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In situ characterization of O-linked glycans of Muc2 in mouse colon

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ABSTRACT

The characterization of mucus O-linked glycans in the proximal and distal mouse colon was performed by conventional histochemical methods and by lectin histochemistry in combination with enzymatic treatment (PNGase, α1,2 fucosidase, sialidase digestion), with and without prior desulfation. We demonstrated the presence of sialo- and sulfomucins in both the proximal and distal colon of the mouse. In the distal colon the sulfomucins were clearly prevalent, although there were always sialomucins with sialyl residues linked α2,6 to the subterminal galactose. Sialic acid was poorly O-acetylated, especially in the distal colon. The lectin binding pattern indicates a massive presence of fucose α1,2 linked to galactose in O-glycans and smaller quantities of fucose linked α1,6 to N-acetylglucosamine in the core of N-linked glycans. Lectin histochemistry also demonstrated the presence of glycosidic residues of N-acetylglucosamine, N-acetylgalactosamine, and galactose in oligosaccharide chains of highly sulfated mucins.

Introduction

The colonic epithelium of mammals is covered by a protective mucus layer, mainly composed of mucin glycoproteins, secreted by goblet cells. The mucus layer plays several roles including: lubrication, modulation of water and electrolyte absorption, and protection of the underlying epithelium from mechanical and chemical stress (Kim and Ho, 2010). The mucus also plays a key role in accommodating highly concentrated commensal intestinal microbes (the microbiota) with essential metabolic functions and which are in continuous crosstalk with mucosal cells (Becker and Lowe, 2003). In addition, the colonic mucus layer provides the protection from colonization and invasion by pathogenic bacteria; the mucins act as ligands for targeting of leucocytes to endothelial cells (Robbe et al., 2004).

Intestinal mucins are high-molecular weight glycoproteins with a high content of clustered O-linked oligosaccharides to tandem repeat peptides rich in threonine, serine, and proline. N-linked oligosaccharides are also present, but are quantitatively a minor constituent, although they may be functionally important. MUC2 is the major colonic secretory mucin in humans and mice (Van Klinken et al., 1999). The rodent homologue of human MUC2 is designated as Muc2, and has a similar structure motif and cell and tissue type-specific expression. Goblet cells produce MUC2 polymers, which are densely packaged into numerous apically stored granules, and released into the intestinal lumen to form the mucus-gel layer. The mucus layer is divided into 2 distinct sub-layers: an inner layer that firmly adheres to the intestinal mucosa, and an outer layer that can be washed off by rinsing (Bergstrom et al., 2010; Johansson et al., 2011). The dominant structural feature of mucins is their high content of carbohydrate determining the high molecular weight of mature mucins and a large part of their physicochemical properties. Altered mucin glycosylation leading to shorter carbohydrate side chains or altered sialylation has been observed frequently associated with many pathological conditions.

Lectin histochemistry, when combined with enzymatic digestion and chemical treatment, can be a valuable tool to highlight changes in the glycosylation of intestinal mucins

Abbreviations: AAA, *Aleuria aurantia* agglutinin; AB, alcian blue; ConA, *Canavalia ensiformis* agglutinin; DBA, *Dolichos biflorus* agglutinin; GSA-II, *Griffonia simplicifolia* agglutinin; HID, high-iron diamine; MAA, *Maackia amurensis* agglutinin; PAS, periodic acid-Schiff; PB, periodic acid borohydride; PBS, phosphate buffered saline; PNA, *Arachis hypogaea* agglutinin; SNA, *Sambucus nigra* agglutinin; TBS, Tris buffered saline; UEA-I, *Ulex europaeus* agglutinin.

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(Desantis et al., 2011) and thus is valuable for diagnosis of diseases. The mouse is frequently used as animal model to investigate the mechanisms of development of intestinal disease induced by supply of drugs, diets, and genetic modification (Erdelyi et al., 2009; Fu et al., 2011; An et al., 2007). Relatively few studies have been performed on the mucus oligosaccharide chains in goblet cells of normal mouse colon and little is known about the distribution of the various glycans in the different tracts of the colon or the glandular crypts. Kandori et al. (1996) compared the lectin binding pattern of gastrointestinal mucus cells among wild type and germ-free mice. Tobisawa et al. (2010) carried out a carbohydrate structural analysis by mass spectrometry of the mucus removed from the epithelial surface of the mouse colon by mechanical scraping. We have studied the mucin *in situ*, *i.e.* in the goblet cells, and through lectin histochemistry in combination with enzymatic treatment we tried to formulate hypotheses about the structure of the mucin oligosaccharide chains, with particular reference to the terminal glycosidic sequences, to the sialic acid presence, to its acylation and links with subterminal sugars. We also considered the possible differences of lectin binding within the glandular crypts and between the proximal and distal colon. The aim of our study was to establish a valuable baseline for further lectin histochemical studies to evaluate alterations of mouse colon mucins under different physiological, pathological or experimental conditions, with possible translational value in humans.

Material and methods

Animals and tissue collection

Six adult male mice, strain C57BL/6J (weighing 25–35 g: Harlan, S. Pietro al Natsione, Italy) were kept in individual cages under controlled conditions of temperature and humidity and a constant 12-h light/dark cycle. Animals had access to standard mouse chow and tap water *ad libitum*. The mice were treated in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, Bethesda, MD, USA) and the experimental protocols were approved by the University of Bari.

Following anesthesia with ether/oxygen, animals were sacrificed by cervical dislocation. The colon was rapidly removed and washed in TBS pH 7.5, 0.1 M. Two samples were collected from each animal, one immediately after the cecum (proximal colon) and the other at 2 cm from the anus (distal colon). The samples were fixed in 10% neutral buffered formalin, dehydrated in a graded series of ethanol, than embedded in paraffin wax. Sections were serially cut at 5 μm.

