The disease entity of cystic fibrosis (CF) was initially based on the observed mucus phenotype. The term cystic fibrosis emanated from the cysts observed in the pancreas after blocking the exocrine ducts, and mucoviscidosis alluded to the viscous mucus found in the lungs. After the discovery of a dysfunction of the CF transmembrane regulator (CFTR) channel transporting chloride in the 1980s (Quinton, 1983; Riordan et al., 1989), there has been little progress in understanding the link between CFTR dysfunction and the mucus phenotype. The reason for this gap in knowledge has not been a result of lack of understanding of the CFTR channel itself (Riordan, 2005) but rather that the knowledge of mucins and their formation has been developing slower. An important reason for this slow progress has been the difficulties of working with mucins because of their large size, repetitive gene, and high glycosylation. Typical for mucins is the presence of long rod-like mucin domains that have non-conserved sequences rich in the amino acids proline, threonine, and serine (PTS) densely covered by O-glycans. There are several types of mucins in which the gel-forming ones form the actual mucus gel (Thornton et al., 2008; Johansson et al., 2011). These mucins have one or several mucin domains in the central part and cysteine-rich domains in their N and C termini that take part in their oligomerization. Out of the human gel-forming mucins, the MUC2 in the intestine and MUC5AC in the lungs and stomach have the highest sequence similarities in their less glycosylated ends. Both are synthesized and released by goblet cells in the surface epithelia and can either form a two-layered attached mucus, as found in the stomach and colon, or a loose and easily removable mucus, as in the lung and small intestine (Atuma et al., 2001; Johansson et al., 2008).

Using knowledge from the von Willebrand coagulation factor and biochemical and electron microscopy studies, we have recently presented a model of how the MUC2 mucin is stored in the regulated secretory vesicles of goblet cells (Sadler, 2009; Ambort et al., 2012). The N-terminal MUC2 forms six-sided rings (also five- and seven-sided) at the high Ca²⁺ concentration and low pH of these vesicles (Ambort et al., 2012). From each of the corners, the mucin...
intestinal problems and the CF mice lack major lung problems, we have focused on understanding the relation between lack of CFTR and mucus properties in the distal small intestine. Bicarbonate has emerged as an alternative ion to chloride that can pass through the CFTR channel (Quinton, 2008). CF mutations associated with a severe clinical phenotype have an abolished bicarbonate transport, whereas those associated with a milder clinical pancreas phenotype retain a residual bicarbonate transport (Choi et al., 2001). Today it is accepted that bicarbonate can be transported by the CFTR, although less efficiently than chloride. This led to the hypothesis that bicarbonate is the missing link between CFTR and mucus stagnation (Quinton, 2008).

Previous observations of improper mucus secretion in the CF mouse small intestine (Garcia et al., 2009), and our experience in analyzing the mucus and mucin in the colon (Johansson et al., 2008, 2010), prompted us to study the small intestine in more detail. We now demonstrate a crucial role for bicarbonate in neutralizing the pH and removing Ca\(^{2+}\) for unpacking the mucin at secretion. The normally unpacked mucus is easy to remove by gentle aspiration and is also permeable to 2-µm beads. However, the mucus in the Cftr\(^{-}\)F508 mice remains attached, cannot be easily removed, is denser, and is less permeable to beads. The Cftr\(^{-}\)F508 mucus can, however, be transformed to an almost normal mucus by a high concentration of NaHCO\(_3\) or EDTA, pH 7.4, implying novel CF therapies.

Figure 1. The ileal mucus of the Cftr\(^{-}\)F508 mouse is attached to the epithelium and cannot be easily aspirated. (A) Carnoy-fixed ileal sections from WT (n = 7) and CF (n = 7) mice were immunostained for Muc2 (green) and nuclei (blue). Arrows point to mucus attached to goblet cells. Bars, 100 µm. (B) Transmission electron micrographs of CCh- and PGE\(_2\)-stimulated ileal explants of WT (n = 2) and CF (n = 2) mice. Empty denotes space empty from mucus between the epithelial cell microvilli and mucus layer. Note the distended crypt with condensed material in CF mice. Bars, 2 µm. (C) Bright field images of WT mouse ileal tissue mounted in the horizontal chamber with charcoal to visualize the upper surface of the transparent mucus layer before and after aspiration. See Video 1 to watch removal of charcoal labeled mucus (arrow points at easily distinguished crypt opening). (D) Same experiment as in C, but on ileal explant from the CF mouse. See Video 2 to watch the difficulty in removing the thick and opaque mucus covering the villi (arrows). (E) Representative confocal images of a single villus from ileal tissue (red) from WT (n = 3) or CF (n = 4) mice mounted in the horizontal chamber overlaid with 2 µm fluorescent beads (green) for 40 min. Experiment illustrates mucus penetrability. Bars, 50 µm.

domains of three mucins are extending and held together at their C-termini. These rings formed by MUC2 N termini organize the packing of the mucin as a result of Ca\(^{2+}\) and pH effects on this part of the mucin (Ambort et al., 2012). Ca\(^{2+}\) ions are also, as suggested before, important for shielding the negative charges of the glycans found on many, but not all, mucins (Verdugo et al., 1987; Nordman et al., 1997).

