Increased levels of mucins in the cystic fibrosis mouse small intestine, and modulator effects of the Muc1 mucin expression

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Malmberg, Emily K., Karin A. Noaksson, Mia Phillipson, Malin E. V. Johansson, Marina Hinojosa-Kurtzberg, Lena Holm, Sandra J. Gendler, and Gunnar C. Hansson. Increased levels of mucins in the cystic fibrosis mouse small intestine, and modulator effects of the Muc1 mucin expression. Am J Physiol Gastrointest Liver Physiol 291: G203–G210, 2006. First published February 23, 2006; doi:10.1152/ajpgi.00491.2005.—The mouse model (Cftrtm1UNC/Cftrtm1UNC) for cystic fibrosis (CF) shows mucus accumulation and increased Muc1 mucin mRNA levels due to altered splicing (Hinojosa-Kurtzberg AM, Johansson MEV, Madsen CS, Hansson GC, and Gendler SJ. Am J Physiol Gastrointest Liver Physiol 284: G853–G862, 2003). However, it is not known whether Muc1 is a major mucin contributing to the increased mucus and why CF/Muc1−/− mice show lower mucus accumulation. To address this, we have purified mucins from the small intestine of CF mice using guanidine chloride extraction, ultracentrifugation, and gel filtration and analyzed them by slot blot, gel electrophoresis, proteomics, and immunoblotting. Normal and CF mice with wild-type (WT) Muc1 or Muc1−/− or that are transgenic for human MUC1 (MUC1.Tg, on a Muc1−/− background) were analyzed. The total amount of mucins, both soluble and insoluble in guanidine chloride, increased up to 10-fold in the CF mice compared with non-CF animals, whereas the CF mice lacking Muc1 showed intermediate levels between the CF and non-CF mice. However, the levels of Muc3 (orthologue of human MUC17) were increased in the CF/Muc1−/− mice compared with the CF/MUC1.Tg animals. The amount of MUC1 mucin was increased several magnitudes in the CF mice, but MUC1 did not appear to be a major mucin. The amount of insoluble mucin of the large intestine was also increased in the CF mice, an effect that was partially restored in the CF/Muc1−/− mice. The thickness of the firmly adherent mucus layer of colon in the Muc1−/− mice was significantly lower than that of WT mice. The results suggest that MUC1 is not a major component in the accumulated mucus of CF mice and that MUC1 can influence the amount of other mucins in a still unknown way.

Muc2: proteomics; CFTR

THE MAIN CLINICAL MANIFESTATION of cystic fibrosis (CF), caused by mutations in the cystic fibrosis transmembrane conductance regulator gene (CFTR), is accumulation of mucus on epithelial surfaces in pancreas and the respiratory and gastrointestinal tracts (4). All CF mouse models suffer from intestinal goblet cell hyperplasia and severe intestinal mucus obstruction, but they do not display the respiratory pathology that is the dominant feature of CF in humans (33, 38). The intestinal phenotype of CF mice is similar to the human CF lung phenotype as mucus adhesion and obstruction are commonly observed (7, 33, 38). We have previously suggested that the severe intestinal phenotype was due to bacterial overgrowth, comparable with the human lung phenotype (14). This was also recently suggested from a study showing bacterial overgrowth of certain specific bacteria in the CF mice small intestine (21). Both studies also proposed that antibiotic treatment could rescue the animals, again reminiscent of the human lung phenotype. It is thus likely that we can learn more about the human disease by using the CF mouse intestine as a model.

Mucins are the major protein constituent of mucus (15, 26). They are large molecules characterized by abundant and variable O-linked glycans attached to hydroxy amino acids clustered in what are called mucin domains. A main function of mucins is to protect the epithelial cells by binding bacteria both by nonspecific trapping and by binding to specific glycans. The mucins are subdivided into two major groups depending on their structural features: membrane bound or secreted, gel-forming mucins (15). The matrix in the mucus gel is made up of the polymerizing mucins (MUC2, 5AC, 5B, 6, and 19). Of these, the MUC2 mucin is dominate in the intestine (11, 12, 16). This mucin is also found in small amounts in the lungs of CF patients (8). MUC2 is one of the best-studied secreted mucins (2, 9, 11), known to dimerize in the endoplasmic reticulum via its COOH termini, become heavily O-glycosylated in the Golgi, and polymerize via the NH2 termini by disulphide-bonded trimerization (9). The formation of an additional covalent bond renders the mucin insoluble (12), a property that would be beneficial in the intestine but devastating in the lungs. The MUC2 mucin thus forms enormously large, netlike molecules constituting the intestinal mucus matrix.

