sodium channel, ENaC, is under negative regulation by CFTR (3–5, 35–37). In the absence of CFTR, as is the case in CF, ENaC is relieved from CFTR inhibition in lung epithelial cells, leading to an increase in Na\(^+\) transport. To test for the possibility that altered sodium transport could play a role in affecting organellar acidification in CF, we first established whether H\(^+\)-ATPase played a role in hyperacidification of the cellubrevin endosomes in CF cells. Treatment with baflomycin A\(_1\) abrogated hyperacidification of cellubrevin-labeled compartments in CF cells (Fig. 5b). Next, a role for sodium transport in hyperacidification was tested. The addition of amiloride, a sodium channel inhibitor, led to an increase in pH of 1 unit in cellubrevin labeled endosomes of IB3-1 CFTR mutant cells after 2 h of incubation (Fig. 5c). This observation is consistent with sodium transport, (i.e. sodium efflux from the organelles) playing a role in determining the pH of the cellubrevin endosomal compartment. Additional experiments (Fig. 5d) with acetylstrophanthin, an Na\(^+\)/K\(^+\)-ATPase inhibitor, and ion substitution studies (data not shown) confirmed the role of Na\(^+\) conductance in organellar hyperacidification in CF.

**Recycling of Transferrin Is Affected in CF Cells—**Previous studies have indicated that altering endosomal pH can affect the function of the recycling endosome (38, 39). To assess the functionality of cellubrevin recycling endosome in CF, we examined recycling of transferrin in CF and CFTR-corrected cells. IB3-1 cells and their CFTR-corrected derivatives, C38 and S9, were allowed to take up 125I-labeled transferrin. The kinetics of transferrin recycling is shown in Fig. 6. There was no difference in recycling after 15 min. However, after 60 min, recycling of transferrin in IB3-1 (CFTR mutant) cells was reduced by 21% (p = 0.0244) compared with the CFTR-corrected C38 cells and by 16% (p = 0.0054) relative to S9 cells. In contrast, endocytosis of transferrin or fluid phase endocytosis (Fig. 7, inset) was not different in CF and CFTR-corrected cells and was equally sensitive to the inhibitor of bulk endocytosis,
cells were allowed to endocytose 125I-labeled transferrin for 45 min at 37°C, excess transferrin was removed by washing at 4°C, and recycling was measured after 15 and 60 min at 37°C. Shown are mean values ± S.E. (n = 3). After 60 min, recycling was reduced in IB3-1 (CFTR mutant) cells by 21% (p = 0.0244) compared with the CFTR-corrected C38 cells and by 16% (p = 0.0054) relative to S9 cells. Inset, fluid phase endocytosis (measured by HRP uptake) is not affected in CF cells (IB3-1 versus C38, p = 0.4966) and is equally sensitive to wortmannin (WM) in both CF and normal cells, IB3-1 versus C38, p = 0.7954; C38 versus wortmannin C38, p = 0.0001; IB3-1 versus wortmannin IB3-1, p = 0.0001.

wortmannin (27) These results indicate that endosomal recycling is impaired in CF bronchial epithelial cells.

DISCUSSION

The studies reported here were inspired by previous models (11, 12) in which altered pH in intracellular organelles was predicted in CF. However, our observations that the recycling endosomal compartment is hyperacidified in mutant CFTR IB3-1 and CFT1 cells is at variance with the previously published values for CF nasal polyep cells reporting slight alkalization of the endosome (pH 6.5 in CF versus pH 6.3 in CFTR corrected cells) (11). Others have observed no differences in endosomal acidification of Swiss 3T3 fibroblasts (15), CFPAC-1 (17), or Chinese hamster ovary cells (13) transfected with either functional or nonfunctional CFTR. The discrepancies between these studies and our findings can be explained by the different cell types investigated, as in our work human bronchial and tracheal epithelial cells derived from CF patients were tested. It is known that, depending upon the cell type, CFTR may have either positive (4, 37) or negative (40) regulatory effects on sodium channels, and so the cell type selection for testing is critical.

It is important to note that our data cannot be easily explained by the previously proposed action of CFTR as a chloride channel in the context of organellar acidification (11). Instead, regulatory functions of CFTR must be invoked, such as the CFTR-dependent inhibition of the sodium conductance in human respiratory epithelial cells (3, 4, 35–37, 40). In this model, excess positive charge, caused by the accumulation of H+ in the lumen of the organelles, may be compensated by Na+ efflux into the cytosol, thus dissipating the electrogenic charge differential (41) and allowing the H+-ATPase to develop a greater transmembrane pH gradient. In the context of charge gain or loss, the impact of Na+ exit is equivalent to the influx of Cl– with the net effect of relieving the proton pump from the inhibition associated with the build up of membrane potential. In normal cells, inactive sodium channels, and most likely active Na+/K+-ATPase along with potassium channels, increase the interior positive membrane potential and thus counteract acidification. In CF cells, in the absence of CFTR-dependent inhibition (3–5, 35–37), the probability for open state of the sodium channel increases and Na+ efflux compensates for the H+–associated positive charge build-up, thus neutralizing the membrane potential and facilitating H+-ATPase action and vesicle acidification. Independent studies show that the TGN, another compartment through which CFTR and sodium channel ENaC traffic in normal cells, is also hyperacidified in CF (42).

Because, as shown here, the function of the recycling endosome is affected in CF, this defect may have repercussions on endocytic and plasma membrane trafficking processes in this disease. For example regulation of plasma membrane signaling events by endocytosis, the availability of receptors and the duration of signals may be altered in CF. This may potentially contribute to the well recognized deficiencies in pro- and anti-inflammatory signaling in CF (28). In addition, the endosomal pathway may affect the interactions of respiratory epithelial cells with the bacterial pathogens responsible for recurring respiratory infections in CF (1, 43). Of particular interest in CF may be the repercussions of altered pH in the recycling endosome on membrane flow to the points of bacterial entry into epithelial cells, because recycling is reduced in CF lung epithelial cells. For example, phagocytosis is inhibited when delivery of membrane from the recycling endosome to the nascent phagosome is obstructed (44, 45). Thus, the dysfunction of the recycling endosome in CF could affect the uptake of microorganisms by cells and explain the reduced bacterial phagocytosis reported for CF epithelium (46). In addition, hyperacidification of the recycling endosome, and potentially that of other compartments, may have effects on other fundamental cellular functions including the transcytosis of biologically active molecules and the pH homeostasis of both intracellular and extracellular environments. The phenomena described here suggest the existence of new physiological links between the CFTR defect, via organellar hyperacidification, and pathogenesis in CF.

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