Cystic Fibrosis Transmembrane Conductance Regulator and H$^+$ Permeability in Regulation of Golgi pH

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Summary

This paper reviews experiments from this lab that have tested the hypothesis that pH of the Golgi (pH$_G$) of cystic fibrosis (CF) airway epithelial cells is alkaline compared to normal, that this altered pH affects sialyltransferase and other Golgi enzymes controlling biochemical composition of the plasma membrane and that altered surface biochemistry increases bacterial binding. We generated a plasmid encoding a modified green fluorescence protein-sialyltransferase (GFP-ST) chimera protein that was pH-sensitive and localized to the Golgi when transfected into HeLa cells and also CF and normal or cystic fibrosis transmembrane conductance regulator- (CFTR)-corrected airway epithelial cells [1]. Digital imaging microscopy of these Golgi-localized probes showed that there was no correlation between pH$_G$ (6.4-7.0) and the presence of CFTR, whether cells were in HCO$_3^-$/CO$_2$-containing or in HCO$_3^-$/CO$_2$-free solutions. Activation of CFTR by raising cell [cAMP] had no effect on pH$_G$. Thus, CFTR seemed not to be involved in controlling pH$_G$. Experiments on HeLa cells using an avidin-sialyltransferase chimera in combination with a pH-sensitive fluorescent biotin [2] indicated that even in cells that do not express CFTR, Cl$^-$ and K$^+$ conductances of the Golgi and other organelle membranes were large and that pH$_G$ was controlled solely by the H$^+$ v-ATPase countered by a H$^+$ leak. A mathematical model [3] was applied to these and other published data to calculate passive H$^+$ permeability (P$_{H+}$) of the Golgi, endoplasmic reticulum, *trans*-Golgi network, recycling endosomes and secretory granules from a variety of cells. An organelle's acidity was inversely correlated to its calculated P$_{H+}$. We conclude that the CFTR plays a minor role in organelle pH regulation because other (Cl$^-$ and/or K$^+$) channels are present in sufficient numbers to shunt voltages generated during H$^+$ pumping. Acidity of the Golgi (and perhaps other organelles) appears to be determined by the activity of H$^+$ pumps countered by H$^+$ leaks.

As proposed in the CF-Golgi pH hypothesis [4, 5], CFTR could affect pH$_G$ through its ability to conduct Cl$^-$ and thereby control membrane potential and the ability of the H$^+$ v-ATPase to acidify the lumen. This hypothesis further proposed that alkalinization of the normally somewhat acidic Golgi, *trans*-Golgi and *trans*-Golgi network would alter the activities of resident sialyltransferase, sulfotransferase and fucosyltransferase, thereby leading to changes in the chemical properties of membrane and secreted glycoproteins (e.g., mucus) and glycolipids (asialo-GM1, proposed to be an important bacterial binding site on the

membranes of airway epithelial cells [6, 7, 8, 9]).

Previous tests of the hypothesis [10, 11, 12, 13] could be criticized because: i) human airway epithelial cells may be different from fibroblasts, lymphocytes and non-airway epithelial cells from other species with regard to pH_G regulation, ii) no previous comparisons of pH_G were performed on genetically matched $\Delta F508$ and wild type CFTR-expressing respiratory cell lines, and iii) it was important to measure the pH of Golgi cisternae where sialyltransferase and the other critical enzymes reside because pH regulatory mechanisms may differ in different organelles [14].

This paper summarizes experiments designed to directly test the organelle pH-CF hypothesis [1]. A ratiometric, GFP-based pH sensor (so-called pH-sensitive excitation ratiometric GFP: pHERP) was genetically targeted to the Golgi with sialyltransferase in matched $\Delta F508$-CF and CFTR-corrected $\Delta F508$-CF human airway epithelial cells. Measurements in CF tracheal and nasal epithelial cells and in CFTR-expressing tracheal and bronchial epithelial cells were used to determine the role of CFTR in the control of pH_G. These measurements were compared to previous measurements in HeLa cells made with a membrane-permeable, pH-sensitive fluorescein-biotin derivative that was targeted to the Golgi using a chimera of sialyltransferase-avidin [2].

