Gel-forming mucins form distinct morphologic structures in airways

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Gel-forming mucins, the primary macromolecular components of airway mucus, facilitate airway clearance by mucociliary transport. In cystic fibrosis (CF) altered mucus properties impair mucociliary transport. Airways primarily secrete two closely related gel-forming mucins, MUC5B and MUC5AC. However, their morphologic structures and associations in airways that contain abundant submucosal glands and goblet cells are uncertain. Moreover, there is limited knowledge about mucins in airways not affected by inflammation, infection, or remodeling or in CF airways. Therefore, we examined airways freshly excised from newborn non-CF pigs and CF pigs before secondary manifestations develop. We found that porcine submucosal glands produce MUC5B, whereas goblet cells produce predominantly MUC5AC plus some MUC5B. We found that MUC5B emerged from submucosal gland ducts in the form of strands composed of multiple MUC5B filaments. In contrast, MUC5AC emerged from goblet cells as wispy threads and sometimes formed mucin sheets. In addition, MUC5AC often partially coated the MUC5B strands. Compared with non-CF, MUC5B more often filled CF submucosal gland ducts. MUC5AC sheets also accumulated in CF airways overlying MUC5B strands. These results reveal distinct morphology and interactions for MUC5B and MUC5AC and suggest that the two mucins make distinct contributions to mucociliary transport. Thus, they provide a framework for understanding abnormalities in disease.

mucus | cystic fibrosis | lung | asthma | COPD

Mucus propelled by ciliary activity (mucociliary transport, MCT) is an important host defense that removes particulates from airways (1–3). The predominant macromolecular components of airway mucus are two secreted mucins, MUC5B and MUC5AC (1, 4–7). These gel-forming mucins are long, heavily glycosylated proteins with similar domain organization and amino acid sequence. Previous studies described biochemical properties of these and related mucins (1, 4, 6, 8). In human airways, MUC5B is produced in submucosal glands and goblet cells, and MUC5AC is produced in goblet cells (1, 4, 5, 9). In mouse lungs, MUC5B and MUC5AC are expressed primarily in club cells (1, 10). Mice with a disrupted *Muc5b* gene accumulated mucus in the upper airway, whereas mice with a disrupted *Muc5ac* gene lacked apparent respiratory abnormalities (10). Together, these results suggest that MUC5B and MUC5AC may have different functions.

Mucin abnormalities may contribute to lung disease (1, 5). In asthma and models of airway hyperreactivity, mucus contains increased levels of MUC5B and MUC5AC, airways exhibit goblet cell hyperplasia, and *MUC5AC* transcripts are increased whereas *MUC5B* transcripts are decreased (1, 4, 5, 11–13). Chronic obstructive pulmonary disease manifests increased mucin production (9, 13). Variations in the *MUC5B* gene promoter/enhancer region have been associated with interstitial pulmonary fibrosis (14). In advanced cystic fibrosis (CF), airways show goblet cell hyperplasia and submucosal gland hypertrophy, and imaging of radiolabeled particles deposited in the lung indicates that MCT is reduced (1, 3). The MCT reduction is greater as the severity of the disease increases, consistent with the finding that reduced MCT has not been detected in young people with CF (15, 16).

Porcine models of CF develop airway disease that replicates that in humans (17–20). At birth CF pig lungs lack airway infection and inflammation yet display disrupted MCT, indicating a primary host defense defect (21). In vivo studies of spontaneously breathing newborn CF pigs revealed impaired movement of insufflated microdisks after cholinergic stimulation of submucosal gland secretion (21, 22). Microdisks traveled up the airways at rates that varied substantially even over the same airway region and even in the same pig. Moreover, in CF airways, some microdisks did not move at all, whereas others sped by in close proximity. The variability in microdisk behavior in both non-CF and CF suggested substantial heterogeneity in airway mucus traveling over the airway surface. In ex vivo studies, we used fluorescent nanospheres (functionalized with carboxylate, sulfate, or amine) to label mucus arising from submucosal gland ducts (21). In CF, the mucus strands sometimes failed to break, and as a result, they remained attached to ducts, halting MCT. Preventing Cl^- and HCO_3^- secretion in non-CF pigs also partially prevented mucus from breaking free from submucosal glands, directly linking loss of CFTR and impaired

Significance

Mucus propelled by cilia is key for removing particulates from lungs by mucociliary transport. The major structural components of airway mucus are two gel-forming mucins, MUC5B and MUC5AC. These mucins exhibit distinct morphologic structures. MUC5B is secreted by submucosal glands in the form of strands. MUC5AC is secreted by goblet cells as threads and thin sheets. After emerging onto the airway surface, the two mucins associate to form MUC5B strands partially covered with MUC5AC. These distinct morphologic structures likely enable efficient mucociliary transport. In cystic fibrosis, strands become entangled, MUC5B often fills submucosal gland ducts, and MUC5AC sheets are larger, are more abundant, and overlie strands. Disrupted anion secretion in cystic fibrosis alters mucin morphology, which will impair mucociliary transport.