Conventional histochemistry

In order to characterize acidic groups, rehydrated sections were stained with the following histochemical methods:

- Alcian blue pH 2.5 and periodic acid-Schiff (AB-PAS) to reveal both acidic and neutral glycans (Mowry and Winkler, 1956).
- High iron diamine-alcian blue pH 2.5 (HID-AB pH 2.5) to demonstrate simultaneously sulfated and non-sulfated acidic glycans (Spicer et al., 1965).
- Periodic acid borohydride, potassium hydroxide and periodic acid-Schiff (PB-KOH-PAS) to reveal O-acylated sialic acid (Culling et al., 1975).

Conventional histochemistry using the HID/AB (pH 2.5) method was performed as previously described (Spicer et al., 1965). Briefly, sections were dewaxed and rehydrated, then immersed overnight (18 h) in a solution of N,N-dimethylmetaphenylene diamine dihydrochloride, N,N-dimethylparaphenylene diamine dihydrochloride, and fresh ferric chloride (60%, w/v) solution. After washing in running tap water, sections were counterstained with 1% AB in 3% acetic acid (pH 2.5) for 30 min and then dehydrated, and mounted.

Lectin histochemistry

Binding of nine lectins (3 biotinylated, 1 alkaline phosphatase labeled, 4 FITC conjugates) was assessed to characterize the structure and distribution of glycosidic chains. The choice of lectins has been made on the basis of the literature about the probable structure of the oligosaccharide chains of mucins in the colon of mammals. The common names, sugar specificities and concentrations of the lectins used are shown in Table 1.

For binding with FITC-conjugated lectins (PNA, DBA, UEA-I, ConA, Vector Laboratories, Burlingame, CA, USA), sections were incubated for 1 h at room temperature with the FITC-lectin solution in Hepes pH 7.5, 10 mM. Sections were subsequently rinsed in the same buffer and mounted in N-propyl gallate in 50% glycerol.

For AAA phosphatase alkaline labeled lectin (Vector Laboratories, Burlingame, CA, USA) rehydrated sections were incubated for 1 h at room temperature with the conjugated lectin in TBS pH 7.4, 0.1 M. The sections were then rinsed in TBS and incubated in substrate working solution (BCIP/NBT alkaline phosphatase substrate Kit IV, Vector) for 15 min at room temperature. The endogenous alkaline phosphatase activity has been inhibited by the addition of levamisole to the working solution. The sections were washed in TRIS buffer pH 9.5, 0.1 M for 5 min, dehydrated, cleared in Histolemon (Carlo Erba Reagents, Rodano, Italy) and mounted with Entellan® (Merck, Darmstadt, Germany). For MAA, SNA and GSA-II biotinylated lectins (Vector) rehydrated sections were immersed in 3% v/v hydrogen peroxide in methanol for 20 min at room temperature to eliminate endogenous peroxidase. Sections were then incubated with the lectins at a concentration of 15 μg/ml in TBS pH 7.4 for 1 h at room temperature. After rinsing in TBS the sections were subsequently incubated with horseradish peroxidase labeled streptavidin (Vector) at a concentration of 2.5 μg/ml for 30 min. Peroxidase was then revealed with diaminobenzidine–HCl

Table 1
Lectins used and their carbohydrate specificities.

Acronym of lectins	Lectin source	Sugar specificity	Dilution (μg/ml)	Inibitory sugar
PNA	*Arachis hypogaea*	Galβ1,3GalNAc	20	Gal
DBA	*Dolichos biflorus*	GalNAc	20	GalNAc
AAA	*Aleuria aurantia*	Fucα1-6GlcNAc; Fucα1,3(β1,4) GlcNAc	10	L-Fuc
UEA-I	*Ulex europaeus*	Fucα1-2Gal	20	L-Fuc
MAA	*Maackia amurensis*	Neu5Acα2,3Galβ1,4GlcNAc	15	NeuAc
SNA	*Sambucus nigra*	Neu5Acα2,6Gal(NAc)	15	NeuAc
ConA	*Canavalia ensiformis*	Man, Glc	20	Methyl α-mannose
GSAII	*Griffonia simplicifolia*	GlcNAc	15	GlcNAc

Abbreviations: Fuc, fucose; Gal, galactose; GalNAc, N-acetylgalactosamine; Glc, glucose; GlcNAc, N-acetylglucosamine; Man, mannose; Neu5Ac, neuraminic acid (sialic acid).

(Sigma–Aldrich, St. Louis, MO, USA) at a concentration of 5 μg/ml with 3% v/v hydrogen peroxide for 10 min at room temperature. Finally, the sections were dehydrated through graded alcohols, cleared in Histolemon (Carlo Erba Reagents), and mounted in Entellan®.

Lectin binding was also tested after desulfation and after PNGase digestion (see below). PNA and DBA staining was performed with and without pretreatment with neuraminidase, to remove sialic acid (see below). UEA-I binding was also tested after α1,2 fucosidase digestion (see below).

Enzyme treatment

Binding with PNA and DBA was carried out in adjacent sections with and without sialidase digestion also after desulfation. To remove terminal sialic acid, sections were incubated with 1 U/ml sialidase from *Clostridium perfrigens* (Neuraminidase Type V, Sigma–Aldrich) for 48 h at 37 °C, in a moist chamber, in 0.1 M acetate buffer pH 5.3, containing 10 mM $CaCl_2$. Lectin binding was performed with and without enzymatic pretreatment using PNGase to remove N-linked glycans. Digestion was carried out by incubating sections with the enzyme at 6 U/ml in Tris buffer pH 9.0, 0.1 M, 150 mM NaCl, 2.5 mM EDTA for 48 h at 37 °C in a moist chamber (Alonso et al., 2003).

Binding with UEA-I was carried out with and without α1,2 fucosidase digestion. To remove terminal α1,2 fucose sections were incubated in the enzyme diluted 1:200 in 0.2 M PBS, pH 5.0, for 48 h at 37 °C in a moist chamber (Liquori et al., 2007).

