Mucus Associated Proteins and their Functional Role in the Distal Intestine

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Cover illustration: Mouse colon section stained for Muc2 and Zg16

Till min kära familj

Abstract

The mammalian intestine, especially the large intestine, harbors complex societies of beneficial bacteria coexisting with the host. This is a mutualistic relationship, where the host provides nutrients and a favorable environment while the bacteria in return ferment indigestible polysaccharides to short chain fatty acids. The host needs to keep the bacteria at a safe distance from the intestinal cells to prevent disease. The first line of defense hindering these microorganisms from invading the underlying epithelium is a mucus layer built by the highly polymeric and heavily *O*-glycosylated MUC2 mucin, the main structural component. In the colon this mucus barrier is made up of two layers with similar composition. The outer layer is loose and easy removable while the inner is more dense and firmly adherent to the underlying epithelium. The inner layer is impermeable to bacteria and therefore separates the bacteria that reside in the lumen from the epithelial cells. In order to obtain a better understanding of the function and structure of this dynamic barrier we analyzed the mucus using proteomics based approaches to identify novel mucus components

The focus of this thesis has been trying to understand the specific protective role of the MUC2 assosiated proteins in the mucus layer. Three proteins were chosen for further studies based on their abundance and production by the mucus secreting goblet cell.

AGR2 belongs to the protein disulfide isomerase family, and has been proven important for proper MUC2 production. Using molecular biology tools and cell culture experiment it was shown that AGR2 does not covalently bind the MUC2 terminal recombinant proteins and that secretion of the molecule is dependent on an internal cysteine residue.

The mucin-like protein FCGBP is a highly repetitive molecule that contains 13 von Willebrand D domains. Eleven of these contain an autocatalytic cleavage site that forms a new reactive C-terminus after cleavage, which occurs early during biosynthesis.

ZG16 is a lectin-like molecule that has now been shown to bind peptidoglycan, the major bacterial cell wall component, via its carbohydrate recognition domain. It was shown that ZG16 is not bactericidal, but that it binds and aggregates Gram-positive bacteria and translocate them further out in the mucus. ZG16 is also able to bind to enterocytes via a protein receptor implying a novel sensory function.

In summary, the results from this thesis demonstrate that these MUC2 associated proteins are important to form a functional protective mucus layer that prevents bacteria to reach the epithelium and by this cause disease.

Keywords: intestine, mucus, bacteria, MUC2, AGR2, FCGBP, ZG16

Papers in this thesis

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

I. Rodríguez-Piñeiro AM, Bergström JH, Ermund A, Gustafsson JK, Schütte A, Johansson ME, Hansson GC. (2013) **Studies of mucus in mouse stomach, small intestine, and colon. II. Gastrointestinal mucus proteome reveals Muc2 and Muc5ac accompanied by a set of core proteins**. Am J Physiol Gastrointest Liver Physiol. 1;305(5):G348-56.

II. Bergström JH, Berg KA, Rodríguez-Piñeiro AM, Stecher B, Johansson ME, Hansson GC. (2014) **AGR2, an Endoplasmic Reticulum Protein, Is Secreted into the Gastrointestinal Mucus**. PLoS One. 11;9(8):e104186.

III. Bergström JH, van der Post S, Johansson ME, Hansson GC, Bäckström M. **The vWD domains in the mucus associated protein FCGBP is cleaved during early biosynthesis**. *Manuscript*

IV. Bergström JH, Gustafsson IJ, Johansson ME, Hansson GC. **ZG16 is secreted by goblet cells and bind enterocytes**. *Manuscript*

V. Bergström JH, Birchenough GM, Katona G, Schütte A, Ermund A, Johansson ME, Hansson GC. **Gram-positive bacteria are held at a distance in the colon mucus by the lectin-like protein ZG16**. *Manuscript*

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Abbreviations

AGR2	Anterior gradient 2 protein
B-EDA	Biotinylated ethylenediamine
CD	Crohn's disease
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane regulator
CK	Cystine knot
DC	Dendritic cell
DSS	Dextran sodium sulfate
DTT	Dithiothreitol
ER	Endoplasmatic reticulum
FCGBP	IgGFc-binding protein
GlcNac	N-Acetylglucosamine
hBD	Human β-defensin
HNP	Human neutrophil peptide
IBD	Inflammatory bowel disease
ILF	Isolated lymphoid follicle
IEL	Intraepithelial lymphocyte
LRP1	Low density lipoprotein receptor 1
LRP2	Low density lipoprotein receptor 2
LPS	Lipopolysaccharides
LTA	Lipoteichoic acid
PDI	Protein disulfide isomerase
PGN	Peptidoglycan
RegIIIα	Regenerating islet-derived protein 3-alpha
sPGN	Solubilized peptidoglycan
TLR	Toll-like receptor
MLN	Mesenteric lymph nodes
MUC	Mucin
MurNac	N-Acetylmuramic acid
UC	Ulcerative colitis
vWD	von Willebrand D
WT	Wild type
ZG16	Zymogen granule protein 16

Introduction

Intestinal epithelium

The small intestinal epithelium is composed of a monolayer of cells that form invaginations named crypts and villi protruding into the lumen. At the base of the crypts reside the stem cells responsible for the maintenance and quick renewal of the epithelium (1). The stem cells proliferate and differentiate to the cell types that give rise to the epithelium as they move upwards to the villus tip, eventually undergoing apoptosis and shedding into the lumen after a period of about four days (2). Four different cell types are usually considered to make up the intestinal epithelium, enterocytes, goblet cells, enteroendocrine cells and Paneth cells. The dominating cell type is the enterocyte, responsible for nutrient uptake. Goblet cells are secretory cells scattered throughout the epithelium and produce mucus. Enteroendocrine cells produce hormones and the long lived Paneth cells, found in the bottom of the crypts produce antimicrobial peptides. There are, however, at least three additional cell types observed, M-cells, cup cells and tuft cells. M-cells are found on the mucosal lymphoid follicles and function as an interface between the lumen and the underlying immune cells (3). The wine glass shaped cup cells are fairly abundant, but their function is yet to be deduced (4). Also the role of the tuft cell has been an enigma, however the recent identification of tuft cell specific markers will allow further characterization of this cell type (5). The architecture of the colon is similar to the small intestine except for the lack of villi, and Paneth cells and the more numerous goblet cells.