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MCT (21, 23, 24). These functional observations suggest that mucus forms discontinuous structures rather than a homogeneous layer on the airway surface.

Despite the importance of mucins, knowledge of the in situ structural morphology of mucins on the airway surface in health and disease is limited. First, few studies have examined mucins on the airway surface. Second, mucus has been studied on cultured cells, but they lack submucosal glands and material cannot flow onto or leave the surface. Third, mice, though valuable for many studies, have only a few submucosal glands in the proximal trachea, most mucin is secreted by club cells, and mice with a disrupted *CFTR* gene do not develop airway disease like that in people with CF (25). Fourth, it is difficult to obtain mucus from normal lungs, and sputum obtained from people with airway disease may contain increased proteases, accumulated DNA, and other confounders (26). Moreover, isolation, processing, and storage of mucin can alter its structure and function (4).

Whether the morphologic appearances of MUC5B and MUC5AC differ or what distinct roles they might play in airways remain uncertain. Our goal was to examine the morphology of these mucins as they are secreted onto the airway surface. We studied pigs because their airways are similar to humans (27), including the presence of submucosal glands, and they develop disease that mimics human CF (17, 19, 20). We used newborns to avoid secondary effects of the disease. In previous studies, we excised tracheas and covered the apical surface with saline to clamp pH, ionic composition, and liquid levels (21). Here, we studied airways without submersion or rinsing the apical surface.

Results

WGA Lectin Preferentially Labels MUC5B, and JAC Lectin Preferentially Labels MUC5AC. We made the empirical observation that wheat germ agglutinin (WGA) lectin preferentially labeled MUC5B, and jacalin (JAC) lectin preferentially labeled MUC5AC. We found that mucous cells in the acinus of submucosal glands labeled with anti-MUC5B antibody and colocalized with WGA labeling (Fig. 1*A*). In contrast, neither anti-MUC5AC antibody nor JAC labeled the submucosal gland cells (Fig. 1*A*). The presence of MUC5B but not MUC5AC in submucosal glands is consistent with previous studies in humans and pigs (1, 4, 5, 28).

In cells of the surface epithelium, we found that JAC colocalized with anti-MUC5AC antibody (Fig. 1 *A* and *B* and Fig. S1*A*) and WGA colocalized with anti-MUC5B antibody (Fig. 1 *A* and *B*). En face images showed that ~73% of goblet cells labeled only with JAC, about 17% labeled with WGA alone, and about 10% labeled with both WGA and JAC (Fig. 2 *A* and *B*). Despite substantial variability at different locations within individual airways, these data suggest that MUC5AC is the predominant mucin produced by goblet cells. These results are consistent with earlier studies in porcine and human airways (1, 4, 5, 28). In addition, WGA labeling did not colocalize with anti-MUC5AC antibody, and JAC labeling did not colocalize with anti-MUC5B antibody (Fig. S2 *A* and *B*). Note, however, that WGA and JAC are not specific only to these mucins; these lectins also show some staining of membranes and other structures (Fig. S2*C*).

In subsequent studies of secreted mucins on the airway surface, we used WGA and JAC to identify MUC5B and MUC5AC, respectively. Like in cells, we found that WGA, but not JAC, colocalized with anti-MUC5B antibody. JAC, but not WGA, colocalized with anti-MUC5AC antibody. However, we did not rely on lectin staining alone; for all key studies, some experiments were done with mucin antibodies.

Strands of MUC5B Mucus Emerge from Submucosal Glands and Associate with MUC5AC. We found that WGA-linked fluorophore labeled mucus emerging from submucosal glands (Fig. 3*A*). WGA colocalized with MUC5B immunostaining (Fig. S3), as predicted based on production of MUC5B by submucosal glands. Mucins exit gland ducts not as a homogeneous tube or stream but rather as a strand of multiple MUC5B filaments (Fig. 3*B*). At the point of exit, strands varied from 5 to 50 μm in diameter. After that point, the diameter of strands sometimes increased. Their shape also sometimes changed, becoming less cylindrical, adopting a ribbon-like appearance, or fanning out. After leaving the duct, strands extended toward the larynx and in a ventral direction, consistent with the direction of cilia beating (22).

JAC and anti-MUC5AC antibody also labeled mucus strands (Fig. 3 *A* and *B*). This seemed surprising because the submucosal glands produced MUC5B but not MUC5AC. To test if mucin strands emerging from the glands labeled with JAC, we examined strands in the ducts at the level of and just below the airway surface. Emerging strands labeled with WGA but not JAC, indicating they were composed of MUC5B (Fig. 3*C*).

Scanning electron microscopy also revealed strands of mucus emerging from submucosal gland ducts extending toward the larynx (Fig. S4). Mucus strands were comprised of individual filaments, consistent with the lectin and antibody labeling.

