Evidence against Defective trans-Golgi Acidification in Cystic Fibrosis*

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Defective organelle acidification has been proposed as a unifying hypothesis to explain the pleiotropic cellular abnormalities associated with cystic fibrosis. To test whether cystic fibrosis transmembrane conductance regulator (CFTR) participates in trans-Golgi pH regulation, intraluminal trans-Golgi pH was measured in stably transfected Swiss 3T3 fibroblasts (expressing CFTR or ΔF508-CFTR) and CFTR-expressing and nonexpressing epithelial cells. trans-Golgi pH was measured by ratio-imaging confocal microscopy using a liposome injection procedure to label the lumen of trans-Golgi with fluid phase fluorescein and rhodamine chomophores (Seksek, O., Biwersi, J., and Verkman, A. S. (1995) J. Biol. Chem. 270, 4967-4970). Selective labeling of trans-Golgi was confirmed by colocalization of the delivered fluid phase fluorophores with N-[6-(7-nitrobenzo-2-oxa-1,3-diazol-4-yl)amino]caproyl]-sphingosine. In unstimulated fibroblasts in HCO₃⁻ buffer, trans-Golgi pH was 6.25 ± 0.04 (mean ± S.E.; n = 80, vector control), 6.30 ± 0.03 (n = 74, CFTR) and 6.23 ± 0.06 (n = 60, ΔF508) (not significant). After stimulation of plasma membrane Cl⁻ conductance by 8-(4-chlorophenylthio)-cAMP (CPT-cAMP), trans-Golgi pH was 6.42 ± 0.07 (n = 22, control), 6.47 ± 0.07 (n = 20, CFTR), and 6.35 ± 0.07 (n = 22, ΔF508) (not significant). Similarly, significant pH differences were not found for control versus CFTR-expressing cells in 25 mM HCO₃⁻ buffer. In epithelial cells, which do not express CFTR, trans-Golgi pH was (in 25 mM HCO₃⁻) 6.36 ± 0.04 (n = 33) and 6.34 ± 0.08 (n = 23, CPT-cAMP) in MDCK cells and 6.25 ± 0.04 (n = 18) and 6.24 ± 0.06 (n = 15, CPT-cAMP) in SK-MES-1 cells. In Calu-3 cells, which natively express CFTR, trans-Golgi pH was (in 25 mM HCO₃⁻) 6.19 ± 0.05 (n = 25) and 6.17 ± 0.08 (n = 23, CPT-cAMP). To test whether CFTR expression affects pH in the endosomal compartment in HCO₃⁻ buffer, pH was measured by ratio imaging in individual endosomes labeled with fluorescein-rhodamine dextrans. Comparing control and CFTR-expressing fibroblasts, average endosome pH (range, 5.40-5.53 after 10 min; 4.79-4.89, 30 min) differed by <0.13 unit, both before and after CAMP stimulation. These results indicate that CFTR expression and activation do not influence pH in the trans-Golgi and endosomal compartments, providing direct evidence against the defective acidification hypothesis.

Although the cystic fibrosis (CF) genotype is clearly associated with defective plasma membrane Cl⁻ conductance (1-3), there is little known about the fundamental cellular abnormality that links genotype to cellular defect to clinical disease (for review, see Refs. 4 and 5). A provocative hypothesis to account for many of the observed cellular abnormalities in CF is that the CF genotype is associated with defective acidification of intracellular organelles (6). It was proposed that CFTR provides a conductive pathway across the limiting membrane of certain organelles of the endosomal and secretory pathways, which permits acidification by the electrogenic, vacuolar-type proton pump. The “defective organelle acidification” hypothesis is attractive because it may provide a unifying theme to explain the diverse cellular defects in cystic fibrosis involving abnormal post-translational modifications (decreased sialylation and increased fucosylation) of secreted and surface proteins (7-9) and increased cell adhesion of Pseudomonas aeruginosa (10, 11). For example, decreased protein sialylation has been proposed to result from decreased activity of pH-dependent sialyltransferases in the trans-Golgi (12). The principal evidence supporting the hypothesis is (6): (a) the pH in early endosomes and trans-Golgi in CF cells, measured by DAMP immunoelectron microscopy, was 0.2-0.3 pH unit of alkaline relative to normal cells; and (b) ATP-dependent acidification in cell-free endosomal preparations suggested a functional Cl⁻ channel in endosomes from normal but not CF cells.