Mucus layer

The human body uses barriers as the first line of defense to withstand the constant external threats of bacteria, parasites, chemical agents and mechanical stress. The outer surface of the human body, with an area of almost 2 m_2, is lined with a thick layer of dead keratinized cells. The gastrointestinal tract, with a newly revised surface area of 32 m^2, is composed of a single layer of tightly connected epithelial cells (6). The essential gastrointestinal functions of epithelial-, secretion and absorption do not allow the intestine to be shielded by an impermeable layer of dead cells. The cells do however need some kind of protection from the harsh environment in the intestine. The potential threats are not limited to ingested exogenous materials, but also endogenously secreted molecules such as hydrochloric acid, digestive enzymes and bile salt could destroy the epithelium if not protected. The barrier at these sites, often neglected due to a lack of understanding, is instead made up of a viscous mucus layer that covers the epithelium (7). The main structural components of mucus are polymeric and heavily glycosylated glycoproteins called mucins. Due to the hydrophilic nature of the attached glycans, mucins are able to bind ample amounts of water giving mucus its gel-like properties. The barrier is further reinforced by

immunoglobulins, especially secretory IgA, and antimicrobial peptides secreted from the epithelium into the mucus (8, 9). The mucus layer is constantly renewed and moved outwards towards the lumen in order to keep threats away from the epithelium, in the colon the time between translation and secretion is about 5 hours (10). The use of mucins to protect the epithelium is not new, and gel-forming mucins can be traced all the way to early metazoan (11). The properties and thicknesses of the gastrointestinal mucus layer vary along the gastrointestinal tract reflecting the physiology of the separate regions (12, 13). Measurements of the intestinal mucus barrier in live anesthetized rats show a continuous mucus from the stomach to the colon with a thickness that ranges between 200 and 800 µm (7). In the stomach, with its high acid concentration, the mucus has two layers with a dense inner layer attached to the underlying cells. Here the mucus acts as a diffusion barrier creating a proton gradient from the highly acidic stomach lumen towards the gastric mucosa (14). In the small intestine, where nutrient uptake is the main function, the mucus is not attached and can be easily removed (12). Constant secretion of mucus, water and antimicrobial peptides together with peristaltic movement traps potential harmful components and moves them away from the epithelium and transports them distally (15).The main function of the distal part of the intestine is dehydration and storage of fecal material. The mucus layer in the colon is two layered with a dense attached inner layer impermeable to the large bacterial community that resides in the outer mucus (16).

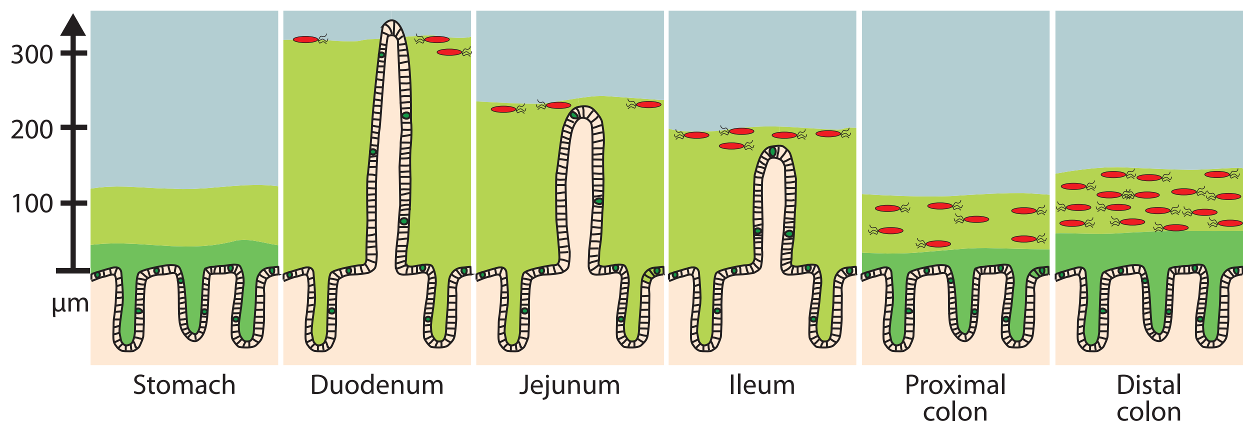
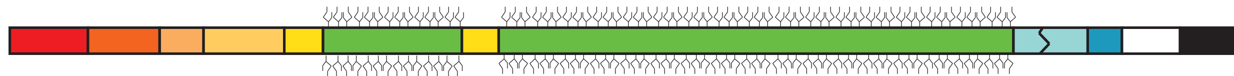


Figure 1: Schematic representation of the gastrointestinal mucus system. Dark green represent inner mucus and light green outer mucus. Bacteria depicted in red. Axis to the left shows the thickness of the mucus as measured in mice. Figure adopted from (17).

Mucins

Mucins are a divergent group of glycoproteins with high sequence similarity including the PTS domains which contains a high proportion of proline, threonine and serine amino acids often arranged in tandem repeats (18). The hydroxyl groups of these threonine and serine residues act as attachment sites for a large number of *O*-linked glycans. When the glycans are attached, these domains become what are called mucin domains and the molecular mass is increased ten-fold and give the mucin domains an

outstretched, rigid conformation much like a bottle brush. The heavy glycosylation shields the protein backbone making the mucins resistant to acid and the pancreatic digestive enzymes (19). The large mucin family can be divided into two subfamilies; the membrane bound mucins (MUC1, MUC3, MUC4, MUC12, MUC13, MUC16, MUC17, MUC20 and MUC21) (20-29), and the secreted mucins (MUC2, MUC5AC, MUC5B, MUC6, and MUC7) (30-36).