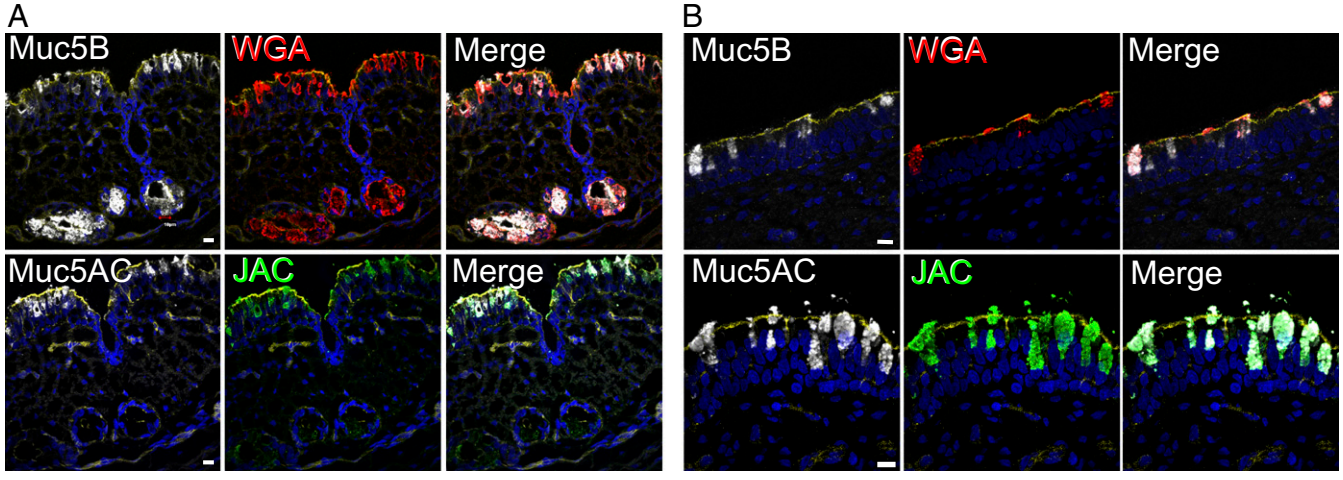

Fig. 1. WGA and JAC lectins preferentially label MUC5B and MUC5AC. Figure shows labeling of trachea from newborn pigs by fluorophore-linked lectins and anti-mucin antibodies. (*A*) Images are tracheal sections showing submucosal glands labeled with anti-MUC5B antibody (white) and WGA (red) (*Top*) or anti-MUC5AC antibody (white) and JAC (green) (*Bottom*). Also shown are actin labeling (phalloidin, yellow) and nuclei labeling (DAPI, blue). (Scale bar, 10 μm.) (*B*) Images are airway surface epithelium showing goblet cells labeled with anti-MUC5B antibody (white) and WGA (red) (*Top*) or with anti-MUC5AC antibody (white) and JAC (green) (*Bottom*). Also shown are actin labeling (phalloidin, yellow) and nuclei labeling (DAPI, blue). (Scale bar, 10 μm.)

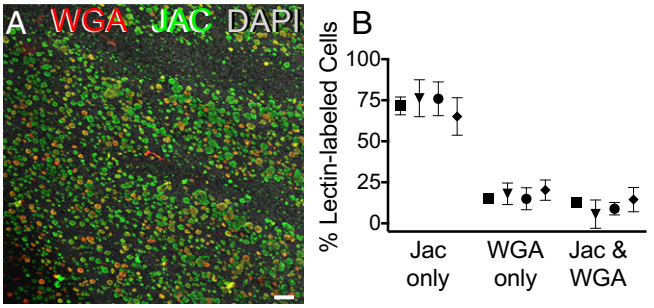

Fig. 2. WGA and JAC lectins label surface goblet cells. (*A*) *En face* image of excised airway surface epithelium labeled with WGA (red), JAC (green), and nuclei (DAPI, gray). (Scale bar, 50 μm.) In subsequent *en face* images, red and green mucin staining in goblet cells, with proportions varying in individual fields, can be seen below secreted mucin. (*B*) Percentage of goblet cells in airway surface epithelium labeled by JAC (MUC5AC), WGA (MUC5B), or both. Each symbol represents average of experiments on epithelia from one animal, and error bars indicate SD.

Threads and Sheets of MUC5AC Are Released from Goblet Cells onto the Airway Surface. The JAC lectin and anti-MUC5AC antibody detected wispy threads that were distinct from the mucus strands (Figs. 3 *A* and *B* and 4*A*); henceforth, we refer to MUC5AC in this form as "threads." The MUC5AC threads were ~1–4 μm in diameter. Threads often joined MUC5B strands (Fig. 3 *A* and *B*). We also sometimes observed small thin sheets of JAC-labeled material (arrow in Fig. 4*A*); these were much more prominent in CF (Fig. 5*B*). In contrast to the frequent appearance of MUC5B strands attached to submucosal gland ducts, we seldom observed MUC5AC threads emanating from goblet cells (Fig. 4*A*). The lack of attachment of MUC5AC threads to surface cells suggests that MUC5AC is rapidly released from goblet cells after it is secreted. In contrast to MUC5AC, we rarely detected MUC5B threads; Fig. 4*B* shows a rare example.