A testable prediction of the defective acidification hypothesis is that organelar pH in cells expressing CFTR is lower than that in nonexpressing or CF cells, particularly after CFTR stimulation by CAMP agonists. In addition, CFTR should be present and functional as a Cl⁻ conductance in the endosomal and trans-Golgi compartments of CFTR-expressing cells. Several studies have been carried out to define the role of CFTR in acidification of vesicular compartments of the endosomal pathway. Using transfected Chinese hamster ovary cells, Lukacs et al. (13) concluded that CFTR is functional in a mixed (early and late) endosomal compartment, but that CFTR expression does not influence endosome pH. Biwersi and Verkman (14) reported a similar conclusion for the early endosomal compartment in transfected 3T3 fibroblasts and T84 colonic epithelial

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1 The abbreviations used are: CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; FS, fluorescein sulfonate; SR, sulforhodamine 101; CCCP, carbonyl cyanide m-chlorophenylhydrazone; C₆-NBD-ceramide, N-[6-(7-nitrobenzo-2-oxa-1,3-diazol-4-yl)amino]caproyl]-sphingosine; CPT, 8-(4-chlorophenylthio); SPQ, 3-(6-methoxyquinolino)-propanesulfonate; Cl₂Cf-TMR-dextran, 5(and 6)-carboxy-2'-6'-dichlorfluorescein-5-(and 6)-carboxyteramethylrhodamine-dextran; MDCK, Madin-Darby canine kidney; PBS, phosphate-buffered saline.
trans-Golgi Acidification in Cystic Fibrosis

Golgi or endosomal pH.

Acetic, and 100 mg/ml streptomycin. For microinjection, cells were microinjected with liposomes containing 0.34 mg of bovine serum albumin. The solution was pre-mixed with 10 nM bafilomycin A1 in PBS at 23°C to inhibit the vacuolar proton pump.

Preparation of Liposomes—Multilamellar vesicles were prepared by dispersing dry dioleoylphosphatidylcholine (40 mg/ml) in 25 ml HEPEs, 115 mM KCl, and 2.5 mM MgCl2, containing SR (5 mM) and FS (0 or 30 mM) or rhodamine B-dextran (40 mg/ml), pH 7.2. Multilamellar vesicles were then frozen in liquid nitrogen and thawed five times. Small, uniform-sized liposomes were obtained by passing 100 μl of the liposome suspension through polycarbonate membranes (Nucleopore, Pleasanton, CA) of decreasing pore size (20 times through a 50-nm pore membrane then 20 times through a 100-nm pore membrane) and rinsed with PBS (140 mM NaCl, 3 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 1.5 mM KH2PO4, and 8 mM Na2HPO4, pH 7.4). 

Reagents—Fluorescein isothiocyanate (FITC), 5(and 6)-carboxytetramethylrhodamine-dextran (Cl2Cf-TMR-dextran; Molecular Probes, Eugene, OR). Defatted bovine serum albumin, monensin, CCCP, nigericin, bafilomycin A1, forskolin, human placental collagen, and 8-(4-chlorophenylthio) (CPT)-cAMP were obtained from Sigma, and dioleoylphosphatidylcholine was from Avanti Polar Lipids (Alabaster, AL). 3-(1-Hydroxyethyl)3-hydroxy-5-nitrobenzo-2-oxa-1,3-diazole-4-sulfonic acid (N-(6-[(7-nitrobenzo-2-oxa-1,3-diazol-4-yl)amino]caproyl)-sphingosine (C6-NBD-ceramide), and rhodamine B-dextran (Molecular Probes, Eugene, OR) were purchased from Molecular Probes (Eugene, OR). Defatted bovine serum albumin was obtained from Miles Laboratories (Indianapolis, IN). Proteinase K (Worthington Biochemicals, Freehold, NJ) was purchased from Boehringer Mannheim (Indianapolis, IN). DAPI (fluorescent dye for DNA stained) was purchased from Sigma (St. Louis, MO).