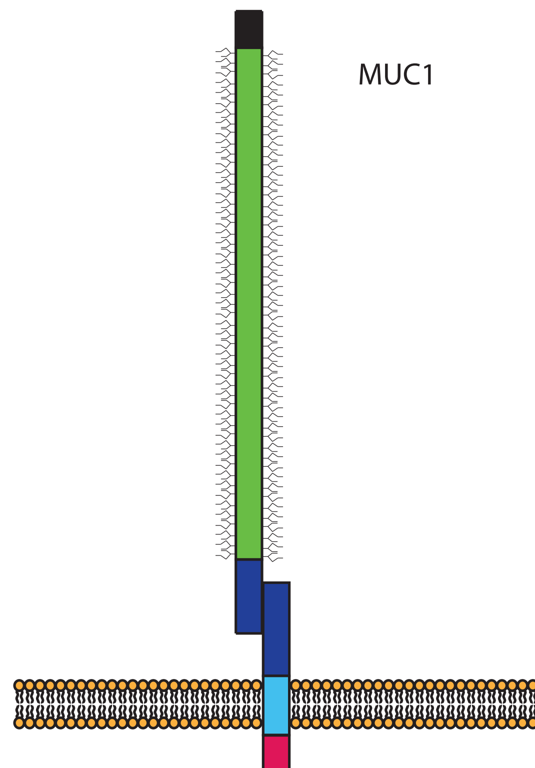


Figure 2: Schematic representation of mucins and their domain structure. Gel-forming mucins are represented with MUC2 and transmembrane mucins with MUC1.

Membrane bound mucins

The membrane tethered mucins are all type 1 transmembrane proteins, and they all share the same domain structure while the length, sequence and glycosylation pattern differs. The three main components are the large highly glycosylated extracellular mucin domain, a transmembrane domain and a cytoplasmic domain (37). They are generally found attached to the apical side of polarized epithelial cells where they form the glycocalyx, but can after proteolytic cleavage or alternative splicing be secreted from the cell. Most transmembrane mucins (MUC1, MUC3, MUC12,

MUC13, MUC16 and MUC17) harbor an extracellular domain called SEA (sea urchin-enterokinase-agrin) which is cleaved during folding by an autocatalytic mechanism (38, 39). The two resulting peptides are however held together by noncovalent bonds when the proteins are presented on the cell surface. The force required to pull the SEA domain apart is lower than the force necessary for pulling the protein out of the cell membrane. This mechanism has been hypothesized to be a cell protective mechanism to hinder cell membrane rupture if the cell is exposed to extensive force (40). MUC4 contains NIDO-AMOP-vWD domains intead of the SEA domain and these domains are cleaved in the GDPH sequence within the vWD domain (41). As constituents of the glycocalyx, the transmembrane mucins may also limit bacterial invasion. Increased susceptibility to enteroinvasive *Escherichi coli* was observed after reduction of endogenous MUC17 expression in intestinal epithelial cell cultures and MUC1 shows a protective effect against *Helicobacter pylori* infection in the stomach (42, 43). Apart from this protective role, membrane bound mucins are hypothesized to act as receptors and sensors of the extracellular environment, sending signals through their cytoplasmic tail to the cell nucleus resulting in gene transcriptional changes (44, 45). The transmembrane mucins expressed in the intestine are MUC1, MUC3, MUC4, MUC12, MUC13 and MUC17 (46, 47).

Secreted mucins

The secreted mucins are gel-forming, with the exception of the small monomeric MUC7 mucin found in the saliva (30). Gel-forming mucins are the main components of the mucus layer that covers the mucosal surfaces of the gastrointestinal, urogenital and respiratory tracts. The genes of the human gel-forming mucins are, with the exception of MUC19, clustered on chromosome 11p15.5 (48). MUC19 is instead found on chromosome 12q12, and although it is not expressed in humans, it is expressed in pigs, horses, cow and rat (31, 49). There are large similarities in the sequence as well as in the domain structure of the gel-forming mucins (18). All harbor a central assembly with one or more mucin domains heavily decorated with complex *O*-glycans making the molecule outstretched and rigid. The mucin domain is flanked by N- and C-terminal cysteine rich domains involved in polymerization. These domains are shared with von Willebrand factor, involved in blood clotting, and are designated von Willebrand D (vWD) domains. All gel-forming mucins contain three full and one truncated N-terminal vWD domains, or more correctly denoted vWD assemblies (50), an additional C-terminal vWD domain is also found in MUC5AC, MUC5B and MUC2. The far C-terminal also contains an additional cysteine rich domain designated cysteine-knot (CK). All secreted mucins except MUC6, contain various numbers of small CysD domains which are interspersed in the mucin domain and able to form non-covalent dimers held together by strong hydrophobic forces (51). Despite the high similarity, the small differences observed in the sequences of members of the group may be adaptations to the specific needs of different mucosal surfaces. The gel-forming mucins expressed in the gastro-intestinal tract are MUC2

(small and large intestine), MUC5AC (stomach), MUC5B (mouth) and MUC6 (stomach and small intestine) (52-55).

Biosynthesis of MUC2

Due to the large size and complexity of the mucin family most of the knowledge of the biosynthesis of these molecules has come from studies were specific domains have been expressed and observed. Up to date, no full length gel-forming mucin has been produced recombinantly. In the intestine, specialized secretory cells called goblet cells produce these complex molecules. The main mucus protein in the intestine is the MUC2 mucin. The human MUC2 apoprotein contain 5,179 amino acids, giving a theoretical mass of 500 kDa. In the ER the molecule undergoes *N*-glycosylation, folding and dimerization through the C-terminal CK domain, increasing the mass to $>$ 1 MDa (56-59). In the *cis*-Golgi the protein encounters a number of polypeptide-*N*-acetylgalactosaminyl-transferases that initiate *O*-glycosylation by the attachment of *N*-acetylgalactosamine to the hydroxyl groups of serines and threonines (60). As the protein is transported through the Golgi, additional glycosyltransferases elongate and branch the glycan chains creating complex structures that vary in length, composition and structure (61). The glycosylated dimer now has a molecular mass of about 5 MDa. In the *trans*-Golgi the protein is sorted to the secretory pathway and the vWD3 domain in the N-terminal forms trimers through disulfide bonding resulting in large polymers with a potential mass of $\geq$100 MDa (62, 63). Another characteristic of the MUC2 mucin is its insolubility in chaotropic salts like guanidinium chloride (64, 65). The insolubility occurs in conjunction with a formation of a non-reducible bond of up to date unknown character (66). The MUC2 mucin is cleaved by an autocatalytic process in the GDPH sequence of the C-terminal vWD4 domain (67). The cleavage is enhanced by the low pH found in the later secretory pathway and after cleavage the protein is still held together by a disulfide bond spanning the cleavage site. The role of this cleavage is not known but the generation of a reactive anhydride in the newly formed C-terminal allows additional crosslinking. The N-terminal contains the information that directs the MUC2 polymer to the regulated secretory granule of the goblet cell where it is subsequently densely packed (68). The condensation of the molecule in the secretory granules is Ca^{2+}-dependent, Verdugo *et al*. proposed that calcium was necessary to shield the negative charges on the attached glycans to allow packing (69). However, this is not correct, as it was observed that the N-terminal of MUC2 form large aggregates of concatenated rings in the presence of Ca_{2+} and low pH suggest that the packing is highly organized (68). With relatively flat concatenated N-terminal rings as a base, the mucins extend perpendicular and are connected at the other end by the dimerized C-termini. The 3D reconstruction of the MUC2 N-terminal suggests that the rod-like condensed polymers are further packed end to end in the mucin granules (63). Upon secretion the Ca^{2+}-ions are removed and the pH is raised by sodium bicarbonate provided by the cystic fibrosis transmembrane regulator (CFTR) (70). During exocytosis the packed mucin polymer is unfolded