MUC1 was the first transmembrane mucin to be cloned, but the numbers have since increased to the presently known nine transmembrane mucins (MUC1, 3A, 3B, 4, 12, 13, 15, 16, and 17; Refs. 3, 10, 13, 23, 25, 36, 37). Despite the near completion of the human genome, several of these genes are not fully sequenced. The transmembrane mucins are located at the apical side of the epithelial cells, and all of them seem cleaved in their extracellular domain close to the membrane, although still held together by noncovalent forces. Most of the transmembrane mucins are expressed in the intestine, including the MUC3, MUC12, and MUC17 mucin family (3, 10, 23, 25, 35–37). These mucins are found at the 7q22 locus of the human genome, but the corresponding mouse locus has a large gap.

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and only one gene is found. Muc3 is the only mouse mucin annotated in this locus. Muc3 shows the highest sequence similarities to the human MUC17 mucin and is located close to the Trim56 gene, where the human MUC17 gene is located. It is likely that the mouse Muc3 is the orthologue of the human MUC17. We have thus chosen to designate the mouse mucin of this locus “Muc3(17).”

In the context of CF, the MUC1 mucin has attracted the most attention as the mRNA levels are increased the most (up to six times), whereas other mucins analyzed showed similar or decreased levels (22). However, the increased mRNA levels of the MUC1 mucin was not due to the normal forms of MUC1, but instead due to two splice variants (14). One variant encoded a secreted mucin named MUC1/SEC (32), lacking the transmembrane domain. The other variant encoded the Muc3(17) protein, where the human MUC17 mucin was not due to the normal forms of MUC1, but instead due to two splice variants (14). The importance of the Muc1 mucin in the mucus accumulation of the CF mice was further substantiated by the observation that CF mice lacking Muc1 (Muc1 designates the mouse and MUC1 the human MUC1 mucin) show less mucus accumulation. In addition, introduction of the human MUC1 in the CF/Muc1−/− mice, creating the MUC1.Tg transgenic CF mice (CFM), recapitulated the Muc1-expressing CF mice (14).

The mucus layer of the rat small intestine is loosely adherent, and it is possible to totally remove it by light suction. In contrast, a 100-μm thick firmly adherent mucus layer that is not possible to remove without damaging the epithelial cells covers the large intestine (1). In addition, a loosely adherent mucus layer easy to remove is located on top of the adherent layer. The mucus layer of the rat intestine can extend up to 800 μm from the epithelial cells.

As discussed, accumulation of mucus was found in the intestine of CF mice, but this was observed only as an accumulation of Alcian blue-stained material. The composition of the accumulated mucus has not been studied. Here we have isolated and purified mucins, soluble or insoluble in guanidinium chloride (GuHCl), from the small intestine of CF and non-CF transgenic mice and analyzed the relative amounts of mucins as well as relative amounts and distribution of the MUC1, Muc2, and Muc3(17) mucins. To illuminate the suggested modulating role of Muc1 for the mucus levels of the intestine, we have also measured the mucus thickness in mouse colon in vivo.

## MATERIALS AND METHODS

**Animals.** All mice in this study were inbred on the C57BL/6 background, and the CF mice were of the C57BL/6J-Tm1Unc/C57BL/6J Unc genotype. The ages of the relatively healthy CF mice studied ranged from 9 to 24 wk, and they were all maintained on a liquid mouse diet. The different genotypes are listed in Table 1. All animals were kept under standardized conditions of temperature (21–22°C) and illumination (12:12-h light-dark cycle). Animal experimental procedures were approved by the Swedish Laboratory Animal Ethical Committee in Uppsala and were conducted in accordance with guidelines of the Swedish National Board for Laboratory Animals.