Control experiments on HeLa cells showed that pH_G measured using sialyltransferase-pHERP (pH_G 6.4-6.6) was nearly identical to values obtained using Golgi-targeted fluorescein and GFP-based pH sensors [2, 15, 16]. Respiratory epithelial cells had more alkaline pH_G (6.7-7.0) than HeLa cells. We do not know the source of this difference between HeLa cells and airway epithelial cells. However, it was clear that pH_G of airway epithelial cells did not correlate to the presence or absence of CFTR: cells from $\Delta F508$ patients (cystic fibrosis tracheal cell line, number 2 clone (CFT2) and human cystic fibrosis nasal epithelial cell line (JME)) had pH_G = 6.7 and 7.0, while those expressing wild type-CFTR (WT)-CFTR (CFT1-CFTR and human bronchial epithelium cell line (HBE)) had pH_G = 6.9 and 6.7. When comparing the genetically matched CFT1 to CFT1-CFTR cells, the $\Delta F508$-expressing CFT1 cells were 0.2 pH units more acidic than the WT-CFTR-expressing CFT1-CFTR cells. This small difference was statistically significant, but this seems unlikely to be physiologically important. There was no significant difference in cytosolic pH (pH_C = 7.4-7.6) among the various cells.

The CFTR is activated by protein kinase A, and the effects of cAMP on pH_G have been controversial [12, 16]. We tested the effects of cAMP in CFT1 and CFT1-CFTR cells and found no change in pH_G in response to forskolin whether experiments were performed in the presence of 25 mM HCO_3^- and 5% CO_2 or in HCO_3^--free solutions, indicating that CFTR was not involved in regulation of steady state pH_G in either control conditions or during stimulation with cAMP.

Since CFTR seemed not to be important for regulation of pH_G, what ion transport mechanisms were involved? Measurements in HeLa cells have provided an explanation. Bafilomycin, the well known inhibitor of the H^+ v-ATPase, caused an immediate alkalinization of the Golgi, indicating that this pump was certainly active and that it operated to counter a H^+ leak, which was also an important determinant of pH_G [2, 16, 17, 18, 19, 20 21]. The roles of Cl^- and K^+ counterion conductances in controlling pH_G in intact cells have been tested by removal of Cl^- from the extracellular solution and by adding valinomycin (K^+-ionophore), neither of which had any effect on pH_G in control conditions or during bafilomycin treatment; valinomycin also had no effect on bafilomycin-induced dissipation of pH_G in Cl^--free solutions (not shown). Further experiments determined the relative magnitudes of counterion conductances in Golgi membranes by measuring rates of apparent H^+ leak out of and into the organelles

using constant transmembrane pH gradients under conditions in which either Cl⁻ or K⁺ conductances would be expected to be rate limiting. K⁺-free solution would be expected to slow passive H⁺ leak out of the Golgi lumen into the cytosol if K⁺ were the major conductance of the membranes. Cl⁻-free solution would be expected to slow H⁺ flux into the organelle lumen from the cytosol if Cl⁻ were the major conductance of the membranes. Rates of passive H⁺ leak across the Golgi in bafilomycin-pretreated, plasma membrane-permeabilized (using streptolysin-O: SL-O) cells was insensitive to the presence of either K⁺ or Cl⁻ [2], indicating that K⁺ and Cl⁻ conductances were both large and not rate limiting on their own for determining rates of H⁺ flux across the membrane. For example, in Cl⁻-free solutions, K⁺ could still shunt any potentials, and *vice versa*. These and other [17, 18] results lead to the important conclusion that the potential across the Golgi membrane is likely to be less than 10 mV.

The Golgi membrane appeared to have large enough conductances to both K⁺ and Cl⁻ to limit H⁺ movement [1, 2]. Thus, we and others [17] have concluded that the acidity of the Golgi was limited by H⁺ pumping and the H⁺ "leak" or "permeability" pathway. Since CFTR regulates other ion transport pathways [22, 23, 24, 25], we measured this H⁺ permeability in Golgi's of HeLa and in CF and normal cells. Bafilomycin-treated cells were acid-loaded (40 mM NH₄Cl for 20 min, followed by Na⁺-free Ringer's). When Na⁺-containing Ringer's was returned to the chamber, pH_C recovered rapidly to pH 7.4, and pH_G recovered at a slower rate, which was determined by the Golgi's buffer capacity, permeability to H⁺, the ratio of the surface area to the volume and the cytosol to-Golgi pH gradient. Figure 1a shows typical results for the recoveries of pH_C and pH_G in HeLa cells as the outside solution was switched from Na-free to Na-containing Ringer's. Similar experiments have been performed in CFT1 and CFT1-CFTR cells, and there were no significant differences in the rates of passive H⁺ leak, indicating that the H⁺ leak was unaffected by the presence or absence of CFTR [1].