CF is characterized by chronic infections of the lungs. This involves typical bacteria such as Pseudomonas aeruginosa, one of the most prevalent bacteria contributing to the shortened CF life expectancy. Because of the dominating problem with the lung disease, there has been less focus on other problems. Meconium ileus, intestinal obstruction at birth, is characteristic for CF. In adult life, many CF patients also suffer from DIOS (distal intestinal obstruction syndrome), which is caused by obstruction of the distal small intestine (O’Sullivan and Freedman, 2009). Interestingly, all mouse models lacking a functional Cftr channel have no major symptoms from their lungs, but just like the human patients the animals suffer from intestinal problems (Grubb and Boucher, 1999). This was originally believed to be only caused by obstruction of the distal small intestine, but it has become clear that these mice also develop bacterial overgrowth that may become lethal if not given laxatives, liquid diet, or antibiotics (Thomsson et al., 2002; Norkina et al., 2004a,b). Because mice and humans with dysfunctional CFTR have similar or identical small
RESULTS

The mucus of the small intestine in the CftrΔF508 mice is attached to the epithelium and is impenetrable

None of the CF mouse models have any major lung phenotype, but all have the intestinal problems with mucus accumulation and bacterial overgrowth requiring special food or laxatives in the drinking water. We have chosen to use the CftrΔF508 mouse because this is the model that resembles the most common human CF mutation (van Doorninck et al., 1995). Tissue sections of the small intestine stained for the Muc2 mucin indeed show that the mucin is not only trapped in the ileal crypts of the CF animal but also seems to be attached to the surface epithelium (Fig. 1 A).

To further analyze the difference between WT and CF ileum, electron microscopy was performed. In the WT animals, the mucus was seldom found in close contact with the microvilli of the epithelial cells (Fig. 1 B). In contrast, in CF mice the mucus was typically found in close contact with the cells. The ileal crypts were typically thin and almost empty of mucus in the WT, whereas the CF crypts were filled with dense mucus material. Thus the CF mice show a characteristic phenotype with adherent and dense mucus as compared with WT mice.

To study the secreted mucus and its properties in more detail, we developed an Ussing-type explant system where small tissue samples are mounted horizontally between two plastic plates with a hole of 2.5 mm. This system allows mucus to form on the mucosal side of the explant tissue (Johansson et al., 2010; Gustafsson et al., 2012). Fig. 1 C shows a top view of ileum from a WT mouse where the villi are extending toward the viewer. As mucus is normally transparent, it was not possible to localize the mucus upper surface and observe if the villi were covered. However, the mucus surface can easily be visualized by allowing charcoal to sediment onto its surface (Fig. 1 C). This mucus is easily aspirated with a thin pipette as shown in Video 1. The aspiration leaves the villi free of mucus except at the outer edge of the chamber, where mucus has been trapped between the two plastic plates. That all the mucus has indeed been removed together with the charcoal is reflected by the fact that the crypt openings can now be seen (Fig. 1 C, arrow). New charcoal added sedimented down between the villi, confirming that the mucus had been removed (unpublished data). Thus, the mucus normally found in the ileum was not attached.

In contrast, the ileal mucus of the CF mice was less transparent and the villi not as easy to distinguish (Fig. 1 D, arrow). After addition of charcoal, we tried to remove the mucus in the same way as in WT mice. However, the mucus now proved impossible to remove. It seemed attached to the epithelium, being more streaky, and only followed the pipette to a minor extent as shown in Video 2. The mucus covered with charcoal also remained on top of the villi as shown in Fig. 1 D (CF: After). Moreover, unlike in normal intestine, the crypt openings remained invisible and the villi were still not easily distinguished (Fig. 1 D, CF: After). When charcoal was added again, it did not sediment down between the villi (unpublished data). Thus, the mucus of the CF mice is attached to the epithelium and cannot be easily removed.

As the mucus can act as a molecular sieve (Johansson et al., 2010; Gustafsson et al., 2012), we tested the penetrability of the mucus. The mucus was overlaid with fluorescent beads with a diameter of 2.0 µm, and bead penetrability was measured by z-stacks in a confocal microscope after 40 min of incubation (Fig. 1 E). In WT, these beads (green) sedimented through the mucus and were found on the epithelium (red) both between and on top of the villi. In contrast, in CF mice the beads did not pass through the mucus. Instead, they were trapped in and on top of the mucus and thus never reached the crypt openings. This finding suggests that the CF mouse has altered properties as compared with mucus in WT small intestine.