**Isolation of soluble and insoluble mucus.** Mucosa from small intestine was scraped and placed in extraction GuHCl (6 M GuHCl, 5 mM EDTA, 0.01 M NaH2PO4; pH 8.0) and homogenized using a Dounce homogenizer, pestle B. Samples were stirred overnight at 4°C and centrifuged at 18,000 rpm at 10°C for 30 min. Supernatants containing the soluble mucins were saved. Pellets dissolved in extraction GuHCl and stirred overnight at 4°C. The washing was repeated three times, and all supernatants were pooled. The final pellets containing the insoluble mucins were dissolved in reduction GuHCl (6 M GuHCl, 5 mM EDTA, and 0.1 M Tris-HCl, pH 8.0, with 10 mM DTT) and stirred at 37°C for 5 h. To alkylate the samples, iodoacetamide (2.5 molar excess of DTT) was added and stirred overnight.

**Density gradient ultracentrifugation.** Mucins (soluble and insoluble) were prepared according to Carlstedt et al. (6) by dialysis against water, lyophilized, dissolved in nuclease digestion buffer (50 mM NaH2PO4, pH 7.0; 5 mM MgCl2; 1 mg RNase; and 0.5 mg DNase), and incubated at 37°C overnight. Samples were dialyzed against water as before, lyophilized, and dissolved in extraction GuHCl. CsCl was added to 0.6 mM (1.39 g/ml starting density) diluted to 4 M GuHCl with 10 mM NaH2PO4, 5 mM EDTA, pH 8 and ultracentrifuged in a Beckman centrifuge (25 × 89 mm) at 40,000 rpm, 15°C, 72 h in a 70Ti rotor. Twenty-four fractions were taken from the bottom. Mucin peaks were identified by periodic acid-Schiff (PAS) staining and density and A230 measurements. Fractions covering the mucin peak were pooled and dialyzed, lyophilized, and dissolved in 0.2 M GuHCl; CsCl was added to 1 mM (starting density 1.5 g/ml), and centrifugation was performed as before. Fractions covering the mucin peak were pooled, dialyzed, lyophilized, and dissolved in H2O with 0.05% NaN3.

**Gel filtration.** Gel filtration was performed using a Superose 6 10/30 column (GE Healthcare). The column was connected to a Pharmacia HPLC 2248 pump and a 2238 Uvicord S2 detector. As eluting buffer, 4 M GuHCl with 50 mM Tris-HCl (pH 7.0) was used. Flow was set at 0.25 ml/min, and 0.5-ml fractions were collected.

**Glycan detection.** Glycoproteins were detected with the PAS or DIG glycan detection kit (Roche). PAS-stained or immunostained membranes were scanned using video densitometry.

**Gel electrophoresis and proteomics.** The purified insoluble and soluble mucins were separated by agarose-polyacrylamide gel electrophoresis, visualized by Alcian blue, and blotted to nitrocellulose membranes (30). For proteomics, the bands were cut out, digested with trypsin, and analyzed by nano-HPLC-MS and tandem mass spectrometry (MS/MS) using a Thermo LTQ-FT instrument and a 50 μm × 20 cm Kromasil C-18 column eluted with an acetonitrile gradient in water at a flow of 200 nl/min. Significant peptides have a mass within 2 ppm of predicted mass and a supportive MS/MS derived sequence.

**Antibodies.** MUC1 protein was detected using MAb 214D4 (kindly provided by J. Hilkens, Utrecht, The Netherlands) against the tandem repeat region and MAb CT2 against the cytoplasmic tail of MUC1. The Muc3(17) protein was detected using Muc3(17)-S1, -S2, and -C1 polyclonal antisera raised against the peptides SQEYSEKLQDRK-SEEFSFNKTIT, KYTPGFNTLDTVKVNLETIKKNT, and

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Muc1 designates the mouse mucin, and MUC1 designates the human MUC1 mucin. WT, wild type; MUC1. Tg, Muc1−/− mice expressing a human MUC1 transgene; CFM, MUC1.Tg transgenic cystic fibrosis (CF) mice.