Chemical treatment

Desulfation preceding lectin staining and sialidase digestion was performed by a sequential methylation–saponification. Sections were immersed in 0.15 N HCl in methanol for 5 h at 60 °C and than in potassium hydroxide 1% in 70% ethanol for 15 min at room temperature.

Controls

Controls for lectin histochemistry were performed by: (1) substitution of the lectin solution with the buffer alone; (2) preincubation of the sections with the corresponding hapten sugar inhibitor at a concentration of 0.2 M. The inhibitory sugars, α-methylmannopyranoside, L-fucose, D-N-acetylglucosamine, D-N-acetylgalactosamine and N-acetylneuraminic acid were purchased from Sigma–Aldrich. The effectiveness of the desulfation technique was confirmed by subsequent abolition of the positivity to HID. Controls for enzymatic digestion were performed by the substitution of the enzymes with buffer solution alone under the same incubation conditions to determine the influence of the enzyme-free buffer (Plendl et al., 1989) were also performed.

Semi-quantitative analysis

The images were captured using an Eclipse E600 photomicroscope equipped with epifluorescence and a DMX 1200 digital camera (Nikon Instruments SpA, Calenzano, Italy). The intensity of the histochemical staining was assessed by two independent observers and classified as follows: no labeling (0), weak labeling (1), moderate labeling (2), strong labeling (3).

Table 2
Distribution of neutral and acidic glycans and acetyl substituent in sialic acid in the crypts of the mouse colon.

	AB-PAS	HID-AB	PB-KOH-PAS
Proximal colon			
Upper crypt	Purple 3	Brown 2/Blue 1	2
Lower crypt	Purple 2	Blue 2/Brown 1	2
Distal colon			
Upper crypt	Purple 1	Brown 3/Blue 1	1
Lower crypt	Purple 1	Blue 1/Blue 1	0/1

AB-PAS: red, neutral glycans; blue, acidic glycans; purple, mixed neutral and acidic glycans. HID-AB: brown, sulfated glycans; blue, carboxylated glycans. PB-KOH-PAS stained red, O-acylated sialic acid. 0 = negative; 1 = weak; 2 = moderate; 3 = strong staining.

Results

Conventional histochemistry

Neutral and acidic glycans

The goblet cells of the whole colon were PAS positive, stained with AB at pH 2.5 and appeared purple with the AB-PAS procedure (data not shown). Glandular crypts reacted with both HID (brown)-AB pH 2.5 (blue). HID stained the goblet cells mainly in the upper part of the proximal colon crypts (Fig. 1A) and in the whole crypts of the distal colon (Fig. 1B).

Acetyl substituent in the sialic acid

Red staining with PB-KOH-PAS was more evident in the goblet cells of the proximal colon crypts (Fig. 1C) than in the distal colon crypts (Fig. 1D).

Table 2 illustrates the distribution of carbohydrate moieties visualized by the different staining methods, and the results of the PB-KOH-PAS method for the identification of acetyl substituent in sialic acid. Histochemical results revealed the presence of sulfomucins and sialomucins throughout the whole colon of the mouse. In the distal colon sulfomucins are clearly prevalent, although there are always sialomucins. Sialomucins are mainly located in the lower part of the glandular crypts, and are particularly abundant in the proximal colon. Sialic acid was poorly O-acetylated, above all in the distal colon.

Lectin histochemistry

Direct visualization of sialic acid residues (SNA and MAA lectins)

The terminal sialic acid can be detected directly by binding to two lectins: SNA, for Neu5Ac linked α2,6 to Gal/GalNAc, and MAA, for Neu5Ac linked α2,3 to Galβ1,4GlcNAc. Goblet cells in both proximal (Fig. 1E) and distal colon (Fig. 1F) were bound to SNA lectin, whereas they were not stained with MAA lectin (Fig. 1G and H). SNA affinity was more intense in the proximal than in the distal colon (Fig. 1E and F). SNA and MAA binding revealed sialyl residues linked to galactose *via* α2,6 in the mouse colon mucus.

The binding pattern of these two lectins is reported in Table 3.

Table 3
Direct visualization of sialic acid residues.

	SNA	MAA
Proximal colon		
Upper crypt	1	0
Lower crypt	2	0
Distal colon		
Upper crypt	1	0
Lower crypt	1	0

0 = negative; 1 = weak; 2 = moderate; 3 = strong staining.

