Defective goblet cell exocytosis contributes to murine cystic fibrosis–associated intestinal disease

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Introduction

Cystic fibrosis (CF), an autosomal recessive disease, is caused by loss-of-function mutations in CF transmembrane conductance regulator (CFTR) (1), an epithelial anion channel largely responsible for transepithelial secretion of chloride and bicarbonate. One of the more perplexing manifestations of CF is mucoviscidosis. Often used synonymously for CF, mucoviscidosis describes the pathological manifestation mucoviscidosis, which is the secretion of tenacious, viscid mucus that plugs ducts and glands of epithelial-lined organs. Goblet cells are the principal cell type involved in exocytosis of mucin granules; however, little is known about the exocytotic process of goblet cells in the CF intestine. Using intestinal organoids from a CF mouse model, we determined that CF goblet cells have altered exocytotic dynamics, which involved intrathecal granule swelling that was abruptly followed by incomplete release of partially decondensed mucus. Some CF goblet cells exhibited an ectopic granule location and distorted cellular morphology, a phenotype that is consistent with retrograde intracellular granule movement during exocytosis. Increasing the luminal concentration of bicarbonate, which mimics CF transmembrane conductance regulator–mediated anion secretion, increased spontaneous degranulation in WT goblet cells and improved exocytotic dynamics in CF goblet cells; however, there was still an apparent incoordination between granule decondensation and exocytosis in the CF goblet cells. Compared with those within WT goblet cells, mucin granules within CF goblet cells had an alkaline pH, which may adversely affect the polyionic composition of the mucus. Together, these findings indicate that goblet cell dysfunction is an epithelial-autonomous defect in the CF intestine that likely contributes to the pathology of mucoviscidosis and the intestinal manifestations of obstruction and inflammation.

Recent breakthroughs in CF mucoviscidosis research have focused on the critical role of HCO$_3^-$ in mucus biology and the underlying deficit of CFTR-mediated HCO$_3^-$ transport. Studies of isolated mouse intestine provide indirect evidence that approximately 50% of stimulated mucus release requires CFTR-dependent HCO$_3^-$ secretion (7). It was hypothesized that HCO$_3^-$ secretion during exocytosis may be important for mucus expansion by neutralizing protons and precipitating Ca$^{2+}$ ions that, during granule condensation, shield the repulsive forces of high-density anionic sites on mucin glycoproteins (8). Subsequent studies by Gustafsson et al. demonstrated that a high concentration of luminal HCO$_3^-$ (115 mM), intended to recapitulate stimulated HCO$_3^-$ secretion by adjacent CFTR-replete enterocytes, significantly decreased the adherence of mucus to the surface of the CF mouse ileum (9). Moreover, it was recently shown in CF mouse ileum that a high luminal HCO$_3^-$ concentration provides unfolding of MUC2 that is likely necessary for cleavage by the brush border metalloendopeptidase meprin β and subsequent release of mucus from the mucosal surface of the intestine (10).

Direct involvement of CFTR in goblet cell function has been controversial, largely due to the limitations of immunolocalization. Studies of human tracheal gland mucous cells and murine gallbladder columnar cells demonstrated CFTR expression in the cellular membrane/intracellular compartments and mucin granule membrane, respectively (11, 12). However, subsequent studies were unable to find significant CFTR expression in goblet cells of human airways and intestine (13, 14) or mouse colon (15). An early study reported a role of CFTR in regulating organellar pH by purportedly providing counter anions to support
organellar acidification by the vacuolar H^+ -ATPase (V-ATPase) (16). Others have both supported (17, 18) and refuted (19–21) the contention that lysosomal/trans-Golgi pH is alkaline in CFTR-deficient cells. Recently, studies of human airway epithelium showed immunolocalization of CFTR with MUC5AC in isolated mucin granules and, further, demonstrated a CFTR-dependent Cl^- conductance that enhanced granule acidification (22). Granule pH can affect mucus biology in several ways. An acidic pH in the secretory pathway of mucus-producing cells is required for autocatalysis (23), N-terminal trimerization of MUC2 (8), and granule organization (24). It is also known that an acidic granule pH facilitates K^+ /Ca^{2+} ion exchange at the granule membrane during stimulation of exocytosis, which may signal granule release as well as contribute to the initiation of exocytosis (25). In other secretory cells, an alkaline pH of the secretory granules is known to slow the rate of exocytosis (26). Thus, the question of mucin granule pH in CF goblet cells has important implications with regard to mucoviscidosis.

Few studies have investigated the dynamics of CF goblet cell function in a well-differentiated epithelium. Studies of human airway epithelial explants found that CF goblet cells had normal rates of degranulation, as estimated en face by temporal image subtraction, in response to luminal purinergic agonists (ATP, UTP) (27). However, an assessment of epithelial mucus in endobronchial biopsies found a 50% increase in the mucus volume of CF airway goblet cells and evidence of luminal mucus anchored to goblet cell thecal contents (henceforth referred to as luminal “blebbing”) (28). A similar phenomenon occurs in the CF intestine, where ultrastructural studies have shown evidence of abnormal goblet cell exocytosis that manifests as mucus blebbing of partially decondensated mucin granules (29). Studies at the level of the goblet/mucous cell take on added significance in that recent organ-level studies of a porcine CF model show that impaired mucus detachment from submucosal gland ducts is primarily responsible for the deficit of mucociliary clearance in CF respiratory disease (30).