- **Table 1. Genotype and designation of the different mouse strains analyzed in this study**
Animal preparation for intravital microscopy. The mice were anesthetized with spontaneous inhalation of isoflurane (Forene; Abbott Scandinavia, Kista, Sweden). The inhalation gas was administered continuously through a breathing mask (Simtec Engineering) and contained a mixture of 40% oxygen, 60% nitrogen, and ~2.2% isoflurane. Body temperature was maintained at 37°C by means of a heating pad controlled by a rectal thermistor probe. A catheter containing heparin dissolved in isotonic saline was placed in the left carotid artery to monitor blood pressure. The jugular vein was cannulated for continuous infusion of Ringer solution at a rate of 0.35 ml/h. The colonic preparation for in vivo microscopy in rat has been described in detail previously (1). In this study we adapted this method to the smaller size of the studied species. Briefly, exteriorization of the proximal part of the descending colon through a midline abdominal incision was followed by a lengthwise incision along the antimesenteric border using electric cautery. The animal was placed on its left side on a Lucite table with a part of the colon loosely draped over a truncated cone at the center of the table, with the mucosal surface facing upwards. A mucosal chamber, with a hole in the bottom corresponding to the position of the cone, was placed over the mucosa exposing ~0.07 cm² of the mouse mcosa through the hole. The mucosal chamber did not touch the mucosa, in order to not impair blood flow, and the edges of the hole were sealed with silicon grease (high vacuum grease; Dow Corning, Wiesbaden, Germany). The mucosal chamber was filled with 3 ml of 0.9% NaCl maintained at 37°C by means of circulating warm water in a jacket in the bottom of the chamber. The colonic mucosa was observed through a stereomicroscope (model MZ12; Leica, Heerbrugg, Switzerland) and was transilluminated with light from a 150-W light source guided by fiber optics.

Measurements of the mucus thickness in vivo. Mucus thickness was measured with micropipettes connected to a micromanipulator (Leitz, Wetzlar, Germany) with a digmatic indicator (IDC Series 543; Mitutoyo, Tokyo, Japan). Glass tubing (borosilicate tubing with 1.2 mm OD and 0.6 mm ID; Frederick Haer, Brunswick, ME) was pulled with a pipette puller (pp-83; Narishige, Tokyo, Japan) to a tip diameter of 1–3 µm. To prevent mucus adhering to glass, the pipettes were siliconized by dipping the tip of the micropipette into a silicone solution (MS1107, 25% acetone) followed by drying at 100°C for 30 min. The luminal surface of the mucus gel was visualized by placing graphite particles (activated charcoal, extra pure; Merck, Darmstadt, Germany) with a digmatic indicator (IDC Series 543; Mitutoyo, Tokyo, Japan) on the gel, and the colonic epithelial cell surface was visible through the microscope. The micropipette was inserted into the mucous gel at an angle of ~30 deg to the surface. The angle was measured with a protractor, and the same angle was maintained throughout an experiment. The distances, traveled by the micropipette from the luminal surface of the mucus gel to the epithelial cell surface were measured, and a mean value was calculated. The mucus thickness, which is the vertical distance between the cell surface and the luminal mucus surface, was then calculated. The mean value of 3–4 different measurements was taken as one thickness value.

The loosely adherent mucous layer was removed by gentle suction with a thin polyethylene cannula connected to a syringe. This procedure was conducted under supervision through a stereomicroscope to avoid contact with the epithelium.

Statistical analysis. Values are means ± SE. For statistical evaluations of the in vivo mucus measurements, analysis of variance (ANOVA) for repeated measures was used, and ANOVA for multiple comparisons was performed when comparing data between groups. ANOVA was followed by the Fisher protected least-significant difference test. To compare single values, the Student’s t-test for paired or unpaired data was used. The differences were regarded as significant at P < 0.05.

RESULTS

Increased amount of mucus in the small intestine of CF mice. The mucosa was scraped from the small intestine of CF (Cftr<sup>+/−</sup>/Cftr<sup>+/−</sup>) and non-CF transgenic mice with wild-type (WT) Muc1, Muc1<sup>−/−</sup>, or human MUC1. The genotypes and nomenclature used are explained in Table 1. The mucosal scrapings were dissolved in GuHCl and separated into GuHCl soluble and insoluble fractions followed by purification of the mucins by three rounds of isopycnic density gradient ultracentrifugation on cesium chloride gradients using the protocol of Carlstedt et al. (6). From each round of centrifugation, fractions were collected, and mucin peaks were identified by PAS staining and density and A<sub>280</sub> measurements. The obtained mucin fractions were analyzed for composition by carbohydrate and amino acid analysis (Table 2). The relative proportion of protein (17–20%) and carbohydrate (80–84%) was similar in the different insoluble mucin fractions. The soluble fractions require further purification by gel filtration (not performed for WT mice and CF mice), revealing that the mucins in these fractions have a high carbohydrate content (>90%, Table 2).

