Intestinal mucins from cystic fibrosis mice show increased fucosylation due to an induced Fuc α 1-2 glycosyltransferase

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In gene-targeted mouse models for cystic fibrosis (CF), the disease is mainly manifested by mucus obstruction in the intestine. To explore the mucus composition, mucins insoluble and soluble in 6 M guanidinium chloride were purified by three rounds of isopycnic ultracentrifugation from the small and large intestines of CF mice (Cftr^{m1UNC}/Cftr^{m1UNC}) and compared with wild-type mice. The amino acid composition was typical of that for mucins and showed increased amounts of the insoluble (2.5-fold increase) and soluble (7-fold increase) mucins in the small intestine of the CF mice compared with wild-type mice. Mucins from the large intestine of both wild-type and CF mice showed a high but constant level of fucosylation. In contrast, the insoluble and soluble mucins of the small intestine in CF mice revealed a large increase in fucose, whereas those of wild-type mice con-

tained only small amounts of fucose. This increased fucosylation was analysed by releasing the O-linked oligosaccharides followed by GC-MS. NMR spectroscopy revealed that the increased fucosylation was due to an increased expression of blood group H epitopes (Fuc α 1-2Gal-). Northern-blot analysis, using a probe for the murine Fuc α 1-2 fucosyltransferase (Fut2), showed an up-regulation of this mRNA in the small intestine of the CF mice, suggesting that this enzyme is responsible for the observed increase in blood group H-type glycosylation. The reason for this up-regulation could be a direct or indirect effect of a non-functional CF transmembrane conductance regulator (CFTR) caused by the absence of CFTR channel.

Key words: MS, O-glycosylation.

INTRODUCTION

Cystic fibrosis (CF) is one of the most common inherited lethal diseases among Caucasians, and is due to mutations in the gene coding for a CF transmembrane conductance regulator (CFTR) [1,2]. CF patients suffer from excess mucus production in the lungs combined with an increased susceptibility to lung infections by certain bacteria and to mucus plugging in other organs, such as pancreas and usually less severely the gastrointestinal tract. The main function of the CFTR protein is as a cAMP-regulated chloride transport channel, acting on the apical surface of epithelial cells. Although more than 1000 CFTR mutations have been reported, the deletion of the triplet encoding Δ Phe⁵⁰⁸ is by far the most common. The main effect of the Δ Phe⁵⁰⁸ CFTR is that the mutation causes a defect in the folding of the protein, and thus only a small amount reaches the apical cell surface [3].

The primary link between CFTR and the pathogenesis of CF is believed to be its role as a chloride transporter. By contrast, theories explaining the inability to clear the lung infections in CF have been suggested, which are based on contradictory results regarding the composition of the airway surface liquid (ASL). One theory suggests lower amounts of ASL in the CF patient, causing the mucus to stick to the cilia and giving an impaired mucociliary clearance [4], and the other an increased salt concentration in ASL inactivating natural bactericidal factors [5]. Presently, it is still an open question whether the viscous mucus observed in CF is due to altered hydration or is caused by modifications of any of the mucus components. Alterations in mucus glycosylation have been consistently reported [6,7]. How-

ever, there are no studies defining the precise glycosyltransferases that are the basis for these alterations.

Mucins are the major protein components of the mucus layer that protects the epithelial layers of our body. Mucins consist of 50–80% carbohydrate with a highly diverse repertoire of oligosaccharides O-linked to serine and threonine residues, clustered on the protein backbone (mucin domains). Apart from maintaining a gel-like viscosity of the mucus, an important function of mucins is probably to interact with bacteria, which often use saccharide epitopes as attachment sites. These types of interactions can, e.g. retain the normal bacterial flora of the large intestine by acting as attachment sites and nutrition source, and also serve to trap and expel pathogens. An altered mucin glycosylation in CF airways has mainly been suggested from studies in non-natural systems, such as immortalized airway epithelial cells, xenograft models or tissue staining with lectins [7]. There is a lack of well-controlled comparative studies involving structural characterization of glycans and studies of specific glycosyltransferases. This is largely due to the difficulties in obtaining sufficient amounts of tissue from healthy individuals, as well as the persistent lung infections in CF airways.

So far, ten CFTR knockout mouse models have been described [8]. In all these models, mice display a CF phenotype in the intestine, manifested by intestinal mucus obstructions. The intestinal problems limit their lifespan, but at the same time there is little or no phenotype in the lungs of these animals. The CFTR knockout mice offer a possible way to study, in detail, mucin glycosylation alterations in an organ affected severely by CF. In the present study, we have analysed the mucin composition and

Abbreviations used: ASL, airway surface liquid; CF, cystic fibrosis; CFTR, CF transmembrane conductance regulator; ESI-MS, electrospray ionization mass spectrometry; GalNAc, N-acetylgalactosamine; GdmCl, guanidinium chloride; NeuGc, N-glycolylneuraminic acid; PNA, peanut agglutinin; UEA 1, *Ulex europaeus* agglutinin.