CF mice secrete a denser mucus than WT mice

The mucus thickness on the mounted explant tissue can be measured with a glass pipette attached to a micrometer as
described previously (Atuma et al., 2001; Johansson et al., 2010; Gustafsson et al., 2012). The initial mucus thickness measured from the top of the mucus down to the crypt opening was found to be ~210 µm, with ~60 µm above the villi tips (Fig. 2 A). The mucus was then aspirated from the WT mice. For the CF mice, the mucus had to be removed by a more vigorous procedure, including scraping and extensive aspiration, still leaving intact villi (unpublished data). When the secretagogues carbachol (CCh) and prostaglandin E$_2$ (PGE$_2$) were added to the basal perfusate, the mucus thickness increased. This phenomenon was not observed in the absence of these secretagogues (Fig. 2 A). The mucus layer grew to ~200 µm in 20 min, and an additional 50 µm the next 20 min. No difference in the mucus growth was observed between the WT and CF mice (Fig. 2 A).

The mucus secreted after 40 min of stimulation with CCh and PGE$_2$ was measured in the WT and CF mice, followed by standardized aspiration of the mucus (Fig. 2 B). The mucus in WT animals was readily removed, but in the CF mice the mucus was not possible to aspirate and the mucus thickness remained unaltered.

Because the mucus secreted from the CF mice was less transparent and adherent to the epithelial surface, we wondered whether the mucus contained more Muc2 mucin than mucus secreted from WT mice. The secreted mucus and the apical buffer were collected from WT and CF mice and analyzed using composite agarose-polyacrylamide electrophoresis (Fig. 2 C). The analysis showed that the collected mucus contained 2.6× more Muc2 glycoprotein in the CF animals compared with the controls (Fig. 2 D). CF mice have previously been shown to have 2.5× more insoluble mucus (Muc2 mucin) in the small intestine (Malmberg et al., 2006). The CiftrΔF508 mice have also an increased number of goblet cells that were more densely filled (van Doorninck et al., 1995), something which was confirmed in our animal stock. The mean number of goblet cells per crypt in WT mice was 6 ± 0.4 and in CF mice 8 ± 0.8 (n = 7, P = 0.038). Together, the results suggest that the small intestine of CF mice have an increased mucin production. As the mucus thicknesses were similar in the WT and CF mice (Fig. 2 A), the CF mice probably had mucus with higher Muc2 content, something which could explain the less transparent mucus observed in Video 2.

The CF mucus phenotype can be generated in WT mice by inhibiting bicarbonate secretion

Because HCO$_3^-$ secretion is known to be impaired in CF, the question was raised of whether the CF mucus phenotype could be generated by removal of HCO$_3^-$ transport in WT tissue. To test this, we kept the apical HCO$_3^-$ concentration at 23 mM, as before, but removed HCO$_3^-$ from the serosal buffer. The thickness of the apical mucus after stimulation with PGE$_2$ and CCh was, as before, ~200 µm, but the mucus could not be aspirated and almost 100% remained attached to the epithelial surface (Fig. 3 A). Fig. 3 B and the Video 3 show that the mucus and the carbon particles remained on the mucus surface after aspiration. This suggests that HCO$_3^-$ is important for the secreted mucus properties and that HCO$_3^-$ has to be transported from the serosal side through the epithelium to reach the apical surface where it affects the mucus properties.

We initially tried to inhibit CFTR in the explants by using two known CFTR inhibitors (GlyH-101 and 172), both separately and in combination (Ma et al., 2002; Muanprasat et al., 2004). However, we were never able to reach more...
than ~70% inhibition of the forskolin response, attributed to CFTR activation (Bell and Quinton, 1992), and thus these inhibitors were not sufficiently effective in the explant system. Instead, we added 5 mM serosal barium chloride, 0.2 mM apical DIDS, or 0.2 mM serosal DIDS to inhibit basolateral potassium channels (Burleigh, 2003), apical anion transport (PAT1 and DRA; down-regulated in adenoma; Jacob et al., 2002), and basolateral anion transport (Cl/HCO\textsubscript{3}\textsuperscript{−} exchange and Na\textsuperscript{+}/HCO\textsubscript{3}\textsuperscript{−} co-transport; Zhao et al., 2005). In the presence of BaCl\textsubscript{2} or DIDS, mucus secretion was induced by PGE\textsubscript{2} and CCh and the response was recorded for 40 min. Pretreatment with BaCl\textsubscript{2} did not affect the thickness of the secreted mucus, but almost all of the mucus remained attached after aspiration (Fig. 3 C). Pretreatment with DIDS did not affect the thickness of the secreted mucus when added to the apical or serosal side (Fig. 3 C). Basolateral DIDS induced an intermediate mucus phenotype where ~50% of the secreted mucus remained attached after aspiration. Apical DIDS did not affect mucus adherence (Fig. 3 D). The results suggest that cAMP-mediated secretion and serosal HCO\textsubscript{3}\textsuperscript{−} uptake is important for formation of normal mucus.