Compared with WGA labeling, JAC and anti-MUC5AC antibody labeled the exterior of strands (Fig. 3 *A* and *B*). The appearance of MUC5AC threads on the surface of MUC5B strands indicates that MUC5AC associates with MUC5B strands after they exit from the duct orifice onto the airway surface. Thus, mucus strands have a MUC5B core and a partial coating of MUC5AC.

The Appearance of the Mucins Differs in CF and Non-CF Airways. Previous studies showed that abnormal mucus impaired MCT in newborn CF pigs (21). However, *MUC5B* and *MUC5AC* transcripts, Western blotting of mucin protein, goblet cell numbers, and mucus glycosylation did not differ by genotype (28, 29). In addition, controlling the solution volume and maintaining pH at 7.35 on the apical surface did not prevent the mucus abnormality or impaired MCT (21). Those observations focused attention on mucus produced below the surface in submucosal glands. However, those studies were done with airways submerged in saline, they did not identify the mucin, and they could not reveal morphologic aspects. Thus, we hypothesized that mucins would exhibit abnormal morphology in ex vivo airways not submerged in saline. We administered methacholine in vivo to stimulate mucus secretion and then removed and examined airways. Like mucins in non-CF airways, in CF airways we observed mucins in strands and threads and MUC5AC partially covering the surface of MUC5B strands (Fig. 5 *A* and *B*).

However, the morphologic appearance of the mucins differed between the two genotypes in several ways. First, in CF airways, MUC5B strands often remained attached to the ducts from which they emerged. In addition, strands emanating from different submucosal gland ducts often merged and appeared entangled (Fig. 5*A*). This pattern is consistent with earlier functional experiments showing that mucus strands from CF ducts sometimes failed to break and then leave submucosal gland duct openings (21). That defect produced the appearance of aggregated mucus strands on the surface of submerged CF tracheas. In contrast, in non-CF airways, MUC5B strands were less tangled (Fig. 5*A*).

Second, in CF, MUC5AC often appeared as thin sheets overlying MUC5B strands (Fig. 5*B*). In contrast, in non-CF, MUC5AC sheets were rarely observed overlying MUC5B strands.

Third, compared with non-CF, CF submucosal glands were more often distended with mucin from the acinus up through the duct to the airway surface (Fig. 6 *A* and *B*). These findings suggested that ducts of CF submucosal glands would be filled with mucus more frequently than non-CF ducts. To test this prediction, we counted ducts filled with WGA-labeled mucin. Compared with non-CF, a greater fraction of CF ducts were filled with MUC5B (Fig. 6 *B* and *C*). Finding more mucus-filled ducts in CF is in seeming contrast with previous studies showing that CF submucosal glands are smaller and secrete less liquid than non-CF (28, 30, 31). However, less liquid secretion together with more mucus filling of the ducts suggests that the mucus has abnormal biophysical properties, a conclusion consistent with earlier studies (21).