In the present study, goblet cell function during stimulated exocytosis was investigated using WT and Cfrt-KO mouse intestine. The Cfrt-KO mouse recapitulates the major aspects of human CF intestinal disease, including goblet cell hyperplasia, adherent secreted mucus, low-grade bowel inflammation, and overt obstructive disease that is prevented by osmotic laxative maintenance therapy (2, 31–34). Real-time evaluation of the degranulation process was possible through the use of small intestinal organoids (enteroids), which provide visual access to the longitudinal axis of mature goblet cells within the native setting of the crypt/lower villus, i.e., surrounded by enterocytes and other cell lineages of the intestinal epithelium (35, 36). Cfrt-KO enteroids also provide the opportunity to evaluate the epithelial-autonomous effect of CFTR loss on goblet cell function by eliminating confounding factors inherent to the CF intestinal environment, i.e., dysbiosis, inflammation, and submucosal/systemic disease alterations. Our studies provide evidence of altered exocytotic dynamics, incomplete mucus discharge, ectopic intracellular granule location, and increased pH of mucin granules in Cfrt-KO goblet cells. Further, it is shown that provision of high luminal HCO_3^- concentration to Cfrt-KO goblet cells improves exocytosis and granule decondensation, supporting the possibility that oral bicarbonate therapy may be useful in the treatment of CF intestinal disease.
The contents (also see higher magnification in Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI73193DS1). Using video microscopy to follow the exocytotic process in real time (Figure 1D and Supplemental Video 1), CCH stimulation of exocytosis in WT enteroids was observed to be an orderly process resulting in the loss of granules from the apical to basal pole of the theca in a central radial pattern, i.e., cupping. A wispy, translucent stream from the luminal thecal border was occasionally observed, but accumulation of degranulated mucus, i.e., a mucus bleb, at the luminal border was not observed.

In contrast (see Figure 1E and Supplemental Video 2), the granule cluster of Cftr-KO goblet cells treated with CCH initially underwent an increase in size, often with visible expansion of granules within the theca. Subsequent expulsion, sometimes appearing as a rupture of the apical membrane, occurred with formation of a transparent mucus bleb at the luminal surface of the goblet cell. Cupping of the goblet cell theca was not observed. Notably, ectopic exocytosis with formation of a transparent mucus bleb was occasionally evident at the basal aspect of the epithelium.

Results
Postexocytotic mucus retention by Cftr-KO goblet cells is an epithelial-autonomous effect of CFTR loss. Similar to the evidence of goblet cell mucus retention in human CF intestine (29), mucus bleb attachment to the content of goblet cell thecae is frequently observed in intestinal sections from Cftr-KO mice (10). As shown by the comparison to WT intestine in Figure 1A, the histological appearance of Cftr-KO intestine following carbachol (CCH) stimulation of exocytosis typically includes goblet cells that appear distended with mucus and others in which the granule cluster retains a connection with secreted mucus. The abnormal environment of the CF intestine (dysbiosis, inflammation, dysmotility, deficiencies of innate immunity, obstructive disease) may affect goblet cell function (2, 32, 33, 37–43); therefore, stimulated goblet cell exocytosis was compared in primary enteroid cultures taken from WT and Cftr-KO sex-matched littermates. As shown in Figure 1, B and C, goblet cells in crypts from the WT enteroids exhibited cavitation at the luminal pole following treatment with CCH, whereas Cftr-KO goblet cells often form luminal mucus blebs attached to the thecal contents (also see higher magnification in Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI73193DS1). Using video microscopy to follow the exocytotic process in real time (Figure 1D and Supplemental Video 1), CCH stimulation of exocytosis in WT enteroids was observed to be an orderly process resulting in the loss of granules from the apical to basal pole of the theca in a central radial pattern, i.e., cupping. A wispy, translucent stream from the luminal thecal border was occasionally observed, but accumulation of degranulated mucus, i.e., a mucus bleb, at the luminal border was not observed. In contrast (see Figure 1E and Supplemental Video 2), the granule cluster of Cftr-KO goblet cells treated with CCH initially underwent an increase in size, often with visible expansion of granules within the theca. Subsequent expulsion, sometimes appearing as a rupture of the apical membrane, occurred with formation of a transparent mucus bleb at the luminal surface of the goblet cell. Cupping of the goblet cell theca was not observed. Notably, ectopic exocytosis with formation of a transparent mucus bleb was occasionally evident at the basal aspect of the epithelium.
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The Altered dynamics of CCH-stimulated exocytosis in Cftr-KO goblet cells. To ensure uniform luminal and basolateral membrane exposure of the enteroids to a physiological Kreb’s bicarbonate Ringer’s (KBR) solution (25 mM NaHCO₃, 5% CO₂), a series of studies was performed in which the interiors of gel-immobilized enteroids were superfused using a glass micropipette (1–2 μl/min, see Figure 2A). The micropipette was inserted into a puncture of the enteroid central cavity opposite of the flow direction of the bath superfusion (~3 ml/min). Luminal superfusion was denoted by a visible distention of the lumen and the flushing of debris from the enteroid interior at the puncture site. Goblet cells for analysis were chosen for morphological maturity based on cell position above +4, small granule size relative to Paneth and “intermediate” cells, and a granule cluster positioned primarily in the apical 30% of the cell.

Postacquisition morphometric analysis of the goblet cell granule area before and during exposure to 100 μM CCH identified landmark events during the exocytotic process. Exocytosis in WT enteroids occurred in 87.5% (7 of 8) of the goblet cells and was observed as a progressive disappearance of individual granules from the apical pole. Exocytosis in the Cftr-KO enteroids occurred in 57.1% (8 of 14) of the goblet cells and typically resulted in the formation of a luminal mucus bleb during loss of granules from the apical pole (in 1 additional case, compound exocytosis was entirely localized to the basal aspect of the epithelium). As shown in Figure 2, B and C, the cross-sectional granule areas of WT and Cftr-KO goblet cells were identical under basal conditions. Following CCH exposure, the Cftr-KO granule area increased in size, whereas WT goblet cells exhibited a small, nonsignificant increase in granule area (time points 0 → 1). The increased area for each genotype occurred within approximately the same time period (Figure 2B), thus yielding a greater overall rate of increase in the Cftr-KO goblet cells (Figure 2D, basal-maximum). There was no corresponding change in cell volume as indexed by cell height (44) (WT: basal = 50.3 ± 2.7 μm and maximum = 50.9 ± 2.6 μm, NS; Cftr-KO: basal = 52.6 ± 1.6 μm and maximum = 53.2 ± 1.8 μm, NS; n = 8–12). The small increase in granule area in WT goblet cells was immediately followed by individual granule loss from the apical pole, which continued until a steady-state minimal granule area was attained (~4 minutes). In contrast, Cftr-KO goblet cells underwent a significant delay after attaining maximal granule area before there was evidence of degranulation, i.e., luminal bleb formation and/or visible loss of granular area (time points 1 → 2: WT = 0.55 ± 0.45 minutes; Cftr-KO = 1.77 ± 0.47 minutes, P < 0.05; Figure 2B). Exocytosis by Cftr-KO goblet cells was an abrupt, almost forceful, event that contrasted with the orderly progress of WT exocytosis. Hence, the rate of granule area change from initiation to conclusion of exocytosis was 3 times the exocytotic rate in WT goblet cells (Figure 2D, exocytosis-minimum). Luminal mucus blebs were only observed on the Cftr-KO goblet cells and formed simultaneously as the granule area decreased to a minimum that was similar in magnitude to that of WT goblet cells (Figure 2, B and C). Cupping of the apical thecal area in Cftr-KO goblet cells was not observed (see also transmission electron microscopy [TEM] in Figure 3E). The Cftr-KO luminal mucus blebs attained a 2D area (208.0 ± 42.0 μm²) that was
approximately 5-fold greater than the loss of granule area ($\Delta$basal to minimum = $-$38.6 ± 9.6 $\mu$m$^2$), suggesting a greatly reduced exocytotic expansion of Cftr-KO mucin granules as compared with an expected expansion of 350- to 600-fold (45).