Most of the properties of the attached mucus of the Cftr\textDeltaF508 small intestine are normalized by high bicarbonate

Because HCO\textsubscript{3}\textsuperscript{−} transport was required for formation of a normal mucus in WT mice, we hypothesized that increasing the concentration of NaHCO\textsubscript{3} on the apical side of the epithelium would normalize the mucus phenotype in CF mice. The mucus on the explants was carefully removed and apical buffers with 23, 69, 92, and 115 mM NaHCO\textsubscript{3}, pH 7.4, were added. Mucus secretion was stimulated by serosal perfusion of the secretagogues CCh and PGE\textsubscript{2}. After 40 min, the thickness of the mucus was measured with paired controls (23 mM NaHCO\textsubscript{3}). The total mucus thickness was similar for all HCO\textsubscript{3}\textsuperscript{−} concentrations (see Fig. 5 A). It should be pointed out that the surface of the small intestinal mucus is very loose, fluffy, and poorly held together. The surface mucus floats out to the sides and dissolves into the apical buffer. This means that the mucus is normally not observed far above the villus tips, something that may explain why no increased mucus thickness was observed with the higher HCO\textsubscript{3}\textsuperscript{−} concentrations. The formed mucus was then aspirated from the paired tissues with control (23 mM) and experimental mucosal HCO\textsubscript{3}\textsuperscript{−} solutions, and its thickness was measured after adding charcoal (Fig. 4 B, Remaining). The mucus remained attached in 70 mM NaHCO\textsubscript{3}, but in 92 mM NaHCO\textsubscript{3} ~50% of the mucus could be removed. At 115 mM NaHCO\textsubscript{3} ~75% of the mucus could be removed, which is similar to what is observed in WT (Fig. 4 A).

Bicarbonate buffers are in equilibrium with CO\textsubscript{2} in the air, and thus the actual HCO\textsubscript{3}\textsuperscript{−} concentrations in the buffer were not as in the original buffer. As described in the Materials and methods, we have measured the actual HCO\textsubscript{3}\textsuperscript{−} concentrations in the working buffers. The buffer labeled 23 mM was actually ~21 mM, 69 was ~64 mM, 92 was ~84 mM, and 115 was ~104 mM HCO\textsubscript{3}\textsuperscript{−}. The HCO\textsubscript{3}\textsuperscript{−} concentrations given are the ones used for preparing the original buffer.

To illustrate the importance of a functional CFTR to obtain apical HCO\textsubscript{3}\textsuperscript{−} during mucus release, the serosal liquid was replaced with a buffer containing 115 mM NaHCO\textsubscript{3} and the mucosal fluid was kept at 23 mM NaHCO\textsubscript{3} in CF mice. No significant amount of mucus could be removed with 115 mM NaHCO\textsubscript{3} on the serosal side (Fig. 4 B, gray bar), suggesting that CFTR is the only efficient route for HCO\textsubscript{3}\textsuperscript{−} through the cell. The effect of apical HCO\textsubscript{3}\textsuperscript{−} on the secreted mucus is further illustrated in Fig. 4 C and Video 4, where it is shown that the mucus can easily be removed if secreted into a buffer containing 115 mM NaHCO\textsubscript{3}.

![Figure 4. The CF mucus phenotype can be normalized by apical HCO\textsubscript{3}\textsuperscript{−}.](image)
To test the hypothesis that the NaHCO₃ works by chelating Ca²⁺, we tested whether another Ca²⁺-chelator, EDTA, had the same effect on the mucus. Mucus was secreted into an apical buffer containing either 10 mM or 20 mM EDTA. The total mucus thickness was measured, followed by aspiration of the mucus and measurement of the remaining thickness. The total mucus thickness did not differ between control and EDTA-treated tissue, but the remaining mucus thickness after aspiration was reduced to values similar to WT in the group treated with 20 mM EDTA (Fig. 4 D). This argues for Ca²⁺-chelation being important for normal mucus release.

The properties of already formed CftrΔF508 mucus can be normalized by adding bicarbonate

To address if NaHCO₃ could normalize already secreted mucus, mounted CF and WT ileal explants were apically exposed to a buffer containing 115 mM NaHCO₃. In the WT mice, the mucus thickness remained intact, whereas in the CF mice, the mucus thickness increased by 57% to ~300 µm in 30 min (Fig. 5 A). Thus, the addition of NaHCO₃ causes already formed mucus in the absence of a functional CFTR to expand in volume. This is in contrast to mucus secreted into a high NaHCO₃ buffer (Fig. 4 A), suggesting that already formed CF mucus has a larger tendency to hold together in this experimental set up. This mucus also gained WT properties as the mucus became penetrable to fluorescent beads like in the WT mucus in about half of the studied tissues (Fig. 5 B). In the remaining tissues, the beads passed into the mucus but did not reach the crypt openings. When the mucus formed on the CF explants was incubated with 115 mM NaHCO₃ for 30 min and overlaid with charcoal, it was also possible to aspirate the mucus as for WT explants (Fig. 5 C and Video 5). When charcoal was added again, it sedimented down in between the villi. The CF explant tissue was also studied by electron microscopy after addition of NaHCO₃ to the already formed mucus (Fig. 5 D). The mucus was now found to be mostly separated from the epithelium like in WT, but sometimes the mucus was in contact with the epithelial cells (Fig. 5 D, arrowhead). It was also typical that many crypts were greatly distended as if the trapped mucus in this compartment had expanded (Fig. 5 D, right). Together, these experiments suggest that the properties of the mucus formed in the absence of CFTR can be partially normalized with NaHCO₃.