Calibration—FLIM and FCS data were analyzed using a custom program based on routines written by R. P. Launay and C. Goehring (University of California, Santa Barbara, CA) for the program FLIM-FCS (Max-Planck-Institut, Göttingen, FRG) and written in C. The program allows determination of the lifetimes of signal decay from individual pixels in the image. These lifetimes can be translated into pH values using pH-dependent decay curves obtained from control samples. The pH dependence of the decay was determined by varying the pH of the control sample (PBS plus 10 mM Hepes, pH 5.4-7.4) over the range of interest (pH 5.4-7.4). The pH sensitivity of the decay was determined by fitting the decay curves to a single-exponential decay function and determining the pH dependence of the decay constant.

Calibrations were performed as described previously (14). Brefeldin A was dissolved in DMSO to a final concentration of 10 mM, and aliquots were stored at -80°C. The solution was thawed and diluted in PBS prior to use.

Fusion Activity—The fusion activity of each liposome was determined by measuring the amount of FITC and/or Rh-dextran incorporated into the cell membrane. Briefly, after microinjection with liposomes containing 50 nmol of C6-NBD-ceramide or FS and SR, cells were incubated for 30 min at 37°C and mounted in the FS-to-SR signal ratio was plotted as a function of polyno-

In this study, we have applied the liposome fusion method to compare pH in Swiss 3T3 fibroblasts expressing CFTR, ΔF508-CFTR, or no CFTR, as well as in epithelial cells that natively express CFTR (Calu-3 cells; Ref. 22) and those that do not (MDCK and SK-MES-1 cells; Ref. 23). The influence of CFTR expression on the pH in Swiss 3T3 fibroblasts expressing CFTR, MDCK cells (ATCCCCL34) were obtained from the American Tissue Culture Collection. MCF-7 cells (ATCC HTB 55) were provided by Dr. Jonathan H. Widdicombe, and MCF-7 cells (ATCC HTB 55) were provided by Dr. Michael Welsh. MDCK cells (ATCC CCL34) were obtained from Calu-3 cells (ATCC HTB 55) were obtained from Dr. Cary M. Widdicombe. Cells were stained with 10 nM bafilomycin A1 in PBS at 23°C to inhibit the vacuolar proton pump. To set trans-Golgi luminal pH equal to extracellular pH, cells were then incubated for 10 min with 10 mM bafilomycin A1 and 10 μM calyculin A. The cells were then washed 3 times with PBS and incubated for an additional 15 min at 37°C in the presence of 5 mM CPT-cAMP and 0.5 mM CaCl2, titrated to specified pH values. The FS-to-SR signal ratio was plotted as a function of polyno-

In situ pH Calibration—In situ pH calibration was performed using glass capillary electrodes prepared from thin-walled filament capillaries (FHC, Brunswick, ME) drawn to a fine tip (0.5-μm hole diameter) with a vertical needle puller (Kopf, Tujunga, CA) and back-filled with injection solutions. Filled needles were mounted in the holder of an Eppendorf 5170 microinjection pump, and cells were injected with a volume of ~4 × 10−12 ml using an Eppendorf 5242 microinjector over 0.5 s at an injection pressure of 100 kilopascals. During the procedure, the cells were visualized on a Nikon inverted epifluorescence microscope equipped with a ×40 air objective.

