

The MUC2 mucin

-A network in the intestinal protective mucus

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Cover illustration: Mouse colon stained by anti-MUC2C3 and anti-rabbit-FITC.

Till min kära familj

ABSTRACT

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The intestine is covered by mucus that is the first line of defence of the epithelium. The main structural component of the intestinal mucus is the MUC2 mucin. This is a large glycoprotein with two long and heavily *O*-glycosylated mucin domains. Our studies of the biosynthesis have revealed that MUC2 forms large disulphide linked networks starting with C-C terminal dimers. In the late secretory pathway the N-terminal of MUC2 forms trimers within a core fragment resistant to trypsin cleavage. The MUC2 assembly creates an enormous network with an ability to resist protease degradation. It is important that the mucus is resistant to the intestinal digestive enzymes.

In colon, the real challenge is to wield the large number of bacteria in the normal flora. Immune tolerance has been studied intensely, but the contribution of the mucus in the protective function has been neglected largely due to the technical difficulties to work with the large mucin glycoproteins. The mucus in colon is made up of two mucus layers. In mouse the inner mucus layer is 50 μm thick and firmly attached to the epithelium. This is a compact, insoluble and stratified mucus layer with a high Muc2 concentration. The firm layer is converted to a 100 μm , soluble, loose overlaying mucus layer that is expanded in volume by proteolysis. The mucus turnover is fast and in colon the luminal mucus layers are renewed in hours. The composition of the mucus was investigated by proteomics and was found to be similar in the two mucus layers, indicating a common source. One of the components identified, Fc gamma binding protein (Fcgbp) was shown by purification of the mucus in guanidinium chloride to be covalently attached to Muc2. The binding may be mediated by potential autocatalytic cleavage sites that generate new reactive C-termini in Fcgbp. The disulphide stabilized Fcgbp could thus be a cross-linker of the Muc2 network.

Bacteria in colon were detected in the outer loose mucus layer by *in-situ* hybridization using a 16S rRNA general bacterial probe. This mucus is likely to be a good habitat for bacteria providing binding sites and energy. The inner compact firm mucus is impervious to bacteria, making it a protective barrier for the enormous bacterial load. The mucus is through this mechanism a part of the innate immunity to keep the homeostasis in colon.

The protective function of mucus argues for that defects in the mucus can be a cause of inflammation. In fact, mice with the Muc2 gene disrupted do not produce mucus and develop spontaneous colitis. In these animals the epithelium is in direct contact with the colonic flora, bacteria enter deep into the normally sterile crypts and penetrate the epithelial cells. An overt immune reaction to the bacteria is an obvious cause of the inflammation. In wild type mice Dextran sulphate (DSS), a highly sulphated glucose polymer, is used to induce colitis and is the most common UC model. DSS exposure resulted in alterations in the mucus long before any signs of inflammation were observed. The inner mucus allowed bacteria to penetrate as early as after 4 h of exposure, with a massive bacterial penetration into the inner mucus after 12 h. The mechanisms behind this colitis model were not known until now when our observations suggest that a defect mucus layer is likely to have triggered the inflammation. The importance of the inner mucus for epithelial protection argues for defective mucus as a possible cause of ulcerative colitis.

Keywords: mucus, Muc2, trimer, proteomics, intestine, colitis, DSS

PAPERS IN THIS THESIS

The thesis is based on the following papers, which are referred to in the text by their Roman numerals:

- I. **Johansson, M. E. V.**, Godl, K., Lidell, M. E., Morgelin, M., Karlsson, H., Olson, F. J., Gum, J. R. Jr., Kim, Y. S., and Hansson, G. C. (2002). The N terminus of the MUC2 mucin forms trimers that are held together within a trypsin-resistant core fragment. *J. Biol. Chem.* 277, 47248-56.
- II. **Johansson, M. E. V.**, Phillipson, M., Petersson, J., Velchich, A., Holm, L., and Hansson, G. C. (2008). The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria. *Proc. Natl. Acad. Sci. U S A.* 105, 15064-9.
- III. **Johansson, M. E. V.**, Thomsson, K. A., and Hansson, G. C. (2009). Proteomic analysis of the two mucus layers of the colon barrier reveal that their main component, the Muc2 mucin, is strongly bound to the Fcgbp protein. *J. Proteome Res.*, *in press*.
- IV. **Johansson, M. E. V.**, and Hansson, G. C. (2009). Mucus turnover in the intestine measured by *in vivo* labeling of glycoproteins. *Manuscript*.
- V. **Johansson, M. E. V.**, Gustafsson, J., Petersson, J., Holm, L., Sjövall, H., and Hansson, G. C. (2009). The colitis inducing agent Dextran sulfate disrupts the colon inner mucus gel and allows bacteria to penetrate before inflammation is observed. *Submitted manuscript*.

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ABBREVIATIONS

AgPAGE	Agarose-polyacrylamide gel electrophoresis
CF	Cystic fibrosis
Cftr	Cystic fibrosis transmembrane conductance regulator
CHO	Chinese hamster ovary
CID	Collision induced dissociation
CK	Cysteine knot
COS	CV-1 (simian) origin carrying SV40 genetic material
CysD	Cysteine rich domain
DSS	Dextran sodium sulphate
EGF	Epidermal growth factor
EM	Electron microscopy
ER	Endoplasmic reticulum
ESI	Electrospray ionization
FISH	Fluorescent <i>in-situ</i> hybridisation
GalNAc	N-acetylgalactosamine
GalNAz	N-azidoacetylgalactosamine
GuHCl	Guanidinium chloride
HPLC	High pressure liquid chromatography
IBD	Inflammatory bowel disease
LC	Liquid chromatography
MALDI	Matrix-assisted laser desorption/ionization
MS	Mass spectrometry
PAGE	Polyacrylamide gel electrophoresis
PAS	Periodic acid-Schiffs
PDZ	Post synaptic density, disc large, and zonula occludens-1 domain
PSM	Porcine submaxillary mucin
PTS	Proline, serine and threonine rich domain
SEA	Sperm protein, Enterokinase and Agrin domain
SIBO	Small intestinal bacterial overgrowth
SNARE	SNAP (soluble NSF protein) receptor
TAMRA	Tetramethyl rhodamine
TLR	Toll-like receptor
TNBS	Trinitrobenzene sulfonic acid
UC	Ulcerative colitis
vWA, B, C, D	von Willebrand A, B, C, D domain
vWf	von Willebrand factor

INTRODUCTION

Epithelium and mucus

All organisms have to acclimatize to fit their surrounding environment. One important function is to protect itself from hazards such as infectious agents or physical and chemical damages. Our outer surface, the skin, is protected by layers of dead keratinized cells, whereas the epithelial surfaces within the body lining the lungs, the gastrointestinal tract as well as the reproductive and urinary tracts are composed of a single cell layer. The cells in the epithelium are tightly connected and form a barrier towards the lumen, but will also allow for selective transport over the epithelium. The mucosal surfaces are exposed to the outer environment which is especially harsh in colon where a large number of bacteria reside. The epithelial cell barrier is very vulnerable and it is protected by an overlaying mucus layer. Mucus is a viscous gel-like material with mucins, large glycoproteins, as the main component. The protection by a mucus gel is evolutionary old and molecules with sequence similarities to mucins coding for long stretches with numerous prolines, serines and threonines, known as PTS domains have been identified in primitive animals as *Nematostella* (1). The preservation of these molecules and their functions argues for a vital biological role for the organism. The properties and composition of the mucus differ in different organs depending on the function of the underlying epithelium. An example is in the lungs where there is a water air interface and regular clearance of mucus is needed. The gastrointestinal tract on the other hand is exposed to many different compounds and microorganisms and therefore need a more persistent protection, but also need to allow uptake of nutrients.

The intestine and mucus

The small intestine is a 5-7 m long tube with the main function to digest and absorb nutrients from ingested food. In the duodenum, the gastric juice is neutralized and pancreatic and bile secretions are added to facilitate the digestion. This creates a harsh milieu for the epithelium. The small intestinal epithelium is organized in villi to produce as large a surface as possible in contact with the ingesta. There are two different compartments of the intestinal epithelium, the crypt with proliferative stem cells and Paneth cells, and the villus with enterocytes, enteroendocrine cells and mucus filled goblet cells as shown in Figure 1 (2). The number of microorganisms is not high in the proximal intestine, but the load is higher further down in the ileum where the bacteria are monitored by the host Peyer patches (3). The

goblet cells produce mucus and in the intestine this is largely made up by the MUC2 mucin. This mucus need to allow absorption of nutrients and help to move the luminal material distally. The motility, driven by the motor complex, and the liquid secretion are both important for the function of the small intestine.

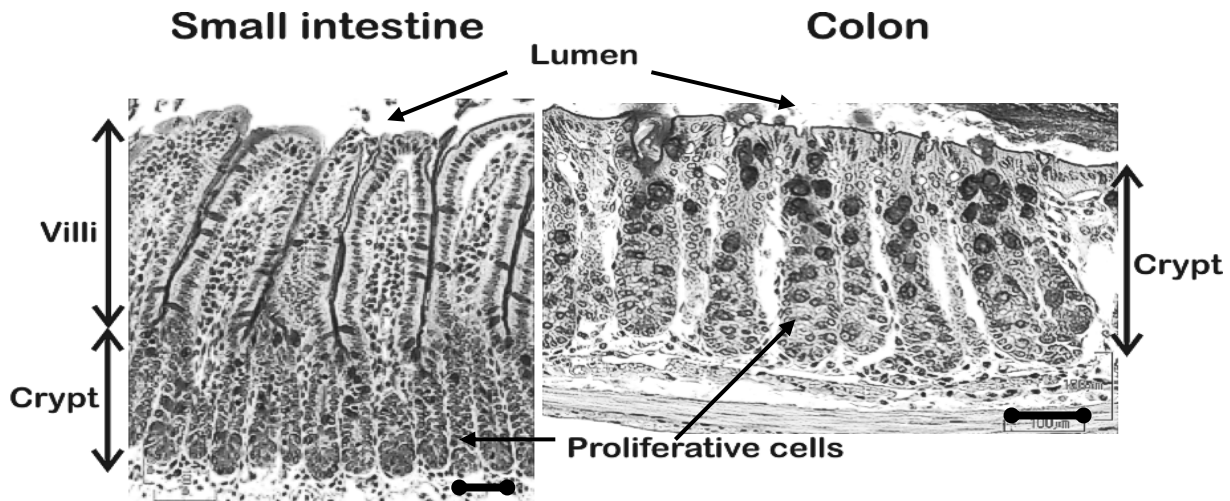


Figure 1 Histology of the mouse small intestine and colon stained with Alcian blue and PAS showing dark goblet cells. Scale bars are 100 µm.