Ectopic granule location in Cftr-KO goblet cells. In the enteroid studies, CCH-induced exocytosis and luminal mucus bleb formation by Cftr-KO goblet cells was often accompanied by the delayed appearance of a similar bleb at the basal aspect of the enteroid (Figure 3A and Supplemental Video 2). The translucent appearance of the basal bleb and occasional observations of an apical-to-basal wave of cell distortion before formation of a basal bleb suggested ectopic basolateral exocytosis in the enteroids. However, the epithelial-laminin interface in enteroid culture does not recapitulate basement membrane composition in vivo (46); therefore, studies were performed using WT and Cftr-KO intestine. Freshly excised loops of small intestine were placed in warmed, oxygenated baths (37°C, 95% O$_2$:5% CO$_2$) containing 25 mM HCO$_3$-KBR with or without 100 $\mu$m CCH. After 20 minutes, the intestinal loops were collected, fixed, and sectioned for MUC2 IHC, periodic acid-Schiff (PAS) histology, or TEM. As shown in Figure 3B, MUC2 staining of WT goblet cells, whether untreated or CCH treated, was confined to the goblet cell thecal area, with light staining extending to the apical aspect of the nucleus. Epithelial staining was not observed in the absence of primary MUC2 antibody (Supplemental Figure 2). CCH treatment resulted in the formation of a MUC2-positive mucus layer on the epithelial surface and reduced granule area in WT goblet cells. In contrast, as shown in Figure 3C, 8.6% (9/105, $n$ = 6 sections) of untreated Cftr-KO goblet cells in both villus and crypt compartments exhibited MUC2 staining extending from the apical pole to the basement membrane. In some cases, the basement membrane protruded into the lamina propria. Luminal bleb formation over MUC2-stained cells was often apparent. CCH treatment increased the proportion of goblet cells showing basal extension of MUC2 staining to 18.2% (28/209, $n$ = 6 sections). Histologically, PAS-positive granules in some Cftr-KO, but not WT, goblet cells were located at or basal to the level of the cell nucleus (see Supplemental Figure 3).

At the ultrastructural (TEM) level, untreated WT goblet cells had the expected appearance of a granule-filled theca located just apical to the nucleus (Figure 3D, left). Following CCH, most WT goblet cells exhibited cavitation at the apical pore with remaining granules near the apical margin of the nucleus, and rarely, amorphous material was observed near the midregion of the nucleus (Figure 3D, right). In untreated Cftr-KO intestine (Figure 3E, left), goblet cells had features similar to WT goblet cells, but occasional Cftr-KO goblet cells had luminal blebs and amorphous material extending past midnucleus to the basal aspect of the cell (additional examples in Supplemental Figure 4). Following CCH (Figure 3E, right), Cftr-KO goblet cells commonly had granules/amorphous vacuoles causing displacement or distortion of the shape of the nucleus. The amorphous granules/vacuoles often extended to the basement membrane; however, breach of the basolateral plasmalemma or basement membrane could not be conclusively identified. In contrast to WT goblet cell superfused with 115 mM HCO$_3^-$-KBR. Left panel, enlarged granule cluster 2.92 minutes after CCH treatment; panel 2, normal-appearing progression of degranulation after CCH treatment; panel 3, appearance of transparent luminal blebs (white arrow) as mucin granules accumulate; panel 4, stationary granular bleb after exocytosis.
cells, cavitation at the apical aspect of the granule cluster was not apparent, whereas luminal blebs and/or granule compaction at the apical pole was observed.

Partial correction of abnormal exocytosis in Cftr-KO goblet cells by luminal superfusion with a high bicarbonate concentration. Studies by Gustafsson et al. have shown a positive effect of high luminal HCO$_3^-$ concentration on the adherence of surface mucus in the Cftr-KO intestine (9). To determine whether high luminal HCO$_3^-$ has a beneficial effect on the dynamics of Cftr-KO goblet cell function, enteroids were superfused on the luminal membrane with a KBR solution containing 115 mM NaHCO$_3$ gassed with 5% CO$_2$, which by luminal superfusion with a high bicarbonate concentration (57.1%, P < 0.05). The cross-sectional area of luminal goblet cells developing a luminal bleb decreased to 25.0% as compared with WT (time points 1–2: WT = –0.15 ± 0.41 minutes; Cftr-KO = 1.04 ± 0.26 minutes, P < 0.05; Figure 4A). Granule area in the Cftr-KO goblet cells decreased more during exocytosis and at a greater rate as compared with WT (Figure 4C, exocytosis-minimum), but remained slightly larger at the completion of exocytosis (minimum, Figure 4, A and B). Most importantly, the percentage of Cftr-KO goblet cells developing a luminal bleb decreased to 25.0% as compared with Cftr-KO goblet cells superfused with 25 mM HCO$_3^-$ (75.3%, P < 0.05). The cross-sectional area of luminal blebs was also reduced by approximately one-half (compare Figure 2C and Figure 4B) and appeared more granular and opaque. As shown by stills from a video sequence in Figure 4D (see also Supplementary Video 3), the enlarged Cftr-KO granular area superfused with 115 mM HCO$_3^-$ and exposed to CCH began exocytosis in an orderly process similar to that of WT goblet cells. However, as exocytosis proceeded, decondensation of released granules apparently did not keep pace, resulting in the formation of small, transparent luminal blebs and the accumulation of a granular mass at the cell surface.