The microinjection procedure—Cell microinjection was performed using glass capillaries prepared from thin-walled filament capillaries (FHC, Brunswick, ME) drawn to a fine tip (0.5-μm hole diameter) with a vertical needle puller (Kopf, Tujunga, CA) and back-filled with injection solutions. Filled needles were mounted in the holder of an Eppendorf 5170 microinjection pump, and cells were injected with a volume of ~4 × 10−12 ml using an Eppendorf 5242 microinjector over 0.5 s at an injection pressure of 100 kilopascals. During the procedure, the cells were visualized on a Nikon inverted epifluorescence microscope equipped with a ×40 air objective.

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results

Fig. 1 shows the labeling of transfected 3T3 fibroblasts and MDCK epithelial cells after microinjection with liposomes containing fluid phase fluorescent probes. Selectivity of trans-Golgi labeling was demonstrated by colocalization of liposome-delivered SR with the trans-Golgi-specific lipid phase marker C6-NBD-ceramide (Fig. 1, A and B). The liposome delivery method worked for every cell type tested; similar micrographs showing colocalization of SR and C6-NBD-ceramide were obtained for Calu-3 and SK-MES-1 cells (data not shown). For pH determination, cells were microinjected with liposomes containing the fluorophores FS and SR. Fig. 1, C and D, shows colocalization of FS and SR fluorescence in 3T3 fibroblasts and MDCK cells; similar results were obtained for SK-MES-1 and Calu-3 cells (not shown). There was no differential photobleaching or dye leakage under the conditions of our experiments.

After a 30-min incubation at 37 °C to permit delivery of the fluorescent probes, the specific labeling of the trans-Golgi lumen remained stable for more than 3 h at 23 °C. However, further incubation at 37 °C caused a rapid disappearance of the signal (∼15 min), which could be due to dye leakage or downstream trafficking. To distinguish between these possibilities, liposomes containing rhodamine B-dextran 10,000 (a high molecular weight and membrane-impermeant chromophore) were microinjected, and trans-Golgi was stained with C6-NBD-ceramide (Fig. 2). Early after microinjection (5–15 min), little specific labeling was observed. Thirty minutes was found to be the optimal incubation time for labeling. Subsequently, trans-Golgi fluorescence declined over 45–90 min, and the labeling pattern became less distinct. A few cells (∼5%) were still observed with good trans-Golgi labeling at 60 min. We note that the ability to follow the fate of high molecular weight fluorescent probes in the lumen of the secretory compartment should enable the study of secretory trafficking by fluorescence microscopy.

The cell types used for subsequent trans-Golgi pH measurements were examined for functional expression of CFTR at the plasma membrane using the SPQ fluorescence method in which cytoplasmic Cl− was measured in response to Cl−-nitrate exchange and CPT-cAMP addition (14). SPQ measurements showed strong CPT-cAMP-stimulated Cl− efflux in CFTR-transfected fibroblasts and Calu-3 cells but little change in Cl− efflux in the other cell types (Fig. 3). These functional results are consistent with the known CFTR expression in the transfected fibroblasts and Calu-3 cells (22, 23, 27).

trans-Golgi pH measurements were performed on 3T3 fibroblasts and several types of epithelial cells by ratio-imaging confocal microscopy, as described and validated previously (21). FS (pH sensitive) and SR (pH insensitive) were delivered together in the microinjected liposomes. Absolute trans-Golgi pH was calculated by quantitative image analysis from the FS-to-SR signal ratio, followed by a two-point in situ calibration in which the trans-Golgi and solution pH were set equal using buffers (pH 7.8 and 5.8) containing high K+ and ionophores (Fig. 4, inset). No significant difference was found between the trans-Golgi pH of mock-transfected (pH 6.25 ± 0.04), CFTR-transfected (pH 6.30 ± 0.03), and ΔF508-CFTR-transfected (pH 6.23 ± 0.06) 3T3 fibroblasts in HCO3−-free buffer (Fig. 4). Similarly, in 25 mM HCO3−, the trans-Golgi pH of mock-transfected fibroblasts (6.22 ± 0.07) was not significantly different from the trans-Golgi pH of CFTR-transfected fibroblasts (6.28 ± 0.07). However, after addition of CPT-cAMP, the trans-Golgi pH in the three cell types increased in HCO3−-free buffer by 0.1–0.2 pH unit, whereas no significant change was found in HCO3−-containing buffer (see “Discussion”).

