Novel MUC1 splice variants contribute to mucin overexpression in CFTR-deficient mice

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Cystic fibrosis (CF) is caused by defects in the CF transmembrane conductance regulator (CFTR) ion channel. Some of the clinical manifestations of the disease are mucus accumulation in the respiratory and gastrointestinal tracts. Normally, a layer of mucus covers the surface of the gastrointestinal tract, which is indispensable to maintain the integrity of the intestinal mucosa. In CF, the mucus deposits (seemingly a mixture of several mucins) are one of the pleiotropic manifestations of the disease. The excessive mucus results in severe intestinal obstruction at birth, known as meconium ileus. Intestinal obstruction can also occur later in life. Several mucins have been found to be important in the normal or diseased intestine. These include the gel-forming secreted mucins MUC2, MUC5B, and MUC5AC (4, 10, 11, 49) and the nongel-forming membrane bound or secreted mucins MUC1, -3, -4, -12, -13, and -17 (6, 13, 19, 39, 44, 50, 52). Some biochemical and immunological analyses of mucins obtained from CF patients suggest abnormalities in mucus glycosylation, production, and secretion, but there is a lack of knowledge of specific alterations in mucin expression. It is unclear how the absence of a functional CFTR protein could modify the pattern of expression of some epithelial mucins. The defective ion transport in CF may contribute to a reduction in the volume of airway surface liquid (29) or alter the salt content of the airway surface liquid (43), and either of these situations would change the milieu into which the mucus is secreted. Other studies on CF mice have suggested that the absence of CFTR in the lungs and intestines perturbs normal differentiation of a secretory cell population (7, 12, 22). These situations would potentially facilitate abnormal mucin/mucus production.

CFTR mutant mice (Cftrtm1Unc/Cftrtm1Unc), called CF mice, exhibit intestinal goblet cell hyperplasia and dilated crypts with excessive mucus accumulation and obstruction mimicking human meconium ileus, but they do not show mucus accumulation in the lungs (45, 55). Interestingly, when CF mice lacking the mucin Muc1 were analyzed, the intestinal mucin accumulation was decreased compared with Muc1-expressing CF mice (34). The human mucin is designated as MUC1, whereas the mouse homologue is designated Muc1. Despite the fact that Muc1 is expressed at low levels in the normal intestine and that it was originally described as a transmembrane mucin, it appears to play a role in normal levels of intestinal mucus in CF mice (34). MUC1 has a large extracellular domain (1,000–2,000 amino acids), a hydrophobic membrane-spanning domain, and a tyrosine-phosphorylated cytoplasmic domain of 72 amino acids (17, 18, 27). MUC1 is overexpressed in mammary tumors (18, 27), in colon cancer (3, 10), and in other malignancies (56), and soluble forms exist in tissue culture supernatants and body fluids (1, 6). The MUC1 cytoplasmic tail (CT) interacts with many proteins involved in signal transduction and cell adhesion, including β-catenin, p120ctn, and α-catenin (1, 12, 22). The presence of MUC1 in the cytoplasm suggests that it may have an important role in the regulation of cell growth and differentiation.

Novel MUC1 splice variants and the previously described secreted MUC1 mRNA and protein of two novel splice variants, CT80 and CT58, were both transmembrane proteins with cytoplasmic tails different from the normal MUC1. The MUC1-CT80 and MUC1/SEC forms are found spanning domain, and a tyrosine-phosphorylated cytoplasmic tail (17, 18, 27). MUC1 is overexpressed in mammary tumors (18, 27), in colon cancer (3, 10), and in other malignancies (56), and soluble forms exist in tissue culture supernatants and body fluids (1, 6). The MUC1 cytoplasmic tail (CT) interacts with many proteins involved in signal transduction and cell adhesion, including β-catenin, p120ctn, and α-catenin (1, 12, 22). The presence of MUC1 in the cytoplasm suggests that it may have an important role in the regulation of cell growth and differentiation.

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glycogen synthase kinase-3β (GSK-3β), c-src, Grb2/SOS, PKCδ, epidermal growth factor receptor (EGFR), erbB2, erbB3, and erbB4 [23–26, 33, 37, 38, 41, 53].