Luminal superfusion with 115 mM HCO$_3^-$ also altered the dynamics of exocytosis by the Cftr-KO goblet cells. Granule area in both WT and Cftr-KO goblet cells increased slightly at the same rate after CCH exposure (Figure 4C). WT goblet cells began exocytosis at this point, whereas there was still a significant delay before exocytosis by Cftr-KO goblet cells (time points 1–2: WT = –0.15 ± 0.41 minutes; Cftr-KO = 1.04 ± 0.26 minutes, P < 0.05; Figure 4A). Granule area in the Cftr-KO goblet cells decreased more during exocytosis and at a greater rate as compared with WT (Figure 4C, exocytosis-minimum), but remained slightly larger at the completion of exocytosis (minimum, Figure 4, A and B). Most importantly, the percentage of Cftr-KO goblet cells developing a luminal bleb decreased to 25.0% as compared with Cftr-KO goblet cells superfused with 25 mM HCO$_3^-$ (75.3%, P < 0.05). The cross-sectional area of luminal blebs was also reduced by approximately one-half (compare Figure 2C and Figure 4B) and appeared more granular and opaque. As shown by stills from a video sequence in Figure 4D (see also Supplementary Video 3), the enlarged Cftr-KO granular area superfused with 115 mM HCO$_3^-$ and exposed to CCH began exocytosis in an orderly process similar to that of WT goblet cells. However, as exocytosis proceeded, decondensation of released granules apparently did not keep pace, resulting in the formation of small, transparent luminal blebs and the accumulation of a granular mass at the cell surface.

Luminal superfusion of both WT and Cftr-KO goblet cells with 115 mM HCO$_3^-$ appeared to mimic the early stages of CCH-induced exocytosis. To more directly evaluate the effect
of 115 mM HCO₃⁻, the luminal superfusate for WT and Cftr-KO enteroids was acutely switched from 25 mM HCO₃⁻ to 115 mM HCO₃⁻ KBR solution (Figure 5, A and B). As expected, switching to the higher luminal HCO₃⁻ concentration reduced granule area in the WT goblet cells (–19.6%) and increased granule area in Cftr-KO goblet cells (+33.3%). The decrease in granule area of WT goblet cells was consistent with a moderate induction of exocytosis. Spontaneous exocytosis was visibly appreciable in some WT goblet cells (Figure 5C). The paucity of the WT exocytotic events and extended recording times confounded a direct measurement of WT exocytotic rate, but the rate of change in granule area after switching to 115 mM HCO₃⁻ KBR was –5.6 ± 1.9 μm²/min (P < 0.05 vs. 0; n = 6–7). Although the change in granule area as a measure of exocytosis may be affected by unappreciated changes in granule or cell hydration, the longitudinal axis of the granule cluster in WT goblet cells decreased, whereas the transverse axis did not change (Δalong axis = –2.3 ± 0.4 μm, P < 0.05; Δtransverse axis = –0.5 ± 0.3 μm, NS), which is consistent with changes in granule cluster morphology in human colonic goblet cells undergoing stimulated exocytosis as compared with nonresponding goblet cells (47). The composition of the 115 mM HCO₃⁻ KBR, as compared with 25 mM HCO₃⁻ KBR, involved substitution of 90 mM HCO₃⁻ for 90 mM Cl⁻. To determine whether decreased luminal Cl⁻ concentration contributed to the observed effects, the luminal superfusate for WT and Cftr-KO enteroids was acutely switched from 25 mM HCO₃⁻ KBR to 25 mM HCO₃⁻ KBR in which 90 mM Cl⁻ was replaced with the impermeant anion isethionate. As shown in Figure 5D, the switch to isethionate KBR did not affect the granule area of either WT or Cftr-KO goblet cells. The effects of high luminal HCO₃⁻ concentrations on goblet cell function may have physiological relevance. It is possible that high HCO₃⁻ concentrations are achieved by Cftr-dependent transport at the luminal surface of goblet cells in vivo, especially when confined by an unstirred layer within the crypt or the recessed apical membrane of goblet cells on villi (see Figure 5E and refs. 48, 49).
Mucin granules in Cftr-KO goblet cells have an alkaline pH relative to WT. Mucin granule pH reflects the end point of the acidic secretory pathway and influences granule organization, mucin packaging, and granular Ca²⁺ signaling/polyionic composition (8, 23–25). In other secretory cells, granule pH is known to significantly alter the rate of exocytosis (26). Since CFTR plays a significant role in intestinal epithelial pH physiology (35, 50), we asked whether mucin granule pH differed in Cftr-KO goblet cells. Using LysoSensor, a fluorescent ratiometric pH indicator (pH range = 3.5–6.0), and Limar3D imaging, the mucin granule pH was compared between WT and Cftr-KO goblet cells at the apical, middle, and basal thirds of the granule cluster (Figure 6A). As shown in Figure 6B, the granule pH in the Cftr-KO goblet cell granule cluster was significantly alkaline at each level as compared with WT. An apical-to-basal gradient with decreasing pH was observed in the Cftr-KO but not WT granule cluster. As an independent validation of alkaline granule pH in Cftr-KO goblet cells, the intensity of quinacrine staining of mucin granules from our studies of intracellular pH (pHi, see below) was compared between enteroids from WT and Cftr-KO sex-matched littermates. Quinacrine is a fluorescent weak base that accumulates into acidic secretory granules, including mucin granules (26, 35); it was used in the pHi studies to identify goblet cells. Since quinacrine uptake and intensity are reduced by alkalizing secretory granule pH (26), it would be predicted that quinacrine intensity is less in alkaline Cftr-KO mucin granules. These measurements revealed that intensity of quinacrine-loaded granule clusters in Cftr-KO goblet cells was reduced relative to WT in all pairs by 24.8% ± 0.1% (P < 0.05, 64 and 78 goblet cells, respectively, from n = 6 WT and Cftr-KO littermate pairs). To confirm that LysoSensor was sensitive to pH changes in the granules, enteroids were treated with bafilomycin A1 (10 nM, 4 hours) to inhibit the V-ATPase of secretory granule membranes (24, 51). Bafilomycin A1 significantly increased granule pH in WT goblet cells, but was without effect on Cftr-KO granules (490/550 ratio: WT vehicle = 1.30 ± 0.05; WT bafilomycin A1 = 1.52 ± 0.03, P < 0.05; Cftr-KO vehicle = 1.49 ± 0.05; Cftr-KO bafilomycin A1 = 1.48 ± 0.03, NS; n = 3). Acute treatment with CFTR inhibitor-172 alkalinizes crypt epithelial pH in WT enteroids (35). To evaluate whether mucin granule pH is similarly affected, granule pH was measured in WT enteroids after a 1-hour treatment with 25 µM CFTR inhibitor-172. However, mucin granule 490/550 ratio was unaltered as compared with that in WT goblet cells treated with DMSO vehicle (CFTR inhibitor-172: 1.34 ± 0.11; DMSO: 1.32 ± 0.08, n = 7–9 goblet cells from 3 WT mice).