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glycosylation of the intestinal mucins obtained from the insoluble gel-forming and soluble mucins [9] in CF mice and wild-type controls. Structural studies showed an increased fucosylation (Fuc α 1-2 linkage) due to the induction of a specific fucosyl-transferase, Fut2.

MATERIALS AND METHODS

Materials and antibodies

$^2\text{H}_2\text{O}$ was purchased from Glaser (Sweden). The rabbit antiserum anti-gpA (PH497) obtained by immunization with HF-treated glycopeptide A from rat Muc2 was described previously [10]. This antiserum reacts with the small central Cys-rich domain of Muc2, as was shown by its use in cloning the rat Muc2 mucin from a bacterial phage library [10], and its selective staining of the early secretory pathway of goblet cells in the mouse small intestine [11]. Deletion of the Muc2 gene from mice using homologous recombination resulted in loss of staining with PH497 antiserum, confirming its specificity for the mouse Muc2 gene [12]. DNase I (EC 3.1.21.1, type IV), RNase A (EC 3.1.27.5, type I-A), biotinylated *Ulex europaeus* agglutinin (UEA I), NeuAc, *N*-glycolylneuraminic acid (NeuGc) and muraminic acid were purchased from Sigma. The peanut (*Arachis hypogaea*) agglutinin (PNA) lectin was from ICN (Aurora, OH, U.S.A.).

Animals

CF mice homozygous for the Ser⁴⁸⁹ → X mutation (Cftr^{tm1UNC}/Cftr^{tm1UNC}) [13] were generated and weaned on liquid diet as described previously [14]. The CFTR mutation contains an in-frame stop codon in exon 10. Only 'healthy' looking 8–19 weeks old mice were used. Control mice (C57Bl/6) were maintained on liquid diet for 7 days before being killed. Three preparations, each from the small intestine of 14–15 wild-type and 6–7 CF mice, were analysed. For the preparation of large-intestinal mucins, pools of 13–15 wild-type and CF mice were used.

Isolation and purification of mucins

The mucosa of small and large intestine (caecum and colon) of pools of mice were scraped into 6 M guanidinium chloride (GdmCl) (40 ml for small and 20 ml for large intestine/10 animals). After slow stirring, the insoluble mucins were recovered by centrifugation and the pellets were washed (20 and 10 ml respectively) three times and solubilized by reduction followed by alkylation [9,15]. The soluble mucins recovered from the first two washes were treated in the same way. The dialysed and freeze-dried material was nuclease-digested [50 mM sodium dihydrogen phosphate (pH 7)/5 mM MgCl₂, 1 mg/ml RNase, 0.5 mg/ml DNase, 37 °C overnight]. After dialysis, the mucins were subjected to three rounds of ultracentrifugation in caesium chloride, two in 4 M GdmCl and one in 0.2 M [16]. Mucin peaks in the collected fractions were identified by slot blot stained by periodate–Schiff, Alcian Blue [17] and biotinylated lectins (UEA I and PNA). Aliquots of mucin fractions were applied on nylon membranes, dried and deglycosylated with gaseous HF at room temperature overnight [18], and the membrane was immunostained with the anti-gpA antiserum (PH497) [10]. Combined fractions were dialysed against water and freeze-dried. Amino acid analysis was performed as described previously [9] with an Alpha Plus amino acid analyser (Pharmacia Biotech, Uppsala, Sweden). The combined supernatants containing the mucins soluble in 6 M GdmCl from the small intestine were prepared by three rounds of density-gradient centrifugation as described above.

Lectin assay

Mucins (10 µg/ml in PBS) were coated in polystyrene microtitre wells (Maxisorb, Nunc, Denmark) overnight at 37 °C. Conditions for blocking, incubation and detection were as described previously [18]. Biotinylated lectins (UEA I or PNA) were used at a concentration of 1 µg/ml. Europium-labelled streptavidin was used for detection using the DELFIA method [18] and measured in an Arcus Fluorometer (Wallac, Turku, Finland).

Release and fractionation of oligosaccharides

Oligosaccharides were released from purified insoluble mucins (1–3 mg) with 0.05 M potassium hydroxide and 1 M sodium borohydride [9], desalted and fractionated into neutral, sialic acid- and sulphate-containing oligosaccharides [19]. Briefly, the released and desalted oligosaccharides were applied to an anion-exchange column, the neutral oligosaccharides were eluted with methanol, followed by elution of the sialylated species after an on-column derivatization of the carboxyl groups to methyl esters and, finally, the sulphated species were eluted with pyridinium acetate. The methyl esters were converted into methyl amide derivatives before permethylation.

Analysis of oligosaccharide fractions

The neutral oligosaccharides were permethylated and analysed using high-temperature GC and GC-MS [20]. For electrospray ionization mass spectrometry (ESI-MS), the mixtures were diluted in 0.15 mM NaCl in methanol, and analysed by syringe infusion at 10 µl/min to a single quadrupole LC-MS mass spectrometer, API 150EX (PerkinElmer Sciex, Thornhill, Canada) equipped with a Turbo Ionspray source. The instrument parameters were set as follows. Positive mode: ion-spray voltage, 4.3 kV; orifice voltage, 30 V; scan parameters, step 0.1 a.m.u.; dwell time, 1 ms; scan range, *m/z* 200–2000.