In the colon, the enormous bacterial load constitutes the major challenge, but the mechanical forces on the epithelium also require a good mucus protection. The mucus of the colon only needs to be permeable to small molecules such as water and short chain fatty acids. The normal bacterial flora harboured in the colon lives in a mutualistic relationship with the host that has evolved over a long time. Colon serves as an anaerobic bioreactor for bacteria that are able to degrade indigestible polysaccharides, but the bacteria also gain energy from the host mucus (4;5). The colonic epithelium has crypts with stem cells, mucus-producing goblet cells and enterocytes as a sealed sterile compartment. The cells in the surface epithelium have migrated up from the crypts (Fig. 1).

The thickness of the mucus throughout the gastrointestinal tract has been measured in rats as shown in Figure 2 (6). The mucus in colon and stomach consists of both an inner layer which is firmly attached to the epithelial surface, and an overlaying thicker loosely attached mucus layer. In the small intestine on the other hand only one, removable layer is observed. The difference in organisation of the mucus layers is likely to be the result of the different demands of the tissues. The intestinal goblet cells produce and secrete the mucus which is a

mixture of molecules with different functions, but with largely unknown composition. The intestinal epithelium also secretes other types of defence molecules such as antimicrobial peptides, defensins. Immunoglobulins are also found in the intestinal lumen as secretory IgA and IgG (7;8).

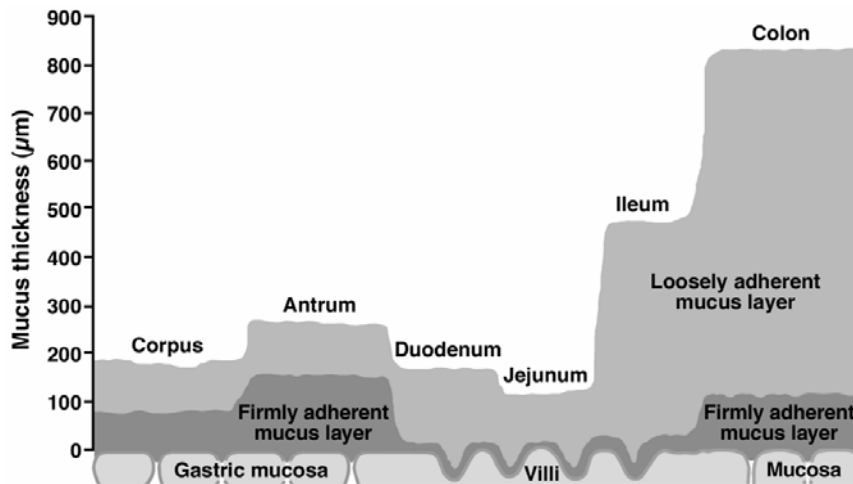


Figure 2 Mucus thickness measured throughout the gastrointestinal tract of rat (adapted from Atuma et. al. 2001) (6).

Mucins

Mucins are glycoproteins containing serine-, threonine- and proline-rich repeated sequences, so called PTS domains, serving as attachment sites for *O*-linked carbohydrates. The many glycans attached to these sequences gives the mucin domain its “bottle brush” structure. The heavy glycosylation, 50-90% of the mass, results in a protease-resistant mucin domain, an important feature for the protective properties (9). Two functionally different families of mucins have been described, the transmembrane and the gel-forming mucins (10). The transmembrane mucins are all type I transmembrane proteins differing in length, sequence and glycosylation. MUC1, MUC3A and B, MUC4, MUC12, MUC13, MUC16, MUC17 and MUC20 have been identified so far (11-19). All contain a mucin domain and most of them EGF and SEA domains (20). A majority of the transmembrane mucins are cleaved during biosynthesis, but the two products remain associated. This is best studied for MUC1 where the SEA domain is cleaved early in biosynthesis by forces generated by folding and the two parts are held together within the SEA domain (21).

In the intestine the MUC3, 12 and 17 family as well as MUC13 and MUC4 mucins are expressed (22). These molecules reach far away from the cell surface suggesting a function as sensors of the luminal milieu. The cytoplasmic tails of most transmembrane mucins could be

involved in different intracellular signalling cascades (20;23). The MUC3, 12 and 17 mucins can also interact with other molecules via scaffolding proteins using PDZ motifs in the C-terminal end of their cytoplasmic tails (24). For many of the transmembrane mucins there are also splice variants generating secreted molecules contributing to the luminal mucus (25-29).

The secreted mucins are all gel-forming, with the exception of the small secreted MUC7 mucin found in saliva (30). The gel-forming mucins form complex large gel-like structures and they are the main components of the mucus protecting the epithelial surface. There are four known gel-forming mucin genes located as a gene cluster on chromosome 11p15 (31). These four genes encode *MUC6*, *MUC2*, *MUC5AC* and *MUC5B* in this order (32-38). Another gene, *MUC19* have been identified at another chromosomal location (39) but it is probably silent in humans. The four gel-forming mucin genes are expressed in different organs, indicating tissue specific functions. Gel-forming mucin genes have been identified in many species and as many as 27 are found in *Xenopus* (1). All the gel-forming mucins are thought to assemble into polymeric molecules via their C- and N-terminal regions that contain conserved von Willebrand D (vWD) domains, rich in cysteines (40). The vWD domain organisation is a feature which is evolutionary preserved with mucin-like homologues found in a range of metazoan species (1). The main sequence differences between these mucins are found in the mucin domains which are interrupted by short cysteine-rich domains (CysD). These are only two CysD domains in the MUC2 sequence, but this domain occurs regularly in both MUC5AC and MUC5B. The CysD domain contains a conserved tryptophane motif that can be mannosylated and it shows homologies to a domain found in human cartilage intermediate layer protein (CILP) and Oikosin 1 from *Oikoplura dioica* (41). The function of this domain is not known, but the different numbers of vWD domains in the different mucins indicates different properties of the resulting mucus gel and could be coupled to the tissue specific expression. The heavy glycosylation is the common feature for the mucin domains but the sequence, which is often repeated, is not conserved between the mucin except for the abundance of serine, threonine and proline residues. The genomic organisation of the mucin domain as one long exon is also a feature common for most mucin genes. There are exceptions in the mucin domain organisation of some mucins identified in the frog, fish and chicken genomes. The mucin domain is here encoded by one exon per repeat sequence (42). This could be an evolutionary intermediate or it could have evolved to generate variable lengths of the glycosylated domain by splice variation.

MUC2 and its domain structure

The *MUC2* gene was identified in 1988 (43) and the complete sequence was known in 1994 (34). This was the second mucin sequence to be completed, an extremely difficult task regarding the long and repetitive nature of the gene. MUC2 is a gel-forming mucin homologous to the other members of this family, that also share many domains with the vonWillebrand factor (vWf) (40). The vWf is expressed in blood and is involved in blood clotting where it mediates adhesion of platelets to the connective tissue and also carries the factor VIII (44;45). The domains vWB, vWC, vWD and cysteine knot are also found in the MUC2. All the domains are cysteine-rich and the cysteine positions are well conserved between all the proteins.

The vWD-domain is found in a number of molecules including the mucins MUC2, 5AC, 5B, 6 and 19 and also in the porcine submaxillary mucin (PSM) (46). Other proteins that contain vWD-domains are Zonadhesin, Tectorin, Hemocytin, Otogelin, SCO-spondin and a partial repeat in Crossveinless 2 (47-52). The function of the vWD is not known, but a theme in many of these proteins is the domain structure D1-D2-D'-D3-XX-D4 organization (40). The D1- D2-D3 combination is found early in evolution and is already at this point associated with a PTS domain (1).

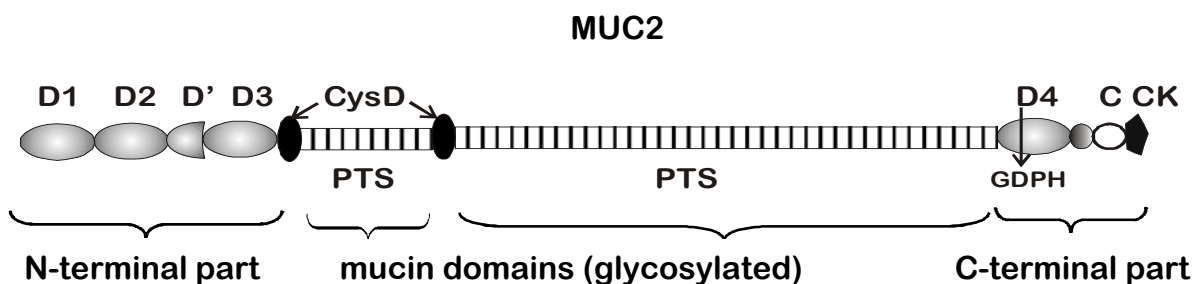


Figure 3 The domain structure of the MUC2 mucin. D, vWD with the GDPH cleavage site in D4 marked, PTS, proline, threonine and serine rich domain, CysD, cysteine rich domain, C, vWC and CK, cysteine knot.

A common pattern found in many of the vWD domains is the CXXC motif described in thiol disulphide oxidoreductases as in thioredoxin and protein disulphide isomerase (53). The multimerisation of these molecules through disulphide bonds occurring in the later secretory pathway might thus be based on an intramolecular mechanism (40). The CXXC motif in the vWD2 domain of MUC2 is however not intact which is also true for the PSM.

MUC2 mucin domain contains two CysD domains, one close to the cysteine rich N-terminal part and one inserted between the two PTS domains. A splice variant lacking the PTS domains has been identified (54). This is the only splice variant known for MUC2.