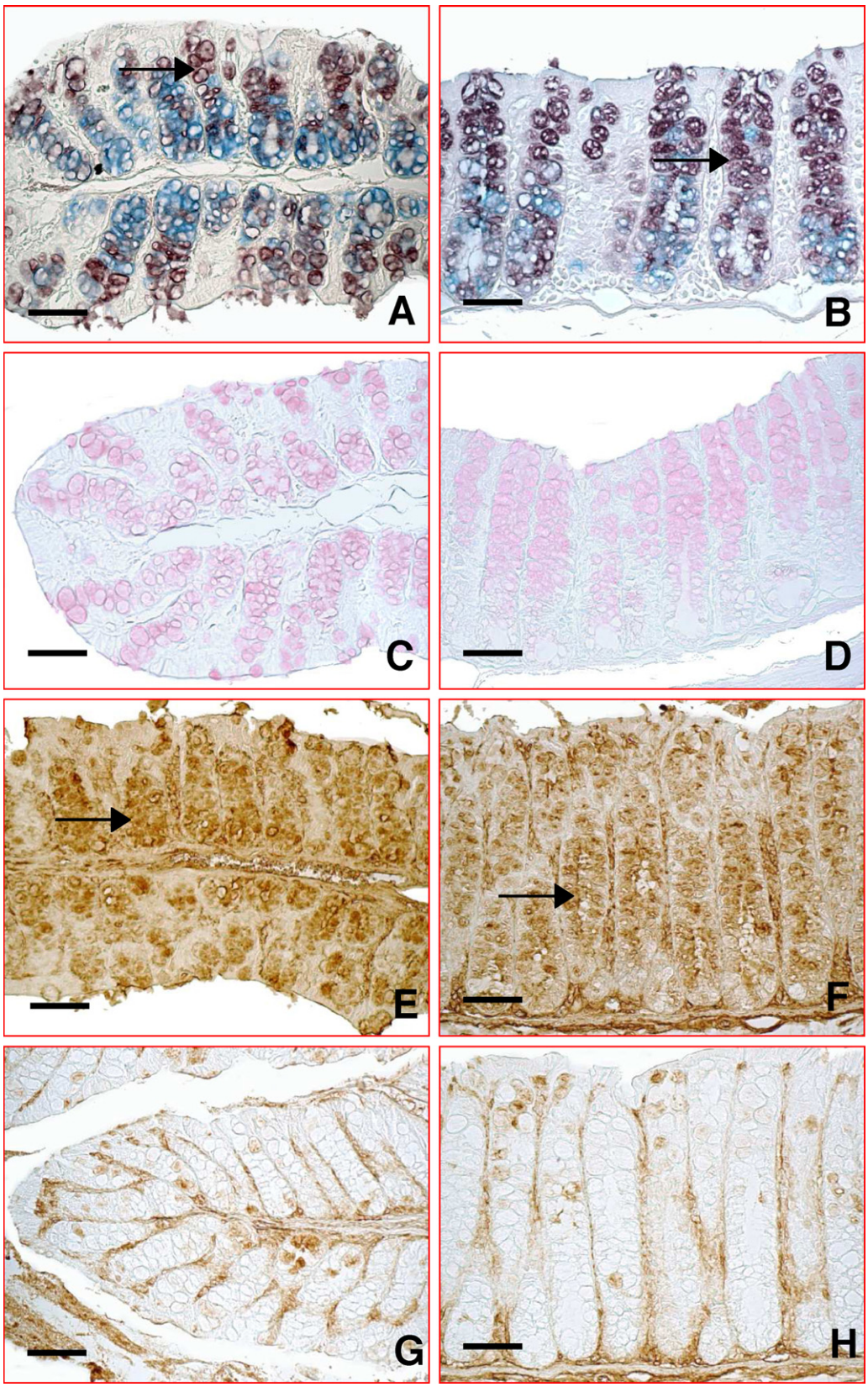

Fig. 1. (A and B) HID-AB pH 2.5 method for the demonstration of both sulfated and carboxylated acidic glycans. (A) Proximal colon. Goblet cells produced sialylated and sulfated mucins. Goblet cells in the upper part of the glandular crypts contained mainly sulfomucins (arrow). (B) Distal colon. Sulfomucins were predominant throughout the glandular crypts. (C and D) PB-KOH-PAS method to reveal O-acylated sialic acid. (C) Proximal colon. O-acylated mucins were observed in the glandular crypts. (D) Distal colon. In the glandular crypts a reduced content of O-acylated sialic acid was observed. (E and F) Staining with SNA and MAA lectins for direct visualization of sialic acid. In both proximal (E) and distal (F) colon, goblet cells bound SNA lectin. Binding was more evident in the lower part of the glandular crypts in the proximal colon (arrow). (G and H) In both proximal and distal colon, binding with MAA was not observed (H). (A and B) HID-AB pH 2.5; (C and D) PB-KOH-PAS-hemalum; (E and F) SNA biotinylated lectin; (G and H) MAA biotinylated lectin. Scale bars = 50 μm.

Table 4
Identification of subterminal residues linked to sialic acid in the crypts of the mouse colon.

	PNA	D/PNA	S/PNA	D/S/PNA	DBA	D/DBA	S/DBA	D/S/DBA
Proximal colon								
Upper crypt	0[a]	2	0[a]	3	3	3	3	3
Lower crypt	0[a]	2	0[a]	3	3	3	3	3
Distal colon								
Upper crypt	0[a]	3	0[a]	3	1	1	1	1
Lower crypt	0[a]	1	0[a]	3	1	1	1	1

[a] Binding was observed only on the Golgi complex; D, desulfation; S, sialidase digestion.
0 = negative; 1 = weak; 2 = moderate; 3 = strong staining.

Staining with PNA lectin to detect terminal galactose and to indirectly demonstrate terminal sialic acid bound to subterminal galactose

In both the proximal and the distal colon labeling with PNA was evident in the whole glandular crypts only after desulfation (Fig. 2A and B). Without desulfation, lectin binding was evident only in the Golgi complex (Fig. 2A and B, inserts). PNA binding increased after desulfation-sialidase digestion (Fig. 2C and D), but not solely with sialidase digestion in both the proximal (Fig. 2C, insert) and the distal (Fig. 2D, insert) colon.

Staining with DBA lectin to detect terminal N-acetylgalactosamine and to indirectly demonstrate terminal sialic acid bound to subterminal N-acetylgalactosamine

Goblet cells of the proximal colon stained intensely with DBA lectin (Fig. 2E). In the distal colon goblet cells showed weak affinity for this lectin (Fig. 2F). Sialidase digestion did not affect DBA staining (Fig. 2E and F, inserts).

Results of the staining with PNA and DBA lectins are reported in Table 4. These results indicate the presence of sialic acid bound to subterminal galactose in oligosaccharide chains of highly sulfated mucins.

Staining with AAA to detect fucose linked α1,6 to N-acetylglucosamine in N-linked glycans or fucose linked α1,3 or α1,4 to N-acetyllactosamine.

In both the proximal (Fig. 3A) and the distal colon (Fig. 3B) goblet cells showed weak affinity for AAA lectin. PNGase digestion abolished AAA staining.

Staining with UEA-I lectin to detect fucose α1,2 linked in O-linked glycans

In the proximal colon (Fig. 3C) the affinity for UEA-I was stronger than in the distal colon (Fig. 3D). α1,2 fucosidase digestion drastically reduced UEA-I binding in both proximal (Fig. 3C, insert) and distal colon (Fig. 3D, insert).