Monosaccharide composition analysis

Neutral monosaccharides were analysed as described previously [20]. NeuAc and NeuGc were quantified after acidic hydrolysis in 100 µl of 0.1 M HCl at 80 °C by high pH anion-exchange chromatography with pulsed amperometric detection and muraminic acid as internal standard. The column (Carbopac PA10, 4 mm × 250 mm, Dionex Corp., Sunnyvale, CA, U.S.A.) with a guard column Carbopac PA10 (4 mm × 50 mm) was eluted isocratically at 1 ml/min with 0.1 M sodium acetate and 0.05 M sodium hydroxide (50% solution; J. T. Baker, Deventer, The Netherlands). The methyl esterified residues in the acidic oligosaccharide fractions were converted to carboxyl groups by 0.1 M sodium hydroxide (50 °C, 2 h), before acid hydrolysis.

Proton-NMR spectroscopy

Mixtures of released and desalted oligosaccharide mixtures from purified insoluble mucins from control and CF mice were analysed. Deuterium exchange was performed twice in 0.5 ml of 99.95% $^2\text{H}_2\text{O}$ (Aldrich, Milwaukee, WI, U.S.A.) for 30 min, followed by 0.5 ml of 99.98% $^2\text{H}_2\text{O}$ for 30 min, with freeze-drying of the sample between each exchange. Samples were analysed by a 500 MHz instrument (Varian Assoc. Inc., Sunnyvale, CA, U.S.A.) with the sample kept at 25 °C. Assignments were done using standard single-pulse one-dimensional experiments and two-dimensional-COSY experiments.

Northern-blot analysis

Total RNA was extracted from the small- and large-intestinal scrapings of pools of control and CF mice using the TRIzol method following the manufacturer's instructions (Life Technologies, Gaithersburg, MD, U.S.A.) and homogenized using a Polytron electric homogenizer. Approximately 30 µg of total RNA was electrophoresed, blotted and probed with ³²P-labelled probes (Rediprime, Amersham-Pharmacia BioTech) as described previously [10,11]. The Fut2 probe was a 214 bp DNA fragment from mouse Fut2 open reading frame, specifically reacting only with the Fut2 transcript [21] (GenBank® AF214656, bases 2190–2403). Specific probes for the mouse Fut1 and Sec1 Fucα1-2 transferases were also used [21]. A probe for β-actin was used as a standard.

RESULTS

Purification and chemical composition of mouse mucins

The mucosa from the small and large intestines of CF and control mice was scraped into 6 M GdmCl. It is known that some mucins, largely the MUC2 mucin, are insoluble in this chaotropic salt before reduction [9,22]. The mucins insoluble and soluble in the 6 M GdmCl respectively were purified from the scrapings by three rounds of isopycnic ultracentrifugation in caesium chloride. Fractions collected from the last ultracentrifugation (0.2 M GdmCl) were slot-blotted and revealed by periodate–Schiff and Alcian Blue staining. The insoluble mucin peaks from the small intestine of CF and wild-type mice had a density of 1.55

Figure 1 Isopycnic density-gradient centrifugation of insoluble mucins from the small intestine of wild-type (WT) and CF mice

The third CsCl density-gradient ultracentrifugation made in 0.2 M GdmCl is shown. Relative staining by periodate–Schiff (●), Alcian Blue (■) and with the antiserum anti-gpA raised against rat Muc2 (PH497) after deglycosylation by HF (◆) (the weak reactivity at low density in fraction 5 is due to a technical artifact). The preparation shown is from 15 wild-type and 6 CF mice. The bars show the fractions combined for further studies.

Table 1 Relative amino acid composition of intestinal mouse mucins

The results from the insoluble mucins are the mean of three analyses for the small intestine and two for the large intestine. The results from the soluble mucins are the mean for two analyses. The value are given as means ± S.D.

	Insoluble				Soluble	
	Small intestine		Large intestine		Small intestine	
	Wild-type	CF	Wild-type	CF	Wild-type	CF
Ser*	27 ± 0.6	28 ± 1.0	30 ± 7.1	30 ± 5.7	36 ± 0.6	32 ± 0.2
Thr*	52 ± 1.5	51 ± 0.6	48 ± 2.1	49 ± 4.2	50 ± 1.5	52 ± 5.7
Pro*	21 ± 1.5	21 ± 0.6	23 ± 5.7	21 ± 1.4	15 ± 0.9	16 ± 5.4
Ser/Thr/Pro of all amino acids (mol%)	75 ± 2.9	71 ± 6.8	68 ± 19	71 ± 13	43 ± 1.0	40 ± 8.7

* Relative amount in mol % of amino acid in relation to the total amount of Ser, Thr and Pro.