The cysteine rich C-terminal part of MUC2 contains a fourth vWD domain with the sequence GDPH that is known to undergo to autocatalytic cleavage in an acidic environment (55). The new C-terminus forms a reactive anhydride between the carboxyl group and the aspartate side chain as shown for the pre- α -inhibitor heavy chain (56). This reactive C-terminus can bind to hydroxyl groups or primary amines. This might potentially create new bonds to another MUC2 mucin or to other molecules, but specific targets have not been identified. In the C-terminal part of MUC2 there is also a vWB domain but this is not assigned as a domain in the Pfam database and has no known function. The vWC domain, also found in MUC2, is in some proteins named cysteine rich domain, but is not the CysD domain. The vWC is found in many proteins including Chordin, connective tissue growth factor, Thrombospondin 1, Procollagen II, Neuralin, Kielin and Crossveinless-2 (57). The structure of this domain is elucidated (58) and it is known to bind to the transforming growth factor- β superfamily members with extracellular regulatory functions. This makes the vWC domains in MUC2 interesting, as a site for potential protein-protein interactions. The most C-terminal domain, the cysteine knot (CK), is found in many different dimerizing proteins as the gel-forming mucins, the vWf, Norrin, NGF (Nerve growth factor), TGF- β 2 (Transforming growth factor- β 2), PDGF-BB (Platelet derived growth factor-BB) and VEGF-B (Vascular endothelial growth factor-B) (59-62). The cysteine knot domain contains 11 conserved cysteines that are involved in the formation of disulphide-linked dimers. The MUC2 domain structure is presented in Figure 3.

MUC2 biosynthesis

MUC2 is the main mucin expressed in the intestine but lower amounts of transcripts are reported in testis, prostate, trachea and stomach (63). The biosynthesis of the vWf, which is similar to MUC2, is well studied and can serve as a model for the biosynthesis of MUC2. The vWf forms dimers as an early step during biosynthesis in the endoplasmic reticulum (ER) (44;59;64). The N-glycosylation is co-translational and needed for further processing. The correctly folded dimeric vWf pass the quality control system of the ER and enters the Golgi apparatus where trimming of N-glycans and O-glycosylation takes place. This is followed by a linear N- to N-terminal dimerisation in the vWD3 domain at low pH (65-69). Two cysteines

that are especially important in the multimer formation have been identified (70). The multimeric vWf is cleaved, probably by Furin, removing the vWD1 and vWD2 domains, referred to as a propeptide (71). The propeptide controls the intermolecular disulphide bond by forming a disulphide linked intermediate with the vWD3 domain which is rearranged in the late Golgi (72). The multimers are condensed and packed into Weibel-Palade bodies which is the name of the secretory vesicles storing the vWf (73), a process that involves the associated propeptide (74). The multimers form tube-like complexes with repeating propeptides and vWD³-D3 dimers with the C-terminus looping out from the helix resulting in densely packed molecules (75).

The biosynthesis of gel-forming mucins has been thought to be processed in concordance with the vWf, but there have been observations indicating differences. The biosynthesis of PSM that share domain features with MUC2 have been studied (46). The C-terminal of PSM expressed in COS cells forms dimers within the last 90 amino acids, the CK domain (76). The multimerisation occurring in the N-terminal part do not however follow the path of the vWf instead it is suggested to be assembled into trimers when expressed as a recombinant N-terminal protein (77).

Studies of the MUC2 mucin biosynthesis in the human colonic cell lines LS174T showed dimerisation in the ER (78). The rat Muc2 C-terminal part, expressed as a fusion protein, forms dimers and the dimerisation is inhibited by removal of the CK domain (79-81). The correct processing in the ER involves the chaperones Calnexin and Calreticulin as a part of the quality control system (82). We have made a construct of the human MUC2 C-terminal part fused to the Green fluorescent protein (EGFP). Expression of this fusion protein in CHO-cells produce dimers in the endoplasmic reticulum (83). When we expressed the same construct in LS174T cells it forms heterodimers between the recombinant protein and the endogenously expressed MUC2 (83). The transport out from the ER of the MUC2 mucin is shown (LS 174T cells) to be dependent on its *N*-glycosylation (78). The same conclusions were drawn from experiments using the rat orthologue with more precise data on individual *N*-glycans playing a role in the dimerisation and not only for ER exit (79;84).

In the Golgi apparatus the *O*-glycans are attached to the hydroxyl groups of the serines and threonines in the mucin domain. The initial step is the attachment of an *N*-acetylgalactosamine (GalNAc) by a polypeptide-*N*-acetylgalactosaminyl-transferase. There are 20 such transferases known in human and they are all initiating the *O*-glycosylation but have different substrate specificities (85;86). Elongation and branching by the glycans by other glycosyltransferases then creates a variety in length, composition and structure of the

glycans presented on the mucin. This facilitates a large variation in potential binding sites. The dense glycosylation can generate multivalent binding sites something that may be important as carbohydrate-protein interactions are normally weak. In colon the level of sialylation and sulphation is high, but the inter-individual variation is surprisingly small (87).

MUC2 can form a polymer complex that becomes insoluble. The insolubility, even in chaotropic solutions as guanidinium chloride, is a property described for intestinal mucins before the MUC2 nature was known (88). During the biosynthesis another event takes place in the MUC2 mucin, the formation of a non-reducible bond (89;90). This starts shortly after initiation of *O*-glycosylation, but the molecular mechanism is not understood (89). The non-reducible and stable dimer is found in the insoluble MUC2 complex.

Other posttranslational events taking place in MUC2 are cleavages of peptide bonds. MUC2 has not been observed to cleave off the N-terminal two vWD domains corresponding to the vWf propeptide, but another furin cleavage has been reported in the far C-terminal end of rat Muc2. Here, the cleavage site RTRR, 13 amino acids from the C-terminus, is not inhibiting the dimer formation and the reason for this cleavage is not understood (81). A better characterized cleavage is the GDPH cleavage in the vWD4 domain already mentioned.

Studies of the multimerisation of MUC2 are technically difficult. The size of the glycosylated dimer is about 5 MDa and the multimers can potentially be more than 100 MDa, which is not easy to study. The size, oligomeric nature, high number of attached glycans, viscosity and insolubility together restrict the usage of available techniques.

The newly synthesised mucins are stored in regulated secretory vesicles that are released after stimulation. The large molecules need to be densely packed to fit into the vesicles. The structure of the packed molecules is not known, it could possibly be tubes as for the vWf, but the mucin vesicles do not show the cigar-shape as for the Weible-Palade bodies. The packing requires both acidic pH and a high calcium ion concentration to shield the anionic mucus matrix (91-93). The calcium ion concentration is high but might also be exchanged to potassium ions where the mucus matrix might act as an ion exchanger during secretion (94). The densely packed mucins within the granule are making up the matrix of the vesicles. This mucin matrix is at least partly immobilized, but allows some diffusion of molecules within this matrix meshwork (95;96). The biosynthesis and storage of mucins in the secretory vesicles of the goblet cells involves many more proteins. However, more knowledge is necessary to achieve a better understanding of these mechanisms.

Secretion

The goblet cell granules store the mucus until release. The mucus release process is not well understood, but recent studies of the airway goblet cells reveal several proteins involved in the secretory process. It is mediated via the second messengers diacylglycerol (DAG) and Inositol trisphosphate (IP3) that triggers Ca^{2+} release from the ER. This affects the membrane protein Synaptogamin 2, a Ca^{2+} sensor for the SNARE complex, on the mucin granules and mucin release by secretion is finally mediated via the Munc13 and Munc18 and additional proteins in the SNARE complex (97). Secretion causes hydration of the previously condensed mucin material by the binding of water to the large amounts of mucin carbohydrates and this creates the viscous gel. If the hydration is a passive or a more active and controlled process is not known. Massive mucin secretion is triggered by stimulation, but a smaller basal secretion can also be observed (98). The release can be triggered by a wide variety of stimuli. Cholinergic agonists, neuropeptides, nucleotides, hormones, immune system mediators and nitric oxide can all result in secretion (99). There are contradictory data depending on cell types, method or organs used in these experiments. Acetylcholine and histamine have been shown to cause mucus release of colonic goblet cells, but no effect was observed using peptide hormones or prostaglandin E2 (100;101). Bacterial products such as lipopolysaccharide can also evoke mucus secretion. The variable responses of the different cells indicate a complex system sensitive to different stimuli.

Colon and bacterial mutualism

The large intestine is the habitat of our microbiota made up by an enormous number of microorganisms. This is a system of mutualism where both the host and the bacteria are benefiting from the coexistence developed during evolution (4). The host gains energy from indigestible polysaccharides. The bacteria have a favorable anaerobic environment, with a large source of energy from undigested food or the carbohydrate-rich mucus (5). Bacteria can secrete enzymes to liberate the glycans that can be used, but are also able to reprogram the glycan expression in the host (102).

The microbiome of colon is diverse with *Fermicutes* as the dominating division and *Bacteriodes* that account for one fourth of the normal species (103). The composition has been revealed in recent years by the development of ribosomal 16S rRNA sequencing (104). The sequence variation between species in the amplified product is used to classify the bacteria. The bacterial composition will give some knowledge, but a functional understanding

of bacterial products is crucial to understand the host-bacterial interplay. The microbial selection is largely made by the host as shown by microbiota transplants to germ free hosts of differing species. The host selects an for them almost normal composition similar to the microbiota in conventionally raised animals (105).

The host can monitor and respond to the normal bacteria or bacterial products in a way to maintain the homeostasis. Pattern recognition receptors as the Toll-like receptors (TLR) and NOD-like receptors on both epithelial and immune cells can respond to bacteria (103). The TLR family are surface or endosome transmembrane receptors responding to lipopolysaccharide, peptidoglycan, flagellin and double stranded RNA. The responses mediate signalling through NF- κ B, resulting in transcriptional activation of genes with appropriate response elements. The TLRs signal through the common mediator MyD88 and animals deficient in MyD88 expression in both epithelial and immune cells do not develop spontaneous colitis, but show an enhanced susceptibility to induced colitis (106). Abolishing the NF- κ B activator NEMO, only in epithelial cells, results in spontaneous inflammation. The inflammation is prevented by depleting MyD88 in both epithelial and immune cells. These different effects reflect the variable responses of epithelial or immune cells. The MUC2 promotor has a NF- κ B response element and NF- κ B signalling has been suggested to regulate MUC2 expression (107). This indicates a link between the bacteria residing in colon and the mucus production.