DISCUSSION

Using explants from the small intestine of CF mice, we have now shown that a sufficiently high concentration of bicarbonate, normally provided by the CFTR, is necessary for proper unfolding of the MUC2 mucin at secretion. The MUC2 mucin is packed in the goblet cell granulae as a result of the low pH and high Ca²⁺ in these vesicles (Ambort et al., 2012). The packed MUC2 has to be quickly dissolved at secretion by the removal of Ca²⁺. In the WT ileum, the secreted mucus was easy to aspirate and was fully permeable to 2-µm fluorescent beads. In contrast, the mucus in the CftrΔF508 mice was not easily aspirated and beads could not penetrate. The same mucus phenotype was found when no HCO₃⁻ was present on the serosal side of WT explants, implying that epithelial HCO₃⁻ secretion is required for proper mucin expansion. The HCO₃⁻ concentration close to the apical side of the epithelium necessary for normal mucus expansion was estimated to be ~100 mM.

The N-terminal part of the MUC2 mucin carries the necessary information for sorting the mucin to the regulated secretory pathway (Ambort et al., 2012). Here, the MUC2 mucin forms disulfide bonded N-terminal trimers
The high Ca$$^{2+}$$ and low pH (6.2) in these secretory vesicles triggers the MUC2 trimers to form six-sided noncovalent rings that become concatenated into large aggregates (Ambort et al., 2012). In this way, MUC2 is stored highly condensed in the secretory vesicles. To unfold these aggregates, the pH has to rise and, most importantly, the Ca$$^{2+}$$ ions must be removed. That HCO$$^3$$– is able to mediate the unpacking is suggested from the observation that the large MUC2-N concatemers were dissolved when treated with NaHCO$$_3$$ (Ambort et al., 2012). When this happens, the trimeric MUC2 N termini will start to separate, the ring structure dissolves, and the mucin starts to unfold. Our previous studies on the unpacking of the MUC2 N termini also suggested that HCO$$^3$$– is at least as effective as the known Ca$$^{2+}$$-chelator EDTA (Ambort et al., 2012). The reason for HCO$$^3$$– being that effective is probably that it reacts with Ca$$^{2+}$$ to form insoluble CaCO$$_3$$ at the same time as it raises the pH. The formation of CaCO$$_3$$ will quickly remove free Ca$$^{2+}$$ and disaggregate the MUC2 mucin. This allows the mucin to expand the estimated 1,000× to form the normal mucus.

If mucin is secreted in the absence of a functional CFTR channel or in the absence of basolateral HCO$$^3$$– ions that can supply the CFTR channel, the mucin cannot expand normally. The mucin also remains attached to the epithelium and cannot be easily removed. The mechanism behind this attachment is not understood, but the rapid effect of HCO$$^3$$– directly added on secreted mucins suggests a direct chemical effect on the MUC2 mucin. In the electron microscopic pictures of the CF epithelium, denser areas of mucus were observed as shown and could be expected if the mucin was not fully expanded. Such areas were less frequently seen in mucin treated with 115 mM NaHCO$$_3$$, suggesting that this treatment can disaggregate the packed mucin. That the CF mucin is trapped and attached was also suggested from studies on the cervical mucin in mice lacking Cfr, where less mucin was secreted into the lumen and found trapped in the glands (Muchekehu and Quinton, 2010). The previous observation of reduced mucin found in the perfusates of intestinal segments in CF mice or in the absence of HCO$$^3$$– (Garcia et al., 2009) is directly consistent with our observation of an attached and less unfolded mucin.

The mucin organized around the Muc2 mucin in the small intestine is normally penetrable to 2-µm beads. This is in contrast to the inner mucus layer in the large intestine, which is impenetrable to these beads (Johansson et al., 2010). Interestingly, the small intestinal mucin formed in the CF mouse is impenetrable to these beads, suggesting that this mucin has other properties than in WT mice. This altered penetrability was reversed by 115 mM NaHCO$$_3$$. The thickness of the CF mucin secreted into 23 mM HCO$$^3$$– upon stimulation was similar to WT mice. However, the WT mucin did not expand further by 115 mM HCO$$^3$$–, whereas the CF mucin expanded, suggesting a higher density of mucin in the CF mucin. In fact, the concentration of Muc2 in the secreted mucin was estimated to be at least 2.6× higher in the CF as compared with the WT. This and the expansion of the CF mucin, but not WT, by 115 mM NaHCO$$_3$$ suggests that the CF mucus is only partly unfolded. The lower penetrability of CF mucus was probably a result of this partial unfolding.

Our results suggest that a concentration of about 115 mM HCO$$^3$$– is necessary for the formation of normal mucus. This is within the physiological range for duodenal bicarbonate secretion and below the 140 mM HCO$$^3$$– found in the pancreatic duct (Park et al., 2010). It is higher than the estimated maximum concentrations possible to generate by Cl$$^-$/HCO$$^3$$– exchangers, further suggesting that CFTR is the major supplier of HCO$$^3$$–. Furthermore, the CFTR permeability for HCO$$^3$$– can be regulated as it was increased by activation of the WNK1-OSR1/SPAK kinase pathway in the pancreas (Park et al., 2010). As the CFTR expressing enterocytes are localized next to a goblet cell, a high HCO$$^3$$– concentration in the immediate vicinity of mucin secretion could be expected. A substantial amount of mucus secretion takes place at the crypt openings where the HCO$$^3$$– could emanate from the crypts. For surface goblet cells, adjacent enterocytes could contribute with the HCO$$^3$$–. This high concentration will probably be quickly diluted and thus not reflected in the lumen.