and 1.54 g/ml respectively in 0.2 M GdmCl, as shown in Figure 1. The corresponding soluble mucins had densities of 1.53 and 1.51 g/ml respectively (results not shown). The chemical staining showed symmetrical peaks that overlapped the staining with the lectins UEA I (only for the CF mice) and PNA (only for the wild-type) (results not shown). After deglycosylation with HF, the anti-gpA (PH497) antiserum, reactive with mouse Muc2, revealed a peak partially coinciding with the main insoluble mucin peak. Amino acid analysis of the combined insoluble mucin fractions from the small and large intestines gave a similar composition for the CF and wild-type mucin preparations in which the three amino acids Ser, Thr and Pro together accounted for 71–81 mol %, typical for mucins. The molar percentages of these three amino acids are displayed in Table 1, showing an approximate distribution of 1:2:1 for Ser, Thr and Pro, respectively. The Muc2 peaks did not coincide precisely with the mucin staining (Figure 1), suggesting that Muc2 is only one of several mucins in the purified insoluble mucin fraction of the small intestine. The soluble mucins purified by three rounds of isopycnic ultracentrifugation showed high amounts of the amino acids serine, threonine and proline, although less than those for the insoluble mucins. The relative distribution of these amino acids showed a slightly higher amount of serine and lower amount of proline compared with the insoluble mucins, suggesting that different mucin core proteins comprise the soluble and insoluble mucins.

The combined results from the amino acid and carbohydrate composition analyses (Table 2) showed that the small intestine of CF mice contained about 2.5 times (239 µg/intestine) as much insoluble mucins compared with the wild-type mice (95 µg/intestine). The increase in total amount of soluble mucins was much higher (723 µg/CF intestine compared with 100 µg/wild-type intestine). In the large intestine, the CF mice contained about 1.7 times more insoluble mucins compared with wild-type mice. The insoluble mucins of the small intestine consisted of approx. 81 % carbohydrate and 19 % protein, whereas the soluble fraction consisted of 72 and 28 % respectively, a distribution characteristic of mucins. Monosaccharide composition analysis displayed residues typical for mucin oligosaccharides (NeuAc, Fuc, Gal, GlcNAc and GalNAc). The CF mucins of the small intestine showed increased levels of Fuc in both the insoluble and soluble mucins. Decreased levels of GalNAc and increased levels of NeuAc in the insoluble mucins were also observed. In the large intestine, no significant differences were seen.

Table 2 Chemical composition of intestinal mouse mucins

	Insoluble				Soluble	
	Small intestine*		Large intestine†		Small intestine	
	Wild-type	CF	Wild-type	CF	Wild-type	CF
Mucin ($\mu\text{g}/\text{intestine}$)	95 $\pm$ 43	239 $\pm$ 84	42 $\pm$ 17	72 $\pm$ 14	100 $\pm$ 0.7	723 $\pm$ 301
Protein (%)	19 $\pm$ 2.3	19 $\pm$ 5.7	17 $\pm$ 2.8	14 $\pm$ 1.4	28 $\pm$ 0.7	29 $\pm$ 5.7
Carbohydrate (%)	81 $\pm$ 2.3	81 $\pm$ 5.7	83 $\pm$ 2.8	86 $\pm$ 1.4	72 $\pm$ 0.7	71 $\pm$ 5.7
Fuc‡	0.23 $\pm$ 0.09	0.60 $\pm$ 0.14	0.49 $\pm$ 0.07	0.52 $\pm$ 0.13	0.12 $\pm$ 0.00	0.24 $\pm$ 0.02
Gal‡	1.5 $\pm$ 0.08	1.4 $\pm$ 0.10	1.3 $\pm$ 0.11	1.2 $\pm$ 0.23	1.17 $\pm$ 0.01	1.12 $\pm$ 0.12
GlcNAc‡	0.87 $\pm$ 0.07	0.80 $\pm$ 0.19	0.94 $\pm$ 0.09	0.97 $\pm$ 0.15	0.92 $\pm$ 0.01	1.01 $\pm$ 0.02
GalNAc‡	1.0 $\pm$ 0.19	0.80 $\pm$ 0.11	0.59 $\pm$ 0.02	0.65 $\pm$ 0.03	0.81 $\pm$ 0.01	0.70 $\pm$ 0.05
NeuAc‡§	0.29 $\pm$ 0.05	0.37 $\pm$ 0.02	0.58 $\pm$ 0.07	0.63 $\pm$ 0.02	0.32 $\pm$ 0.00	0.28 $\pm$ 0.08

* The results presented are from the analyses of three separate mucin preparations from 6 to 15 animals. The values are given as means $\pm$ S.D.

† The results presented are from the analyses of two mucin preparations from 13 to 15 animals.

‡ μmol of carbohydrate/mg of mucin. Man was analysed, but not detected (< 50 nmol/mg of mucin).

§ NeuGc was analysed, but was $< 2\%$ of NeuAc (< 50 nmol/mg of mucin).

Figure 2 Lectin binding to purified insoluble mucins with PNA and UEA I

Mucins from small intestine of control (●) and CF (○) mice and large intestine of control (×) and CF mice (■) were coated in microtitre wells, and the bound biotinylated lectins were detected by Eu-labelled streptavidin using fluoroimmunoassay (DELFA). The PNA lectin binds to Gal β 1-3GalNAc epitopes, and the UEA I lectin to Fuc α 1-2Gal epitopes. For PNA, the three curves for small- and large-intestinal CF mice and large intestine of control animals lie on the x axis.