Mucus and disease

Mucus overproduction is a problem in airway diseases like asthma, chronic bronchitis, chronic obstructive pulmonary disease and cystic fibrosis (CF) resulting in insufficient mucus clearance and persistent infections.

In the CF mouse model with a disrupted cystic fibrosis transmembrane conductance regulator gene (*Cfr*) the typical lung phenotype is not present, but the problems in the small intestine remains. These intestinal problems are due to accumulation of a viscous mucus, which retains bacteria and results in small intestinal bacterial overgrowth (SIBO) (108;109). An interplay between different mucins was demonstrated in the CF model as lack of *Muc1* reduced the small intestinal mucus accumulation (110). This mucus reduction cannot be explained by the loss of *Muc1* since it does not contribute largely to the mucus. The accumulated mucus is rather explained by *Muc2* (111). The CF animals also have slower

motility with longer exposure to the bacteria entrapped in the mucus (112). The problem can be reduced by antibiotic treatment and laxatives (112;113).

Mucus is also affected in gastrointestinal infections that cause diarrhea. The mucus is a barrier developed in symbiosis with the normal flora, but pathogens have evolved ways to invade the host and to penetrate the mucus. Mucin expression is altered in the intestine infected with pathogenic *Citrobacter rodentium* (22). A more specific pathogenic mechanism is demonstrated by *Entamoeba histolytica* that secretes a cysteine protease that disrupts the mucus gel by a specific cleavage in the C-terminal part of MUC2 (114).

Inflammation enhances the risk of developing cancer. Mucins are found upregulated in many carcinomas and can be used as diagnostic markers in several cancer forms. The mucins are used by the cancer cells as an anti-adhesive to increase their metastatic potential, as an adhesive to invade tissues, and as a way to escape the immune system (20). Aberrant glycosylation is also observed in mucins expressed by cancer cells. Glycosylation changes can be due to mislocalisation of the glycosyltransferases normally distributed according to the pH gradient in the Golgi (115).

Ulcerative colitis and colitis models

Ulcerative colitis (UC) is one of the inflammatory bowel diseases (IBD). It is a chronic relapsing inflammation with unknown etiology. The inflammation is always initiated in the distal colon and can spread along the large intestine. The disease has a genetic component, but still with low concordance in identical twins (116). Different ideas of underlying mechanisms are proposed such as a less functional mucus barrier, an impaired epithelial barrier, inappropriate immune responses, dysfunctional regulation of the immune system or dysbiosis with an altered balance of the bacterial flora (103;117). The question is if all these mechanisms are actually causing the inflammation or if they are mere results thereof. Changes in the bacterial flora is observed in UC patients (118) and an immune reaction towards the normal flora is the favoured hypothesis, as many susceptible animal models fail to develop colitis in germ free conditions (103).

Mucus serves as the first line of defence for the epithelium and a malfunctional mucus layer might lead to damage or infections. Smaller amounts of produced or secreted mucus, insufficient polymerisation, glycosylation differences or lack of other components could be key factors affecting this barrier function. The lack of Muc2 results in spontaneous colitis and intestinal tumour development in the Muc2 knock-out animal model (119);(120). The knock-

out of Muc2 showed effects on proliferation and apoptosis of the epithelium leading to tumour development. The colitis in this model is more pronounced in the distal colon with an altered morphology and elongated crypts.

In UC patients the mucin transcription is reduced or unaltered (63;121;122) and the MUC2 expression is lowered in patients with active UC, but returns to control levels in patients in remission (122). The mucus is also altered by changes in the glycosylation with reduced sulphation (123;124). The altered glycosylation can change the mucus properties and as well alter the binding sites provided for the colonic bacteria. Impaired barrier function due to changed glycosylation pattern is shown in mice deficient in the production of core 3 *O*-glycans resulting in increased susceptibility to induced colitis (125).

Other mouse strains with genetic defects in the mucosal barrier or inflammatory responses have been generated (117;126). It is interesting to note that only few of the single gene mutations develop spontaneous colitis, but many strains are more susceptible to DSS-induced colitis. Interleukin 2 (IL-2)- and IL-10-deficient mice develop colitis in response to bacteria but with different underlying mechanisms (127). IL-10-deficient mice also produce less Muc2, but the expression is transiently induced by bacteria (128).

Colitis can be chemically induced in animal models and Dextran sulphate (DSS) or trinitrobenzene sulfonic acid (TNBS) are the most commonly used animal UC models (126). The DSS model is used to study acute colitis after exposing the animal to this highly sulphated oligosaccharide administered in the drinking water for 3 to 7 days. The underlying mechanism of this colitis model is unknown. The DSS-induced colitis results in increased apoptosis and decreased proliferation of the epithelium (129).

ASPECTS ON METHODOLOGY

Proteomics

Identification of proteins in a complex mixture is important for a better understanding of which proteins are expressed but it has been technically difficult to achieve. Mass spectrometry methods have now developed and allows for the identification of peptides from numerous proteins present in the same sample. It is thus possible to use 1D-PAGE to separate proteins in the sample, followed by in-gel digestion. The instrument we used was an LTQ-FT spectrometer coupled to a 50 μm LC column, packed with C_{18} particles, separating the peptides by reversed phase chromatography. The interface to the mass spectrometer is an excellent setup developed in-house by Dr Hasse Karlsson that uses the electrospray ionization (ESI) method. The LTQ has a linear ion-trap where the peptide ions are trapped before being analyzed in the ion cyclotron. The data are converted by Fourier transformation to mass spectra with a very accurate mass determination. The selected largest ions are also further fragmented by collision induced dissociation (CID) in the ion trap and the fragment spectra recorded.

The raw MS-data is then compared to theoretically calculated peptide masses from protein sequences available in databases using the Mascot software. The searches identify peptides that are mapped to proteins and different criteria are used to remove false positive hits. The database searches in our studies were performed using the full NCBI non-redundant (NCBI-nr) database containing both full length and fragment protein sequences from all species. We used this approach with respect to the not so well annotated mouse genome. Proteins identified in other species were helpful in determining proteins denoted as unknown in mouse. The mucin genes are among the most complex ones to sequence, as they are long and repetitive. Many gaps in the genome sequences can be found in the locations of the mucin genes. This problem was partly overcome by producing an in-house mucin database containing assembled data from non-annotated sequences in the genome. The mucin genes were assembled by Dr. Tiange Lang (1). The criteria used to identify a protein in these proteomic studies were set, using the large NCBI-nr database, as one peptide identified with 97% significance and an additional peptide with 95% significance. This resulted in Mascot ion score cut off values of 47 and 40, respectively, and these cut-off settings were also used for searches in the smaller mucin database. Several proteins were sometimes found as different entries or a family of proteins can share peptide sequences, but this problem was

overcome by sorting the identified proteins by their gene name using the MGI (Mouse Genome Informatics) nomenclature clustering the entries that shared peptides.

Carnoy's fixation and FISH

Continuous mucus layers can only be observed when special care is taken during the fixation procedure. A key is to avoid water in the fixatives (130;131). Carnoy's fixative has been used to preserve the mucus and the use of methanol as the alcohol component referred to as methanol Carnoy or methacarn, was found to most efficiently preserve the mucus. Care had to be taken to avoid the spreading of luminal material during sectioning and only to use sections where these artefacts were absent.

The bacteria were detected by fluorescent *in-situ* hybridisation (FISH) using a probe complementary to a general sequence in the bacterial 16S rRNA (132) common to many bacterial species (133;134). A probe with the reversed sequence was used as a negative control. DAPI co-staining was used as a positive control to reveal DNA in bacteria.

GalNAz labelling and Click-iT chemistry

The labelling of carbohydrates during biosynthesis with an azido and acetylated monosaccharide was developed by Carolyn Bertozzi (135). Glycans will then get an incorporated modified monosaccharide that can be detected in different ways by click-it-chemistry, attaching labels *in vivo* or *in vitro* (136). The GalNAc analogue GalNAz is used by peptide-GalNAc transferases and will be incorporated in *O*-glycans via the salvage pathway in cell cultures (137). The GalNAz labelling technique can also be used *in vivo* (138). We injected GalNAz intraperitoneal and the pulse was performed for different time periods before fixation of the tissues in Carnoy's fixative. The tissue sections were then incubated with a TAMRA-coupled reagent with an alkyne group that reacted with the azide group of the GalNAz, producing a triazole conjugate, in a Cu-catalyzed reaction. The sections were also stained for MUC2 with a specific antiserum.

AIMS OF THE PROJECT

- To understand the biosynthesis of the MUC2 mucin and how it, together with other components, organizes a functional mucus.
- To understand the role of the mucus layer in the protection of colon from normal bacteria.
- To understand the role of mucus in the pathogenesis of ulcerative colitis.

RESULTS AND DISCUSSION

The N-terminal part of MUC2 forms trimers (Paper I)

Expression of the MUC2 N-terminal part

The biosynthesis of mucins is difficult to study due to their large size. The over 5000 amino acids of the MUC2 apoprotein are after dimerisation and glycan attachment reaching a molecular mass of 5 MDa before multimerisation. After multimer assembly the MUC2 is well over the limit for most available analytical methods. To study the MUC2 biosynthesis in more detail we started by designing fusion proteins of the N- and C-terminal cysteine-rich domains. These domains were assumed to be responsible for the disulphide bond-stabilised oligomerisation. The work was started in close collaboration with Drs Young Kim and Jim Gum at UCSF in San Francisco, who had at the time (1994) just published the full sequence of the MUC2 mucin (12;33;34;43). This was, with the technical means available; an enormous achievement based on many small DNA clones. These PCR products were evaluated and the longest overlapping clones were used for the assembly of the terminal constructs. The N-terminal part was made from three DNA fragments assembled with natural restriction sites and the C-terminal part was assembled from two fragments. The C-terminal part was fused to MUC2's own signal peptide including a predicted signal peptide cleavage site. When the sequences were analyzed, several mutations were identified and corrected by mutagenesis, one by one. Both constructs were placed in several vectors and an expression vector with a SV40 promoter was first used. The plasmids were transfected into COS-7 cells. This gave an extreme overproduction of the recombinant N-terminal protein with intracellular accumulation of extremely large protein aggregates, but the C-terminal protein was not expressed at all. The problems with overproduction and poor folding made us use another promoter and cell line. The absent expression of the C-terminal part was likely due to a non-functional signal sequence and therefore this was replaced. To make the two expression systems similar, the signal sequence was replaced in both constructs, with the murine Igk leader sequence. The SV40 promoter was replaced by the CMV promoter. A myc-tag for detection of the protein with a monoclonal antibody was also inserted and as a last step the EGFP sequence was added in the position where the PTS is normally located. The constructs were used to transfect CHO-K1 cells and stable clones were generated. The expression was verified by fluorescent microscopy, immunoprecipitation and western blot. The clones with

the highest expression showed enormous intracellular vesicles, at first believed to be mucin vesicles. After analysis it was concluded that the large vesicles contained poorly folded non-matured MUC2 due to saturation of the ER because of overproduction. Therefore lower-expressing clones were selected for further studies. The work with the MUC2 DNA fragments was started in 1994 and the cell lines producing the functional recombinant proteins were available in 1999.