Our hypothesis is that Muc1 is participating in the intestinal mucus obstruction in the CF mice, it is consequently increased in the CF intestines, and an alternative shed or secreted MUC1 form is aberrantly produced under the altered environment generated by the absence of a functional CFTR. Here, we demonstrate the contribution of MUC1 to the CF intestinal phenotype. We show 1) the differential expression of MUC1 at the mRNA and protein level in CF MUC1 transgenic (CFM) mice vs. the control MUC1 transgenic (MUC1.Tg) mice with an intact CFTR, 2) histological localization of MUC1 in the intestinal lumen of CFM mice, 3) the presence of increased MUC1/SEC (message and protein) in the CFM mice, and 4) the existence and characterization of a new MUC1 splice variant (MUC1-CT80) that shows elevated mRNA and protein levels in the CFM mice. Additionally, the differential expression of the MUC1 forms along the intestinal tract consistently demonstrate that MUC1 is aberrantly expressed in the small intestine of CFM mice in which expression resembles large intestine autouchthonous MUC1 expression. These findings are consistent not only with a MUC1 role in the CF intestinal phenotype but also suggest that CFTR has the ability to affect the nature and amount of mucus secretion of certain epithelial cells. Whether this is directly related to CFTR function or indirectly due to secondary phenomena caused by the lack of CFTR is still to be defined.

MATERIALS AND METHODS

Animals. All studies were performed on inbred C57BL/6 mice containing either wild-type CFTR or mutant CFTR (Cftrtm1Unc/Cftrtm1Unc) (45), the latter referring to the CF mouse. Mice lacking Muc1 [46] were crossed with CF mice. The resulting CF/Muc1−/− mice were mated with MUC1.Tg mice (40) in a Muc1−/− background, to produce the CFM mouse (Cftrtm1Unc/Muc1−/−, MUC1−/−). The control mice were MUC1.Tg in a Muc1−/− background with a functional CFTR (40). All mice were maintained in specific pathogen-free conditions in a barrier facility at Mayo Clinic Scottsdale (Natalie Schafer Transgenic Animal Facility). Mice 3- to 4-wk-old were weaned onto a mouse liquid diet (product no. F3017; BioServ, Frenchtown, NJ), and sterile water was provided ad libitum. All experimental procedures were conducted according to Institutional Animal Care and Use Committee guidelines. Sixty-five animals were studied, ages 2 wk to 11 mo. CFM, MUC1.Tg, and CF/Muc1−/− mice were age matched in 1–2 days when younger than 8 wk, and older animals were matched within 1–2 wk.

Intestinal lysates. Intestinal lysates were prepared from CFM mice (controls MUC1.Tg and CF/Muc1−/−). The small intestine (defined as the portion directly below the duodenum and above the cecum) was divided into two equal parts; the proximal part was taken as jejunum and the distal part as the ileum. The colon was the portion between the cecum and the anus. Intestinal segments were opened longitudinally and cleaned with cold PBS. The luminal mucus was scraped away from the intestinal lumen and homogenized in Triton X-100 lysis buffer of (in mM): 20 HEPES (pH 8.0), 150 NaCl, 2 EDTA, 2 sodium orthovanadate, and 10 sodium fluoride, with 50 μM ammonium molybdate and 1% Triton X-100, plus complete inhibitor mixture (Sigma, St. Louis, MO). Protein lysates were centrifuged to separate cell debris. Protein concentration was determined using the bicinchoninic acid assay (Pierce) and samples were stored at −80°C.

Antibodies. We used three MAb to recognize MUC1. HMFG-2 (kindly provided by Dr. Joyce Taylor-Papadimitriou, Imperial Cancer Research Fund, London, UK) recognizes the epitope DTR (9) in the extracellular domain (MUC1-EX). B27.29 (kindly provided by Biomira) recognizes the epitope PDTRPAP (36) also in the MUC1-EX. CT2 recognizes an epitope within the last 17 amino acids of the MUC1 cytoplasmic domain (MUC1-CT) (41). A chicken polyclonal antibody (PAb) directed to a synthetic peptide of a unique (underlined) MUC1/SEC sequence (5′-CGGVSIGLS-FMPLP-3′) (44) and a rabbit PAb (CT80) directed to unique (underlined) COOH-terminal sequence of the splice variant MUC1-CT80 (5′-CGKDSEGGTWKTRQAWKR-3′) were produced. The amino acids C or CGG were added to the amino terminal peptide sequences to facilitate their conjugation to keyhole limpet hemocyanin.

Western blots were performed using 25 μg of protein lysate per sample detected with the antibodies HMFG-2, B27.29, and MUC1/SEC, and 200 μg of protein lysate per sample for CT2 and CT80. Samples were separated by SDS-PAGE and transferred to polyvinylidene difluoride membrane (Immobilon-P; Millipore) for Western blot analysis. Negative controls included antibody-specific peptide blocking and Muc1 knockout tissues. Dilutions of the antibodies were as follows: CT2 (supernantant) 1:100, HMFG-2 (supernatant) 1:20, MUC1-SEC 1:1,000 (serum), and CT80 1:1,000 (serum).