Lectin reactivity of purified mucins

To analyse further the glycosylation alterations, the purified insoluble mucins were tested for their interaction with the lectins PNA and UEA I by an immunofluorimetric assay (Figure 2). PNA bound strongly to mucins from the small intestine of wild-type mice, but not to mucins from the CF mice or to mucins isolated from the large intestine of control or CF mice. In contrast, the mucins from the small intestines of CF mice, and mucins from large intestine of wild-type and CF mice, bound strongly to UEA I. The lectin PNA binds Gal β 1-3GalNAc epitopes and UEA I binds terminal Fuc-linked α 1-2. The results obtained are in accordance with the increased levels of Fuc detected on mucins from the small intestine of CF mice. The PNA and UEA I reactivities seem to be mutually exclusive,

Table 3 Monosaccharide composition of oligosaccharide fractions from mouse insoluble intestinal mucins

	Small intestine*		Large intestine*	
	Wild-type	CF	Wild-type	CF
Neutral oligosaccharides†				
Fuc per GalNAcol (mol)‡	0.1	1.1	1.1	1.1
GalNAc per GalNAcol (mol)	0.3	0.3	< 0.1	< 0.1
Average length (residues)§	2.9	4.2	4.2	4.2
Oligosaccharide chains (mol%)	60	50	57	62
Sialylated oligosaccharides†				
Fuc per GalNAcol (mol)‡	< 0.1	1.0	0.5	1.3
GalNAc per GalNAcol (mol)	2.1	1.8	2.0	2.3
NeuAc per GalNAcol (mol)	0.9	1.3	3.3	3.2
Average length (residues)§	9.8	10.9	17.4	17.6
Oligosaccharide chains (mol%)	8	11	5	9
Sulphated oligosaccharides†				
Fuc per GalNAcol (mol)‡	0.1	1.9	1.4	2.1
GalNAc per GalNAcol (mol)	1.0	1.7	1.3	1.6
NeuAc per GalNAcol	0.6	1.0	2.0	2.0
Average length (residues)§	6.8	11.5	13.5	15.9
Oligosaccharide chains (mol%)	32	39	38	29

* The results are from two analyses.

† Oligosaccharides were subfractionated into neutral, sialylated and sulphated species.

‡ Fucose levels relative to the linkage sugar GalNAcol, which was reduced during oligosaccharide release from the protein backbone.

§ Average monosaccharide residue chain length, based upon measured molar amounts of Fuc, Gal, GlcNAc, GalNAc, NeuAc relative to GalNAcol.

|| Percentage molar amounts of GalNAcol distributed in the neutral, sialylated and sulphated fractions.

suggesting that the increased levels of Fuc residues block the PNA epitopes.

Neutral oligosaccharides

To study further the increased fucosylation detected by the lectin assays, the O-linked oligosaccharides of the insoluble mucins were released and separated into fractions with neutral, sialylated and sulphated compounds. The amount of Fuc, Gal, GlcNAc, GalNAc, NeuAc, Man and GalNAcol were quantified (Table 2).

Table 4 Neutral oligosaccharides of insoluble mucins isolated from small and large intestine of control and CF mice

The oligosaccharide sequences were elucidated and semi-quantified by GC and GC-MS. These are listed together with additional ions detected by ESI-MS.

No.	Sequences of neutral oligosaccharides*	Molecular mass (Da)†	Small intestine‡		Large intestine‡	
			Control	CF	Control	CF
N1.1	GalNAcol	307.2	23.8	20.3	25.7	18.5
N2.1	Gal → 3GalNAcol	511.3	38.9	10.6	12.0	12.3
N3.1	Fuc → Gal → 3GalNAcol	685.4	5.0	31.7	23.1	20.4
N3.2	Gal → 3(GlcNAc → 6)GalNAcol	756.4	23.5	6.5	9.5	12.0
N3.3	HexNAc → Gal → 3GalNAcol	756.4	1.3	1.1	2.0	1.4
N4.1	Fuc → Gal → (GlcNAc → 6)3GalNAcol	930.5	2.7	13.7	14.5	19.3
N4.2	Gal → 3(Gal → 4GlcNAc → 6)GalNAcol	960.5	3.3	0.8	2.5	0.7
N4.3	HexNAc → Gal → 3(GlcNAc → 6)GalNAcol	1001.5	0.9	0.6	1.9	1.9
N5.1	Gal → 3(Fuc → Gal → 3GlcNAc → 6)GalNAcol	1134.6	0.2	0.3	1.1	1.7
N5.2	Gal → 3(Fuc → Gal → 4GlcNAc → 6)GalNAcol	1134.6	0.1	3.1	2.7	4.0
N5.3	Fuc → Gal → 3(Gal → GlcNAc → 6)GalNAcol	1134.6	1.4	0.8		
N5.4	Fuc → Gal → HexNAc → Gal → 3GalNAcol	1134.6	0.6	0.4		
N5.5	HexNAc (2), Gal (2), GalNAcol	1205.6	x§			
N6.1	Fuc → Gal → 3(Fuc → Gal → 3GlcNAc → 6)GalNAcol	1308.7	0.1	1.8	1.5	2.3
N6.2	Fuc → Gal → 3(Fuc → Gal → 4GlcNAc → 6)GalNAcol	1308.7	0.3	7.6	3.6	4.2
N6.3	Fuc, Gal (3), HexNAc, GalNAcol	1338.7	x			
N6.4	Fuc, Gal (2), HexNAc (2), GalNAcol	1379.7	x	x	x	
N7.1	Fuc (3), Gal (2), HexNAc, GalNAcol	1482.8	x	x	x	
N7.2	Fuc (2), Gal (2), HexNAc (2), GalNAcol	1553.8		x	x	
N8.1	Fuc (2), Gal (3), HexNAc (2), GalNAcol	1757.9	x	x		