When properly expressed the C-terminal construct produced in CHO-K1 cells showed dimer formation in the ER and secretion of glycosylated dimers. Production in the colon cell line LS174T expressing endogenous MUC2 also revealed heterodimer formation (83).

The N-terminal construct contained the first 1379 amino acids except the signal sequence. The resulting pSNMUC2-MG (plasmid-signal peptide-MUC2 N-terminal part-myc tag-EGFP) vector encodes the MUC2 vWD1-D3 domains and the first CysD followed by myc and EGFP. Metabolic labelling of CHO-K1 expressing SNMUC2-MG followed by immunoprecipitation using the myc-tag showed secretion of a large oligomer after 60 min chase. The secreted non-reduced multimer exceeded the size of the 512 kDa ApoB-100, indicating molecular forms larger than dimers. No multimeric forms were observed within the cell.

The glycosylation status of the intracellular and secreted material was determined by deglycosylation with specific enzymes. This revealed the intracellular material to be an ER retained pool and the secreted form to be *O*-glycosylated. The size difference of the secreted and intracellular material was largely explained by attached glycans. To completely remove all *O*-glycans the fusion protein was exposed to hydrogen fluoride. This resulted in a decreased size but it was still not identical to the size of the intracellular form, possibly indicating other modifications.

The trimer is formed within a trypsin-resistant core fragment

The question of the oligomeric state was tested further by trypsin degradation of the purified non-reduced SNMUC2-MG. This resulted in a resistant core fragment of 85 kDa with a non-reduced multimeric form of 240 kDa. The size difference argues for the formation of a trimer and the size shift between the reduced and non-reduced forms follows the migration of the whole SNMUC2-MG protein. Digestion of the core by other enzymes showed resistance also to Chymotrypsin, Thermolysin and Glu-C, but Pronase resulted in a smaller 60 kDa core and Subtilisin was able to degrade the protein into small peptides.

Mapping of the core fragment was first done by testing its reactivity with different antibodies. Antisera against sequences in the vWD1 (α -MUC2N1) and vWD3 (α -MUC2N3) region were used, as well as the α -myc antibody. All antibodies recognized the whole reduced SNMUC2-MG but only α -MUC2N3 could detect the tryptic core fragment. Edman sequencing revealed EAPTXPD as the N-terminus of the 85 kDa core, a sequence which is found at position 1023 of the MUC2 protein. The rest of the core was identified as peptides by MALDI-MS or ESI-MS after in-gel trypsin digestion of the 85 kDa band. The most C-terminal peptide found ended at the amino acid position 1383. The core fragment thus contained the end of the vWD3, a very small PTS stretch and the first CysD. The trimer formation was further supported when the whole SNMUC2-MG protein was separated by gel filtration and compared to protein standards. The size of the monomeric SNMUC2-MG was determined to about 260 kDa and the multimer was between 690 and 750 kDa, indicating a trimer rather than a dimer. The final proof of a trimeric structure was obtained by electron microscopy (EM) of the purified non-reduced SNMUC2-MG, where the different domains were identified by gold-labelled antibodies. From the observed pictures we concluded that the N-terminal part of MUC2 has a densely packed trefoil-like structure in the vWD3 CysD region, with another globular structure, vWD1-D2, linked to each vWD3 domain via a flexible region (Figure 4).

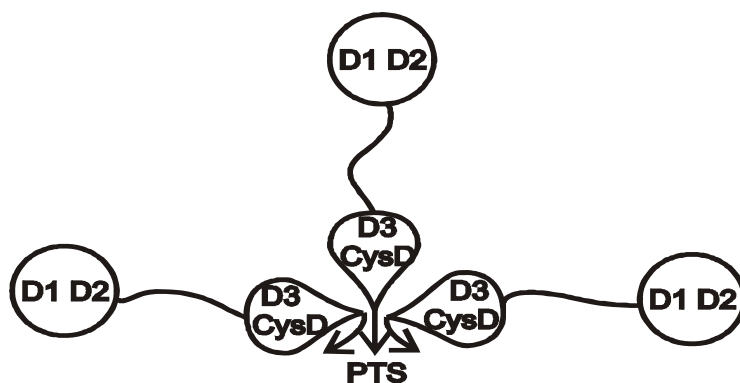


Figure 4 A model of the MUC2 N-terminal trimeric structure.

What is the functional role of trimers?

Contradictory to the common belief we have shown that MUC2 can form multimers as trimers via disulphide bonds between their N-terminal parts. The structure of MUC2 purified from a colon cell line has been visualised by EM, showing both linear and entangled

branched structures (139). This can be considered contradictory to our data which suggests a trimer but could also reflect that different oligomeric processes are possible for different mucins, or even within the same mucin expressed in different cells. MUC5B from saliva is studied by EM and is possibly assembled into linear polymers (140).

The trimeric nature of MUC2 is in concordance with the multimerisation of PSM (77). The ability to form trimers will generate a net-like structure that might be beneficial for the formation of a stratified inner mucus gel. For most of the gel-forming mucins, the multimerisation process is not fully understood. In PSM the N-terminal intermolecular bonds occur between the vWD3 domains. The CXXC motifs are postulated to be important for multimerisation and intermolecular bonds can prevent too early multimer formation, which can lead to accumulation in the ER (40;141). This is supported by experiments where only the vWD3 domain was expressed in CHO-K1, which formed trimers in the ER (Grahn et.al., unpublished). The CXXC motifs, thought to participate in the formation of the intermolecular disulphide bonds, are present in all three vWD domains of both vWf and MUC5B, but vWD2 in both MUC2 and PSM lacks this motif. The function of this motif is not understood for any of these proteins and it could therefore be speculated that it is involved in the regulation of oligomer formation. MUC5B is also cleaved by Furin (142) similar to the propeptide cleavage in the vWf, but this cleavage site is not present in MUC2 or PSM. These discrepancies indicate a possible difference in the assembly of the different mucins.

The vWf is packed in tube structures in the endothelial cell secretory granules named Weibel-Palade bodies. These vesicles have an extended cigar-shaped structure that most likely reflects the long rods of the packed vWf, making this structure optimal for this storage. In contrast, EM images of mucin granules in goblet cells have a spherical appearance, possibly indicating storage of differently organized molecules (98).

One can speculate that the net-like structure formed by the MUC2 polymer is of biological importance for the tissue distribution of this mucin. The trypsin-resistant core fragment must be taken into account in a model where protease-resistance is important to maintain the network even in a protease-rich organ as the intestine. The heavily glycosylated mucin domains are also trypsin resistant (88) and are located in close proximity to the N-terminal resistant core. The more flexible region N-terminal to the tryptic core may be more susceptible to proteolytic degradation, but the oligomeric structure of the Muc2 polymer would still be maintained even if these regions were cleaved. The C-terminal part is also important for the oligomer formation by its dimerisation in the C-terminus. The mucus oligomeric network can only be maintained if most of the C-terminal is intact. The C-

terminal cysteine-rich part can achieve this as it is stabilized by the many intramolecular disulphide bonds. Trypsin is only cleaving the C-terminal construct at two places, but the molecule is still associated by disulphide bonds (unpublished data). The resistance to digestive enzymes and ability to keep the mucin network intact in the intestine is very important for the function of mucus. In this context, it is interesting to note that pathogens have obtained enzymes that are able to dissolve the MUC2 polymer (114).

The two protective mucus layers of colon (Paper II)

The composition of the loose and firm mucus layers

The innate immune system is in focus of the discussion of intestinal homeostasis, but the first line of defence, the mucus is often neglected. A first step to understand the properties of the intestinal mucus was to analyze the mucus from a complex biological system. The main gel-forming mucin in the intestine is the Muc2 and the mucus in colon consists of two layers as measured in rats (6). The mucus closest to the epithelium is firmly attached and the outer layer is loose and non-adherent. We analyzed the the two mucus layers in mouse colon. The mucus thickness was measured *in vivo* revealing a 50 µm firmly attached layer and a 100 µm thick overlaying loose mucus.

The protein composition of both mucus layers was investigated by sensitive nano-LC-MS and MS/MS of tryptic peptides. In-gel trypsin digestion of the proteins in the loose and firm mucus was performed after separation by PAGE and composite AgPAGE. The mucus samples were supplemented with protease inhibitors, important as mucin are easily degraded resulting in fragments after reducing the disulphide bonds (143). All the obtained MS and MS/MS results were searched using the Mascot software against the NCBI nr database including all species. The identification of mucins was improved by using the in-house mucin database. The mucins identified were Muc2, Muc3(17) (human MUC17 orthologue) and Muc13. The transmembrane mucin sequences are not complete in the mouse genome with Muc12 and the orthologue to human MUC3 still missing. The expression of these can therefore not be judged. All of the identified mucins were found in both layers. The Muc3(17) peptides had higher Mascot scores in the loose mucus, possibly reflecting substantial amounts produced in the small intestine and transported distally. The main

component in both mucus layers was the Muc2, the only gel-forming mucin identified in the mouse colon.