Staining with GSA-II lectin to detect terminal N-acetylglucosamine

In the proximal colon the goblet cells of the lower crypts strongly stained with this lectin (Fig. 3E). In distal colon lectin binding was only evident in the deeper part of the glandular crypts (Fig. 3F).

Staining with ConA lectin to detect terminal mannose or glucose residues

Weak affinity for ConA was seen in both the proximal (Fig. 3G) and the distal colon (Fig. 3H). PNGase digestion abolished ConA and AAA staining (Fig. 3G, H, inserts), while scarcely affecting binding with the other lectins.

The lectin binding pattern in the mouse colon is reviewed in Table 5. The lectin binding pattern indicates high presence of fucose α1,2 linked to galactose in O-glycans and smaller quantities of fucose linked α1,6 to N-acetylglucosamine in the core of N-linked glycans. Lectin binding also demonstrates the presence of glycosidic residues of N-acetylglucosamine, N-acetylgalactosamine, and galactose in oligosaccharide chains of highly sulfated mucins.

Controls

Lectin labeling was completely inhibited when the lectins were preincubated with the appropriate hapten sugar or when they were omitted from the incubation medium. Desulfation procedure abolished HID reactivity in control sections containing sulfated mucins.

Discussion

In the present study we characterized the mucus O-linked glycans in the proximal and the distal mouse colon by conventional histochemical methods and by lectin histochemistry in combination with enzymatic treatment. We demonstrated the presence of sialo- and sulfomucins in both the proximal and the distal colon of the mouse. In the distal colon the sulfomucins are clearly prevalent, although there are always sialomucins with sialyl residues linked α2,6 to the subterminal galactose. Sialic acid is poorly O-acetylated, especially in the distal colon. The lectin binding pattern indicates a massive presence of fucose α1,2 linked to galactose in O-glycans and smaller quantities of fucose linked α1,6 to N-acetylglucosamine in the core of N-linked glycans. Lectin histochemistry also demonstrates the presence of glycosidic residues of N-acetylglucosamine, N-acetylgalactosamine, and galactose in oligosaccharide chains of highly sulfated mucins.

Neutral and acidic glycans

We demonstrated by conventional histochemistry (AB pH 2.5, HID-AB) and by lectin histochermistry (SNA, MAA, sialidase-DBA, sialidase-PNA) the presence of sulfomucins and sialomucins in both the proximal and the distal colon of the mouse. In the distal colon the sulfomucins are clearly prevalent, although there are always sialomucins. Sialomucins are mainly located in the lower part of the glandular crypts, and are particularly abundant in the proximal colon. The different pattern of distribution observed within the crypts may be related to a process of maturation of the secretion.

Table 5
Lectin binding in the crypts of mouse colon.

Lectin	Proximal colon		Distal colon	
	Upper crypt	Lower crypt	Upper crypt	Lower crypt
PNA	0	0	0	0
DBA	3	3	1	1
AAA	1	1	1	1
UEA-I	3	3	2	2
MAA	0	0	0	0
SNA	1	2	1	1
ConA	1	1	1	0
GSAII	0	3	0	1

0 = negative; 1 = weak; 2 = moderate; 3 = strong staining.