* The saccharides marked in bold are located on C-6 of GalNAcol. The following assumptions based on Tables 2 and 3 have been made: hexose residues are Gal, deoxyhexose residues are Fuc, *N*-acetylhexosamine linked to GalNAcol is GlcNAc, and *N*-acetylhexosaminitol residues are GalNAcol. The number of monosaccharide units present in sequences detected only by ESI-MS is indicated in parentheses.

† Permethylated oligosaccharides, calculated monoisotopic mass.

‡ The peak area corresponding to each sequence detected by GC and GC-MS was divided by the molecular mass of the specific saccharide, and the results are displayed as (mol %) oligosaccharides.

§ x indicates the presence of additional permethylated oligosaccharides detected as $(M+Na)^+$ ions by ESI-MS.

The molar ratios of Fuc, NeuAc and GalNAc relative to *N*-acetylglactosaminitol (GalNAcol) (linked to the mucin peptide core) give an indication of the average composition of the oligosaccharides and are presented in Table 3. High levels of Fuc relative to GalNAcol were found in the small intestine of CF mice and in the large intestines of wild-type and CF mice. In the small intestine, the increase of Fuc from 0.1 to 1.1 residues per oligosaccharide chain observed in the CF mice accounted for the increase in the calculated average chain length for the three mucin preparations, from 2.9 residues on mucins from small intestine of wild-type mice to 4.2 residues on mucins from small intestine of CF mice and large intestine of wild-type and CF mice. In all four samples, the neutral oligosaccharides accounted for 50–62 mol % of the O-linked oligosaccharide chains on the protein.

The neutral oligosaccharides were permethylated and analysed by ESI-MS, high-temperature GC and GC-MS. ESI-MS, which is not as limited in analytical size range as GC, revealed $[M+Na]^+$ ions of oligosaccharides with up to eight sugar residues (Table 4). Most oligosaccharides from the four samples were consistently detected by both analytical techniques, except for six structures detected only by ESI-MS. The oligosaccharides detected are listed in Table 4, together with their calculated monosaccharide composition. In the four samples, 14 saccharides were sequenced by GC-MS. Ion fragments observed were mainly oxonium and inductive ions [9,11,22]. The core structures of the oligosaccharide were core1 (Gal β 1-3GalNAc-) and core2 [Gal β 1-3(GlcNAc β 1-6)GalNAc-] sequences and the peripheral structures with Fuc linked to Gal-GalNAcol or to type 1 (Gal β 1-3GlcNAc-) or type 2 chains (Gal β 1-4GlcNAc-).

A comparison of the oligosaccharide profiles of the neutral components as revealed by GC is shown in Figure 3. The

structures of components found and a semi-quantitative estimate of their amounts are given in Table 4. The chromatograms of oligosaccharides from small intestine of the CF mice and the large intestine of the control and CF mice were strikingly similar. In contrast, the neutral oligosaccharides from mucins of the small intestine of wild-type mice exhibited a different pattern. In the control mice, fucosylated oligosaccharides accounted for 8.4 mol % of the sequences in the range of mono- to hexasaccharides, whereas in the small intestine of CF mice and in the large intestine of control and CF mice, the fucosylated oligosaccharides made up for 60, 47 and 53 mol % respectively (Table 4). These differences suggest that a fucosyltransferase is expressed at significantly higher levels in the small intestine of the CF mice compared with wild-type animals.

NMR spectroscopy of released mucin oligosaccharides

To obtain additional structural information on the nature of the induced fucosylated compounds, the total mixtures of released oligosaccharides from the insoluble mucins were analysed with proton NMR spectroscopy. From the anomeric region (Figure 4) of mucin oligosaccharides from the small intestine of CF mice, one can deduce that Fuc is mainly linked α 1-2 to Gal (5.22–5.25 p.p.m.) together with minor amounts of Fuc α 1-2 linked to type 2 (5.31 p.p.m.) and type 1 chains (5.19 p.p.m.). Further support (results not shown) for the presence of α 1-2-linked Fuc was found by shift in two-dimensional COSY NMR (Fuc CH₃ protons at 1.24 p.p.m. coupled with Fuc H-5 at 4.23–4.27 p.p.m.) that is typical for this terminal residue [23]. The corresponding results from control mice displayed similar peaks in the regions for Fuc protons, but the peaks were much smaller (results not shown). Thus the NMR results support the interpretation that

Figure 3 High-temperature GC of neutral oligosaccharides released and purified from intestinal insoluble mucins from wild-type and CF mice

SI, small intestine; LI, large intestine. The annotations above the peaks correspond to the sequences listed in Table 4. Chromatographic details are given in the Materials and methods section.

the increased amount of Fuc was due to blood group H-type epitopes (Fuc α 1-Gal).