Using buffer capacity value for Golgi of 20
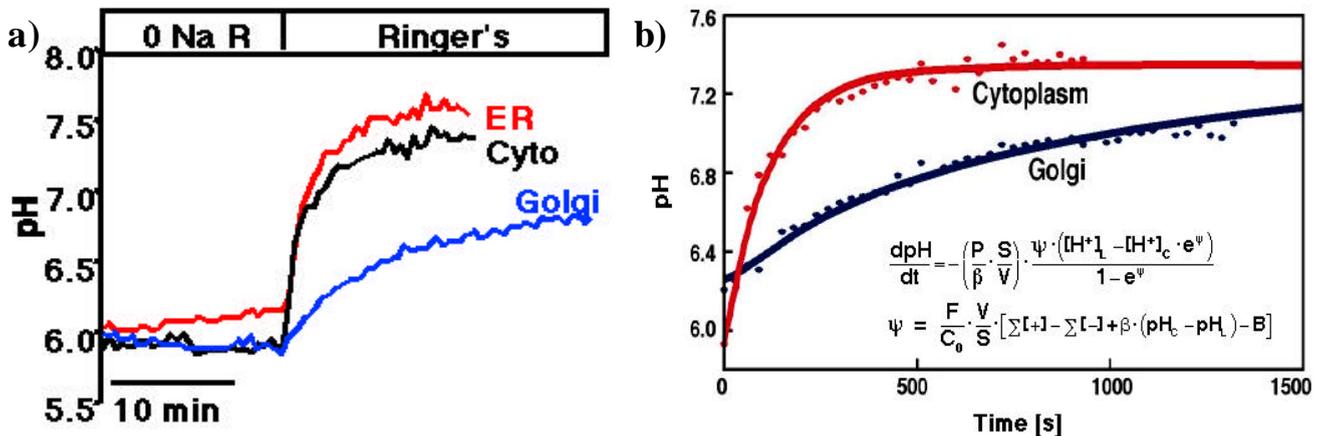
Figure 1 (a) Responses of the ER, Golgi and cytosol to altered pH gradients. HeLa cells were pretreated with bafilomycin, acid-loaded using NH₄ followed by Na⁺-free solution. Readdition of Na⁺ caused pH of the ER and cytosol to recover at the same rates, while the Golgi recovered more slowly. Data taken from [2].
(b) Calculating H⁺ permeability (P) of the Golgi (and other organelles). Time courses of recoveries of pH_C and pH_G (dots, determined in separate experiments) were fit (solid lines) using equations [3] that also incorporate Golgi buffer capacity (β) (determined separately, 20 mM/pH), surface area (S) and volume (V) [26], capacitance (assumed 1 µF/cm²), membrane potential and the difference of cation and ion contents [3]. Similar calculations were performed using measurements of pH of ER (not shown).

mM/pH [2] and surface area and volume measurements obtained from electron microscopy [26], the data from acid-load recovery experiments (Figure 1a) were fit (Figure 1b) using a mathematical organelle pH model [3] to determine the H^+ permeabilities for the Golgi membrane. Although this model makes no assumptions about the membrane potential, the results were consistent with membrane potential being small (less than 10 mV), as predicted above.

We also used another method for determining organelle P_{H+} in a variety of cells. Organelle and cytosolic pH's were obtained either from our own experiments [2, 14] or from published papers [16, 18, 19] during inhibition of the H^+ v-ATPase by bafilomycin, causing organelles to alkalinize while pH_C remained constant. The P_{H+} of the different organelles could then be calculated from the initial rate of increase of organelle pH, assuming buffer capacity of all the organelles was the same, that membrane
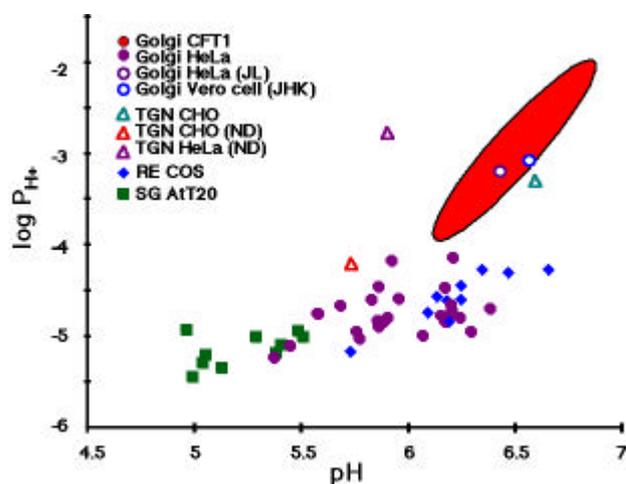
Figure 2. Permeability of organelle membranes to H^+ is directly correlated to steady state pH of the organelle. P_{H+} for the organelles was calculated using the model [3] from time course of effects of bafilomycin on organelle pH assuming cytosolic pH remained constant. Data taken from: our work on Golgi in CFT1 [1] (oval shows range of data) and HeLa [2] cells, recycling endosomes in Cos cells [14], secretory granules in AtT-20 cells (Wu M and Machen T, unpublished) and TGN in CHO cells (Giorgi G and Machen T, unpublished); other data were taken from papers describing steady state pH and effects of bafilomycin as shown: JL [16], JHK [19], and ND [18].