Mucin deglycosylation. Partial deglycosylation of MUC1 was carried out by immersing the immobilized protein on Immobilon-P membrane in liquid trifluromethanesulfonic acid (TFMSA; Aldrich) for 6 h at 4°C (2). The membrane was neutralized by washing in 50 mM Tris-HCl pH 8 for 30 s and blocked before Western analysis as described above.

Immunohistochemistry. Four longitudinal sections of 2 cm each and four transverse sections each of jejunum, ileum, and colon of CFM mice ranging in age from 2 wk to 11 mo were analyzed. Control MUC1.Tg and CF/Muc1−/− mice were analyzed the same way. These tissues were carefully handled to prevent mechanical shearing of the intestinal mucus. Tissues were fixed in methacarn, embedded in paraffin, and 5-μm sections were cut from different areas of the small intestine. Before immunostaining with MAb CT2, tissues were treated with antigen retrieval reagent BD Retrievalen A, pH 6.5, according to the manufacturer’s instructions (Pharmingen, San Diego, CA), blocked for endogenous peroxidase activity, blocked in normal goat serum, and incubated with primary antibodies overnight at 4°C. Slides were washed in enhancing wash buffer (Immunex), incubated with horseradish peroxidase-conjugated secondary antibodies, washed in enhancing wash buffer again, and developed with 3,3′-diaminobenzidine (Santa Cruz Biotechnology, Santa Cruz, CA) and counterstained with Meyers hematoxylin (Sigma). Negative controls included antibody-specific peptide blocking and Muc1−/− intestinal tissues. Antibodies were diluted as follows: CT2, 1:100 and MUC1/SEC, 1:300.

RNA extraction, RT-PCR, cloning, and DNA sequence analysis. Total RNA was extracted from the jejunum, ileum, and colon of CFM and MUC1.Tg mice, using the TRIzol reagent (Invitrogen, Carlsbad, CA). Samples of 20 μg of total RNA were treated with DNase (DNA-free kit; Ambion, Austin, TX) before cDNA synthesis. RT was done as previously described (34). The cDNA was aliquoted and stored at −70°C.
A single batch of cDNA was used for all the PCR results shown here. Each experiment was repeated three times with different batches of cDNA to confirm the consistency of the results. The Expand High Fidelity PCR System (Roche Diagnostics, Indianapolis, IN) was used for all PCR reactions, as recommended by the manufacturer for a 100-μl volume. To compare the presence or absence of transcripts compared with a known RNA, we used a semiquantitative RT-PCR approach featuring low-cycle number amplification (25 cycles) and a mixture of actin primers/actin competimers (Ambion) as amplification reference. For this calibration control, a 2:7.6 ratio of primers/competimers (0.5 μl of premixed actin primers and 1.9 μl of actin competitors) was used for all the RT-PCRs. Amplification conditions included an initial denaturing at 94°C for 5 min followed by 25 cycles of denaturing 94°C (30 s), annealing 57°C (1 min), and terminating 72°C (2 min) for MUC1, and MUC1 splice variants. For MUC1/SEC the annealing temperature was 61°C (1 min). Routine controls for all RT-PCRs included omission of the RT. Amplified products were cloned and sequenced to confirm their identities.

The following primers were used for RT-PCR analysis: For MUC1/SEC, primers were designed from the MUC1 (GenBank accession no. X52228) sequence: P2 (sense), 5'-GGTACCTCCTCTCACCTCCTCAAA-3' (900–923 bp) (32), and P7 (antisense), 5'-GGGGAAGGAAAGGGCGATCTACAC-3' (1089–1066 bp); a 190-bp product was amplified. For classic MUC1 and MUC1 splice variants, primers were designed from the MUC1 genomic sequence (GenBank accession no. M61170 version gi:2055365). For classic MUC1, the primers were Ex5 (sense), 5'-TGCTGGTCTGTGTCGTCTGC-3' (4986–5007 bps) and Ex7 (antisense), 5'-TGGCGAGAAGTGCTGCTCACT-3' (complementary to 6361–6341 bp), generating a 265-bp fragment. To detect MUC1 splice variants, the primers were Ex5 (described above) and InVI (antisense), 5'-CTTCTTTGCTCTACTG-GGG-3' (5575–5734 bp).