Northern-blot analysis

To analyse the enzymic background for the increased fucosylation, Northern-blot analyses with specific probes to the murine fucosyltransferases Fut1, Fut2 and Sec1 [21] were performed. The specific probe to the murine Fut2 fucosyltransferase gave a single band of approx. 3.3 kb that was barely detectable in the small intestine of the control mice, but a distinct band was

Figure 4 Anomeric region of the proton NMR spectrum of the total mixture of released oligosaccharides from the small-intestinal insoluble mucins of CF mice

Partial subtracted 500 MHz one-dimensional ^1H NMR spectrum signals with annotation of Fuc-H1 with suggested origin [21]. R indicates that various branches can be present.

Figure 5 Northern-blot analysis of the expression of the Fut2 transcript in intestinal tissues

RNA was isolated from the small intestine (SI) and large intestine (LI) of wild-type (WT) and CF mice and probed with ^{32}P -labelled Fut2 and actin probes.

observed in the small intestine of CF mice (Figure 5). The same band was also found in the large intestine of both CF and control animals. When comparing loading levels with actin as an internal standard, it is obvious that there is a large increase in the amount of Fut2 message in the small intestine of the CF mice, whereas similar levels were found in the large intestine. The expression levels of the Fut2 fucosyltransferase and lack of Fut1 and Sec1 transcripts (results not shown) are thus in accordance with the biochemical observations, and it is likely that the Fut2 enzyme is responsible for the increased levels of blood group H-type epitopes.

Sialylated and sulphated oligosaccharides

As both sialylation and sulphation have been reported to be altered in CF tissues, the sialylated and sulphated oligosaccharide fractions from the insoluble mucins were also analysed by monosaccharide composition analysis (Table 3). The molar distribution of sialylated and sulphated saccharides was relatively similar in all samples, with 5–11 mol% sialylated and 29–39 mol% sulphated oligosaccharides. Fucose was increased relative to GalNAcol in both the sialylated and sulphated fractions in the small and large intestines of the CF mice. A small increase of NeuAc relative to GalNAcol was found in both the

sialylated and sulphated fractions on the mucins from the small intestine of the CF mice, together with a small increase in the percentage of acidic glycans, from 40 to 50 mol %.

DISCUSSION

The GdmCl insoluble mucins, described previously [9,24] to have MUC2 as a major component, were separated from the GdmCl soluble ones by centrifugation and repeated washing. The mucins from these two fractions of the intestines from CF and wild-type mice were purified. Amino acid composition showed a high relative amount of Ser, Thr and Pro, which is typical for mucins. The peak obtained from antiserum reactivity against Muc2 did not coincide precisely with the chemical staining, suggesting that additional mucins were present in the insoluble fraction of small-intestine mucins from CF and wild-type mice. This result differed from previous studies on rat and human, in which the insoluble fraction was supposed to consist chiefly of Muc2 [9,11,24,25], but is in line with previous studies in mouse [26].

A 2.5-fold increase in the levels of the insoluble and a 7-fold increase in the soluble mucins were found in the small intestine of the CF mice. A slightly smaller increase was observed in the insoluble mucin fraction of the large intestine. Slot-blot analysis of the insoluble mucins stained with the anti-Muc2 antiserum also suggested an increased amount in the small-intestine CF (results not shown). In contrast, we have previously found a significant decrease in Muc2 mRNA levels in CF mice [14]. The present results suggest that both the insoluble and soluble mucins could contribute to the mucus obstruction that has been observed in most CF mouse models [8].

The increased fucosylation in the small intestine was clearly demonstrated by the increased amount of Fuc in the mono-saccharide compositional analyses of the intact insoluble and soluble mucins. This was confirmed, in the released oligosaccharides, by increased binding to the lectin UEA I, and by GC and GC-MS of the neutral oligosaccharides from small-intestinal insoluble mucins. The lectin-binding studies as well as the NMR studies showed that the Fuc was mainly linked α 1-2 to Gal. The two most prominent compounds of this type are when Gal is attached to the GalNAc of core 1 or core 2 structures. The fucosylation and general glycosylation patterns were similar in the large intestine of CF and wild-type mice. In fact, the glycosylation pattern of mucins of the small intestine of the CF mice resembled those of the large intestine, in contrast with what was observed in wild-type mice.