potentials were small and that surface-to-volume ratios were equal to those previously measured [26, 27] or from calculations based on the estimated diameters of spherical organelles such as secretory granules. These data have been summarized in Figure 2, which shows a direct relationship between an organelle's value of P_{H+} and steady state lumenal pH, particularly when comparing different organelles of the same cell type. This direct relationship between pH and P_{H+} is less strict when comparing different organelles among different cell types. In addition, the relationship assumes constant buffer capacity for the organelles, and if buffer capacity increases at low pH [1], then the correlation (slope) will be less steep. However, even with these qualifiers, in general as lumenal pH decreases, the P_{H+} decreases. P_{H+} of the ER, which appears to be so leaky to H^+ that pH of ER and cytosol are nearly always equal [2, 15], was approximately 10^{-2} cm/sec in HeLa cells, while P_{H+} of the moderately acidic Golgi in the same cells was about 10-fold less, and P_{H+} of quite acidic secretory granules in an endocrine cell line (AtT-20) was even smaller. P_{H+} for the Golgi of CFT1 cells was larger than those of other cell types, although differential pump activity may also play a role in the elevated pH_G of CFT1 cells compared to other cells.

Overall, these data were consistent with the idea that the pH attained by different organelles, including the Golgi, was determined only by the rate of acidification by the H^+ v-ATPase countered by the dissipation of this acid accumulation through P_{H+}. Recent experiments have shown that this H^+ permeability in the Golgi is partially inhibited by Zn^{2+} [17], which blocks H^+ channels in the plasma membranes of several cell types [28, 29]. Based on data in Figure 2, it might be proposed that the density of these or other H^+ conductive pathways decreases from the endoplasmic reticulum to the Golgi to secretory granules. The molecular identity of the H^+ leak remains unknown, but is likely to be very

important since it appears to play such an integral role in controlling organelle pH. In contrast, CFTR plays an insignificant role in controlling pH_G because the Golgi's of all cells have multiple K^+ and Cl^- channels, and the presence or absence of CFTR therefore does not affect membrane potential or acidification. Work from other labs [2, 17, 18] similarly indicates that pH_G in CF and normal cells are not different, and that other mechanisms must be involved in generating the altered mucus and surface membrane biochemistry reported by others [6, 7, 8, 9].

Methods

All cells were maintained in tissue culture using typical methods, sometimes including a number of supplements to the media [30].

A pH-sensitive excitation ratiometric GFP (pHERP) was genetically targeted to the sialyltransferase-containing compartment [1]. We created a chimera of two mutant GFPs by placing GFPuv N-terminal to enhanced yellow fluorescence protein (EYFP). The chimera was extremely sensitive to pH when excited at 495 nm, and relatively insensitive to pH at about 440 nm (emission greater than 520 nm). This GFP-GFP chimera (pK 6.5) was coupled to the transmembrane amino acids (1-70) of sialyltransferase. When the plasmid was transfected into cells, pHERP was targeted to the Golgi lumen [1]. We compared measurements of pH_G made with ST-pHERP to those obtained with enhanced green fluorescence protein (EGFP). targeted to the Golgi with galactosyltransferase [16, 20, 21]. When expressed in both CFT1 and CFT1-CFTR cells, galactosyltransferase-EGFP (GT-EGFP) and ST-pHERP yielded similar results when pH_G was perturbed, indicating that ST-pHERP was accurately reporting pH_G. The fact that ST-pHERP was more easily calibrated in terms of pH_G made it preferable over GT-EGFP.