and quickly expands by at least a 1,000-fold (71). This model of packing mucins will, after unfolding, yield a large sheet which is in accordance with the stratified appearance of the inner mucus layer. The large sheets can then be packed on top of each other and held together by hydrophobic interactions via the CysD domains (51). The various number of CysD domains interspersed in the mucin domain could therefore regulate the pore size of the mucin network.

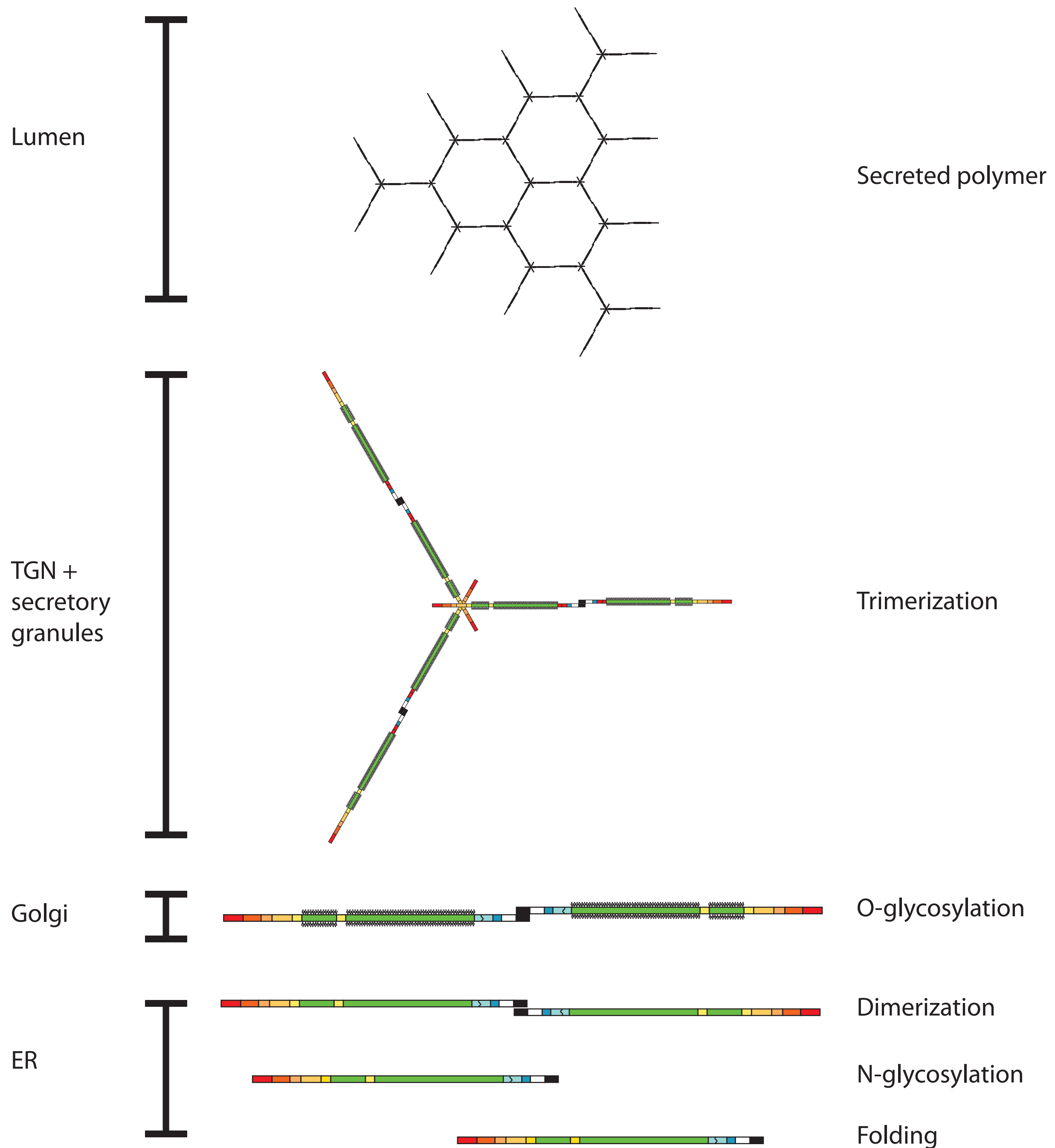


Figure 3: Schematic representation of the biosynthesis of MUC2.

Intestinal microbiota

The fetus is considered sterile and colonization begins during the passage through the birth canal as the newborn is exposed to microorganisms from the mother and surroundings (72). The primary colonizers of the gastrointestinal tract are facultative anaerobic bacteria due to the oxidative environment in the newborn gut. Aerobic respiration of the primary colonizers causes the oxygen levels in the intestine to drop and allows successive colonization by anaerobic microorganisms. Intestinal microbiota composition during the first years of life is relative simple and fluctuates over time and between individuals (73, 74). After three years of age the microbiota is fairly stable and resembles the adult state. Little is known about how the host selects intestinal gut microbes, but the transfer of zebra fish commensals to germ free mice resulted in a mouse-like microbiota suggesting that the mouse is able to select specified bacteria (75). One mechanism that shapes the intestinal flora is the presence of glycans, both ingested and bound to mucins, that bacteria can utilize (76). Approximately 100 trillion bacteria coexist with the host in the human adult intestine, which is ten times the total amount of somatic and germ cells in the body (77). Bacterial counts increase along the gastro-intestinal tract with the highest density in the distal colon (78) (Figure 4). This microbial community is diverse and complex and contains an estimated 1,000 different bacterial species. Each individual has at least 160 species, of which many are shared with other humans (79). Even though the microbial community in the intestine is mainly bacteria, it also includes archea, viruses, fungi and protozoa. The gut microbes are beneficial for the host by degrading ingested polysaccharides and endogenous mucins to short fatty acids, but