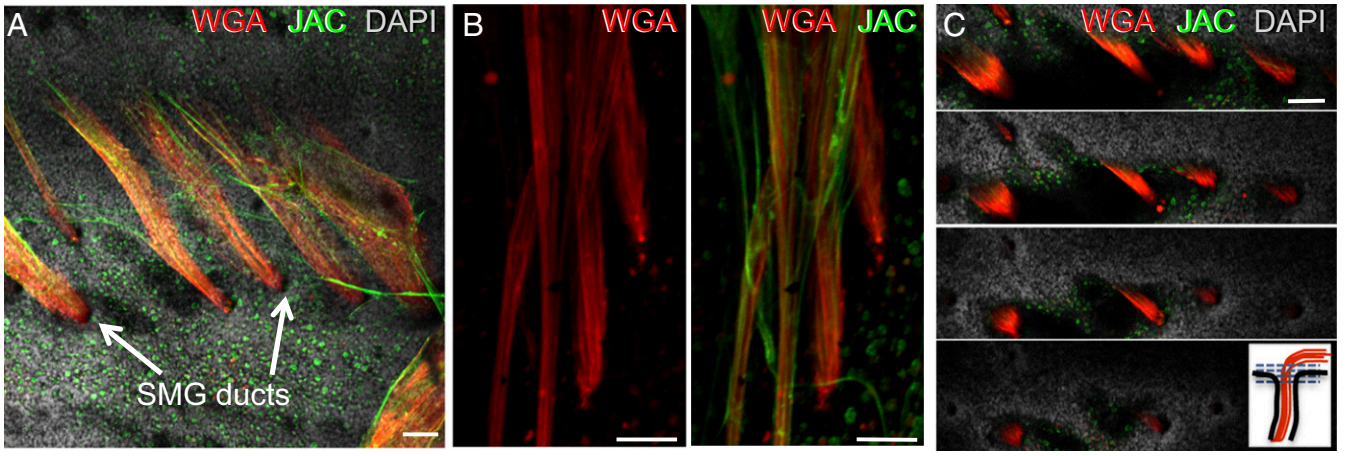

Fig. 3. Mucus emerging from submucosal gland ducts labels with WGA lectin. Images in *A* and *B* are *z* stacks and in *C* are single confocal images of the excised non-CF tracheal surface. WGA is red, JAC is green, and DAPI (nuclei) is gray. (*A*) Airway surface with mucus strands emerging from submucosal gland (SMG) ducts. (Scale bar, 50 μm.) (See also Fig. S6.) (*B*) Image shows that mucin strands are comprised of WGA-labeled filaments. JAC-labeled mucus lies on the surface of the WGA-labeled strands. (Scale bar, 50 μm.) (*C*) Successive single-plane confocal images from the epithelial surface (*Bottom*) to just above the surface (*Top*), as indicated by blue dashed lines in *Inset*. (Scale bar, 50 μm.)

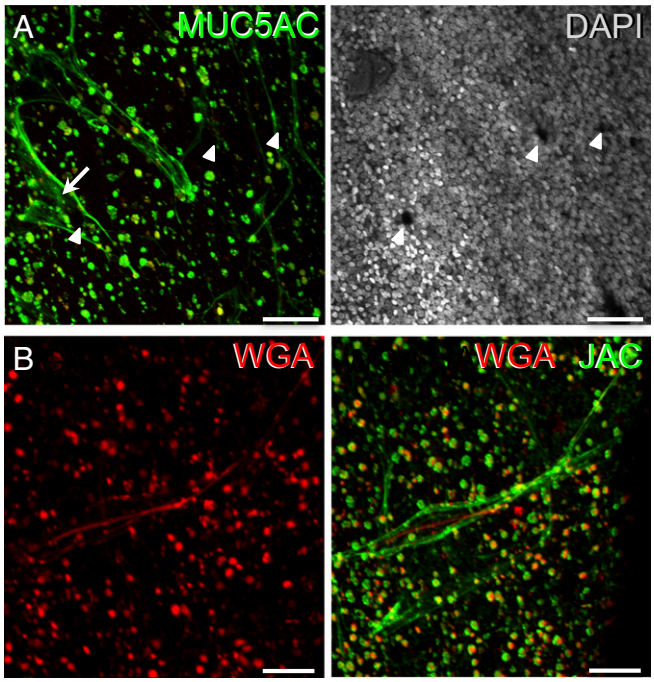

Fig. 4. Mucus from goblet cells forms threads and sheets. Images are *z* stacks of confocal images of excised trachea of non-CF pigs. (*A*) *Left* shows threads of mucin detected by MUC5AC antibody (green), a small sheet of mucus (indicated by an arrow), and position of submucosal gland ducts (indicated by white arrowheads). *Right* shows nuclei (DAPI, gray) to identify the position of submucosal gland ducts (indicated by white arrowheads). A small sheet of mucus is indicated by arrow. (Scale bar, 50 μm.) (*B*) Image of JAC-labeled mucus threads (green) from goblet cells with rare WGA (red) thread. Goblet cells are labeled by JAC and WGA beneath the threads. (Scale bar, 50 μm.)

HCO_3^-/CO_2-Free Saline Plus Bumetanide Produced Mucus Abnormalities in Non-CF Airways. In non-CF airways, inhibiting Cl^- secretion with bumetanide and blocking HCO_3^- secretion with HCO_3^-/CO_2-free solution reproduced some features of CF with impaired breakage and release of mucus from submucosal glands (21, 23, 24). These studies were performed with excised airways submerged in saline. We hypothesized that inhibiting anion secretion in non-CF airways would reproduce morphologic features of mucins in excised, nonsubmerged airways. We found that nominally HCO_3^-/CO_2-free solution plus bumetanide increased the fraction of submucosal gland ducts filled with mucus (Fig. 6 *D* and *E*) without changing pH on the airway surface (Fig. S5). The mucus in ducts was positive for WGA and anti-MUC5B antibody labeling, but it did not label with JAC or anti-MUC5AC antibody (Fig. S1 *A* and *B*). These studies are consistent with earlier work (21, 23, 24) and suggest that loss of anion secretion in submucosal glands alters the properties of mucus.

Discussion

MUC5B Forms Mucus Strands and MUC5AC Forms Mucus Threads and Sheets. Our findings indicate that MUC5B and MUC5AC have distinct morphologic and structural appearances. MUC5B emerges from submucosal gland ducts as a strand-like structure composed of MUC5B filaments (Fig. 7). The MUC5B filaments probably emanate from individual secretory granules or individual mucus-producing cells within the submucosal glands. Multiple filaments passing through the long, thin submucosal gland duct then facilitate formation of the mucin into a strand. The individual filaments are reminiscent of the histopathological appearance of individual mucin filaments produced by the gallbladder and intestine of newborn CF pigs (32).