Since granule pH measurements were performed on intact WT and Cftr-KO enteroids, differences in the intraluminal milieu (pH, ionic content) of the enteroids may have altered granule pH between the 2 groups. To address this, granule pH was measured in enteroids that had been bisected to allow the goblet cell luminal membrane exposure to KBR for 30 to 60 minutes prior to granule pH measurement (Figure 6C). Although the granule 490/550 ratio was increased in both WT and Cftr-KO goblet cells, the Cftr-KO granules remained alkaline relative to WT. The greater 490/550 ratio in this experiment may indicate that intact enteroids normally have acidic luminal content, but this should be interpreted cautiously since the enteroids were provided fresh media after bisection that may not have fully equilibrated with 5% CO₂ tension.

Basal pH is normal in Cftr-KO goblet cells, but markedly alkaline in adjacent enterocytes. Previous studies of Cftr-KO enteroids demonstrated that resting pHi in crypt epithelial cells was alkaline by approximately 0.2 pH units relative to WT enterocytes (35), raising the question of whether goblet cell pHi is normal in Cftr-KO enteroids. To the best of our knowledge, goblet cell pHi has not been previously measured in a well-differentiated intestinal epithelium. Virtual measurement spheres within the plasmalemmal confines of goblet cells and their immediately adjacent enterocytes were placed in reconstructed 3D images from confocal 1-µm slice images of enteroid crypts loaded with quinacrine (to identify the goblet cells) and the pH-sensitive dye SNARF-5F (Figure 6D). Measurements were confined to goblet cells and adjacent enterocytes above the +4 cell position, i.e., inclusive of the transit-amplifying zone. As shown in Figure 6E, goblet cell pHi in WT and Cftr-KO enteroids was almost identical. However, in the Cftr-KO enteroids, the pHi of adjacent enterocytes was significantly more alkaline than the goblet cell.

Discussion
Real-time functional dynamics of goblet cell exocytosis in the small intestine have largely gone unexplored, in part due to the relative inaccessibility of goblet cells in the native intestine and past difficulties with primary culture of a well-differentiated intestinal epithelium. Owing to recent advances in intestinal organoid culture (36) and accurate recapitulation of CF intestinal disease in CF mouse models, enteroids from WT and Cftr-KO mice enable visual access from the apical to basal poles of goblet cells surrounded by absorptive enterocytes in situ. The enteroid model was used to determine whether or not goblet cells function normally when the Cftr-KO intestinal epithelium is isolated from the abnormal CF intestinal environment. We found that goblet cells in Cftr-KO enteroids exhibited alterations in exocytotic dynamics, mucus retention, ectopic granule location, and differences in mucin granule pH. Thus, orderly goblet cell exocytosis is an intestinal epithelial-autonomous function of CFTR. In the absence of CFTR, goblet cell dysfunction likely contributes to the pathology of mucoviscidosis in CF intestinal disease. In contrast to goblet cell function, the phenomenon of goblet cell hyperplasia in the CF intestine is likely a consequence of the altered intestinal environment (dysbiosis, inflammation), since goblet cell numbers in Cftr-KO enteroids were not found to differ from those in WT enteroids (52).

Our data indicate that CCH-stimulated exocytosis by the Cftr-KO goblet cell is characterized by an initial, robust increase in the volume of the granule cluster, as indexed by granule area, which is maintained for a finite period before initiation of exocytosis. Studies of WT goblet cells showed that granule volume also tended to increase initially, but the change was apparently countered by coincident onset of exocytosis and granule loss. Our 2D area measurements of the granule cluster as an index of volume may be confounded by granule reorganization or changes in epithelial cell volume (35, 53). However, direct observation of individual enlarging mucin granules within the Cftr-KO goblet cell is consistent with partial granule decondensation as a contributor to increased granular area. The subsequent exocytotic expulsion of a partially decondensed mucus bleb supports this notion. Moreover, during the early stages of CCH stimulation, the enlarging Cftr-KO
cluster remained granular in appearance (i.e., not a mass of coagulated mucus) and the increased granule area occurred without a change in cell height, an index of cell volume (44). If exposure to a high luminal HCO$_3^-$ concentration can also be regarded as a stimulus of exocytosis, then “swelling” of the granule clus ter prior to exocytosis appears to be a consistent feature of stimulated Cftr-KO goblet cells. Preexocytotic granule swelling and delayed exocytosis are consistent with the slow rate of swelling and diffusivity of mucin granules released by Cftr-KO gallbladder columnar cells (54). Further studies will be necessary to determine whether this abnormality results from a defect intrinsic to the stored granule (e.g., improper processing, imbalance in Ca$^{2+}$ shielding), an alteration in granule membrane electrolyte transport, or a disruption of secretory pore formation in the Cftr-KO goblet cell.