To compare the mucins more accurately, the mucin fractions were further purified by gel filtration in 4 M GuHCl. The amount of mucins was semiquantitatively estimated from the relative PAS staining intensity of the blotted mucins (Fig. 1). The amount of both insoluble and soluble mucins increased about 10-fold in the CF mice. A similar, although decreased tendency, was observed when the less pure mucins were estimated from analysis of the total amount of amino acids and monosaccharides (Table 2). The same type of estimation revealed about a sevenfold increase of soluble mucins and a 2.5-fold increase of the insoluble mucins in the CF mice (mouse Muc1 background) compared with the WT mice (WT). The differences observed in mucin amounts cannot solely be explained by differences in glycosylation, as we have previously observed that the only alteration was an increased fuco-
transgene; CFM, MUC1.Tg transgenic cystic fibrosis (CF) mice.

small intestine were semiquantitatively estimated from the biochemical level, mucins from CF and non-CF (14, 22). To determine whether these effects were also ob-
mucin, but the phenotype returned in MUC1.Tg mice

soluble and insoluble mucins were separated by gel filtration on a Superose 6 column. The mucin fractions were slot blotted on nitrocellulose and stained with periodic acid-Schiff (PAS). The membranes were scanned by video densitometry, and relative amounts of total mucins were semiquantitatively estimated by PAS staining intensity calculated per small intestine. Results are mean values of three independent estimations. The number of animals in each
group was 9–23. MUC1.Tg, Muc1–/– mice expressing a human MUC1 transgene; CFM, MUC1.Tg transgenic cystic fibrosis (CF) mice.

sylation due to an induced FUT2 transferase in the CF mice (34). Taken together, the total amount of mucins was increased in the CF mice irrespective of the method used to estimate the mucin amounts.

Muc1 affects the mucus amounts in the small intestine. According to previous observations of intestinal sections stained with Alcian blue, CF mice accumulated large amounts of mucus that filled the lumen of the intestine. This visual phenomenon was less pronounced in CF mice that lacked the Muc1 mucin, but the phenotype returned in MUC1.Tg mice (14, 22). To determine whether these effects were also observed at a biochemical level, mucins from CF and non-CF small intestine were semiquantitatively estimated from the relative staining intensity (Fig. 1). The CF/Muc1–/– mice showed a lower amount of mucins in both the soluble and insoluble fractions compared with the CFM. The same tendency, although weak, was observed in the less pure mucin fractions shown in Table 2. Irrespective of mouse or human MUC1, the CF animals had compatible mucin amounts (Table 2). Thus decreased mucin levels were observed in the CF mice lacking Muc1 in both the soluble and insoluble fractions.

Gel electrophoresis and identification of mucins by proteomics. To further study the nature of the mucins in the soluble and insoluble fractions from the different mouse genotypes, the purified mucins were subjected to agarose-polyacrylamide gel electrophoresis. The Alcian blue-stained gel revealed a double band in the insoluble fractions (Fig. 2A). Both these bands were identified as Muc2 by trypsin digestion and subsequent nano-HPLC mass spectrometry. That the GuHCl insoluble fraction is made up almost exclusively of Muc2 has been suggested before (5, 12). The upper Muc2 band has previously been observed and suggested to be a nonreducible dimer (2, 12). This means that the relative amounts of Muc2 can be estimated from the results presented for the insoluble fraction in Fig. 1 and Table 2. The soluble fractions are more complex and contain, in addition to the Muc2 bands, a diffuse larger band. When the bands found in the lanes representing the soluble mucins (bands 1–4, Fig. 2A) were trypsin digested and the peptides analyzed by nano-HPLC MS-MS followed by a search in a curated mucin database, several peptides corre-

Fig. 1. Relative amounts of total mucins in the small intestines. Purified soluble and insoluble mucins were separated by gel filtration on a Superose 6 column. The mucin fractions were slot blotted on nitrocellulose and stained with periodic acid-Schiff (PAS). The membranes were scanned by video densitometry, and relative amounts of total mucins were semiquantitatively estimated by PAS staining intensity calculated per small intestine. Results are mean values of three independent estimations. The number of animals in each group was 9–23. MUC1.Tg, Muc1–/– mice expressing a human MUC1 transgene; CFM, MUC1.Tg transgenic cystic fibrosis (CF) mice.
Muc3(17)-C1 antiserum raised against a peptide in the cytoplasmic tail of Muc3(17) showed similar results (data not shown). Staining of identical slot-blot membranes with the one representative out of two independent experiments. Muc3(17) per small intestine were calculated. The results presented here were scanned by video densitometry, and the relative amounts of MUC1 and MUC1 (not shown), supporting previous observations demonstrating that the large increase of MUC1 mucin in the small intestinal mucosa of the CF mice despite the significant increase of MUC1 protein compared with the non-CF mice.