The trans-Golgi pH values of MDCK, SK-MES-1, and Calu-3 epithelial cells were also determined in HCO3−-containing buffer before and after stimulation of CFTR by CPT-cAMP (Fig. 5). Calu-3 cells natively express CFTR (22), and MDCK and SK-MES-1 do not; SK-MES-1 cells were chosen as
Fig. 2. Time course of trans-Golgi fluorescence labeling. CFTR-expressing 3T3 fibroblasts were microinjected with liposomes containing rhodamine B-dextran (SR, M, 10,000). Incubation for indicated times was carried out at 37 °C. Left, cells labeled with C6-NBD-ceramide (NBD); right, rhodamine image of the same cells. Scale bar, 10 μm.

Fig. 3. Functional analysis of CFTR. Time course of SPQ fluorescence. Where indicated, Cl⁻ was replaced by NO₃⁻, and 0.5 mM CPT-cAMP was added. The solution was then switched to 150 mM KSCN to quench SPQ fluorescence.

The pH was next measured in individual endosomes in control and CFTR-expressing fibroblasts in HCO₃⁻-containing buffers. Cohorts of endosomes were pulse labeled with Cl₂CF-TMR-dextran and incubated at 37 °C for a total of 10 or 30 min, and the endosome pH was measured by ratio-imaging microfluorimetry (25). Cl₂CF was chosen because of its good pH sensitivity at low pH values in the range of 4.5–6.0 (pKₐ = 5.3). Fig. 6 shows images of CFTR-transfected 3T3 fibroblasts after 10- and 30-min incubations. Individual fluorescent endosomes are clearly visualized in the fluorescein (left) and rhodamine (right) images. Labeled vesicles (late endosomes and some lysosomes) are larger and located closer to the nucleus at 30 min. Fig. 7A gives averaged endosome pH values measured in the absence and presence of forskolin. Averaged endosome pH values were very similar at each incubation time, with no significant effect of CFTR expression or forskolin activation. To examine the pH values of individual endosomes, the pH distribution was quantified. Fig. 7B shows the pH distribution as the percentage of endosomes in 0.25 (10 min) or 0.2 (30 min) pH unit intervals. There was a unimodal pH distribution for both cell types at 10 and 30 min, with and without forskolin, without evidence of subpopulations of highly acidic endosomes. These results indicate little effect of CFTR expression and activation on endosome pH in HCO₃⁻-containing buffers.

DISCUSSION

The purpose of this study was to test the "defective organelle acidification" hypothesis predicting that the trans-Golgi pH is lower in cells containing functional, activated CFTR than in cells without CFTR. A stably transfected cell line was used, which strongly expressed functional CFTR at the plasma membrane as well as in intracellular vesicles. Previous studies testing whether endosomal acidification is defective in cystic fibrosis were performed in these same cells (14), as well as in stably transfected Chinese hamster ovary (13), T84 (14), and CFPAC-1 (15) cells. CFTR-transfected cells were chosen because direct comparisons could be made with control (vector-transfected) and ΔF508-expressing cells, and because the high CFTR expression levels were predicted to amplify any pH dif-
ferences. In addition, trans-Golgi pH measurements were carried out using several non-CFTR-expressing epithelial cell lines (MDCK and SK-MES-1) and an epithelial cell line that natively expresses CFTR (Calu-3). A liposome injection method developed recently by our laboratory permitted the selective delivery of pH-sensitive fluorophores into the lumen of the trans-Golgi for determination of pH by ratio-imaging confocal microscopy. It was found that CFTR expression and activation did not influence the trans-Golgi pH significantly in any of the cells tested in HCO$_3^-$-containing or HCO$_3^-$-free buffers, with a S.E. of generally better than 0.1 pH unit. Measurements of endosome pH using HCO$_3^-$-containing buffers extended previous observations that the endosome pH is not influenced by CFTR expression and activation. Taken together, these findings provide direct evidence against the defective organelle acidification hypothesis.