RESULTS

Phenotype of the CFM mouse corresponds to the original phenotype of the CF mouse. We (34) previously described that Cftrtm1UNC/Cftrtm1UNC (CF) mice lacking Muc1 exhibited a phenotype with decreased intestinal mucus accumulation compared with the CF mice. To further pursue the role of Muc1 in the mucus accumulation, we introduced the human MUC1 gene into the Muc1−/− mice by mating the CF/Muc1−/− with the MUC1.Tg/Muc1−/− mice to generate the Cftrtm1UNC/Cftrtm1UNC, Muc1−/−, MUC1+ (CFM) mice. This facilitated the detection of MUC1, because there is a lack of antibodies for the extracellular domain of Muc1, and several well-characterized antibodies to human MUC1 are available. The MUC1.Tg mouse contains a 10-kb piece of genomic DNA comprising the entire MUC1 gene and regulatory sequences, and it expresses MUC1 at normal levels, conserving its tissue specificity (40). Intestinal sections of CFM and the control MUC1.Tg mice were stained with Alcian blue and compared with the sections previously obtained from CF and wild-type mice (34). Intestinal mucus accumulation was similar between CFM and CF mice (Fig. 1). The CFM mouse also presents goblet cell hyperplasia and dilation of the crypts filled with mucus as well as the physiological phenotype of early death of the pups as documented with the CF mouse (data not shown) (45, 55). Likewise, the life span, percentage of survival, and general health of the CFM mice housed under the same conditions as the CF mice have proven to be similar for both colonies over a period of 2.5 yr (data not shown).

MUC1-EX is substantially increased in the intestines of the CFM mice. We examined the CFM mice vs. MUC1.Tg mice for MUC1 protein expression along the intestinal lumen. We performed Western blots from intestinal mucus lysates with MUC1 MAbs directed either to the MUC1-EX (B27.29 and HMFG-2) or to the MUC1-CT (CT2). Immunoblotting with MAb to the MUC1-EX revealed a noticeable increase in the levels of MUC1 protein mainly in the jejunum and ileum of the CFM mice (Fig. 2, A and B). The detected MUC1 protein appears heterogeneous in size, probably due to variable O-linked glycosylation. The MAB B27.29, which reacts with the heavily glycosylated MUC1-EX (42), shows the most dramatic MUC1 increase in the small intestine of the CFM mice (Fig. 2A). Furthermore, an identical Western blot probed with MAb HMFG-2, which reacts with an epitope influenced by the length of the oligosaccharide side chains (8), showed no reaction (data not shown). However, when a similarly prepared membrane blot was first partially deglycosylated with TFMSA and then immunoreacted with MAb HMFG-2, MUC1 reactivity was found (Fig. 2B). This reaction with MAb HMFG-2 also showed that MUC1 is augmented in the jejunum and ileum of both animals present very similar amounts of MUC1 (Fig. 2B). The immunoblots with MAb CT2 (Fig. 2C) revealed that the MUC1-CT is also increased in the CFM intestines. In

Fig. 1. Replacement of mouse Muc1 with human MUC1 in cystic fibrosis (CF) mice. Representative Alcian blue-stained intestinal sections of the CF mice expressing the MUC1 gene (CFM) mice (Cftrtm1UNC/Cftrtm1UNC, MUC1+, Muc1−/−) (C and D) display the same intestinal phenotype of luminal mucus accumulation as the CF mouse with its native Muc1 (Cftrtm1UNC/Cftrtm1UNC) (A and B).
this case, MUC1-CT was increased in jejunum, ileum, and colon of the CFM mice. Furthermore, in CFM mice MUC1-CT was more abundant in the colon, whereas MUC1-EX was more copious in the jejunum. In the jejunum of CFM mice, when MUC1-EX showed the highest accumulation (Fig. 2A), MUC1-CT was barely detectable (Fig. 2C).

MUC1-EX is localized abundantly in the crypts and in the mucus secreted into the intestinal lumen. To study the accumulation of MUC1 in the intestine, immunostaining with several MUC1 MAbs was performed. Figure 3, A and E, shows a representative Alcian blue stain of the mucus accumulated in the lumen of the small intestine in the CFM and CF/Muc1/H11002 mice. The immunohistochemical analysis of intestinal sections of CFM mice with MAb B27.29 distinctly showed MUC1 protein, not only on the cell membrane of the villi and crypts, but also in the secreted mucus accumulated in the lumen of the small intestine (Fig. 3B). That the reactivity was not due to nonspecific antibody-mucus interaction was shown by the lack of staining in CF/Muc1−/− mice containing some accumulated mucus but not MUC1 (Fig. 3D). Immunostaining with CT2 also revealed MUC1 in the cell membrane of villi and crypt cells with very faint staining of the luminal mucus (Fig. 3C). Also in this case, the mucus lacking MUC1 did not react with MAb CT2 (Fig. 3G). These results are in agreement with the results obtained in the Western blots of intestinal mucus lysates with MAb CT2. In both, a strong reaction in the small intestine with MUC1-EX was associated with a weak reaction with MUC1-CT (Fig. 2, A and C). Comparable stains performed in colon sections of CFM and MUC1.Tg animals showed no clear difference (data not shown).