**MUC1 modulates mucus amount and thickness in large intestine.** The results suggest that Muc1 is affecting the mucus amount of the intestine. This might be reflected in the mucus thickness, but it is difficult to measure the thickness of the mucus layer in the small intestine as the mucus is also located between the villi. However, this is not the case for the large intestine where the organization with a flat epithelial surface makes such measurements possible (1). First, we measured the amount of insoluble mucins as estimated by amino acid and carbohydrate content in the large intestine. The CF mice displayed an increased amount of mucins compared with the WT mice. The CF/Muc1−/− mice showed mucin levels that were intermediate between the CF and WT mice (Fig. 5A). These results suggest that the large intestine suffers similar consequences when lacking Cftr and Muc1 as already discussed for the small intestine.

The mucus thickness was measured in the colon of live mice as described for rats (1). In short, the intestine is exposed and a micropipette is passed through the mucus layer down to the epithelial cells revealing the total mucus thickness (Fig. 5B, total). The loosely adherent layer of mucus is gently removed by suction, and the thickness of the firmly adherent mucus is measured (first removal). The recovery of the total mucus layer is then measured after 15 and 30 min, followed by removal of the loosely adherent mucus and another measurement of the firmly adherent mucus layer (second removal). The total mucus thickness was similar in the WT and Muc1−/− mice, whereas the firmly adherent layer was significantly lower in the Muc1−/− mice compared with WT mice, both after the first removal and after 30 min. The growth rate of the loosely adherent mucous layer was identical in the WT and Muc1−/− mice (Fig. 5C), and the significant differences in mucus thickness were maintained. These results suggest that the Muc1 mucin can modulate both the mucus amount and thickness of the firmly adherent mucous of the large intestine.

![Graph](http://ajpgi.physiology.org/)

**Fig. 4.** Separation of mucins by gel filtration column chromatography. Gel filtration of the mucins purified from the CFM mice on a Superose 6 column. Fractions (0.5 ml) were collected and analyzed after slot blotting by PAS, the anti-MUC1 MAb 214D4, and anti-Muc3(17)-S2 antiserum as revealed in Fig. 4. Most of the mucins, including Muc3(17), eluted as expected in the void volume, whereas MUC1 eluted later. This suggests that MUC1 is not a dominant mucin in the small intestinal mucosa of the CF mice despite the significant increase of MUC1 protein compared with the non-CF mice.

![Graph](http://ajpgi.physiology.org/)

**Fig. 3.** Semiquantitative estimation of the MUC1 and Muc3(17) mucins in small intestines. Purified soluble and insoluble mucins were slot blotted and immunostained with antibodies against the extracellular domain of MUC1 (MAb 214D4, A) or the extracellular domain of Muc3(17) [pAb Muc3(17)-S2, B] followed by anti-mouse and anti-rabbit secondary horseradish peroxidase-conjugated antibodies developed by chemiluminescence. The staining intensity was scanned by video densitometry, and the relative amounts of MUC1 and Muc3(17) per small intestine were calculated. The results presented here are one representative out of two independent experiments.

Nostaining of identical slot-blot membranes with the Muc3(17)-C1 antiserum raised against a peptide in the cytoplasmic tail of Muc3(17) showed similar results (data not shown).

**MUC1 is not a dominant mucin in the CF mucus.** MUC1 was detected using the MAb 214D4 directed against the extracellular tandem repeat region of the human MUC1 mucin. This MAb reacts well with the high-glycosylated forms found in the intestine (31). Although antibodies against repetitive sequences are not that reliable for semiquantitative estimates, a dramatically increased amount (estimated to more than 1,000-fold) of MUC1 mucin was found in the CFM mice compared with the non-CF mice (Fig. 3A). Most of the MUC1 was detected in the soluble fraction, but some reactivity was also found in the insoluble mucins of the CFM mice. No staining was observed with the MAb CT2, directed against the cytoplasmic tail of MUC1 (not shown), supporting previous observations demonstrating that the large increase of MUC1 mucin seen in the CF mice is due to alternatively spliced variants of MUC1 (14). As MUC1 was increased, it was reasonable to conclude that MUC1 is a major constituent of the increased mucin levels found in the soluble fraction in CF. However, this cannot be the case as MUC1 did not comigrate with any of the Alcian blue-stained mucins in the agarose-polyacrylamide gel as revealed by Western blot (Fig. 2B). This conclusion is further supported by analyses of the mucin fractions by gel filtration and staining of individual chromatography fractions by PAS, the anti-MUC1 MAb 214D4, and anti-Muc3(17)-S2 antiserum as revealed in Fig. 4. Most of the mucins, including Muc3(17), eluted as expected in the void volume, whereas MUC1 eluted later. This suggests that MUC1 is not a dominant mucin in the small intestinal mucosa of the CF mice despite the significant increase of MUC1 protein compared with the non-CF mice.