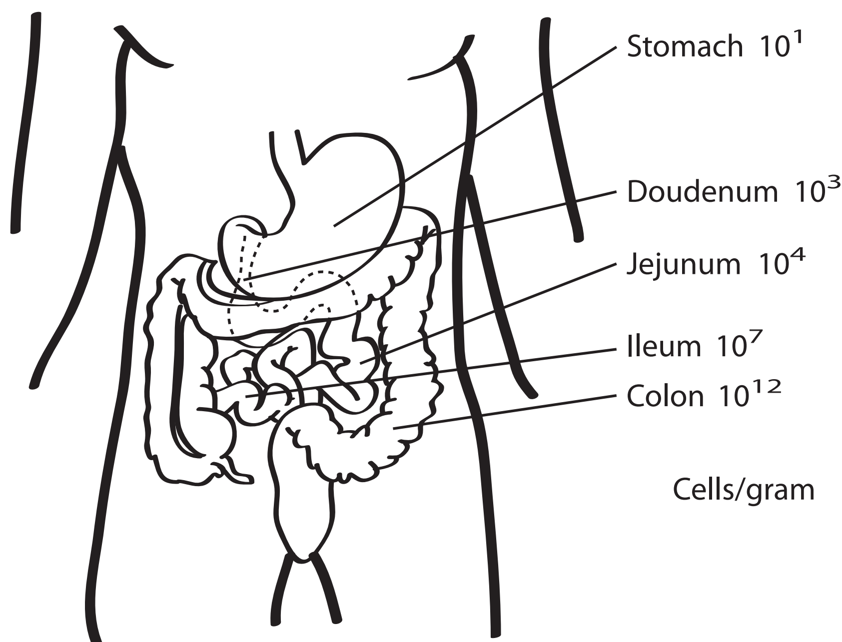


Figure 4: Representation of the bacterial load along the gastrointestinal axes.

are also a potential threat if not kept at a safe distance from the epithelium. This spatial separation is due to the inner firm mucus layer that the bacteria are not able to transverse (80). The recent development of 16S ribosomal RNA sequencing has revolutionized the understanding of the intestinal microbiota due to the difficulties to culture these bacteria. In vertebrates, Firmicutes and Bacteroidetes are the dominating phyla, but members of the Proteobacteria, Verrummicrobia, Actinobacteria, Fusobacteria and Cyanobacteria phyla are also present (81, 82). The bacteria are now considered to be in a mutualistic relationship with the host meaning that both partners experience advantages. The host provides a protected environment and an unlimited source of energy in the form of glycans from ingested food attached to mucins (77). The bacteria provide the host with protection against pathogens and short-chain fatty acids. The microbial community has a large impact on host physiology, where altered composition is implicated in several disease states such as IBD, colon cancer and obesity (83-85).

The mucosal immune system

The main reason for the immune tolerance to the large amount of microbiota observed in the intestine is the spatial separation to the host created by the dense mucus layer. This separation limits the antigenic pressure on the epithelium therefore avoiding the initiation of an aberrant immune reaction. However, alterations in the symbiosis between the host and the bacteria may result in disease making microbiota control essential to maintain homeostasis. The microbiota is monitored both by the innate-, as well as the adaptive immune system. The epithelium was for long considered a silent barrier, however, there is growing evidence of epithelial cells modulating the immune response (86). The enterocytes express pattern recognition receptors that include the structurally homologous toll-like receptors (TLR) and intracellular NOD-like receptors. The pattern recognition receptors recognize a range of conserved bacterial, fungal and viral structures (87). The NF-κB pathway is found downstream of many pattern recognition receptors, activation of NF-κB triggers the expression of genes that modulate the immune cells in an anti-inflammatory mode, including the expression of antimicrobial proteins and immune regulatory cytokines. Interestingly, the MUC2 promoter has an NF-κB response element and is thought to be activated via this pathway (88). The combined effect of mucus and antimicrobial secretion further reduces the bacterial load close to the epithelium. Also, secretion of secretory IgA from B cells, which is then transported through the epithelium, reinforces the barrier and reduces bacterial numbers (89). Antigen uptake responsible for IgA production is thought to be delivered to dendritic cells (DCs) in Peyer's patches and isolated lymphoid follicles (ILFs) by M-cells that continuously sample the luminal bacteria. (90). The traditional view of antigen uptake, presentation and activation of T cells outside Peyer's patches and ILFs is by lamina propria DCs that extend dendrites between the epithelial cells and samples luminal antigens (91, 92). The DCs should then carry the sampled molecular information to the naïve T cells in

the lymph nodes. However, recent observations show that goblet cells can take up luminal material and deliver these to the lamina propria DCs by the formation of goblet cell-associated antigen passages (93, 94). Given the sheer number of intestinal bacteria, it is inevitable that some will breach the epithelial barrier and gain access to the lamina propria. They will encounter a second line of defense made up by resident macrophages that phagocytose bacteria without triggering a strong immune response and thereby limit tissue inflammation (95). Also important in maintaining intestinal homeostasis is another population of immune cells, intraepithelial lymphocytes (IELs). IELs have been shown to be important in repair mechanisms of the intestinal epithelium by the production of keratinocyte growth factor and limiting bacterial translocation (96). In the case of invasion of pathogenic bacteria in the intestinal tissue, a well-coordinated and tightly regulated immune response of the adaptive immune system may also be triggered. Antigen loaded DCs migrate to the mesenteric lymph node and prime naïve T cells to yield Th1 and Th17 effector cells (97). Th2 effector cells are normally absent in healthy individuals free of parasitic infections and therefore not involved in regular bacterial infections. The effector cells will then home to the gut lamina propria at the location of the pathogen via the systemic circulation. At the site of action, the Th1 cells secrete interferon-γ that will enhance macrophage activation and activated macrophages and DCs will in return secrete IL-12 that has a positive feedback on the interferon-γ expression (98). Th17 cells secrete IL-22 that promotes the production of antimicrobials and IL-17 which then promotes neutrophil recruitment and activation. In order to limit the development of inflammatory tissue damage, the immune response is regulated and suppressed by regulatory T cells.