Proteomic analysis revealed many proteins associated with the mucus. The localisation and function of the identified proteins were determined using the Uniprot database and the secreted or transmembrane proteins with large extracellular domains were selected and considered as possible mucus components. Some of the revealed proteins have previously been shown to be associated with mucus secretion. The proteins found among others were Clca3, Clca6, Fcgbp and Agr2. Clca3 is associated with calcium-activated chloride transport (144), but it cannot be a channel as it is secreted (145). It contains a vWA domain that could bind metal ions but the effect of this domain in the mucus is not known. Protein-protein interactions between hCLCA2, another member of this family, and Integrin β_4 have been suggested (146). Clca3 is associated with mucus overproduction in asthma models (147), but contradicting studies imply that this is not true (148) and its role on mucus production and secretion needs to be studied further. The Clca3 is a 110 kDa translation product that is cleaved into two subunits in the ER before secretion. The N-terminal larger molecule contains a vWA domain but the smaller C-terminal part has no known domains. There is also a connection between Clca3 and CF, with downregulated expression of Clca3 in colon and lung of Cfr knock out mice (149), and correction of the low Clca3 expression ameliorated the intestinal disorder (150). Clca6 is a transmembrane molecule of the same family, but it is expressed on the apical surface of enterocytes and co-localized with Cfr (151). The Fcgbp is also expressed within the mucus-producing goblet cells and have a domain structure containing many vWD domains (152). The function of IgG binding could be to link luminal antibodies to the mucus. Agr2 is also expressed in goblet cells (153) and the human orthologue is associated with UC (154). It contains a thioredoxin motif but whether this is functional in the mucus is uncertain. An effect in controlling disulphide bond formation in the ER have been suggested and animals lacking Agr2 expression show no Muc2 protein expression (155). Immunoglobulins and the secretory IgA receptor (Pigr) are additional components involved in the mucus defence system that were identified. The transmembrane molecules could be part of the mucus as shed extracellular domains or originate from detached cells. The overall protein composition of the mucus was found to be identical in the loose and firm mucus, arguing for a common origin. The loose overlaying layer is therefore likely to be the result of a conversion from the firmly attached mucus.

Different properties of the loose and firm mucus layers

The function of the two mucus layers covering the colon epithelium was assessed by studying their properties. The amount of Muc2 mucin was determined in the two layers and the concentration differed by a factor of four, with the higher concentration in the firm mucus. The difference in Muc2 concentration suggests a more compact inner mucus layer. Confocal microscopy of the mucus revealed a stratified inner mucus layer with not yet structured mucus underneath due to the newly secreted Muc2. The inner mucus layer was preserved by fixation of the tissue in Carnoy's fixative. A majority of the Muc2 mucus extracted from the small and large intestine is insoluble even in the chaotropic salt guanidinium chloride (GuHCl), a property formed during biosynthesis and related to the formation of a non-reducible bond (89). The GuHCl solubility of the firm and loose mucus was examined. The inner firm mucus layer was observed to contain insoluble mucus whereas the loose mucus was largely soluble. This argues for different organisation of the two layers.

Proteolytic processing of the firm and loose mucus was tested and the fragments were analyzed by western blot. Fragments from both the N- and C-terminal cysteine rich parts were found after reduction. A 120 kDa fragment corresponding to the C-terminal side of the GD/PH cleaved C-terminal part and an additional fragment of 60 kDa, were both detected in the firm mucus but, the smaller fragment was absent in the loose mucus. The disappearance of the small C-terminal fragment in the loose sample was likely due to further proteolytic degradation. From the N-terminal part, a band of 250 kDa was only observed in the firm mucus and a 200 kDa band was observed both in the loose and firm mucus. The disappearance of the 250 kDa band in the loose mucus indicated further degradation or a complete conversion to the 200 kDa band. These fragments have not been observed previously, but the cleavages were most likely natural as the samples were supplemented with protease inhibitors. These fragments were only detected after reduction and as they were from the cysteine rich terminal parts it is likely that the mucin network was still stabilised by disulphide bonds. Proteolytic processing of the mucus layers have not been addressed before and should be further investigated.

Proteolysis as a possible explanation for the conversion from firm to loose mucus was then tested. During *in vivo* mucus measurements, the firm layer was overlaid with a protease inhibitor cocktail inhibiting serine and cysteine proteases. This markedly reduced the thickness of the mucus over time compared to controls. The non-treated loose mucus contained a lower concentration of Muc2 compared to the inhibitor-treated loose layer. The

conversion from firm to loose mucus was not inhibited, but the volume expansion of the loose mucus was reduced. The lower Muc2 concentration in the loose mucus of the control can be explained by detachment and solubilisation of the mucus followed by release into the overlaying liquid. The proteases involved in these processes could be both of endogenous and bacterial origin, but studies of germ free animals revealed both a firm and a voluminous loose mucus layer. This indicates that enzymes produced by the host were responsible for most of the volume expansion. Western blot analysis of protease inhibitor treated versus the non-treated loose mucus showed differences in both the N-and C-terminal parts. The protease inhibitor treated loose mucus showed similar fragments as the firm mucus. Proteolytic enzymes were identified in the proteomics studies, but no obvious candidates for the observed processing were found.

The theory of volume expansion by proteolytic digestion was tested by incubating a GuHCl extracted mucus pellet with trypsin. The cleavages resulted in a volume expansion showing the effect of proteolysis creating more voluminous mucus as observed *in vivo*. We know from previous unpublished results that trypsin is able to digest the MUC2 in two positions, but that the fragments remain associated via disulphide bonds. Proteolytic cleavage can therefore be a way to alter the mucus properties, something that not necessarily disrupts the Muc2 network.

The inner mucus layer is impervious to bacteria

The characterisation of the colonic mucus revealed two layers with different properties, but the function of this organisation was not clear. An important function of the colon is to harbor for the host beneficial bacteria without eliciting inflammation. The function of the colonic mucus is not accounted for in many studies and was now addressed. To our surprise in the FISH analysis, using a 16S rRNA probe, the bacteria of the distal colon was completely separated from the epithelium. Counterstaining of the mucus using MUC2-specific antiserum showed that the gap between the bacteria and the cells was filled with stratified Muc2 mucus. Thus a mucus barrier is formed that is impervious to the fecal bacteria. We proposed that this is identical to the firm mucus layer. This was supported by the analysis of the loose and firm mucus by semi-quantitative PCR of two sequences in the 16S rDNA. The firm layer contained only minor amounts of bacterial DNA, probably due to technical difficulties, and the loose layer was rich in bacterial DNA. The results show that the inner layer is a protective barrier and the outer a good matrix providing a good habitat for the bacterial colonizers (5). A

spatial separation of the epithelium and the bacteria has been suggested previously in human biopsies, but never clearly shown (156;157). The distal colon has a compact mucus layer protecting the epithelium against the microflora and in addition it protects the vulnerable epithelium from mechanical forces.

The Muc2 barrier is important in homeostasis

The mucus limiting the bacterial exposure to the epithelium will play a role in homeostasis. The Muc2 knock-out model is reported to develop intestinal tumours and spontaneous colitis on some genetic backgrounds (119;120). The state of inflammation could also reflect the bacterial environment in the different animal facilities. The animals show an altered morphology with elongated crypts due to increased number of proliferative cells. The Muc2 deficient animals in our studies developed inflammation with diarrhoea, thickened intestines and rectal prolapses. Weight loss and faecal blood was also observed and infiltrating leukocytes were found by histology. Samples of luminal material did not contain any gel-forming mucin, but the goblet cells were clearly observed by immunostaining for Clca3, also without Muc2 expression. Colonic sections were probed with the 16S rRNA FISH probe to visualize the bacterial content. Massive numbers of bacteria were found in contact with the epithelial cells. Bacteria were also detected at the bottom of the long crypts and intracellular bacteria were found within some of the epithelial cells. The constant and direct exposure of bacteria to the epithelium which is normally shielded is a good explanation for the observed inflammation.

The mucus thus has protective functions in maintaining a balance for the colonic epithelium. Genetic disorders or external disruption can cause a mucus barrier failure and allow the normal flora to enter. Two examples of spontaneous mutations of the Muc2 gene resulting in inflammation have been identified (158). The mutations were located within the cysteine rich sequences in the terminal parts of the Muc2 mucin. One of the mutations was in the CXXC motif of the vWD3 which is important for multimer formation, and the other mutation was located in the vWD4 domain of the C-terminus. Both mutations result in misfolded proteins that give rise to ER stress and smaller amounts of secreted Muc2 mucin. These animals develop spontaneous colitis.

The Muc2 is covalently attached to Fcgbp (Paper III)

Mucus composition and proteins in the insoluble pellet

The total composition of the mucus, identified by proteomics, showed many intracellular components. Cells are constantly renewed in the intestine and the detached cells are intercalated in the mucus layers. The identified proteins also included secreted proteins and bacterial proteins. The majority of the bacterial proteins were found in the loose mucus, another observation in agreement with a bacteria-free firm mucus layer. Low abundant proteins, proteins with many modifications, very small molecules and trypsin resistant proteins could not be detected with the methods used. The complete protein content is difficult to achieve and this is the most detailed description at this time.

The composition of the mucus was further investigated by purification of the Muc2 mucin. The large complex network with a high degree of glycosylation creates problems and special methods have to be used. GuHCl extractions take advantage of the insoluble property of the Muc2 mucin keeping the mucus intact, but solubilize most of the other components. Extensive extractions of colonic mucus in GuHCl revealed only a few components in the mucus fraction. These proteins could be entrapped in the mucus or bound more specifically.

After purification the mucus was reduced and the proteins, released after disulphide bond disruption, were separated by gradient PAGE. This denser gel did not separate large molecules as the Muc2, but small molecules not associated with the Muc2 units after reduction, could be detected. The Muc2 and Fcgbp were observed, but this must be fragments of these molecules since the size did not match the size of the intact molecules.

The large Muc2 mucin units were, after the extensive GuHCl wash and reduction, separated on AgPAGE and tryptic fragments were analyzed by mass spectrometry. The analyses identified only a few proteins, with Muc2, Fcgbp and sodium potassium ATPase found in the duplicate analysis performed. To verify these findings, two different approaches were taken using different sources of material.