Two novel MUC1 splice variants, MUC1-CT80 and MUC1-CT58, were identified. Because the small intestine of CFM mice showed a strong reaction with MUC1-EX accompanied with a weak reaction of its cytoplasmic tail (by immunoblotting and immunohistochemistry), we postulated the presence of a secreted MUC1 form as one of the constituents of the luminal mucus. We used a semiquantitative RT-PCR approach, featuring low cycle number amplification (25 cycles) and actin for amplification reference, to search for transcripts of MUC1 alternate forms and to compare their expression profiles in CFM and MUC1.Tg mice. First, the normal transmembrane form of MUC1 was amplified by using primers unique to this sequence (Ex5-FOR, Ex7-REV). MUC1 levels appear to be increased in both jejunum and ileum of the CFM mice, but not in the colon.

One of the known MUC1 splice variants is MUC1/SEC, which lacks the cytoplasmic tail and has been described in breast cancer cells (44) and in human endometrial glands (1). The MUC1/SEC mucin is partly encoded by intron 2, which results in 11 unique amino acids (VSIGLSFPMLP) at its COOH terminus. We used the P2 and P7 primers to amplify MUC1/SEC, which would not amplify the classic transmembrane MUC1 (Fig. 5A). RT-PCR using 25 cycles revealed MUC1/SEC mRNA in the jejunum, ileum, and colon of CFM mice, but only in the ileum and colon of the MUC1.Tg mice (Fig. 4B). When the RT-PCR number of cycles was taken up to 40, the MUC1/SEC message was also found in jejunum of MUC1.Tg mice (not shown).

The expression pattern of MUC1/SEC and MUC1 appeared fairly similar along the small and large intestines of the CFM mice (Fig. 4, A and B). This pattern differed from the one obtained in the MUC1.Tg mice, in which MUC1 and MUC1/SEC messages were expressed in the colon, very little were expressed in the ileum, and no expression could be detected in the jejunum (Fig. 4, A and B).

Using the same RT-PCR approach, we further screened the region between the MUC1 transmembrane domain in exon 5 and sequences in intron 6 (Fig. 5A). The classic MUC1-CT is comprised of 48 amino acids in exon 6 and 24 amino acids in exon 7. We detected two novel splice variants of the MUC1-CT, a 689-bp transcript called MUC1-CT80 and a 280-bp transcript called MUC1-CT58 (Fig. 4C). The MUC1-CT80 transcript was found in the jejunum, ileum, and colon of the CFM mice but only in the colon of MUC1.Tg mice (Fig. 4C). RT-PCR reactions taken to 40 cycles failed to produce the MUC1-CT80 transcript in small intestines of MUC1.Tg mice (data not shown). The shorter transcript, MUC1-CT58, was observed in
The predicted proteins for the splice variants would have the following general characteristics (shown in Fig. 5). MUC1-CT58 (GenBank accession no. AF423031) and MUC1-CT80 (GenBank accession no. AF423030) share identical coding sequences with MUC1, from 1 to 5265 bp (GenBank accession no. M61170.2 GI: 15321729). Thereafter, the clone MUC1-CT58 has a new exon (exon 6b) located between MUC1 exon 6 and MUC1 exon 7 (Fig. 5A). In MUC1-CT58, the first 420 bp of MUC1 intron 6 are spliced out and the new exon 6b starts at 5686 bps and continues at least 60 more residues within the same MUC1 intron 6 sequence. The clone MUC1-CT80 has a new exon in place of MUC1 exon 6, named 6a (Fig. 5A). MUC1 exon 6 ends at 5265 bp but the new exon 6a continues to 5746 bp. The splice donor and acceptor sites conform to known consensus sequences (see GenBank accession nos. AF423031 and AF423030 for specific sites). The first 48 amino acids at the 5' end of the cytoplasmic tail are the same in MUC1, MUC1-CT58, and MUC1-CT80, because they are coded within exon 6 that is unaltered in the splice variants (Fig. 5B). Exon 6b in the jejunum, ileum, and colon of both types of mice with higher expression in MUC1.Tg mice (Fig. 4C).