A pH-sensitive, fluorescein-coupled biotin derivative (Flubi-2) was targeted to the Golgi lumen of HeLa cells by expressing an avidin-sialyltransferase chimera protein [2]. HeLa cells expressing the ST-avidin chimera protein were loaded with membrane-permeable Flubi-2 (pK_a 6.5; bound selectively to the avidin in the Golgi) to monitor pH. Using a digital imaging microscope [1, 2, 14], pHERP or the Flubi dye was excited alternately at 440 nm and 490 nm (emission greater than 520 nm). An *in vivo* calibration of the fluorescence ratios (490/440) was performed at the end of every experiment [1, 2, 14]. Cytosolic pH was measured using the pH-sensitive dye bis-carboxyethylcarboxy-fluorescein (BCECF) and digital imaging microscopy (from entire cells) as described previously [14, 31]. Measurements on Golgi were taken only from the brightest perinuclear regions.

Air-equilibrated Ringer's solution contained (in mM): 141 NaCl, 2 KCl, 1.5 K_2HPO_4, 1 $MgSO_4$, 10 N-(2-hydroxyethyl)piperazine-N'-(2-ethane)sulfonic acid (HEPES), 2 $CaCl_2$ and 10 glucose brought to pH 7.4 with NaOH. Na-free solutions contained (in mM): 141 N-methyl-glucamine (NMG) base, 2 KCl, 1.5 K_2HPO_4, 1 $MgSO_4$, 10 HEPES, 2 $CaCl_2$ and 10 glucose brought to pH 7.4 with HCl. In some experiments 25 mM NaCl was replaced by an equivalent amount of $NaHCO_3$, and gassing was with 5% CO_2. In other experiments, NH_4Cl (30 or 40 mM) was substituted for 30 or 40 mM NaCl. Calibration solutions contained (in mM): 70 NaCl, 70 KCl, 1.5 K_2HPO_4, 1 $MgSO_4$, 10 HEPES, 10 2-(N-morpholino)ethanesulfonic acid (MES), 2 $CaCl_2$, 10 glucose, 0.01 nigericin, 0.01 monensin and adjusted to various pH values with KOH. Buffer capacities were determined [32] in high K^+/ 0 Na^+ solutions with varying amounts of K acetate or NH_4Cl substituted for KCl. Bafilomycin was used at 100-250 nM. Intracellular cyclic-AMP was increased by perfusing cells with solutions containing either 10 µM forskolin or 10 µM forskolin + 500 µM dibutyryl-cAMP. In some

experiments, plasma membranes were permeabilized with streptolysin-O (SL-O) in an intracellular-like buffer containing only K^+ and Cl^- as the permeant ions [2].

A recently described model [3] was used to calculate H^+ permeability of the Golgi (P_{H+}, in cm/sec). The model requires knowing buffer capacity of the Golgi, surface area and volume of the Golgi. Model calculations indicated membrane potential was less than 10 mV, consistent with the apparent presence of sufficiently large conductances to both Cl^- and K^+. Experimentally determined time courses of changes of pH_G and pH_C were applied to the model to calculate P_{H+}, which was modeled as being due to simple, passive diffusion [33]. P_{H+} was also determined from initial rates of alkalinization following treatment with bafilomycin to block the H^+ pumps (pH_C remained constant). In order to compare our data to previous work, we extracted pH of different organelles *vs.* time from published figures to determine P_{H+} (Figure 2).

Key words Biotin; Cystic Fibrosis; Endoplasmic Reticulum; Fluorescent Antibody Technique; Gene Targeting; Hydrogen-Ion Concentration; Ion Transport; Molecular Probes; Secretory Vesicles

Abbreviations BCECF: bis-carboxyethylcarboxy-fluorescein; CF: cystic fibrosis; CFTR: cystic fibrosis transmembrane conductance regulator; CFT2: cystic fibrosis tracheal cell line, number 2 clone; EGFP: enhanced green fluorescence protein; EYFP: enhanced yellow fluorescence protein; Flubi: fluorescein-coupled biotin derivative; GFP: green fluorescence protein; HBE: human bronchial epithelium cell line; GT: galactosyltransferase; HEPES: N-(2-hydroxyethyl)piperazine-N'-(2-ethane)sulfonic acid; JME: human cystic fibrosis nasal epithelial cell line; MES: 2-(N-morpholino)ethanesulfonic acid; NMG: N-methyl-glucamine; pHERP: pH-sensitive excitation ratiometric GFP; pH_C: cytosolic pH; pH_G: pH of the Golgi; P_{H+}: H^+ permeability; SL-O: streptolysin-O; ST: sialyltransferase; WT: wild type

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