Several caveats should be pointed out in evaluating the strength of our conclusion. The studies were performed in stably transfected cells and a CFTR-expressing epithelial cell line, not in vivo in human airway epithelial cells. For the reasons discussed above, we believe that functional effects of CFTR on trans-Golgi acidification should have been apparent in these systems. The accuracy of the pH measurements here are probably not much better than 0.1 unit; therefore, very subtle changes in pH would not be detected. We believe that such small pH differences, if present, are unlikely to be physiologically important based on the intrinsic pH variability in trans-Golgi of different cell types (for example, between 3T3 fibroblasts and MDCK cells) and the sensitivity of organellar pH to various cytosolic factors (21). Last, it is recognized that the conclusion applies to acidification in endosomes and trans-
Golgi and not to acidification in other subcellular compartments in which CFTR might be localized.

If defective organellar acidification does not account for the multiple cellular abnormalities associated with the cystic fibrosis genotype, then what function of CFTR does? The possibility remains but seems unlikely that the pleiotropic set of defects in CF is a consequence of defective CFTR Cl⁻ conductance at the cell apical membrane. Recent data implicate a role for CFTR in regulating other ion channels, including the epithelial Na⁺ channel (28) and the outwardly rectifying Cl⁻ channel (29). CFTR may itself transport nucleotides (30), water (31), and other substances as well as chloride. The cystic fibrosis phenotype may be associated with defective vesicular trafficking (32) and/or intracellular vesicle fusion (33). Although our negative results here provide no new information about the cellular basis of cystic fibrosis, they underscore the need to investigate the above possibilities, as well as other novel pathophysiological mechanisms.

An interesting finding in these experiments was the effect of CPT-CAMP on trans-Golgi pH. Alkalization was induced by CPT-CAMP in HCO₃⁻-free buffer in all cell types, whereas the trans-Golgi pH was unaffected in HCO₃⁻-containing buffer. The mechanism of CPT-CAMP-induced alkalization cannot be deduced from the available data. Possible mechanisms include cAMP inhibition of the vacuolar H⁺ pump or of a trans-Golgi channel, or CAMP stimulation of a passive H⁺ (OH⁻)⁻coupled transporter. In HCO₃⁻-containing buffer, the absence of a significant cAMP effect on pH might result from the suppression of a CAMP-regulated HCO₃⁻ transport mechanism, which leads to luminal acidification which balances the alkalization. Whatever the mechanistic basis of the CAMP effect, the insensitivity of trans-Golgi pH to CFTR expression and activation indicates that transporters other than CFTR are responsible for the regulation of pH in this compartment.

Our results indicate that the trans-Golgi pH is not affected by CFP expression and CAMP stimulation in CFP-transported and natively expressing cell models. CFTR function as a Cl⁻ channel in trans-Golgi was not studied, because trans-Golgi vesicles cannot be purified, and because there is no effective approach to access the trans-Golgi membrane in intact cell experiments. We believe that CFTR is likely to be functional in trans-Golgi based on evidence that CFTR is functional in endosomes (13) and nuclear and endoplasmic reticulum membranes (34), as well as data for other polytopic membrane-transporting proteins including the related multidrug resistance protein, MDR (35). Finally, it is emphasized here that the principal conclusion of this study rests not on the density and function of CFTR in the trans-Golgi limiting membrane, but on measured trans-Golgi pH, the penultimate determinant of protein processing.

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trans-Golgi Acidification in Cystic Fibrosis

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