The increased rate of exocytosis during formation of the luminal mucus bleb and ectopic granule location may be a related characteristic of Cftr-KO goblet cells. In the Cftr-KO enteroids, goblet cell exocytosis was typically observed as a forceful event. Since exocytosis is mediated by Ca$^{2+}$-dependent processes acting on contractile elements (55, 56), it is significant that past studies have found exaggerated agonist-induced Ca$^{2+}$ mobilization and plasmalemmal Ca$^{2+}$ influx in CF epithelia that may apply to goblet cells (57–61). It was also observed that luminal bleb formation was sometimes followed by ectopic release of mucus to the basolateral side of the epithelium in Cftr-KO enteroids. This phenomenon prompted a search for ectopic goblet cell exocytosis in native Cftr-KO intestine. Although ectopic exocytosis could not be confirmed, we found that 10% to 20% of Cftr-KO goblet cells exhibited ectopic intracellular granule location. Electron microscopy and immunohistochemical and histological imaging of these Cftr-KO goblet cells revealed disruption of cellular morphology, with mucin granules reaching the basal membrane of the cell, suggesting retrograde intracellular granule movement during stimulated exocytosis. Though speculative at this point, the secretory contractions in Cftr-KO goblet cells as luminal mucus bleb forms may force mucin granules toward the basal membrane if the pathway is less resistant. One potential route for retrograde granule movement is goblet cell–associated antigen passageways (GAPs) (62). GAPs normally provide a means for presentation of luminal antigen to submucosal dendritic cells. Together with damage to the goblet cell, disruption of GAPs in this fraction of Cftr-KO goblet cells would likely have immunological consequences and possibly contribute to small bowel inflammation in CF patients and CF mice (32, 42, 43).

A high luminal HCO$_3^-$ concentration improved goblet cell exocytosis in Cftr-KO enteroids and induced a low rate of spontaneous exocytosis by WT goblet cells. For Cftr-KO goblet cells, a luminal 115 mM HCO$_3^-$ concentration increased granule decondensation and reduced luminal mucus bleb formation. Since CFTR-dependent HCO$_3^-$ secretion is often coincident with physiological mucous secretion (7), these findings indicate that the loss of CFTR-dependent HCO$_3^-$ secretion by adjacent enterocytes and perhaps across the granule membrane (22) is an important contributor to the pathogenesis of mucoviscidosis. CFTR-dependent HCO$_3^-$ transport can attain nearly isotonic (150 mM) NaHCO$_3$ concentrations in pancreatic juice (63). Although the local concentration of HCO$_3^-$ at the luminal surface of CFTR-competent enterocytes is not known, it is conceivable that high concentrations are achieved within the restricted space of the crypt or in goblet cell recesses on villi. Thus, proper mucin unfolding and the access to enzyme activity would be available for the subsequent release of mucus from the mucosal surface (10). For WT goblet cells, 115 mM HCO$_3^-$ luminal superfusion induced a moderate rate of exocytosis. Although observation of spontaneous exocytosis was rare, parameters consistently indicating WT goblet cell exocytosis were decreased granular area and morphological changes in the granule cluster indicative of exocytosis (47). Further, 115 mM luminal HCO$_3^-$ greatly increased granule area in the Cftr-KO goblet cell, which was reminiscent of the early stages of CCH stimulation. Given the deficits of HCO$_3^-$ secretion in the Cftr-KO intestine (50, 64, 65), losing local HCO$_3^-$ stimulation may contribute to goblet cell enlargement with stored granules, which is often noted in histological sections of CF epithelia. Despite the positive effects of 115 mM HCO$_3^-$ luminal superfusion, goblet cell dysfunction was still apparent in the Cftr-KO enteroids. After CCH, the initial “swelling” of the granule cluster did not fully normalize after exocytosis and, more importantly, luminal mucus blebs formed on approximately 25% of the goblet cells. The granular appearance of the luminal blebs in this case, as shown in Supplemental Video 3, may be evidence of an exuberant exocytotic rate that exceeds the capacity for granule decondensation.