In contrast to MUC5B, MUC5AC forms thin, wispy threads. In CF, and less commonly in non-CF, MUC5AC appears as thin mucin sheets. Although goblet cells also produce MUC5B, we rarely detected it as threads or sheets. The explanation is uncertain, but perhaps goblet cells secrete less MUC5B.

Production of MUC5B strands coated with MUC5AC may be facilitated by the airway anatomy. When mucus emerges from submucosal gland ducts, it is not released piecemeal. Instead, for a time, it remains anchored at the duct, growing in length as a strand. We propose that wispy threads and sheets of MUC5AC move across the surface, collide with elongating MUC5B strands, and associate with them. The MUC5B strand with associated MUC5AC then eventually breaks and moves up the airway (Fig. 7). The chemical or physical basis of the association between MUC5AC and MUC5B remains uncertain.

It is estimated that the volume of mucus in submucosal glands is ~50 times that in goblet cells (1), suggesting that the main function of glands is to produce large amounts of mucus driven by neuronal stimulation. Our findings suggest that an additional role for mucus production by submucosal glands may be to produce mucus in a specific structural form—that is, strands (Fig. 7). Those strands with associated MUC5AC sheets and threads may have properties that are optimal for trapping and sweeping material from the lung.

CF Alters the Appearance of Airway Mucus. We previously showed that in CF, strands of mucus sometimes fail to break and thus remain attached to submucosal gland ducts (21). Our current results in nonsubmerged airways are consistent with that finding. In addition, we found more MUC5AC sheets in CF than in non-CF. There are several potential explanations. CF airways might have secreted more MUC5AC. MUC5AC might more readily form sheets in CF airways. MUC5AC sheets may be produced similarly in CF and non-CF, but their attachment to stationary MUC5B strands may prevent their movement. A combination of these or other factors is also possible.

Loss of CFTR reduces Cl^- and HCO_3^- secretion (33, 34). These defects decrease the rate of liquid secretion by submucosal glands and reduce the pH of the secreted liquid (30, 31, 35). We

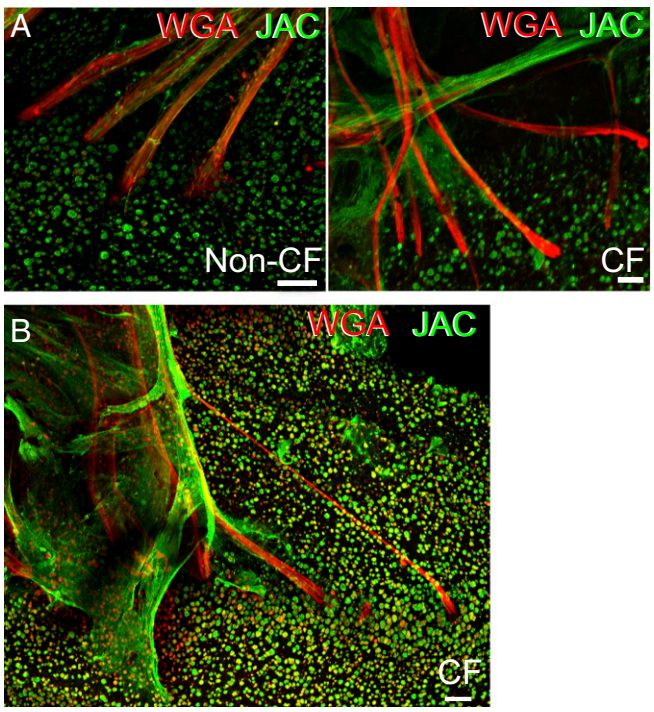

Fig. 5. CF airways showed entangled mucus strands and increased mucus sheets. (*A*) Methacholine-stimulated airways from newborn non-CF (*Left*) and CF pigs (*Right*). WGA is red, and JAC is green. (Scale bar, 50 μm.) (*B*) Large MUC5AC sheet (JAC, green) floating on MUC5B (WGA, red) strands in methacholine-stimulated CF trachea. (Scale bar, 50 μm.)