The predicted proteins for the splice variants would have the following general characteristics (shown in Fig. 5). MUC1-CT58 (GenBank accession no. AF423031) and MUC1-CT80 (GenBank accession no. AF423030) share identical coding sequences with MUC1, from 1 to 5265 bp (GenBank accession no. M61170.2 GI: 15321729). Thereafter, the clone MUC1-CT58 has a new exon (exon 6b) located between MUC1 exon 6 and MUC1 exon 7 (Fig. 5A). In MUC1-CT58, the first 420 bp of MUC1 intron 6 are spliced out and the new exon 6b starts at 5686 bps and continues at least 60 more residues within the same MUC1 intron 6 sequence. The clone MUC1-CT80 has a new exon in place of MUC1 exon 6, named 6a (Fig. 5A). MUC1 exon 6 ends at 5265 bp but the new exon 6a continues to 5746 bp. The splice donor and acceptor sites conform to known consensus sequences (see GenBank accession nos. AF423031 and AF423030 for specific sites). The first 48 amino acids at the 5' end of the cytoplasmic tail are the same in MUC1, MUC1-CT58, and MUC1-CT80, because they are coded within exon 6 that is unaltered in the splice variants (Fig. 5B). Exon 6b in

Fig. 3. Immunohistochemical localization of MUC1-EX in the CFM small intestine. Representative small intestine sections of CFM (A–D), and CF/Muc1<sup>−/−</sup> (E–H) mice were stained with Alcian blue (A and E) to detect the localization of lumenal mucus (L) or were immuno-stained with the MUC1-EX MAb B27.29 (B and F), MUC1-CT MAb CT2 (C and G), and MUC1/SEC PAb (D and H). The CF/Muc1<sup>−/−</sup> mice displayed no staining with either antibody (F, G, and H). Higher magnification of B shows abundant MUC1-EX secreted into the intestinal lumen (red arrow) as well as its localization on the cell membrane (blue arrow). The higher magnification of C shows the intense staining of cell membrane-associated MUC1-CT (blue arrow), which appears scarce in the lumen. Higher magnification of D indicates MUC1/SEC present in the intestinal lumen (red arrows).

Fig. 4. Semiquantitative RT-PCR of transcripts for MUC1 and 3 of its isoforms differentially expressed in the CFM and MUC1.Tg intestines. Representative reactions of a semiquantitative RT-PCR approach featuring low-cycle amplification (25 cycles) and actin as an amplification reference are presented in A–C to show the expression of different MUC1 transcripts in MUC1.Tg and CFM intestines. The arrows point to the RT-PCR amplicons for 265-bp MUC1 (A), 190-bp MUC1/SEC (B), and 888-bp MUC1-CT58 and 280-bp MUC1-CT80 (C). Increased expression of MUC1 (A) and MUC1/SEC (B) is observed in the jejunum, ileum, and colon in the CFM mice, but only in the ileum and colon of MUC1.Tg mice. MUC1-CT58 (C) is observed in the CFM intestines, but only in the colon in the MUC1.Tg mice. MUC1-CT58 is present along the intestines of CFM and MUC1.Tg mice.

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MUC1-CT58 generates a 3' end of the cytoplasmic tail with 10 unique amino acids substituting for the 3' end 24 amino acids of the cytoplasmic tail (Fig. 5B). Exon 6a in MUC1-CT80 generates a cytoplasmic tail with 32 unique amino acids substituting for the 3' end 24 amino acids of the cytoplasmic tail (Fig. 5B). The sites for β-catenin and Grb2 interactions are therefore lacking in both of the splice variants.

Sequencing the newly found transcripts revealed several differences with the published sequence of MUC1 intron 6 (GenBank accession no. M61170 version GI: 2055365). We did extensive resequencing of that region of the genomic DNA with several sense and antisense primers and confirmed that the differences were errors in the published intron 6 sequence. The correct sequence has been submitted to GenBank and the update can be viewed as accession no. M61170.2 GI: 15321729.

MUC1-CT80 is translated into a protein expressed mainly in CFM intestines. To confirm that the MUC1-CT80 transcript was translated into the corresponding protein, a rabbit PAb (CT80) directed to a unique sequence of MUC1-CT80 was used. This antiserum was tested by immunoblotting and immunostaining of CFM, MUC1.Tg, and CF/Muc1−/− intestinal lysates and fixed tissues, respectively. Intestinal lysates were immunoblotted with the CT80 antiserum, revealing several forms of the protein ranging between ~27 and 45 kDa (Fig. 6A). Antiserum was proven to be specific when its reactivity was partially or completely inhibited by preincubating the CT80 antiserum with the immunizing peptide and by the absence of reactivity in CF/Muc1−/− mice (not shown). Nonimmune rabbit serum lacked the MUC1-CT80 bands but showed nonsp-
specific bands below 30 kDa (Fig. 6A). To further determine the specificity of the CT80 antiserum, MUC1 was immunoprecipitated with an antibody to MUC1-EX and blotted with CT80 antiserum and a similar banding pattern was observed (data not shown). The different MUC1-CT80 forms observed in the immunoblot likely represent different levels of MUC1-CT80 glycosylation, phosphorylation, or proteolytic processing. The phenomenon with multiple bands containing the cytoplasmic tail is also observed for the normal MUC1 by using the CT1 and CT2 antibodies (Fig. 2) (41). Multiple bands for MUC1-CT80 were expressed along the whole CFM intestinal tract but only in the colon of MUC1.Tg mice in which a single, diffuse band of ~30 kDa was observed (Fig. 6A). Even with increased amounts of protein, still no CT80 reactivity was found in jejunum and ileum of MUC1.Tg mice. This observation was consistent with the pattern of MUC1-CT80 mRNA seen in both types of mice.