An unexpected finding was that mucin granules within Cftr-KO goblet cells are alkaline relative to WT. Cftr-KO goblet cell pH$_i$ was normal, indicating that goblet cell pH$_i$ is not regulated by plasmalemmal CFTR as it is in adjacent enterocytes. However, the granule pH data raise the possibility that finite amounts of CFTR are present in the secretory pathway/mucin granule membrane and play a role in granule acidification. Some studies strongly dispute the contribution of CFTR to organellar acidification, but others find that CFTR provides a counter-anion permeability that facilitates V-ATPase activity (16–21). Estimates that minimal CFTR activity is required in the secretory pathway (66) are supported by studies showing that low-level transfection with recombinant CFTR corrects abnormal mucin sulfation without significantly increasing cAMP-stimulated Cl$^-$ permeability in CF airway epithelium (67). Further, studies have shown colocalization of CFTR and mucins within gallbladder columnar cells (11), and more recently, studies of mucin granules isolated from human airway epithelium show that CFTR immunolocalizes with MUC5AC and that a CFTR-dependent Cl$^-$ conductance enhances granule acidification (22). Consistent with the latter report, we found that a low concentration of bafilomycin A1 alkalized mucin granules in WT but not Cftr-KO goblet cells, suggesting a deficit of V-ATPase activity in CF. A second possibility is that mucin granule pH$_i$ is indirectly regulated by CFTR via intercellular communication with neighboring enterocytes. Little is known about gap junction–connexin communications between goblet cells and adjacent enterocytes, but extensive interdigitation of the plasma membranes exists at the level of the theca with neighboring alkaline Cftr-KO enterocytes (see Figure 5E, inset). Gap junction–connexin communication provides an ionic conductance in other secretory cells (68). Moreover, gap junctional conductance is increased by an alkaline pH$_i$ (69) and there is evidence of defective regulation in CF epithelium (70). Thus, the possibility is raised that gap junctional communication at the level of the theca may provide HCO$_3^-$ availability or, conversely, a proton sink that affects granule pH in the Cftr-KO goblet cell.
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The alkaline pH of Cftr-KO mucin granules is predicted to have consequences regarding goblet cell exocytosis and a potential contribution to mucoviscidosis. It is not clear whether the degree of alkalinity in the Cftr-KO granules is sufficient to alter the pH-sensitive steps of mucin multimerization and N-terminal cleavage. In vitro, multimerization and N-terminal cleavage of MUC2 occur at up to pH 6.0–6.2 (8, 23), so the pH difference between WT and Cftr-KO granules in a more acidic range may have little effect. More consistent with our observations, it is known that an alkaline granule pH slows or even inhibits exocytosis in secretory cells. In chromaffin granule membranes, dissociation of the multimeric V0 subunit from the V1 subunit of the V-ATPase is an inverse linear function of intragranular pH, and the disassociated V0 subunit is important to formation of the fusion pore (71). An alkaline granule pH also reduces granule Ca\(^{2+}\) signaling by mucin granules, which may delay exocytosis and diminish feedback to the goblet cell of the exocytotic event (25). However, it is the effect of an alkaline secretory path on the polyionic composition of mucins that may cause the greatest disturbance to exocytosis by altering the Donnan equilibrium process of decondensation. In solutions of identical ionic strength in the polyionic composition of mucins that may cause thereby indicating increased mucin-Ca\(^{2+}\) affinity in CF granules relative to mucin granules from normal human airway goblet cells, secretory pathway (77). Increased mucin-Ca\(^{2+}\) affinity would negatively affect alkalinity in the intestinal epithelium. Extrapolated to the CF airways, mucus microbials/trefoil factors (2), and impeded nutrient access (82) at great increased the surface area of mucosal contact in the formation of obstructing impactions (79–81), sequestered secreted antimicrobials/trefoil factors (2), and impeded nutrient access (82) at the intestinal epithelium. Extrapolated to the CF airways, mucus retention by goblet cells may be the cellular basis for impaired mucus detachment from submucosal gland ducts that is responsible for deficient mucociliary transport in the CF pig model (30). In addition to mucus retention, mislocalization of mucin granules within the goblet cell may negatively affect luminal antigen presentation to submucosal dendritic cells and contribute to the inflammatory component of CF intestinal disease (62). Identifying and correcting the process of mucin granule alkalization will shed light on the early stages in the pathogenesis of mucoviscidosis. From an immediate perspective, our data show that provision of a high concentration of HCO\(_{3}^-\) to the mucosal surface greatly improves goblet cell dysfunction. Therapeutic use of HCO\(_{3}^-\), containing solutions for inhalation therapy of CF lung disease has been advocated (9), but ingestion of HCO\(_{3}^-\)-containing medications might also be considered for the treatment of distal intestinal-obstruction syndrome (DIOS) and constipation in CF patients.

Methods

**Animals.** Mice with gene-targeted disruption of the murine homolog of Cfr (Abcc7, Cftr\(^{tm1Unc}\), Cftr-KO) and sex-matched Cftr\(^{+/+}\) or Cftr\(^{-/-}\) (WT) littermates were used (80). Mice were routinely outbred to Black Swiss (Taconic) at generational intervals. Resulting F1 heterozygotes were crossed to generate F2 offspring for experimentation. The mutant mice were identified using a PCR-based analysis of tail-snip DNA, as previously described (33). All mice were maintained ad libitum on standard laboratory chow (Formulab 5008, rodent chow; Ralston Purina) and distilled water containing CoLyte (Schwarz Pharma) laxative to avoid intestinal obstruction in the Cfr-KO mice (33). Mice were housed individually in a temperature- and light-controlled room (22–26°C; 12-hour light:12-hour dark cycle) in the Association for Assessment and Accreditation of Laboratory Animal Care–accredited animal facility at the Dalton Cardiovascular Research Center, University of Missouri.

**Enteroid culture.** The enteroid culture method has previously been described in detail (35). Gel cultures were overlaid with Ham’s F-12 medium containing 5% FBS, 50 μg/ml gentamicin, 125 ng/ml R-Spondin, 25 ng/ml norggin, and 12.5 ng/ml epidermal growth factor. Medium was changed every 3 days. Primary enteroids were passaged at 7 to 10 days after isolation using Cell Recovery solution (BD Biosciences).