Our observations suggest that serosal HCO$$^3$$– is providing the apical HCO$$^3$$– by allowing it to pass through the enterocytes and the apical CFTR channel. The liquid compartment just outside of the epithelial cells will be a key element in mucin expansion as the Ca$$^{2+}$$ ions have to be removed quickly from the mucin and the pH increased to allow expansion of the mucins. As shown here, CCh is a potent stimulator of mucus secretion. CCh treatment of rat intestine also redistributes Cfr from intracellular vesicles to the apical surface membrane of the villus enterocytes at the same time as the Na$$^+$/H$$^+$$ exchanger NHE3 is internalized (Jakab et al., 2011). This could suggest a coordinated behavior of enterocytes and goblet cells for proper mucin secretion in line with the importance of HCO$$^3$$–.

The main phenotype in human CF occurs in the lungs, with stagnant mucus and recurrent severe respiratory infections. The mucus secretory systems of the surface epithelia in the small intestine and lungs show several similarities. The mucus and its major component, the mucins, are formed and secreted by goblet cells. These cells do not express any CFTR channels. Instead, the channel is found in adjacent enterocytes in the small intestine and in ciliated cells in the lungs.

The goblet cells in the airways express the MUC5AC mucin and in the small intestine the MUC2 mucin. These two mucins are structurally the most similar mammalian mucins in their N- and C-terminal parts (Lang et al., 2007). In the normal small intestine, the MUC2 mucin is not attached to the epithelium and is easy to aspirate. Although not formally studied in detail, this is what is expected in the lungs where the mucus is moved by the cilia. In contrast to this situation, both the MUC2 and the MUC5AC mucins can be organized into a two-layered mucus system where the inner layer is attached to the epithelium. MUC5AC forms such mucus in the stomach and MUC2 in colon (Atuma et al., 2001;...
Johansson et al., 2008). How the same gene products can organize themselves differently is not yet understood, but it is interesting to note that the amounts of CFTR in the cells adjacent to the goblet cells are lower in the stomach and colon.

The mucus on the surface of CF human bronchial epithelial (HBE) cultures is attached and not moved by the cilia as in normal cultures (Matsui et al., 1998). This has been attributed to a decreased periciliary liquid (PCL) depth as a result of increased Na\(^{+}\) and liquid absorption. By this, the mucus is trapped in the cilia. Recently, Chen et al. (2010) questioned the increased liquid absorption and lower PCL height by studies on newborn Cfr- deficient pigs. Their conclusion was that it is only the reduced Cl\(^{-}\} and HCO_3\(^{-}\} permeability that initiates the CF airway disease. However, they did not provide any explanation for this phenomenon. The observation that the CF mucus is not moved by the cilia in the HBE cultures and the question of how the CF airway disease is initiated can, however, be explained by our observations of adherent mucus in the absence of HCO_3\(^{-}\}.

There are, thus, several observations that argue for similar CFTR function and its relations to mucus formation in the small intestine and lungs. One of the current corner stones in many CF treatment regimes is hypertonic saline inhalation. As already formed mucus on the epithelium in the ileum was possible to partly or fully correct by 115 mM NaHCO_3, inhalation of NaHCO_3 in a hypertonic solution may be a putative therapy for the CF lung problems.

**MATERIALS AND METHODS**

**Animals.** Homozygous CfrF508 mice on C57BL/6 background (backcrossed for 13 generations) were obtained from the Erasmus MC animal facility, maintained as described, and given regular water 2–3 d before the experiments (van Doorninck et al., 1995). C57BL/6 mice were used as WT controls. The WT mice were either purchased from Taconic or obtained from our in-house breeding program. The animals were between 8 and 16 wk old at the time of experiment and randomly assigned to the respective groups. Ethical approval for the animal experiments was granted by the ethics committee for animal experiments in Gothenburg.

**Tissue fixation and immunostaining.** Dissected pieces of the distal small intestine containing luminal material was fixed in methanol-Carnoy’s fixative and paraffin embedded. The number of goblet cells per crypt was counted in normal cultures (Matsui et al., 1998). This has been attributed to a decreased periciliary liquid (PCL) depth as a result of increased Na\(^{+}\) and liquid absorption. By this, the mucus is trapped in the cilia. Recently, Chen et al. (2010) questioned the increased liquid absorption and lower PCL height by studies on newborn Cfr-deficient pigs. Their conclusion was that it is only the reduced Cl\(^{-}\} and HCO_3\(^{-}\} permeability that initiates the CF airway disease. However, they did not provide any explanation for this phenomenon. The observation that the CF mucus is not moved by the cilia in the HBE cultures and the question of how the CF airway disease is initiated can, however, be explained by our observations of adherent mucus in the absence of HCO_3\(^{-}\}.