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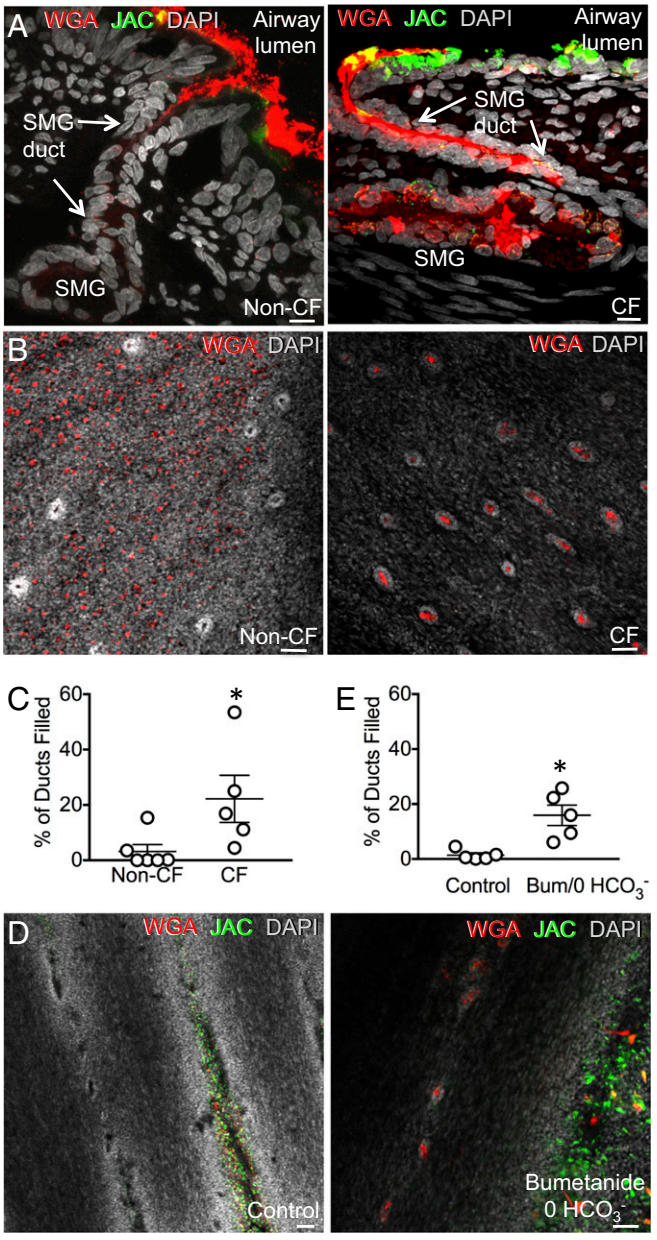

Fig. 6. CF submucosal gland ducts are filled with mucus. (*A*) Images are from pigs treated in vivo with methacholine. WGA (MUC5B) is red, JAC (MUC5AC) is green, and DAPI (nuclei) is gray. Shown are vertical sections of airway excised from non-CF (*Left*) and CF (*Right*) pigs. (Scale bar, 10 μm.) (*B*) *En face* image of excised trachea from methacholine-stimulated newborn non-CF (*Left*) and CF (*Right*) pigs. (Scale bar, 50 μm.) (*C*) Percentage of submucosal gland ducts filled with mucin in excised trachea from non-CF and CF pigs treated in vivo with methacholine. Each data point is from a different pig. Bars and whiskers indicate mean ± SEM. $*P < 0.05$. (*D*) Data are *z* stacks of confocal images at the level of the apical membrane. Excised tracheas from non-CF pigs incubated with methacholine in HCO_3^-/CO_2-buffered saline (control) or Hepes-buffered saline containing bumetanide. WGA, red; JAC, green; DAPI, gray. (Scale bar, 50 μm.) (*E*) Percentage of submucosal gland ducts filled with mucus. Pigs received methacholine in vivo. Each data point is from a different pig. $n = 5$ pigs for each condition. Average number of ducts counted per condition = 340 ± 53. Bars and whiskers indicate mean ± SEM. $*P < 0.05$. (See also Fig. S7.)

found that blocking Cl^- secretion and eliminating HCO_3^- secretion in non-CF airways reproduced some of the abnormalities of CF airways. Whether the abnormalities in CF mucus result from reduced liquid volume, decreased HCO_3^- concentration, abnormally acidic pH, or some combination of these is uncertain (21). A reduced HCO_3^- concentration was reported to contribute to intestinal mucin abnormalities in CF mice (36–38). In CF pigs, a decreased pH, rather than a decreased HCO_3^- concentration, increased airway surface liquid viscosity (29). Earlier studies in CF pigs showed that inhibiting both Cl^- and HCO_3^- secretion in non-CF airways was required to produce mucus abnormalities that resemble those in CF (21, 23, 24). These observations suggest that both liquid volume and the pH or HCO_3^- concentration are important.

This Work Has Advantages and Limitations. We studied an animal model with anatomical and physiological similarities to humans (27). CF pigs develop lung disease that mimics that in humans with CF (19, 20). Because we studied newborn pigs, the properties of mucus were not altered by airway infection and inflammation. We studied mucus rather than sputum, which introduces confounding variables. Because we examined mucus on the surface of freshly excised airways, we avoided alterations that occur with collecting, processing, and storing mucus (4). We studied freshly excised airways at the air–liquid interface without rinsing the surface. The results are similar to those in airways submerged in saline, thus excluding the possibility that decreased airway liquid was responsible for the findings.