The mouse has been shown to have three distinct Fuc α 1-2 transferases, all showing distinct tissue-specific expression patterns on wild-type mouse tissues [21]. The specific probe for the murine Fut2 fucosyltransferase was found to be up-regulated in the small intestines of the CF mice by Northern-blot analysis, whereas Fut1 and Sec1 transcripts encoding other Fuc α 1-2 transferases were not detected. That Fut1 was not detected is in conflict with the previous published results where this transcript was found in the small intestine [21]. The reason for this is probably that only the epithelial cells were used here, and the whole intestine in the previous publication. The Fut2 enzyme shows sequence similarities with the human Secretor (FUT2) enzyme, and the predicted specificity for the induced enzyme on type 1 and type 2 chains as found here is in accordance with the enzyme being the mouse Fut2 glycosyltransferase. The Fut2 enzyme was also recently shown to be responsible for the cyclic altered fucosylation in mouse uterus [21], further proving that the Fut2 enzyme can be under dynamic expression control. Long exposure of the Northern blots revealed a weak hybridization

signal in the small intestine of wild-type mice, which explains the small amounts of fucosylated compounds detected. Upon up-regulation of the fucosyltransferase in the CF mice, there is a high conversion of precursor saccharides into the fucosylated products, probably approaching the upper possible limit. Analysis of the sugar composition in the soluble mucin fraction of the small intestine revealed an increased amount of Fuc (about 2-fold) in CF mice, suggesting that the increased levels of the Fut2 glycosyltransferase affect several types of glycoconjugates. This is expected, because transferases adding terminal sugar residues usually have a wide range of substrate types, and the Fut2 transferase probably is active also on other glycoconjugates.

A large number of CF-related glycosylation alterations in humans have been reported on mucins and membrane-bound glycoconjugates present in tissues, CF-derived cell lines or on purified components [7]. The specific glycosylation alterations observed vary, although a common theme has been increased levels of Fuc, NeuAc and sulphation. One of the hallmarks of CF has been increased sulphation, which was also present in model studies where the problem with infection was eliminated [27]. There is little support for an increased sulphation of mucins in the present study, which would have given a larger proportion of sulphated glycans.

A major unresolved issue is whether glycosylation alterations observed in CF are a primary effect due to a defective CFTR, influencing the glycosylation machinery, or a secondary effect caused by the lack of functional CFTR, as reflected in, e.g. responses to inflammation or infection. Evidence supporting the former is glycosylation alterations observed in CF-derived cell lines and xenografts [27,28]. The recent suggestion that CFTR might be expressed in intracellular compartments of the mucus-producing goblet cells [29] makes glycosylation alterations in goblet cells possible, but does not prove this. Most other studies that have suggested increased fucosylation, sialylation or sulphation in CF mucins suffer from possible secondary effects due to infection or inflammation. Evidence supporting that CF glycosylation alterations could be due to secondary effects of infection is exemplified by a recent demonstration that the severity of lung infections in patients suffering from CF or chronic bronchitis correlated with increased expression of sialyl-Le^x epitopes on bronchial mucins [30]. When Bry et al. [31] studied the effects of colonization of the intestine with specific bacteria in germ-free mice, the expression of blood group H-type epitopes was observed due to the induction of a fucosyltransferase. The enzyme thus induced has recently been shown to be the Fut2 fucosyltransferase [21]. We have recently shown the induction of the Fut2 enzyme and an increased amount of blood group H-type oligosaccharides by 6 days following an infection with the intestinal parasite *Nippostrongylus brasiliensis* in mice (J. M. Holmén, F. J. Olson, H. Karlsson and G. C. Hansson, unpublished work). These observations suggest that the expression of the Fut2 transcript and, thus, the blood group H-type epitopes in mouse can be regulated by type and level of micro-organisms in the small intestine and that Fut2 is readily inducible.

The present investigation is the first to link a CF glycosylation alteration with increased transcript levels of a specific glycosyltransferase. We have no indications that the induction of the Fut2 enzyme in the present study is due to any particular bacterial infection. The CF mice used in the present study are placed on liquid diet at weaning. We have observed that some of the CF mice suffered from diarrhoea after weaning. Treatment of these with the antibiotic metronidazole eradicated the diarrhoea within a few days (M. Hinojosa-Kurtzberg, G. C. Hansson and S. J. Gendler, unpublished work). Importantly, none of the animals treated in this way have been included in the present study

and all the animals used were more than 8 weeks old and thus at least 4 weeks older than the critical period around weaning. This makes it highly unlikely that an infection could be the basis for the increased fucosylation observed in the present study. Intestinal content of CF and wild-type mice were cultured in conventional media to isolate aerobic and anaerobic enteric bacteria, but no major qualitative or quantitative differences in enteric flora were detected (M. Hinojosa-Kurtzberg, G. C. Hansson and S. J. Gendler, unpublished work). However, one cannot exclude the possibility of unconventional micro-organisms colonizing the CF small intestine.

In conclusion, the results presented here demonstrate an increased mucin fucosylation based on the induction of a specific glycosyltransferase Fut2 in the small intestine of the CF mouse, the organ most severely affected by the disease in this species. The small intestine of CF mice could be an interesting model system for *in vivo* studies examining the signalling mechanisms behind the observed glycosylation alterations in CF.

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