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**Fig. 5.** The mucus thickness was measured in the colon of live mice as described for rats (1). In short, the intestine is exposed and a micropipette is passed through the mucus layer down to the epithelial cells revealing the total mucus thickness (Fig. 5B, total). The loosely adherent layer of mucus is gently removed by suction, and the thickness of the firmly adherent mucus is measured (first removal). The recovery of the total mucus layer is then measured after 15 and 30 min, followed by removal of the loosely adherent mucus and another measurement of the firmly adherent mucus layer (second removal). The total mucus thickness was similar in the WT and Muc1−/− mice, whereas the firmly adherent layer was significantly lower in the Muc1−/− mice compared with WT mice, both after the first removal and after 30 min. The growth rate of the loosely adherent mucous layer was identical in the WT and Muc1−/− mice (Fig. 5C), and the significant differences in mucus thickness were maintained. These results suggest that the Muc1 mucin can modulate both the mucus amount and thickness of the firmly adherent mucous of the large intestine.
Muc1 cluster, where only a small part of the expected sequence was found. Such a large gap is found for the Muc3-Muc12-sequence. We have analyzed the mouse genome in more detail, and early death (21). This has prompted us to study mouse intestinal mucus accumulation in more detail. The major small intestinal mucin Muc2 is largely found in a fraction insoluble in GuHCl, where it shows an increase in the CF compared with WT mice. This is in contrast to the previously observed decrease in Muc2 mRNA levels of ileum (22). This discrepancy could be due to regulation of the Muc2 levels at the translational level. However, another possibility is that smaller amounts of Muc2 are actually produced but retained and not passed along the intestine at the same rate as in WT animals.

The amount of soluble mucins was increased 7- to 10-fold in the CF mice compared with wild-type mice (WT). The increase was due to Muc3(17), also previously observed to be increased in CF mice (17), although the present fold increase was less than 2. This means that other mucins in this fraction must make up more of these increased levels. Unfortunately, it has been more difficult to identify the nature of these mucins despite the use of advanced mass spectrometry. The major reason is that the mouse genome is still incomplete when it comes to mucin sequences. In an effort to sort out the correct sequences, we have analyzed the mouse genome in more detail, revealing that several of the known loci for mucins still have large gaps. Such a large gap is found for the Muc3-Muc12-Muc17 cluster, where only a small part of the expected sequences is available. Thus we cannot precisely identify the soluble mucins for the moment.

The only mucin previously shown to display increased mRNA levels in CF mice was Muc1 (22). This increase was significant in the stomach and colon, large in the ileum, but absent in the cecum. More detailed analysis revealed that this was not due to the normal forms of MUC1, but instead, to two splice variants. One of these is the MUC1/SEC lacking a cytoplasmic tail, and the other is the MUC1-CT80 splice variant with an alternate cytoplasmic tail (14). In the present study, only the extracellular domain of MUC1 was quantified, and the observed 1,000-fold increase in the small intestine is probably due to both the above mentioned forms. As the mucins Muc2, Muc3, and Muc5ac, but not Muc1, have shown decreased message levels in CF, it was attractive to assume that the increased protein levels were due to the Muc1 mucin. However, this was not the case as MUC1 was not comigrating with the major mucins when separated by gel electrophoresis and stained by Alcian blue. This dye stains only negatively charged mucins, but even when the more general PAS stain was used for staining the mucins separated by gel filtration, the MUC1 peak did not coincide with the major mucins. Thus it is not likely that the increased mucin levels in CF mice small intestine are only or mostly made up of Muc1.