Human mucus was prepared from biopsies of control patients and purified by extensive GuHCl extractions. The samples included both the mucus and the submucosal tissue of the biopsy. Thus also extracellular matrix components as collagen were identified. In this proteomic analysis MUC2, FCGBP, Amine oxidase and Fibrillin were identified in the duplicate analyses.

Density gradient centrifugation was performed after GuHCl extraction of mouse colonic mucus as an additional purification method. This method separates material that could be entangled in the mucus and have a different density compared to the highly glycosylated mucin. The tryptic peptides analyzed by this approach identified Muc2, Fcgbp and Lactoglobulin. The latter is most likely a contaminant.

The only proteins identified in all these analyses were Muc2 and Fcgbp. The Muc2 peptides were distributed over the whole sequence. The peptide coverage was relatively low due to the trypsin resistance of the mucin domain and the many posttranslational modifications. The number of identified peptides was still high compared to many other identified proteins, due to the many potential peptides in this large molecule. Peptides from both the N- and C-terminal cysteine rich domains indicate that the whole mucin was present in the preparations.

The Fcgbp is a large protein with repetitive vWD domains (152). The mouse sequence is not complete and additional efforts were made to assemble the mouse Fcgbp protein sequence. The protein sequences were aligned to the human sequence. The newly assembled protein sequence was included in the mucin database that was used to identify the peptide coverage of Fcgbp. This protein contains 9 of the 11 vWD domains found in humans. The Fcgbp is expressed in the secretory granules of goblet cells and is postulated to bind to IgG Fc (159;160). The expression in goblet cells was verified in human biopsies by immunostaining.

The molecules removed from the purified mucus by reduction and separation by PAGE revealed many C-terminal peptides of Fcgbp. These peptides originated from bands of sizes not corresponding to the whole Fcgbp and must be fragments. In contrast, the Fcgbp peptides associated with the Muc2 separated by AgPAGE was only from the N-terminal part of the protein. The GuHCl extraction in combination with SDS denaturation and reduction leaves no other explanation to the association observed between Fcgbp and Muc2 than covalent bonds. The Fcgbp binding to Muc2 most likely occur in the N-terminal region of Fcgbp as based on the identified peptides.

Is Fcgbp a cross-linker of mucus?

The human FCGBP sequence was used as a model since its protein sequence is complete. The N- and C-termini are unique, but the central sequence is repetitive including three repeated sequences with three vWD domains each. The vWD domains have potential

autocatalytic cleavage sites in the GDPH sequence, identical to the one in vWD4 of MUC2. The GDPH sequence is found in all of the vWD domains except the last two C-terminal ones. The autocatalytic cleavage at the GDPH site is enhanced by low pH as described previously and could result in binding to both proteins and glycans. Expression of both Fcgbp and Muc2 in goblet cell vesicles, known to have a low pH, makes a covalent binding between them likely. The GDPH cleavage is supported by N-terminal peptides starting with the PH sequences found in fragments from Fcgbp in rat (161). The binding of the N-terminal Fcgbp could be explained by reaction of the GD anhydride with glycans or apoprotein of the Muc2 mucin. The GDPH sequence is not present in the two most C-terminal vWD domains and this part of the molecule will thus not be attached via this linkage. The cleaved parts are possibly held together by disulphide bonds as the C-terminal part of Fcgbp is attached even after GuHCl extractions but removed by reduction. How many of the vWD domains that are cleaved is not known, but the number of potential sites gives rise to a maximum of 11 possibilities. The disulphide bond stabilized structure of the vWD will keep the parts together and it is therefore possible to use it as an anchor with several binding sites. This could be a way to cross-link the mucus.

The vWD is only known as a domain involved in multimerisation processes. The GDPH-containing vWD domains have most likely other functions as the single vWD4 is not involved in multimerisation processes. The MUC2 forms trimers only in the N-terminal part and the CK domain forms dimers in many proteins not containing a vWD domain. Another protein containing five vWD domains is Hemolectin found in invertebrates such as *Drosophila*. Hemolectin has a clotting function and in addition it encapsulates bacteria. (162). The second vWD domain of Hemolectin contains a GDPH site, but no GDPH sequences are present in the vWf.

Turnover of mucus in the intestine (Paper IV)

Labelling of mucin O-glycans in vivo

The composition of the mucus in the intestine and its protective function is linked to the secretion and distal transport. Motility is coordinated with secretion, as nervous control is involved in both processes. In the CF mouse model, with bacterial overgrowth in the small intestine, the transit is decreased (112). The motility has been shown to be increased by

bacterial presence in the intestine (163). The bacteria thus promote clearance and the mucus production and secretion must be coordinated with the transport. To understand the production and function of the two mucus layers protecting the colonic epithelium, an analysis of the mucus turnover was crucial. Another important goal for the turnover experiments was a deeper understanding of the maturation of the two mucus layers with conversion of the firm layer into the loose.

Incorporation of a marker during biosynthesis is one way to monitor the mucus production and secretion. Radioactivity has previously been used to study Golgi function in the intestine, but not the secreted mucus (164;165). The use of *in vivo* radioactive labelling is not attractive and new methods have recently become available. Mucin *O*-glycosylation is initiated by the addition of a GalNAc to serine or threonine and the abundance of attached glycans make them good targets for mucin labelling. Acetylated *N*-azidoacetyl galactosamine (GalNAz) was injected into mice and the modified sugar was incorporated into the glycans during biosynthesis and many glycoconjugates, including mucins, were labelled. Tissue pieces from different parts of the intestine were fixed in Carnoy's fixative and the sections were reacted with a red fluorescent compound (TAMRA) by click-it-chemistry. In the first analysis the samples were fixed 5 h after the GalNAz injection (set to time 0) and the experiment was continued for an additional 2, 4, 6 and 19 h.

Mucus turnover in the small intestine

In the small intestine, goblet cells of the crypts showed stained mucus granulae, with the strongest staining in the cells at the lower part of the crypt. The MUC2 antiserum stained the mucus-filled goblet cells, but also cells at the base of the crypt indicating Muc2 expression in Paneth cells. The time series of GalNAz labelling could be used to describe the production and secretion of mucus in the small intestine as a two-step process. First the production of mucus with GalNAz incorporated occurred in cells of the proliferative crypts. Mucus filled goblet cells migrated up along the villus and the mucus was secreted during this process. The turnover of goblet cells in the ileum was estimated to be less than 19 h. Here, labelled mucus was produced in the goblet cells of the crypts and secretion at the tip of the villus was observed at the longest time monitored (19 h). In the jejunum the turnover was estimated to be twice as long, as labelled cells only migrated half way up the villus in the same time period. The process was even slower in the duodenum, most likely taking several days.

In all parts of the intestine most of the secreted mucus, immunostained for Muc2, was found between the lower villi above the crypts. The mucus observed in the lumen around the upper part of the villi was not attached or continuous as in colon.

Mucus turnover in the colon

In the colonic epithelium synthesis of the new mucus with incorporated GalNAz was visible at time 0 in goblet cells at the bottom of the crypt. In caecum and proximal colon, secretion of labelled mucus was observed already 2 h later.

In the distal colon intracellular staining was found at first in the goblet cells of the lower crypts. The GalNAz-labelled mucus was initially seen in vesicles at the outer rim of the densely packed secretory vesicle mass within the goblet cell. GalNAz incorporation in Muc2 was observed after 2 h, with a clear increase after 4 h of labelling. The number of stained vesicles increased and most of them were stained after 2-4 h. The goblet cells secreted a majority of the GalNAz-labelled mucus after 4 to 6 h. The newly secreted mucus was visible as a not yet expanded material close to the epithelium or as a layer in the middle of the stratified mucus. The mucus could be predicted to be secreted and then moved upwards, converted to the loose mucus and replenished by new mucus secreted from the goblet cells. The total turnover time in colon was a fast process with production and secretion in less than 5 h. This fast turnover could be important as it will limit the bacterial access to the epithelium.

DSS colitis is triggered by bacterial penetration of mucus (Paper V)

A dysfunctional mucus barrier in DSS-exposed colon

DSS is a glucose polymer with a high number of sulphate groups. It is used to induce colitis and is frequently used as a model for UC, but with an unknown mechanism. We now aimed to study the function of mucus in relation to this colitis model. DSS was administered in the drinking water, and short exposure times to DSS were used (4-36 h), with 5 days DSS-administration used as a positive control. The DSS was given at night when the mice were active and the DSS levels in colon were observed as an Alcian blue-stained band on AgPAGE. DSS was accumulated at 12 h, decreased at 24 h and increased again at 36 h. Carnoy-fixed samples of the colon were stained for Muc2 and bacteria were detected using

FISH or identified by DAPI-staining of DNA. A separating stratified mucus layer between the epithelium and the bacteria was present in non-treated mouse colon. The bacterial penetration scores followed the accumulation of DSS in the colon, with some penetration observed already after 4 h and a dramatic increase of bacteria close to the epithelium after 12 h. Histology of the sections, stained with Alcian blue/PAS and Hematoxylin/Eosin, was used to monitor signs of inflammation. The control tissue showed dark blue goblet cells and no signs of inflammation. The tissues exposed to DSS showed no infiltrating inflammatory cells at the earlier time points. After 36 h some infiltrating cells were found, but a dramatic difference in histology due to inflammation was not observed until after 5 days of DSS intake. The localisation of DSS was also analysed by administering FITC-conjugated DSS, showing penetration into the firm mucus after 8 and 12 h.

Explant cultures of mouse colonic tissue were used to measure the mucus thickness. The tissue was allowed to secrete mucus for 45 min and was then exposed to DSS or to Dextran. The measurements showed a reduction of the firm mucus thickness within 15 min of DSS-exposure compared to Dextran-treated controls. A similar decrease in mucus thickness after DSS exposure was observed also on human biopsies. This suggests that the effect of DSS on the firm mucus is a fast process. The penetration of DSS into the firm mucus was also visualized by confocal microscopy cross-sections of DSS-FITC treated mucus. This showed DSS-FITC to be concentrated into pores in the mucus, possibly allowing the bacteria to penetrate at these locations.