Anti-microbial proteins

The secretion of antimicrobial proteins by gut epithelial cells limits bacteria–epithelial cell contact. Antibacterial proteins in the intestine are members of a range of diverse protein families, including defensins, cathelicidins and C-type lectins. Defensins are small, cationic peptides containing disulfide bonds necessary for damaging the bacterial cell wall leading to bacterial death (99). They are further classified as α- and β-defensins dependent on the position of the disulfide bonds. Of the six α-defensins in humans four are produced by neutrophils and named human neutrophil peptides (HNPs 1-4) while the remaining two are produced by Paneth cells and designated human α-defensins (HD-5 and HD-6). The six human β-defensins (hBDs) are expressed by many types of epithelial cells, including enterocytes (100). Apart from the antimicrobial effects directed towards both Gram-negative and Gram-positive bacteria, defensins can also show chemotactic activity (101). LL-37 is the only cathelicidin in humans, and expression is localized to the surface epithelum and upper parts of the crypts in the colon (102). Similarly to the defensins, LL-37 possesses activity to both Gram-negative and Gram-positive bacteria that is driven by electrostatic interactions leading to membrane pore formation and lysis.

(103). C-type lectins are proteins that consist of a carbohydrate binding domain. In humans the main lectin is the regenerating islet-derived protein 3-alpha (RegIIIα), the human ortholog of mouse RegIIIγ (104). The lectin exerts antimicrobial action via binding to peptidoglycan on Gram-positive bacteria. In MyD88$_{-/-}$ mice, where an adaptor protein needed for TLR signaling is absent, the C-type lectin RegIIIγ is not expressed indicating that TLR signaling is needed for expression of this lectin (105). In addition, the Paneth cells express enzymes with antimicrobial properties such as lysozyme and the angiogenin ANG4. Lysozyme is a glycosidase and exerts its antimicrobial effect by cleaving peptidoglycan on Gram-positive bacteria making the bacteria more susceptible to lysis (106). ANG4 belongs to the RNase superfamily and is secreted from Paneth cells upon lipopolysaccharides (LPS) stimulation (107).

Inflammatory bowel disease

Inflammatory bowel disease (IBD) is the common name for two complex diseases, Crohn's disease (CD) and ulcerative colitis (UC). There is an increase in the incidence of both CD and UC in the last 50 years with the highest incidence in northern Europe and North America (108). CD generally involves the ileum and the colon, but can affect the entire intestine in a discontinuous fashion. In genome-wide association studies the autophagy protein ATG16L1 and the pattern recognition receptor NOD2 have been associated with CD (109). This indicates that CD is a result of a dysfunctional host response to the intestinal flora. UC is a chronic relapsing inflammation initiated in the distal colon and can spread proximally in the large intestine. UC patients often suffer from bloody diarrhea, anemia and weight loss, and after longstanding disease they have an increased risk of adenocarcinomas. The treatments for UC available today are 5-ASA, corticosteroids, immune suppressive drugs and antibiotics (110). In severe cases surgical resection of the colon is performed. The etiology of UC is not clear, but clinical and experimental data implicate the commensal microbiota as a major driver of the immune response observed. This is clear by the fact that mice models susceptible to colitis do not develop any symptoms in germ free conditions (87). In addition, a variety of studies have demonstrated dramatic alterations in the luminal composition of the microbiota in patents with IBD (111). The importance of a functional mucus barrier is crucial demonstrated by mice lacking Muc2 which spontaneously develop colitis (112, 113). It is not known which is the proceeding event, inflammation or penetrable mucus, but UC patients have poorer quality mucus than healthy controls allowing bacteria to penetrate the mucus layer (114). A shift in the glycosylation of the MUC2 mucin towards shorter and less complex glycans was observed in UC patients (115). The shorter and less complex glycans could allow faster degradation of the mucus barrier by the commensals as well as an altered selection of the intestinal flora in active disease.

Mouse models with defective mucus

The most severe mucus barrier defect is mice lacking the major structural component, the Muc2 mucin. The absence of mucus, as no compensatory mucin is expressed, allows bacteria to be constantly in close contact with the epithelial cells. This results in spontaneous colitis and further colorectal cancers (80, 112, 113). Also, mutations in the Muc2 gene may result in intestinal inflammation as observed for the two mice strains Winnie and Eeyore (116). These mice were generated by chemical induced mutagenesis and the defects were mapped to the Muc2 gene. The mutations lead to aberrant mucin assembly and ER accumulation resulting in a less developed mucus layer. Not only mutations that influence the protein sequence of MUC2 result in disease, also mutations affecting the post translational modification have an effect. The normal glycan degradation by the bacteria is a relatively slow process with the removal of one monosaccharide at a time. However, in mouse models with less complex glycan structures bound to the mucins due to the deletion of specific glycotransferases, degradation will be faster allowing bacteria to also degrade the inner layer and reach the epithelium (117, 118).

The molecular mechanism behind cystic fibrosis (CF) involves defects, caused by mutations, in the ion channel CFTR (119). In humans, the lung phenotype is the most severe, but intestinal symptoms also occur in CF patents. The disease is characterized by thick and viscous mucus that covers the mucosal surfaces, the viscosity of the mucus obstructs gland- and narrow ducts and impaired clearance causes bacterial overgrowth (120). In the mouse model of CF, the lung phenotype is absent but it suffers from intestinal problems as obstruction caused by the accumulation of viscous mucus and fecal material in the terminal ileum (121). The mucus phenotype observed in CF is due to impaired bicarbonate secretion necessary for proper mucus expansion upon secretion (122). Also mice lacking the sodium-hydrogen exchanger Nhe3, show that an optimal ion milieu is important for proper mucus function. These animals have a mucus layer penetrable by bacteria and develop spontaneous colitis (114, 123). Interestingly, there is also evidence of links between the immune system and the mucus layer. Mice deficient in IL-10, an anti-inflammatory cytokine, develop inflammation that is dependent on the presence of bacteria (124). An investigation into the mucus layer in these animals showed it to be slighter thicker than normal, but the quality were impaired and bacteria could penetrate deep into the mucus towards the epithelium (114). The toll-like receptor 5 is a pattern recognition receptor that recognizes flagellin, a subset of the Tlr5 knock-outs develop spontaneous colitis (125). Inflamed animals deficient in Tlr5 also show a penetrable mucus layer while non-inflamed individuals separated the bacteria from the host (114). One of the most widely used animal models of UC is the administration of Dextran Sodium Sulfate (DSS) in the drinking water to chemically induce colitis. The first effects of the DSS is a more penetrable mucus layer allowing bacteria to penetrate before any signs of inflammation were visible (126).