**Histology, TEM, and IHC.** Freshly excised small intestine was placed in a bath of KBR with or without 100 μM CCH warmed to 37°C and gassed with 95% oxygen:5% CO\(_2\) (pH 7.4) for 20 minutes. As described for histological sections (35), intestine was fixed in 4% paraformaldehyde overnight, embedded in paraffin, and sectioned (5 μm) for PAS staining. As described previously for TEM (2), sections were fixed in a 2.5% glutaraldehyde–2.0% paraformaldehyde solution, postfixed in 1% osmium tetroxide/1% uranyl acetate, and embedded in Epon-araldite (Electron Microscope Sciences). Thin sections were transferred to 200-mesh copper grids before staining with uranyl acetate and lead citrate. Sections were viewed with a JEO1400 TEM at 80 kV accelerating voltage. For MUC2 IHC, tissue was fixed using 4% paraformaldehyde containing 0.1% Igepal, and incubated for 2 hours at room temperature (RT) with rabbit anti-human MUC2 (H-300) primary antibody (Santa Cruz Biotechnology Inc., no. sc-2004). Sections were viewed with a JEOL 1400 TEM at 80 kV accelerating voltage. For MUC2 IHC, tissue was fixed using 4% paraformaldehyde containing 0.1% Igepal, and incubated for 2 hours at room temperature (RT) with rabbit anti-human MUC2 (H-300) primary antibody (Santa Cruz Biotechnology Inc., no. sc-15354) diluted 1:50. Sections were treated with 3% hydrogen peroxide followed by a 1-hour incubation at RT with a 1:500 dilution of goat anti-rabbit IgG-HRP–conjugated secondary antibody (Santa Cruz Biotechnology Inc., no. sc-2004). Sections were counterstained for 20 seconds with a 1:10 dilution of Mayer’s Hematoxylin (Dako). Sections incubated without primary antibody were used to assess nonspecific labeling. Images were acquired using an Olympus IX73 microscope and ImageJ software (http://imagej.nih.gov/ij/).
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Brown, Sutter Instrument Co.) and beveled to an approximately 30-μm tip diameter. Micropipettes were equipped with a PicoPump dual syringe pump (Harvard Apparatus) and inserted into the punctured enteroid epithelium using the ×10 water immersion objective of an upright Olympus BX-51WI microscope. Enteroids were luminally superfused at 1 to 2 μl/min with KBR. In some experiments, 90 mM NaCl was replaced equimolar with NaHCO₃ or Na Isethionate. Visible evidence of luminal superfusion included flushing of debris from the lumen and slight distension of the enteroid and enteroid crypts (see Figure 2A). Experiments were discarded if (a) visual evidence of luminal superfusion was not apparent, (b) the enteroid moved excessively, or (c) hydraulic rupture of the enteroid epithelium was apparent (overall, ~20% of experiments were discarded). Using a ×60 water immersion objective, 1 to 3 goblet cells were imaged in the longitudinal plane to obtain the maximal granule area, a centrally positioned granule cluster, and cell location at the apical one-third of the cell. The polarity of the granule cluster was not different between WT and Cftr-KO goblet cells based on measurements of the longitudinal axis of the granule cluster as a percentage of the total cell height in untreated enteroids bathed in 25 mM HCO₃⁻ KBR (WT cluster length/cell height: 26.4% ± 1.0% vs. Cftr-KO cluster length/cell height: 27.4% ± 1.0%, n = 16–17 goblet cells from 4 WT and Cftr-KO sex-matched littersmates). Paneth cells were identified by larger granules, a centrally positioned granule cluster, and cell location at the crypt base. Baldriculated Paneth cells and intermediate cells (83) with larger granules and theca were strictly avoided. Single goblet cells were imaged in the longitudinal plane to obtain the maximal granule area continuously throughout the experiment using a remote-controlled fine-focus wheel. Enteroids were luminally superfused for approximately 5 to 7 minutes before the addition of 100 μM CCH to the (basolateral) superfusate. Images were acquired at 1- or 5-second intervals for approximately 15 minutes using a Senso-Cam digital camera ( Cooke) and Slidebook 5.0 software (Intelligent Imaging Innovations). Postacquisition analysis of granule area or luminal mucus blebs was performed on a frame-by-frame basis by outlining the perimeter of the granule cluster or bleb with a stylus and obtaining the corresponding area using Slidebook software. Granule or bleb area and the first post-CCH exocytotic event with respective times were measured or observed by a designated operator who was blinded to both mouse genotype and treatment. Granule area measurements were collected at 4 time points during the experiment: 0 (also designated as basal) was the granule area in the frame immediately prior to CCH addition to the basolateral superfusate; 1 (also designated as maximum) was maximal granule area obtained in the frames following CCH exposure; 2 (also designated as exocytosis) was granule area when first visible evidence of exocytosis was observed as a loss of individual granules or a mucus bleb from the granule cluster; 3 (also designated as minimum) was the granule area when granule loss from the granule cluster was no longer observed. The area of mucus accumulated above the goblet cell theca in Cftr-KO enteroids (designated as mucus bleb area) was acquired at the minimum time point by outlining the luminal perimeter of the mucus bleb above a straight line drawn between the apical membranes of enterocytes adjacent to the goblet cell.

Intracellular pH and granule pH measurements. The method for measurement of intracellular pH of enteroid epithelium has been recently described (35). Briefly, enteroids were incubated with the ratiometric fluorescent pH indicator SNARF-5F (Invitrogen, 40 μM, 30 minutes) and counterstained with quinacrine (Sigma-Aldrich, 1 μM, 15 minutes) to identify goblet and Paneth cells. SNARF-5F was excited at 514 μm and images collected at emission wavelengths of 580μm and 640μm; quinacrine was excited at 488μm and images collected at emission wavelengths of 500μm–540μm. Using a TCS SP5 confocal-multiphoton microscope (Leica). For measurement of mucin granule pH, enteroids were loaded with 5 μM LysoSensor Yellow/Blue DND-160, a fluorescent ratio pH indicator (pH range = 3.5–6.0, Invitrogen), for 5 minutes before imaging. LysoSensor dye was excited at 360μm (720μm 2-photon), and dual emissions were collected at 490μm and 550μm. Z stacks of 0.25-μm slices were acquired, and Imaris software (Bitplane) was used for 3D reconstruction of the goblet cell granule cluster and automated placement of virtual measurement spots on the mucin granules (see Figure 6A). The 490μm/550μm emission ratio acquired from measurement spots was averaged either for the entire granule cluster or at the apical, middle, and basal thirds of the granule cluster. Attempts to calibrate the 490μm/550μm ratio of LysoSensor for pH using ionophores (nigericin, monensin) to clamp organellar pH (84) induced goblet cell exocytosis within 2 minutes after exposure. Calibration performed at less than 2 minutes after exposure indicated granule pH within the reported pH range for LysoSensor (WT: 4.61 ± 0.05 vs. Cftr-KO: 4.84 ± 0.02, n = 6), but a steady-state equilibrium at each calibration of pH could not be achieved. Due to this inaccuracy, the more precise measurement of the 490μm/550μm emission ratio is reported.

Statistics. All values are reported as mean ± SEM. Data between 2 groups were compared using a 2-tailed Student’s t test assuming equal variances between groups. Data from multiple treatment groups were compared using a 1-way ANOVA with a post hoc Tukey’s t test. A repeated-measures ANOVA was used for comparisons between different time points in studies of exocytotic dynamics. P < 0.05 was considered statistically significant.

Study approval. All experiments involving animals were approved by the University of Missouri IACUC.

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