These studies also have some limitations. We studied a large airway with submucosal glands, yet small airways lacking glands may also contribute to CF pathogenesis (17, 39). In addition to mucins, other proteins, sugars, and lipids contribute to mucus and may influence MCT (1). We fixed the trachea, which could introduce artifacts; however, the results are consistent with earlier functional studies in living airways and in vivo studies (21). As CF disease progresses, airway remodeling and the mix of proteolytic enzymes, inflammatory cells, and infection may also alter mucus properties and MCT.

For both cells and secreted mucins, the data showed preferential binding of WGA to MUC5B and JAC to MUC5AC. Although preferential lectin labeling provided convenient reagents, caution prevents conclusions about the causes of differential labeling. There are several possibilities. The two mucins might display different glycans. WGA binds to sialic acid and *N*-acetyl-D-glucosamine (40), and JAC has been reported to bind galactose and galactosyl (β-1,3) *N*-acetylgalactosamine on O-glycoproteins (41). However, such determinations are based largely on competition with monosaccharides (42). Binding of lectins also depends on protein hydrophobic interactions, electrostatic interactions, and complex glycan structures that could differ between MUC5B and MUC5AC. Access of the lectins to the two mucins could also differ, just as access of antibodies to mucins may be limited by their glycans. However, the abundance of glycans on mucins (70–80% of mass) can make labeling with lectins more prominent than for other glycoproteins.

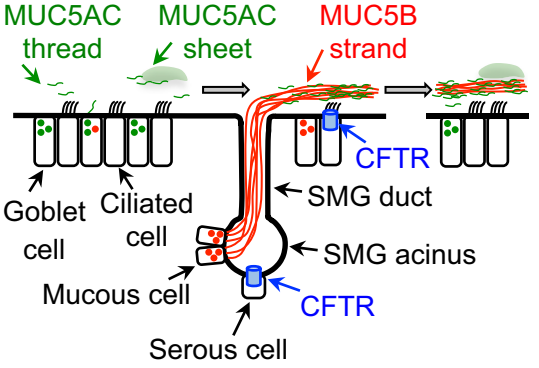

Fig. 7. Model of mucin secretion in pig airway.

These Results Raise Questions for Future Studies. Here we highlight three questions:

First, why do airways have strands, threads, and sheets? Presumably airways evolved these structures to produce the most effective MCT. It could be that strands are required to remove large particulates, whereas threads and sheets are sufficient to remove smaller particulates. That would explain why large airways contain submucosal glands whereas small airways lack submucosal glands and thus presumably lack strands. In this regard, it is interesting that smaller mammals, such as mice and rats, have few submucosal glands in intrapulmonary airways (1). Only relatively small particulates gain access to their airways, and thus, perhaps mucus strands are not required to remove them.

Second, why does MUC5B form strands and MUC5AC form threads and sheets? Is it a function of the mucin—that is, its primary structure or glycan composition—or a function of its site of origin—that is, submucosal glands or goblet cells?

Third, how do the physical structures of MUC5B and MUC5AC relate to abnormalities in disease? In CF, loss of Cl^- and HCO_3^- secretion alters the morphology of mucin strands and sheets and disrupts MCT in large airways (21). Loss of CFTR might also disrupt the structure of mucin threads and sheets in small airways. Mucins may also contribute to other lung diseases, including asthma and chronic obstructive pulmonary disease. Thus, knowledge of mucin structure and biophysical properties may aid understanding of the origins of lung disease and suggest new therapeutic strategies.

Materials and Methods

Animals. We studied non-CF ($CFTR^{+/+}$, $CFTR^{+/-}$) and CF ($CFTR^{-/-}$, $CFTR^{\Delta F508/\Delta F508}$) pigs 8–15 h after birth, as reported previously (19–21). Tracheal segments were obtained between the opening of the right cranial lobe and the larynx. For studies that examined the effect of inhibiting Cl^- and HCO_3^- secretion, segments of trachea were removed from non-CF pigs, wrapped in gauze soaked in either HCO_3^- containing solutions or HCO_3^--free Hepes solution plus bumetanide, and incubated at 37 °C. After incubation, tracheas were cut ventrally, pinned out, and treated for immunocytochemistry. The University of Iowa Animal Care and Use Committee approved all animal studies.

Immunocytochemistry and Scanning Electron Microscopy. Immunocytochemistry of frozen sections of trachea, treated excised tracheal segments, and vertical sections of excised tracheal segments is described in *SI Materials and Methods* and ref. 29. Both sections and segments were subsequently imaged by confocal microscopy. Scanning electron microscopy is described in *SI Materials and Methods*.

Quantitation of Confocal Images. We assessed the percentage of filled submucosal gland ducts using single planes at the membrane level from original confocal images of excised trachea. Ducts were counted by blinded readers on images of airways of paired trachea from individual pigs. We quantified the number of goblet cells stained by JAC or WGA from confocal images of excised trachea.

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