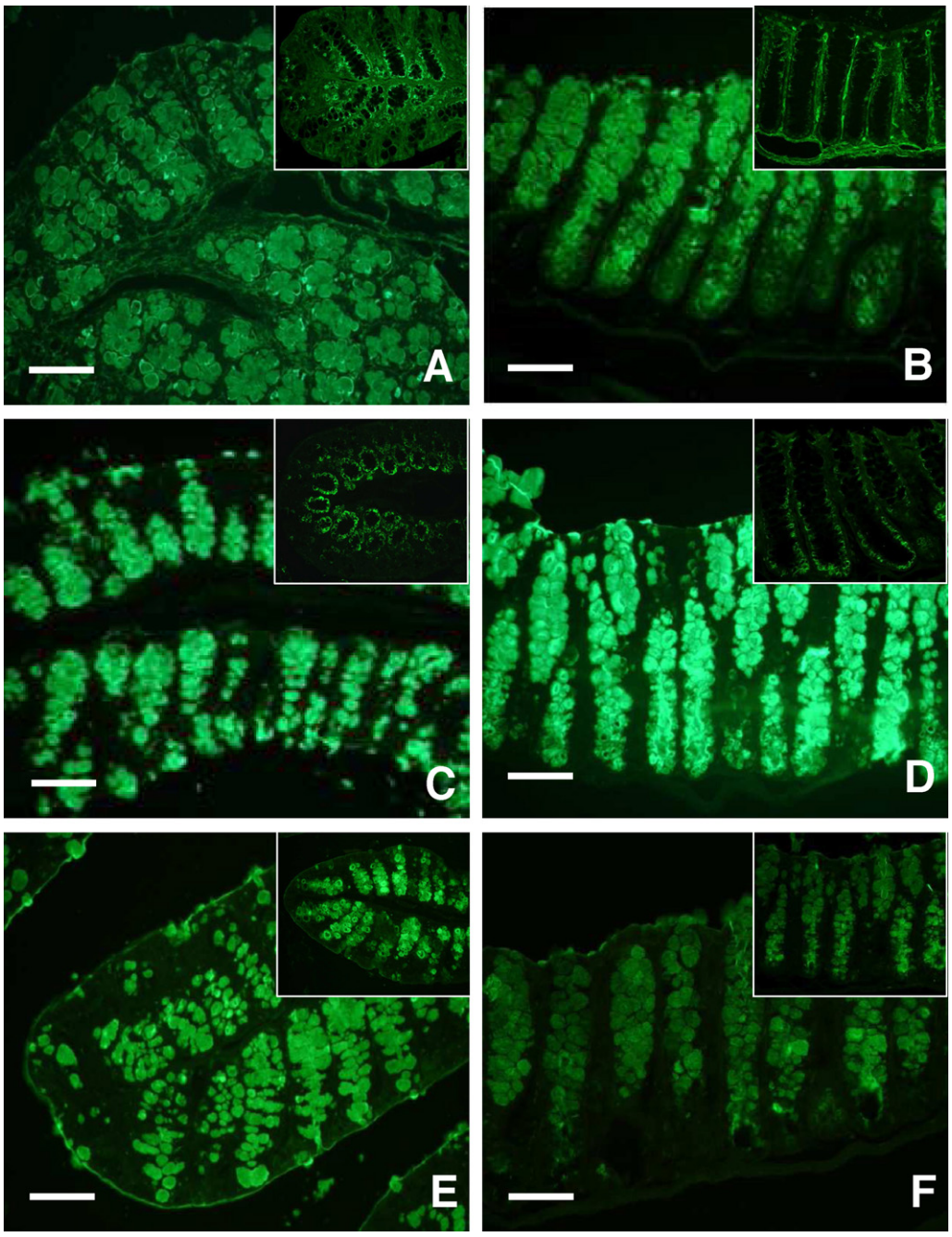

Fig. 2. (A–D) Staining with PNA lectin to detect terminal galactose (A and B) and to indirectly demonstrate terminal sialic acid bound to subterminal galactose (C and D). In both proximal (A) and distal (B) colon binding with PNA in secretory droplets was evident only after desulfation. A, B inserts: without desulfation, lectin binding was evident only in the Golgi complex. PNA binding increased after desulfation-sialidase digestion, but not with the solely sialidase digestion (inserts) in both proximal (C) and distal (D) colon. (E and F) Staining with DBA lectin to detect terminal N-acetylgalactosamine and to indirectly demonstrate terminal sialic acid bound to subterminal N-acetylgalactosamine. (E) Proximal colon. Goblet cells stained intensely with this lectin. Staining did not affect after sialidase digestion (insert). (F) Distal colon. Goblet cells showed weak affinity for DBA. Sialidase digestion did not affect DBA staining (insert). (A and B): desulfation-PNA-FITC; (A and B): insert, PNA-FITC; (C and D): desulfation-sialidase-PNA-FITC; (E): DBA-FITC; (F): sialidase-DBA-FITC. Scale bars = 50 μm.

In the base of the glands, the immature goblet cells are especially rich in sialomucins. Migrating toward the surface of the mucosa, sulfomucins are added to the secretory product of the goblet cells. It is known that sialic acid occupies a terminal position in oligosaccharide chains (Schauer, 1982). By lectin binding with SNA and MAA lectins, we found in the colon mucus sialyl residues linked to the inner sugar residue galactose *via* α2,6 in the mouse colon mucus. In mammals, the goblet cells of the colonic mucosa produce both neutral and acidic mucins. Acidic mucins may be sulfomucins, which are rich in sulfate radicals and sialomucins. Generally, acid mucins are expressed along the whole length of the colon mucosa, but they are clearly predominant in the mucosa of the distal colon and in the rectum (Ueda et al., 1995; Kandori et al., 1996). Sulfomucins and sialomucins have a different pattern of distribution depending on the region of the colon and their location within the glandular crypts (Larsson et al., 2009; Nonose et al., 2009; Pearson and Brownlee, 2010). In the rat (Accili et al., 2008) Neu5Ac linked to Gal or subterminal GalNAc appears to be widely distributed in goblet cells of the colonic epithelium, as in other species of mammals (Karlsson et al., 1997). Results of mass spectrometry analysis