Possible mechanisms for a defective mucus barrier caused by DSS

The mucus is an important protective barrier for the epithelium to avoid contact with the massive number of bacteria in the lumen. This is achieved by a well-organized mucus layer composed of a Muc2 network and other proteins bound to or trapped in the mucus. The network is probably cross-linked by Fcgbp. DSS encounters the mucus from the luminal side and is most likely easily diffusing through the loose mucus. The high degree of sulphation is possibly the key to the ability of DSS to alter the properties of the attached mucus. The mucus in colon is highly sulphated (87;166) and other sulphated oligomers have also been reported to cause colitis, as for example carrageenan (167). The DSS-induced inflammation is most likely elicited by bacteria penetrating the mucus and getting in contact with the epithelium. The mucus is an important barrier in the distal colon as shown by the bacteria-free firm mucus layer in paper II. The importance of a mucus barrier devoid of bacteria is

exemplified by the inflammation in mice with deficient Muc2 expression. The DSS induced colitis is a well known model of UC and we have now shown that the initial step in DSS colitis is mucus alterations allowing bacterial penetration. Our combined results point to a major role for mucus defects in the development of UC.

The mucus protection model of ulcerative colitis

The mucus barrier

The studies in this thesis aimed for a better understanding of the mucus and its protective role in the colon. The observations can be used to create a model which describes the function of this mucus. Mucus is produced by the goblet cells in the epithelium and its main structural component is MUC2. The MUC2 translation product forms an *N*-glycosylated dimer with C-terminal disulphide intermolecular bonds in the ER. The Muc2 then enters the Golgi apparatus where the *N*-glycans are processed and *O*-glycans are attached. The dimers form a network by trimerisation between the N-terminal parts. This process is located in the late secretory pathway possibly after the packaging of the mucin molecules in secretory vesicles. The vesicles are localized in the apical part of the goblet cell, the theca, and the mucus can be secreted constitutively or upon stimulation. The released mucus expands in volume and spreads on the epithelial surface under the existing mucus layer. The mucus expansion can depend on ion concentration or pH, but this process is not fully understood. The secreted and expanded mucus form a structurally stratified layer with a constant addition of newly secreted mucus, creating the layer closest to the epithelium. The mucus is composed of the goblet cell vesicle content but also other secreted molecules and ions. Detached cells will also be a part of the mucus, but if the content of these have any function is not clear. The exact composition of the goblet cell vesicles is not known, but some of the components are known and were also found in this study. The main structural component of the mucus network is Muc2. Clca3, Fcgbp and Agr2 are other proteins expressed by the goblet cell that were identified in the mucus. Trefoil factor 3 is also expressed by the goblet cells and secreted (168) but was not detected, probably due technical limitations. The trefoil factors (Tff) are small molecules associated with wound repair (169). The mucus can possibly be a regulator by binding signalling molecules as for example Tff3. In the instance of lower amounts or damaged mucus these molecules could be released and are able to elicit cellular

responses. Other protective molecules are important in the mucus and of these IgA and IgG were observed. Defensins could also be part of a protective system, but were not detected in this study due to their small size.

The firm mucus is attached to the epithelial cell surface and is constantly renewed from the epithelial side. The mucus is migrating upwards and is by a still unknown mechanism, released from the attached mucus and becomes the loose mucus layer. The thickness of the firm layer is very constant, arguing for a controlled system. The loose mucus is expanding in volume by proteolysis, but the enzymes involved have not been identified. The proteolytic enzymes are most likely of host origin as a voluminous loose layer was observed also in germ free animals. Bacterial enzymes are also degrading the mucus, but they act mainly on the glycans as they are used as an energy source. Enzymes produced by bacteria that are able to cleave the Muc2 protein have not been identified so far. The mucus is renewed within a few hours also contributing to the protective function. The loose layer is where the bacteria reside and propagate. This is probably a way for the host to create a habitat for the selected beneficial bacteria. Binding of bacteria to the many potential binding sites in the loose layer could be a way to control the spatial distribution of the flora. The colon homeostasis is a combination of many factors, but bacterial separation from the epithelium by the mucus is of critical importance. However, this barrier can be broken and when bacteria pass they can elicit inflammation.

Mucus defects as a cause of UC

The results presented here bring forward the importance of the mucus barrier and that a contact between the enormous load of bacteria and an unprotected epithelium can cause inflammation. This is avoided by the dense firmly attached mucus layer that is impervious to bacteria. The most affected segment in UC patients is the distal colon, arguing for a different sensitivity at this location. In the colon only smaller molecules need to reach the epithelium and the protection can be solved by a denser mucus barrier. The water absorption from the fecal material results in more mechanical stress on the epithelium where mucus also plays a role in facilitating this transport. The mucus provides a first line of defence in a more active way than previously believed arguing for a role in the innate immunity. An impaired mucus barrier results in inflammation, as shown in the Muc2^{-/-} mice (paper II), in Muc2 mutated mice (158) or in the DSS colitis model (paper V). Defects in the mucus barrier are therefore a reasonable explanation for the UC inflammation, although UC patients are likely a

heterogeneous group. Therapeutic agents improving mucus properties could be beneficial, if not to cure the disease, but to at least stabilize the remission state of the disease.

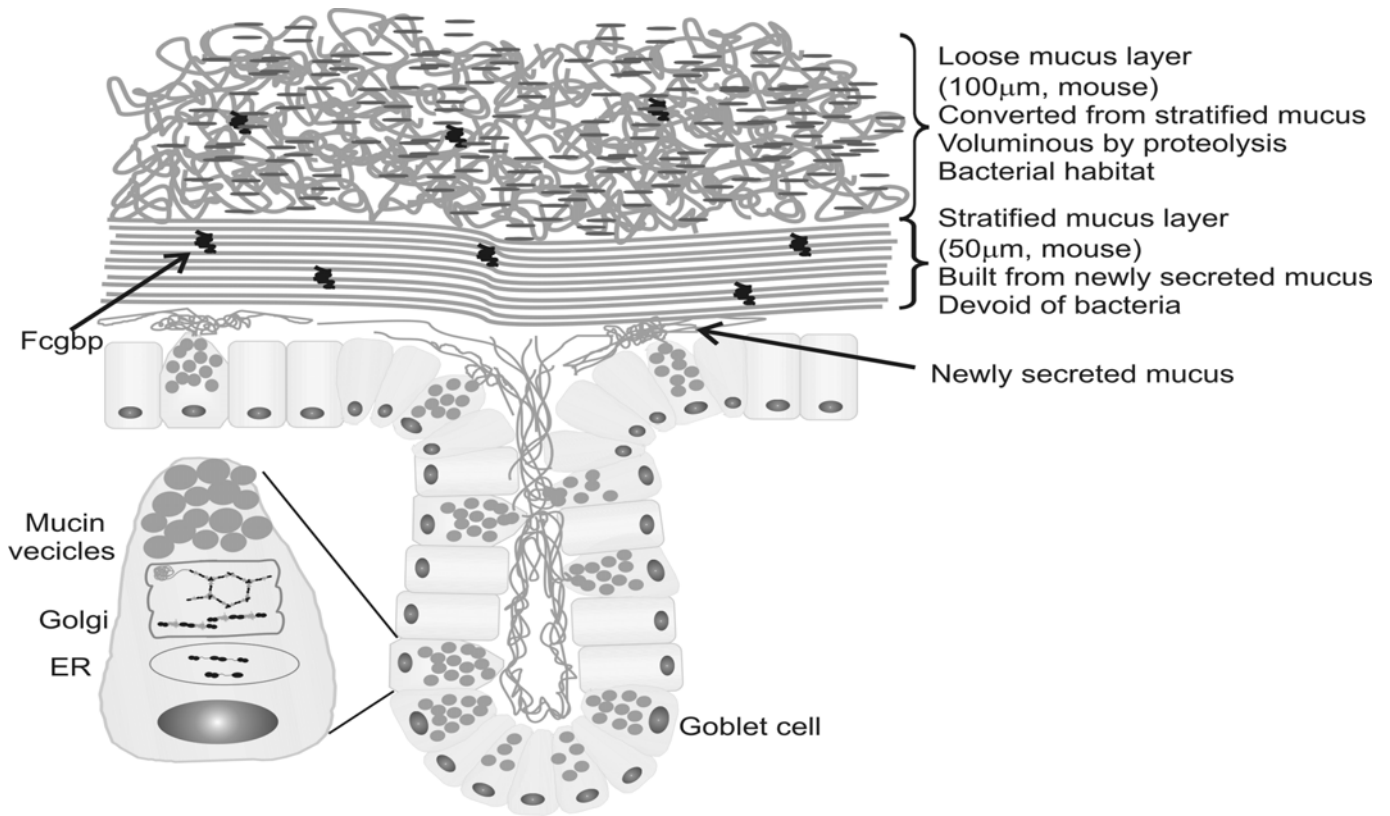


Figure 5 The mucus in colon is built around a Muc2 network of C-terminal dimers and N-terminal trimers. The mucus contains other proteins associated with the mucus, including the Fcgbp which is covalently bound to Muc2. The mucus is secreted from the goblet cells, expanded in volume and creates a structurally stratified mucus layer. This layer is attached to the epithelium and is impervious to bacteria. The firm mucus layer is converted to the outer more voluminous loose layer. In the loose mucus the bacteria (—) of the normal flora are found.

CONCLUSIONS

- The MUC2 multimers are formed by trimer formation via its N-termini in a trypsin resistant core.
- The mucus in colon is built up from the newly secreted material, producing a firmly attached mucus layer with Muc2 as the main structural component. This inner mucus layer is compact and insoluble and is converted to an outer loose mucus layer expanded in volume by proteolysis.
- The mucus composition is similar in the two mucus layers indicating a common source.
- The inner mucus layer is a protective barrier devoid of bacteria. The outer layer is the bacterial habitat.
- In mice lacking the Muc2 mucin, developing colitis, the normal flora is in contact with the epithelium and penetrates the crypts as well as the cells.
- Fcgbp is covalently attached to the Muc2 mucin.
- The mucus turnover in colon is fast and the turnover of goblet cells is within 5 h and the firm mucus layer is renewed within a few hours.
- Colitis induced by DSS is initiated by the mucus alterations that allow bacteria to penetrate the mucus layer.
- Defects of the inner protective mucus layer may be a pathogenetic mechanism to ulcerative colitis.

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