**MUC1/SEC protein expression and histological localization patterns coincide with MUC1-EX.** A chicken PAb (MUC1/SEC) directed to a unique peptide sequence in MUC1/SEC detected protein on immunoblots of intestinal lysates of CFM and MUC1.Tg mice (Fig. 6B). The amount of MUC1/SEC protein decreased from jejunum to colon in CFM intestines. In MUC1.Tg mice, no protein was seen in the small intestine but there is some reaction in the colon. Testing up to 100 μg of protein did not give a positive reaction in the MUC1.Tg small intestine (not shown). CF/Muc1−/− lysates tested with MUC1/SEC were always negative as expected (not shown). We investigated the histological localization of MUC1/SEC in the CFM and CF/Muc1−/− intestines. Figure 3D shows that MUC1/SEC was localized in the intestinal lumen similarly to MUC1-EX (Fig. 3B). The specificity of the MUC1/SEC antibody was corroborated by the absence of staining in the luminal mucus present in the CF/Muc1−/− intestines (Fig. 3H).

**DISCUSSION**

In CF, the major pathological problems are the result of an accumulation of mucus within the respiratory and digestive systems. Despite progress in the identification and study of mucins and their obvious relevance as components of mucus, there is a lack of knowledge of specific alterations in mucin expression in CF. This is due, in part, to the fact that new mucin genes are still being identified (MUC12, -13, -17) (19, 50, 51). In addition, the rodent homologues of all the human mucins have not yet been obtained, which would facilitate the generation of animal models. We have used analyses of both RNA and protein to investigate the participation of MUC1 in the intestinal mucus accumulation in the CFM mouse compared with the MUC1.Tg mouse. Results showed that the extracellular domain of MUC1 is an intrinsic part of the mucus accumulation observed in the intestines of the CFM mice. Western blots revealed an increase in the levels of the MUC1-EX, particularly evident in the jejunum of the CFM mice. This increase was less prominent as one moved distally to the ileum and colon. Because there was no concordant increase in the cytoplasmic tail of MUC1, we investigated the expression of alternate forms of MUC1 mRNA that could account for the apparent inverse proportion of MUC1-EX and MUC1-CT in the small intestine. Both mRNAs and protein expression of novel and previously described MUC1 isoforms matched those of increased MUC1-EX protein in the small intestine of CFM mice.

Alternate splicing is a common event among mucins and several splice variants have been described for MUC1 (5, 30, 32, 44). Therefore, one way to explain the large amount of MUC1-EX in the small intestine of the CFM mice is by the presence of a MUC1 splice variant lacking the cytoplasmic tail, such as MUC1/SEC (44) or any other cytoplasmic tail variant either lacking some or all of the CT2 epitopes. We found roughly similar levels of MUC1/SEC and MUC1 mRNA and protein in the CFM mice, whereas MUC1/SEC was very scarce in MUC1.Tg mice. Messages for the two novel splice variants (MUC1-CT58 and MUC1-CT80) lacking the CT2 epitope were found in CFM and MUC1.Tg mice; the message for MUC1-CT80 showed increased expression in CFM small intestine vs. MUC1.Tg small intestine. Thus it appears that the large increase of MUC1-EX in the small intestine of the CFM mouse may be due to increased transcription of classic MUC1 as well as to other mucins expressing variant cytoplasmic tails and at least one secreted MUC1 form lacking the cytoplasmic tail altogether. Immunoblots corroborated that MUC1-CT80 and MUC1/SEC proteins were expressed. The MUC1-CT80 and MUC1/SEC expression patterns matched that of the MUC1-EX pattern of expression in the small intestine of CFM mice. The fact that MUC1/SEC is overexpressed in CFM intestines, and it is present in the intestinal lumen, indicates that this secreted form is a contributor to the mucus accumulation in such mice. Thus it appears that the large increase of MUC1-EX in the small intestine of the CFM mouse is mediated by increased transcription and translation of classic transmembrane MUC1 and MUC1/SEC as well as the novel cytoplasmic domain variant MUC1-CT80.