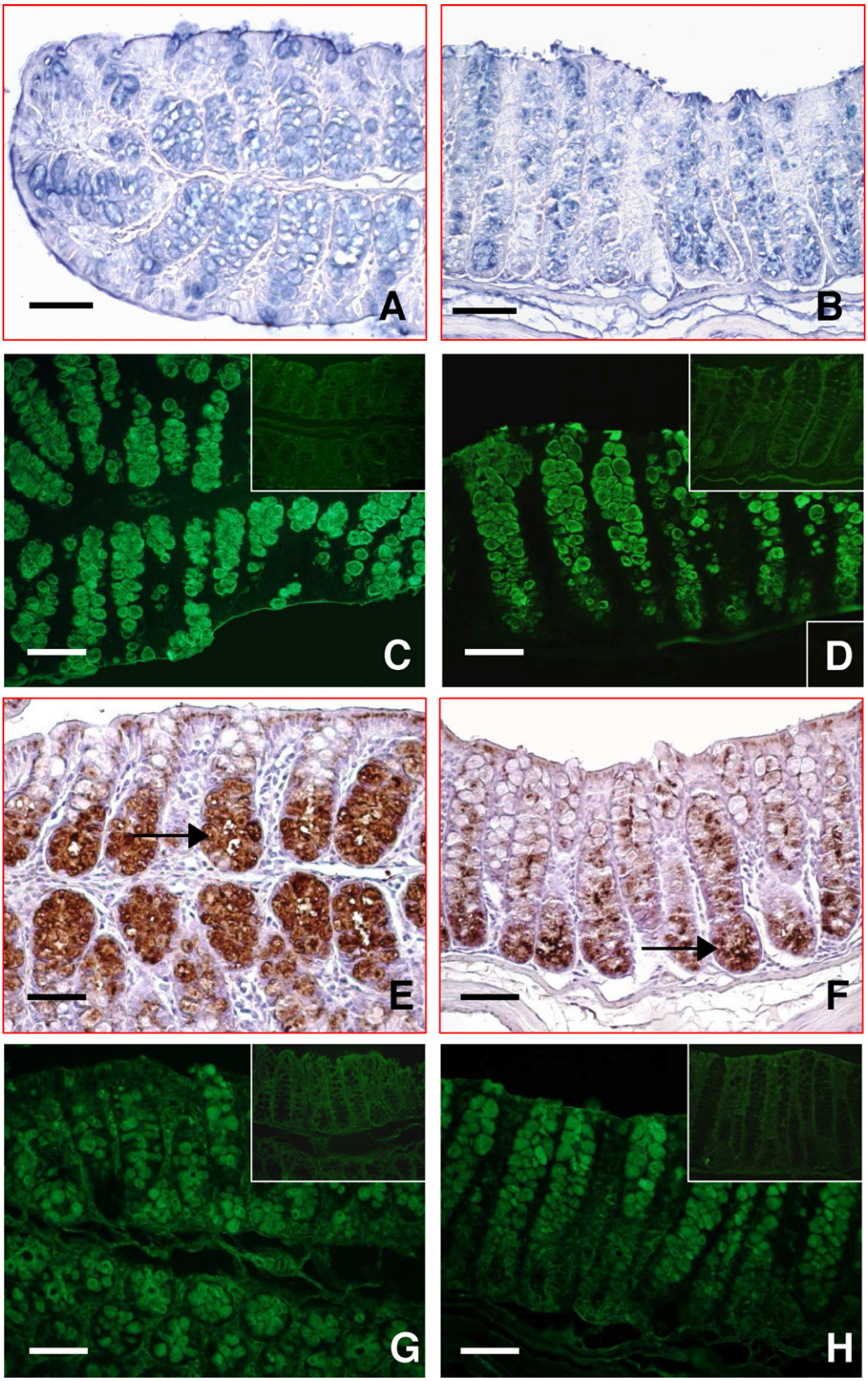

Fig. 3. (A and B) Staining with AAA to detect fucose linked α(1,6) to N-acetylglucosamine in N-linked glycans or fucose linked (α1,3) or (α1,4) to N-acetyllactosamine. (A and B) Both proximal and distal colon goblet cells showed weak affinity for AAA. (C and D) Staining with UEA-I lectin to detect fucose (1,2) linked in O-linked glycans. In the proximal colon (C) the affinity for UEA-I was stronger than in the distal colon (D). α1,2 fucosidase digestion drastically reduced UEA-I binding (inserts). (E and F) Staining with GSA-II lectin to detect terminal GlcNAc. (E) Proximal colon. Goblet cells of the lower crypts strongly stained with this lectin (arrow). (F) Distal colon GSA-II affinity was mainly evident in the deeper part of the glandular crypts (arrow). (G and H) Staining with ConA lectin to detect terminal mannose or glucose residues. Affinity for ConA was seen in both proximal (G) and distal (H) colon. PNGase digestion abolished the lectin binding (inserts). (A and B) AAA alkaline phosphatase labeled lectin; (C and D) UEA-I-FITC conjugated lectin. (E and F) GSAII biotinylated lectin; (G and H) Con A-FITC conjugated lectin. Scale bars = 50 μm.

suggest the presence of sialylated oligosaccharide chains in the Muc2 of the rat with Neu5Ac linked mainly to the sub-terminal dimer α2-6 Gal β1,3GalNAc (Larsson et al., 2009). Kandori et al. (1996) on the basis of histochemical staining (HID-AB) reported that the goblet cells of the mouse colon were mostly positive for sialomucins. In a recent study based on mass spectrometry, Tobisawa et al. (2010) did not report the presence of sialic acid in the mucus covering the colonic epithelium of mice, whereas Hurd et al. (2005) found only the presence of trace amounts of sialic acid. On the other hand, Van Klinken et al. (1999) isolated and characterized the murine colonic Muc2, and their monosaccharide analysis revealed a very high content of sialic acid (22.9%). Also the mucus in the human and rat colon is rich in sialomucins (Milton et al., 1993; Karlsson et al., 1997; Accili et al., 2008). Sialic acid and sulfated residues are responsible for the polyanionic nature of mucins. High negative charge contributes to the stiffness of mucin peptides and permits mucins to adsorb large amount of water (Pearson and Brownlee, 2010). High negative charge of the mucin due to terminal sialic acid also confers resistance to degradation by bacterial glycosidase, because sialic acid must be removed so that exoglycosidase can further degrade the oligosaccharide chains (Milton et al., 1993). O-acetylation of sialic acid increases its resistance to bacterial sialidase. Decreased O-acetylation of colon sialomucins is a common early feature of malignant disorders such as: colorectal cancer, ulcerative colitis, colonic adenoma (Jass et al., 1988; Milton et al., 1993). The sulfate groups in the oligosaccharide chains are typically linked to Gal or GlcNac residues. Sulfomucins play an important protective role acting as a barrier against the mucosal inflammation of colon (Tobisawa et al., 2010). An alteration of the quantitative relationships between sulfated and sialylated mucins is generally associated with intestinal diseases (Nonose et al., 2009). An increase in the expression of sialomucins has been observed in the mucosa of patients with ulcerative colitis (Robbe et al., 2004). The increase of sialic acid from the proximal colon to the distal end is commonly observed and may be tied to a functional gradient in the need for a protective and lubricant function performed by this molecule (Accili et al., 2008). However, our data may indicate that the sulfate groups are mainly responsible for the acidity of the mucus and that from these depend, in particular, in addition to the fucose, the rheological properties of mucus.