Aims of the project

The aim of the thesis is to identify the core mucus proteome along the gastrointestinal tract and further understand the role of selected associated proteins in order to understand the physiology of the mucus barrier in health and disease.

Results and discussion

Paper I

Mucus proteome along the length of the gastro intestinal tract

In depth knowledge of the composition of the gastrointestinal mucus along the length of the system has not been available. Recent improvements in proteomic technologies have now made these kinds of studies feasible. Previous studies of the intestinal mucus using a proteomic approach only sampled mucus from the distal colon (80, 127). Information from different locations that explain the distinct mucus properties along the intestine is lacking. To further investigate the mucus proteome along the gastrointestinal tract, mucus samples were taken from six different intestinal segments (stomach, duodenum, jejunum, ileum, proximal colon and distal colon). In order to study only the mucus without contamination from the underlying epithelium, intestinal tissue explants from six WT C57BL/6 mice (3 females and 3 males) were mounted in a horizontal perfusion chamber and newly secreted material aspirated and analyzed using LC-MS mass spectrometry and bioinformatics tools.

In total 6,934 unique peptides assigned to 1,339 proteins was identified. After removal of reverse entries, contaminants and proteins only identified by modified sites 1,276 mouse proteins with at least one unique peptide remained and were further compared throughout the study. By comparing the different samples using multivariate analysis a high degree of correlation between replicates could be observed, also no protein was significantly different by *t*-test between the replicates, giving validity to the study. A high correlation could also be seen between males and females, showing that gender differences, such as hormones, do not significantly alter the mucus proteome. Increasing anatomical distance between analyzed samples gave lower correlation something that was expected due to the physical differences of the regions.

Gastrointestinal mucus is built around Muc5ac in the stomach and Muc2 in intestine

Of the 1,276 proteins identified 752 proteins were present in all of the samples. There were low numbers of proteins exclusively found in the separate organs, 8 for the stomach, 15 in the small intestine and 8 in the large intestine (Fig. 2A, paper I). The most abundant mucin in the stomach was the gel-forming mucin Muc5ac and peptides were found all the way down to the proximal colon. The distal presence of Muc5ac is likely due to distal propulsion instead of newly secreted material since mRNA and protein expression detected by immunostaining only detect Muc5ac in the surface epithelium of the stomach (128, 129). In all the sampled regions, except for the stomach, Muc2 is the most abundant gel-forming mucin showing that Muc2

was the major structural component at these sites. An expected result since the Muc2 null mice do not have any compensatory mucus secretion in the absence of Muc2 and spontaneously develop colitis due to the absence of an intestinal mucus layer (130).

Muc2 is the only gel-forming mucin in the intestine

No peptides of the mucin Muc5b, the main mucin component in the lungs and saliva or Muc6, the main mucin in the stomach glands, were identified. It has been suggested previously that Muc6 does not mix with the surface mucus in the stomach (131), as former assessment of the composition did not reveal the molecule (132). The extracellular parts of the transmembrane mucins are heavily glycosylated making mass spectrometry based discovery difficult due to the few tryptic peptides generated. The most abundant transmembrane was mucin Muc13, a molecule known to be enriched in shed vesicles from the enterocytes and therefore not likely to be important for mucus formation (133). Single peptides of Muc3(17), a transmembrane mucin expressed by enterocytes, was identified in 4 of the 6 distal colon samples. As a constituent of the glycocalyx Muc3(17) can be shed from the cells by mechanical force and end up in the mucus layer (40).

Mucus associated proteins

To identify proteins with possible importance for mucus production and function, relative abundance of the proteins throughout the gastrointestinal axes was calculated. The proteins were then compared by cluster analysis to reveal components that have similar expression profiles as the Muc2 mucin (Fig. 3, paper I). Fcgbp is a highly repetitive mucin like molecule that contains 13 von Willebrand D domains, 11 of these include a GDPH cleavage site that, after cleavage, generates a reactive C-terminal. Fcgbp is produced by goblet cells and co-purification with Muc2 in the presence of guanidium chloride suggested non-reducible bond formation between the two molecules that further crosslink the mucus (127). Clca1, another protein expressed by goblet cells (Fig 5, paper I), has been incorrectly described as a Cl^--channel and more recently to be involved in controlling Cl^--secretion (134, 135). However, as the secreted Clca1 protein is so abundant in the mucus, it probably has other as-yet-not-understood functions. Zg16 is a small molecule with a carbohydrate binding domain that was first identified in pancreatic zymogen granules (136). Immunostaining with Zg16 specific antiserum detects it both in the goblet cells and the mucus.

To conclude, the mucus proteome along the alimentary tract is relatively conserved and do not explain the different properties of the mucus observed. Instead, the loose mucus found in the small intestine has recently been shown to be dependent on proteolytic processing by the membrane associated metalloprotease meprin β (137). The identification of proteins with expression profiles that follow the expression of the main structural component implies that these associated proteins play an important role in mucus production or function.

Paper II

AGR2 is highly abundant and important for proper mucus formation

To further understand the protective properties of the mucus layer, proteins of interest identified in the proteomic based analyses were selected and more carefully studied. One molecule always present is the 154 amino acid small Anterior Gradient 2 protein (AGR2). This protein is part of a family with three members first identified to be involved in the control of the cement gland and brain development in Xenopus (138). AGR2 is produced in the secretory cell types of the intestine; goblet cells, Paneth cells and enteroendocrine cells (139). It is postulated that AGR2 is a member of the endoplasmic reticulum (ER) protein disulfide isomerase (PDI) family as it harbors a central cysteine residue that is part of a CXXS motif (140). PDIs aid protein folding and assembly by catalyzing the formation and shuffling of cysteine disulfide bonds in the ER. A non-conventional ER-retention signal with the sequence KTEL is found in the C-terminal suggesting that AGR2 is located within the ER (141). AGR2 may also have extracellular effects since studies using recombinant AGR2 suggested that it could bind to the outside of cells (142). Polymorphisms in the AGR2 gene have been associated with increased risk of ulcerative colitis (143). In the Agr2$^{-/-}$ mice gastrointestinal alterations such as goblet cell depletion, abnormal Paneth cells and hyperplasia of the glandular stomach mucosa were observed (144-146).