The functional significance of alternate splice variants in mucins has not been studied, but other transmembrane proteins use this strategy with alternate cytoplasmic domains to switch signaling pathways (14, 31). MUC1-CT80 is lacking the region known to directly interact with β-catenin (53), which potentially could have a large impact on signaling events involving the MUC1-CT. Because MUC1-CT80 is overexpressed in the CFM small intestine in which the main MUC1-EX and MUC1/SEC increase is noticed, it seems possible that MUC1-CT80 is participating in some signaling leading to an atypical mucus secretion into the intestinal lumen. However, more studies are necessary to assess the function of the MUC1-CT splice variants. It is known that the MUC1-CT can participate in a wide variety of signaling events, but until now, these studies have mainly dealt with adhesion and transfor-
accumulation of cancer cells. One can suggest that the altered CFM intestine epithelium may trigger distinct signaling events via alternative splicing of the MUC1 gene.

The discordant MUC1-EX and MUC1-CT in the small intestine of CFM mice could also be due to shedding or proteolytic processing of the classic MUC1. Mature MUC1 results from the posttranscriptional processing of a large precursor that is cleaved in the endoplasmic reticulum, producing a noncovalently bound heterodimer (28, 35). Although this cleavage does not directly lead to the release of the MUC1-EX, it may provide a mechanism for MUC1 shedding and a concomitant degradation of the cytoplasmic tail. A second proteolytic cleavage could also liberate MUC1-EX from the cell membrane. The feature of a proteolytic cleavage associated with posttranslational processing of membrane-associated mucins is shared by at least two other mucins of the same class, MUC4 and epiligrin (47). Accordingly, the heterodimeric presentation of MUC1 and the other transmembrane mucins may provide a mechanism for rapid shedding of the mucin domain into the lumen where it appears to participate in the accumulation of mucus detected in CF.

The major phenotype in the CF mouse occurs in the small intestine, an organ also affected in human CF patients (15). How the lack of a functional CFTR favors the increase of MUC1, in particular, and the increase of mucus secretion in general, remains unresolved. Considering our results of MUC1 message and protein expression in the CF intestines, it appears that the CFM small intestine takes on a resemblance to the normal colon, but it does not present histological signs of colonic differentiation. We have found that mucin fucosylation was also altered in the CF mouse small intestine and resembled fucosylation in the normal colon (48). This resemblance of the CFM small intestine to the colon could be reflecting a subtle alteration in the differentiation of some epithelial cells, leading to atypical mucus secretion. Crypt enterocytes of the small intestine are the cells most abundantly expressing CFTR, although it is expressed in the villus cells as well. One would expect that the lack of a functional CFTR could affect water secretion. It has been suggested that reduced airway-surface liquid in CF patients may create underhydrated mucus and impaired mucociliary clearance. By inference, any reduced secretion of water into the intestinal lumen in the CFM mouse could result in impaired mucus clearance. However, a dehydrated environment could, in principle, also generate a stress signal with the potential power to activate a cascade of protective events to maintain the integrity of the luminal epithelium. Mucus production may be increased as a primary line of defense within such a protective response. Our observations and those of other researchers favor the idea that there is increased expression of mucins and increased secretion of mucin contributing to the intestinal mucus accumulation as well as an altered environment in which such mucus is less readily cleared. Our results clearly point out that MUC1 is part of the mucus accumulated. Recent findings of Khatri et al. (20), using quantitative immunoassays and immunohistochemistry with a variety of anti-Muc3 antibodies, revealed that the ectodomain, but not the cytoplasmic domain, of Muc3 is increased largely in the small intestine of CF mice. This pattern of expression for Muc3 is similar to what we observed for MUC1. Given the identification of message and protein for Muc4, -12, and -13 in the intestines of control mice (39, 50, 52), it is likely that these mucins also participate in the mucus accumulation in CF intestines.

The exact composition of the intestinal mucus accumulated as a result of a nonfunctional CFTR is currently not known, but it is clear that such mucus contains a mixture of mucins, some of them traditionally considered not important intestinal mucins, such as MUC1. Considering our present data of increased MUC1 mRNA and protein and that MUC1 expression is regulated at the level of transcription (16, 21, 54), we establish that MUC1 is not only accumulated in the CFM intestinal lumen due to an impaired mucus clearance but to its increased expression as well. We hypothesize that MUC1 in the CF intestines is not only contributing to the mucus accumulation through the shedding and/or secretion of its extracellular domain but also via its proven role in signal transduction (23–26, 33, 37, 41, 53). MUC1 may signal to the cell interior an altered extracellular milieu, which would provoke a response intended to maintain the integrity of the intestinal mucosa under the stressful environment generated by the absence of CFTR.

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MUC1 SPlice VARIANTS IN CF MUCUS