**Tissue explants.** The distal ileum from WT and CfrF508 mice were dissected and flushed with ice-cold 95% O_2/5% CO_2 Krebs solution (116 mM NaCl, 1.3 mM CaCl_2, 3.6 mM KCl, 1.4 mM KH_2PO_4, 23 mM NaHCO_3, and 1.2 mM MgSO_4), pH 7.4, and kept on ice during transportation (30 min). The tissue was opened along the mesenteric border, the longitudinal smooth muscle was removed, and the tissue was divided into two pieces and mounted between the two plastic sheets of the horizontal Ussing-type perfusion chamber (Johansson et al., 2010; Gustafsson et al., 2012). The two adjacent parts of the tissue were analyzed in parallel. The serosal chamber was constantly perfused with 95% O_2/5% Krebs solution containing 10 mM glucose, 5.1 mM Na-glutamate, and 5.7 mM Na-pyrurate. The apical chamber was filled with 150 µl likewise 95% O_2/5% CO_2-bubbled Krebs solution where glucose was substituted with 10 mM d-mannitol. After bubbling with 95% O_2/5% CO_2, the pH of these solutions was 7.4. The solutions were added at room temperature, and the chamber was gradually heated to 37°C over 10 min and kept at this temperature during the experiment.

Mucous thickness was measured using a glass capillary connected to a micro-manipulator and the mucus surface was visualized by charcoal particles. Initial mucus thickness was measured from the mucus surface to villus tip. After removal of the mucus layer, the villus height was assessed by measuring the distance between the villus tip and the surface epithelium in between the villi. The micropipette was gradually lowered toward the epithelial surface and the level of the epithelium was determined as the point where the tip of the pipette and the epithelial surface were in the same focal plane. Total mucus thickness was calculated by adding the villus height to the initial mucus thickness. Five measurements were made for each time point, and the mean thickness was calculated and used as a single value. The adhesiveness of the mucus layer was assessed by comparing the total mucus thickness to the mucus thickness remaining after aspiration. The aspiration procedure was standardized. The mucus was aspirated using a small plastic Pasteur pipette (PP-101, outer tip diameter 0.9 mm, inner tip diameter 0.7 mm, max volume 800 µl; Cell Projects). The tip of the compressed pipette was placed on the edge of the chamber opening and slowly opened over 3 s to aspirate the apical chamber solution and the loose mucus. The size of the pipette allows for removal of the whole apical solution in one step. The apical chamber was refilled with 150 µl Krebs solution, charcoal particles were added, and the remaining mucus thickness was measured. For a better visualization of the mucus adhesiveness in the videos, the mucus was aspirated using a P-200 (Gilson Pipetman) set to 150 µl with a yellow tip (no. 70.760.502, Sarstedt). In this way, the mucus adhesiveness can be visually illustrated, as the thinner inner diameter allows slower aspiration to show how the CF mucus follows the pipette tip. Mucus secretion was induced by serosal stimulation using a combination of 10 µM CCh and 10 µM PGE_2 (Sigma-Aldrich; Muchekuehu and Quinton, 2010).

The inhibitory effects of the CFTR inhibitors Gly-H101 (50, 100, and 500 µM) and 172 (5 µM) were tested by pretreating the tissue with the respective inhibitors for 20 min, followed by stimulation with forskolin (apical 10 µM), a known activator of CFTR-mediated transport (Bell and Quinton, 2010).

**Using-like perfusion chamber.** Ileal mucus properties were analyzed in an Ussing-like horizontal perfusion chamber (Johansson et al., 2010; Gustafsson et al., 2012) composed of an apical (volume 150 µl) and serosal (160 µl) chamber with a circular opening of 4.9 mm\(^2\). The chamber was mounted in a heating block connected to a temperature controller (Harvard Apparatus), allowing the experiments to be performed at 37°C. The apical solution was kept unstirred to avoid disturbances to the mucus gel, whereas the serosal chamber was constantly perfused at a rate of 5 ml/h. Trans-epithelial potential difference (PD) was measured during the whole experiment using Calomel electrodes (Ref201; Radiometer) connected to the tissue bath via agar bridges (4% agar, 0.9% NaCl). Junction potential was corrected for background PD in the empty chamber and correcting for the voltage difference with an external battery. Asymmetries as a result of altered buffer composition were checked, but this had no effect on background PD.
addition of small amounts (~10 μl for the 115 mM NaHCO₃-buffer) of concentrated HCl. All handling of the gassed solution was done to minimize evaporation of CO₂ by keeping tubes closed when possible. We also made a control experiment with the different HCO₃⁻ buffers and let tubes stand open for 1.5 h (experimental time). The pH did not shift during this time. 10 and 20 mM EDTA was added to Ca²⁺-free 95% O₂/5% CO₂-bubbled KREB solution. The pH was adjusted to 7.4 using concentrated HCl.