Fucose (Fuc)

L-Fucose is a monosaccharide that is a common component of many N- and O-linked glycans and glycolipids produced by mammalian cells. This monosaccharide frequently exists as a terminal modification of glycan structures, and it is possible to characterize and localize fucose residues with two lectins: *Ulex europaeus* (UEA-I) lectin and *Aleuria aurantia* (AAA) lectin. It is generally assumed that AAA binds preferentially to Fuc α1,6-linked to the proximal GlcNAc of the N-linked glycans, and to terminal Fuc with an α1,3 or α1,4 linkage in O-glycans (Kochibe and Furukawa, 1980; Yamashita et al., 1985; Osawa and Tsuji, 1987; Spicer and Schulte, 1992). UEA-I is considered to have high affinity for Fuc with an α1,2 linkage (Pereira and Kabat, 1974). In mice, we observed a moderate binding with AAA lectin that remains unchanged after digestion with PNGase. This result suggested the presence of fucose linked α1,6 to N-acetylglucosamine in N-linked glycans. The UEA-I binding pattern indicates the presence of fucose α1,2 linked to galactose in O-glycans. This is confirmed by the abolition of reactivity after digestion with α1,2 fucosidase. The reactivity remains unchanged after digestion with PNGase. In the mice colon, high fucosylation of the mucins was previously observed by lectin histochemistry (Kandori et al., 1996). Hurd et al. (2005) and Tobisawa et al. (2010) have indicated by mass spectrometry the presence of at least six different fucosylated O-linked oligosaccharide chains in the mucus covering the colonic epithelium of the mouse. Our data are consistent with the proposed fucosilate structures of oligosaccharide chains (Hurd et al., 2005; Tobisawa et al., 2010). Also in humans, the MUC2 is heavily fucosylated (Larsson et al., 2009). Robbe et al. (2003) observed in normal humans, a reduction of fucose from the small intestine to the large intestine, associated with an increase in acidity. On the other hand dramatic changes in the expression of fucosylated oligosaccharides have been observed in cancer and inflammation (Robbe et al., 2003; Moriwaki and Miyoshi, 2010). Fucose and sialic acid in mucus are known to be associated with the viscous property of mucus. Carbocysteine normalizes the viscous property of mucus through regulation of fucosylated and sialylated sugar chain on airway mucins. The fucosylated glycans contribute to the construction of the autochthonous gut microbial community, providing a favorable ecological niche for commensal microorganisms that are able to use fucose as a carbon source (Becker and Lowe, 2003). For instance, *Bacteroides thetaiotaomicron* is a major component of the adult intestine and lives in the lower part of the gut and feeds on fucose residues of mucins. Its metabolic function in humans is to degrade plant polysaccharides, a very essential capability for the human gut, and it is important in the formation of the intestinal mucosal barrier, which helps protect the host against pathogenic invasion (Becker and Lowe, 2003). In ulcerative colitis, loss in fucosylation as well as in sulfation, has also been noted (Pearson and Brownlee, 2010). It is possible to think that the fucosylation is not only useful as a tumor marker, but also a possible factor that determines the characteristics of the cancerous cells (Moriwaki and Miyoshi, 2010). It is noteworthy that *B. thetaiotaomicron* and other species such as *Methanobrevibacter smithii*, as well as other aberrant gut microbiota have been recently implicated in essential metabolic steps linked with the onset of obesity (Samuel and Gordon, 2006; Conterno et al., 2011).

N-acetylgalactosamine (GalNAc)

In this study, binding with the DBA, unchanged after digestion with sialidase, suggests the presence of terminal GalNAc in O-linked oligosaccharide chains which, probably, belong to Muc2. In mice, the binding with the DBA was also observed by Kandori et al. (1996), while Tobisawa et al. (2010) did not report the presence of terminal GalNAc in the mucus covering the colonic epithelium of the mouse. In mammals, including humans, the presence of GalNAc, both in the core and in the terminal position, has well been demonstrated (Karlsson et al., 1997; Larsson et al., 2009). GalNAc plays a significant role in defence against pathogens. It is important, for instance, in preventing amoebic invasion of the colon. In fact the amoebic binding to Gal and GalNAc residues of Muc2 inhibits parasitic adhesion to the underlying epithelium protecting its integrity.

N-acetylglucosamine (GlcNAc)

The binding with the GSA-II which we observed, shows the presence of oligosaccharide chains with terminal GlcNAc in goblet cells of the lower third of glandular crypts. Our data are consistent with those reported by Kandori et al. (1996) who showed histochemically the presence of GlcNAc in the colon mucus of mice. In mice, using analysis by mass spectrometry, Tobisawa et al. (2010) identified in the mucus covering the colonic epithelium four different types, two sulfated and two non-sulfated, of oligosaccharide chains with terminal GlcNAc. In humans (Larsson et al., 2009) and rat (Karlsson et al., 1997) the colonic mucins are rich in terminal GlcNAc terminal. The GlcNAc is particularly important in relation to acidification of mucins. In fact, sulfate groups are linked to the Gal or GlcNAc of oligosaccharide chains.

Galactose (Gal)

In this study we observed PNA binding sites in the goblet cells only after desulfation, which revealed the presence of terminal Gal in oligosaccharide chains of highly sulfated mucins. It is known that the high presence of sulfate groups prevents the binding of some lectins (Parillo et al., 2001). Use of mass spectrometry has been indicated the presence of at least five different sulfated oligosaccharide chains with terminal Gal in the mucus covering the colonic epithelium (Tobisawa et al., 2010). Oligosaccharide chains with terminal Gal in goblet cells of both humans and rats have also been found (Karlsson et al., 1997; Larsson et al., 2009).

In conclusion, we found some differences between our data and that obtained by Tobisawa et al. (2010) using mass spectrometry. These differences may be due to the different methodological approach. While we have analyzed the mucin within the goblet cells, Tobisawa et al. (2010) analyzed the mucus covering the mucosal epithelium, which may have been altered by bacterial action or contamination. Our study on normal mice colon may establish a valuable landmark for further lectin histochemical studies to evaluate alterations of intestinal mucins under different physiological, pathological or experimental conditions. Lectin histochemistry, in combination with enzymatic digestion and chemical treatment, can be a useful tool for the identification of structural alterations of intestinal mucins, usually associated with intestinal diseases. Studies in pathophysiologically relevant models of mucin composition in animal intestine with translational value in humans might prove useful in a number of conditions including metabolic changes, inflammation and tumors.

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