The accumulation of mucus in both ileum and colon, estimated by Alcian blue staining of tissue sections, has been shown to depend on Muc1 expression, as the Muc1−/− mice showed considerably less mucus (22). The dependence of Muc1 was further substantiated when the human MUC1 gene was inserted into the Muc1−/− mice and the phenotype of mucus accumulation reappeared (14). When the MUC1.Tg animals were analyzed in the present study, the amounts of insoluble and soluble mucins were similar to the levels in the WT mice expressing Muc1 and the mucins levels dependent on Cftr expression. This as well as other studies in breast tissues (27) suggest that the human MUC1 reflects the function of the WT Muc1.

The lower amounts of mucins in the Muc1−/− mice probably includes Muc2, as this is the major and only identified mucin in the insoluble fraction and a major component of the soluble mucin fraction. This was, however, not the case for the specific mucin Muc3(17), which instead was increased. As the latter mucin is a transmembrane mucin, just as MUC1, it is possible that this is due to a compensatory phenomenon. As Muc1 does not seem to be a major constituent of the mucus in the small intestine, the lower amounts of total mucins in the CF/Muc1−/− mice compared with the CF mice (both with WT Muc1 and human MUC1) must be explained in another way. One possibility is that Muc1 tethers or anchors the mucus layer in some way to the epithelial cells. This is possible for the normal splice variant of MUC1 as well as one of the CF upregulated forms of MUC1, the MUC1-CT80. However, the second CF-upregulated MUC1, MUC1-SEC lacking the transmembrane domain, cannot anchor the mucus layer. As discussed, MUC1 is only a minor component and at the same time considerably shorter than other mucins, as for example Muc3(17), anchored in the epithelial cell. Thus it is less likely that MUC1 acts as a tether of the mucus gel in the gastrointestinal tract.

The effect of MUC1 on mucins and mucus favors a hypothesis that MUC1 is involved in the regulation of mucus amount.

Fig. 5. Muc1 affects the in vivo mucous thickness in colon. A: The insoluble mucins from scrapings of the large intestine were purified by three rounds of ultracentrifugation, and the amino acid and carbohydrate compositions were measured as described in Table 2. The estimated amount of purified mucins is expressed as micrograms of amino acids and sugar residues per animal (n = number of animals). B: The thickness of mucus in colon was measured in anesthetized animals. Total, the initial mucus thickness; first removal, firmly adherent mucus after gentle removal of the loosely adherent mucus layer; 15 min, mucus thickness after 15-min reconstitution; 30 min, mucus thickness after 30-min reconstitution; second removal, firmly adherent mucus layer after removal of the loosely adherent layer after 30 min. The thickness is expressed in micrometers, and the asterisks express significant differences in the thickness of the WT mice. This is in contrast to the previously observed decrease in Muc3(17), anchored in the epithelial cell. Thus it is less likely that MUC1 acts as a tether of the mucus gel in the gastrointestinal tract.
and mucus formation. This hypothesis is supported by the observed effect of Muc1 on the mucus thickness as studied in live animals. When Muc1 is absent, the firmly adherent mucus layer is significantly thinner. The molecular nature of the loosely and firmly adherent mucus layers is still not known, but the observed effects of an absent Muc1 is well in line with the effects on mucus amounts in both the small and large intestine. The mucus thickness measurements in vivo with normal Cftr suggests a more general role for Muc1 on mucus formation and that the lack of Cftr in the CF mice is exaggerating the Muc1 effects. The present observations suggest a scenario where MUC1 is taking part in the regulation of mucus amounts. This is not impossible as MUC1 has been shown to take part in several intracellular signaling pathways (19, 24, 27). However, most of what is known about MUC1 signaling is from studies of tumor tissue, and further information from normal functional studies is urgently needed (24, 28, 29). How MUC1 is involved in a potential outside-in signaling in normal tissues is currently not well-understood, but the domain organization of MUC1 could suggest a model. The extracellular domain reaches far out from the cell, and the cleaved SEA domain is situated close to the membrane. The structure of this domain has recently been determined, and it suggests that separation of the two parts of this domain might be sensed by the cell via interacting proteins (20). The cytoplasmic tail can also take part in signaling by its capacity to bind several signaling and adaptor proteins (19, 24, 27). Further studies in transgenic mice expressing truncated forms of MUC1 should illuminate the role of MUC1 in regulation of mucus amounts and the mucus accumulation in CF.

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