Bicarbonate buffers. The actual concentration of bicarbonate ions, pH and pCO₂ in the apical buffers used in our experiments was estimated using an ABL 700 (Radiometer). Buffers with 23, 69, 92, and 115 mM NaHCO₃ were prepared with substituents (mannitol, glutamate, and pyruvate), aerated with carbogen gas (5% CO₂, 95% O₂), and pH adjusted to 7.4 using HCl. The buffers are mentioned throughout the text with the original HCO₃⁻ concentration. The tubes containing the buffers were left open and exposed to the air while samples were taken every 5 min during 1 h directly into the Radiometer apparatus. All values were stable after 10 min aeration. The 23 mM buffer contained 21 mM HCO₃⁻, after 1 h, the 69 mM buffer contained 64 mM HCO₃⁻, and the 92 mM buffer contained 84 mM HCO₃⁻. The HCO₃⁻ concentration in the buffer with 115 mM HCO₃⁻ was out of range but could be calculated from the pH and pCO₂, (kPa), using the formula 0.031 × 7.5 × pCO₂ (10^(pH-5.9)), where 7.5 represents the conversion constant between kPa and mm Hg. The actual HCO₃⁻ concentration in the 115 mM buffer was calculated to 104 mM. The pCO₂ in the buffers were 5.5 kPa in the 23 mM HCO₃⁻ buffer, 12 kPa in the 69 mM buffer, 15 kPa in the 92 mM buffer, and 19 kPa in the 115 mM HCO₃⁻ buffer, whereas the pH was 7.3 in the 23 mM buffer and 7.5 in the other buffers.

Confocal microscopy of mucus penetrability in WT and CftrΔF508 mice. Ileal mucus penetrability was studied as previously described (Johansson et al., 2010). In brief, ileal tissues were stained using CellTrace Calcein Violet, AM (1 μg/ml in basolateral perfuse; Invitrogen). After 20 min of incubation, 2 μm green fluorescent beads (FluoSpheres; Invitrogen) were added to the apical surface and allowed to sediment down through the mucus layer for 40 min. Confocal images were taken in an XY stack with an optical section of 2.8 μm in 10-μm intervals using an LSM 700 imaging system and a 20×/1.0 DIC water objective. Images were processed using the ZEN 2009 software and the Z-axis projection was used for presentation.

SDS-agarose composite gel electrophoresis for quantification of mucins. Mucus from WT and CF mice was aspirated from an identical surface area of ileal tissue stimulated with CCh and PGE₂ for 40 min. Complete EDTA-free protease inhibitor (Roche) was added to the sample. Samples were reduced with 100 mM dithiothreitol in SDS-sample buffer at 95°C for 45 min, followed by incubation at 37°C for 2 h. The samples were applied to a composite agarose-polyacrylamide gel and separated on ice at 4°C at 30 mA/gel for 3 h (Schulz et al., 2002). For quantification of the mucins, the gel was stained with SYPRO Ruby protein gel stain (Bio-Rad Laboratories), bands were quantified using a Molecular Imager VersaDoc MP 4000 reader (Bio-Rad Laboratories), and intensities were analyzed using Image Lab Software version 3.0 (Bio-Rad Laboratories). After quantification, the gel was stained for negatively charged mucins with Alcian blue (Schulz et al., 2002).

Transmission electron microscopy. WT and CftrΔF508 ileum tissue were prepared for transmission electron microscopy (Hjalmarsson et al., 2004). The specimens were fixed in Karnovsky fixative (2% paraformaldehyde and 2.5% glutaraldehyde in 0.05 M, pH 7.2, sodium cacodylate buffer) for 24 h, followed by sequential staining using 1% OsO₄ for 4 h, 1% tannic acid for 3 h, and 1% uranyl acetate overnight. Samples were dehydrated and embedded in epoxy resin (Agar 100). Ultrathin sections (50 nm; Reichert Ultracut E) were collected on mesh copper support grids. The sections were contrasted using lead citrate and tannic acid and examined in a 902 electron microscope (Carl Zeiss).

Statistics. Results are presented as mean ± SEM. Comparisons between two groups were made using the Mann-Whitney U test. Comparisons between three or more groups were made using the Kruskal-Wallis test followed by Dunn’s post-hoc test.

Online supplemental material. Video 1 shows aspiration of mucus visualized with charcoal from WT mouse ileal tissue mounted in the horizontal chamber. Video 2 shows the difficulty in aspirating mucus visualized with charcoal from CF (CftrΔF508) mouse ileal tissue mounted in the horizontal chamber. Video 3 shows the difficulty in aspirating mucus visualized with charcoal from WT mouse ileal tissue mounted in the horizontal chamber in the absence of serum HCO₃⁻. Video 4 shows the aspiration of mucus secreted into apical buffer containing 115 mM HCO₃⁻ on CF (CftrΔF508) mouse ileal tissue mounted in the horizontal chamber. Video 5 shows the aspiration of already formed mucus treated with 115 mM HCO₃⁻ on CF (CftrΔF508) mouse ileal tissue mounted in the horizontal chamber. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.2012562/DC1.

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