AGR2 does not form covalent disulfide bonds with MUC2

To study AGR2 function, the protein was cloned and specific amino acid alterations were introduced in the sequence by site specific mutagenesis. The cysteine at position 81 in the potential CXXS motif were exchanged to a serine (C81S), the C-terminal ER retention signal KTEL altered to the canonical ER retention signal KDEL (T173D) or the ER retention signal (ΔKTEL) removed. Using transient transfections in COS-7 and HEK-293T cells, AGR2 was previously suggested to covalently interact with the MUC2 mucin (144). As the truncated MUC2 plasmids used originated from our lab, we decided to further address this observation. We used stable selected CHO-K1 clones expressing the MUC2 N-termini (SNMUC2-MG) or MUC2 C-termini (SMG-MUC2C), respectively (57, 62). The stable clones were selected as transient transfection of these plasmids resulted in ER accumulation and no secretion of the proteins. Cell lysates from transient transfections of WT and mutant versions of AGR2 in MUC2 expressing CHO-K1 cells were separated on non-reducing polyacrylamide gels, western blotted and immunostained with an anti-AGR2 antibody or with anti-GFP for the MUC2N and MUC2C GFP fusion products (Fig. 1, paper II). In these gels, no AGR2 containing bands were apparent at higher molecular mass than the AGR2 monomer. Co-immunoprecipitation with anti-AGR2 antibody or anti-GFP antibody in transfected cell lysates did not show any non-reducible bond between AGR2 and MUC2. This together suggests that no

covalent disulfide bond is formed between AGR2 and MUC2. The discrepancy from the previous study maybe explained by ER retention of the MUC2 parts in the cell lines used and formation of unnatural disulfide bonds.

AGR2 does not aid MUC2 production in cell cultures

As AGR2 is postulated to participate in ER protein folding, we wanted to examine if MUC2 folding were enhanced in our cell culture model. Both MUC2 terminal plasmids encode proteins that harbor cysteine rich domains and are extremely challenging for the cells to produce as observed with the ER accumulation in transient transfected cells. By transfecting WT AGR2 into cells with stable expression of the MUC2N construct and selecting clones with different expression levels of the AGR2 protein we could study if AGR2 levels in the cells could modulate MUC2N production. MUC2N secretion could be observed in the parent no-AGR2-transfected and the clone with the lowest AGR2 expression (Fig. 2, paper II). However, no secreted material was observed in the higher AGR2 expressing clones. This suggested, in contrast to our hypothesis, that AGR2 did not promote folding maturation, or secretion of MUC2. Instead the results suggest that AGR2 retained MUC2 in the cells. The MUC2 mucin cysteine rich domains are almost identical to cysteine rich domains in the blood clothing protein, von Willebrand factor (18). This protein is produced in endothelial cells, a cell type where AGR2 is not expressed suggesting that AGR2 is not required for synthesis of this molecule. This further argues against AGR2 being essential for MUC2 biosynthesis and secretion.

Agr2$^{-/-}$ mice have a defective mucus layer

In the normal state, the distal colon mucus separates the intestinal bacteria from the epithelial cells and disruption of the mucus layer yields bacteria in contact with the epithelium, something that trigger inflammation (80). To investigate the mucus integrity in Agr2 deficient mice, Carnoy fixed distal colon sections were immunostained with a Muc2 specific antisera detecting the mature protein. In the Agr2$^{-/-}$ mice less filled goblet cells and a more unstructured inner mucus layer were observed compared to WT controls. The unstructured layer in the Agr2$^{-/-}$ mice also allows bacteria closer to the underlying cells as observed by DNA staining, however no bacteria were detected in the crypts. This suggests that despite the lower quality mucus, secretion and constant mucus flow is sufficient to keep bacteria at a distance. However, if the system is challenged, as with DSS, bacteria are able to elicit inflammation as shown by Park *et al.* (144). This indicates that Muc2 is produced, but the amount that passes through the secretory machinery might be insufficient to form a well functional mucus layer. Using an antiserum detecting the Muc2 apoprotein an increased ER accumulation and more punctuated staining, resembling a fragmented ER is found. It is evident that AGR2 is involved in ER function as suggested by numerous publications (145, 147, 148), however the mechanism behind this is not understand and requires further attention.

An internal cysteine is important for protein secretion

Increasing evidence indicate that AGR2 is more than an ER resident protein. Using mass spectrometry analyses of colon mucus samples spiked with heavy labeled peptides standards, the molar ratio was calculated for known mucus associated proteins (Fig. 5C, paper II). Agr2 turned out to be the most abundant of the proteins compared. Together with western blot analysis of the mucus with an anti-AGR2 antibody it was shown that AGR2 is secreted out into the mucus. Also, a recent publication suggests AGR2 is a protein with extracellular function that is able to bind to cell membranes (142). This can explain AGR2 involvement in cancer progression as well as its role in the control of normal mammary lobular gland development (149, 150). How is AGR2 able to escape the ER despite the ER retention signal? WT AGR2 is not secreted out in the media in our cell culture model. Mutation of the central cysteine in the CXXS motif to a serine resulted in a protein that was secreted despite the presence of the KTEL sequence (Fig 6B, paper II). Together the results suggest that the cysteine is coupled to ER retention and needs to be modified or transiently bound to another molecule to allow ER exit.

To conclude, AGR2 is an important molecule for proper mucus. It is also evident that AGR2 has effects on ER morphology and function. Further, the high levels of extracellular AGR2 and the role of the single cysteine residue for ER retention suggest extracellular functions.

Paper III

The mucin-like protein FCGBP

In the hierarchical cluster analysis of the mucus proteome along the gastrointestinal tract the Fc γ-binding protein (Fcgbp) shows a similar expression profile to the Muc2 mucin (Fig. 3, paper I). The close co-regulation of the expression suggests that FCGBP is important for proper mucus formation and function. The molecule is a large mucin-like protein composed of 5,405 amino acids but lacks the PTS domains that are the hallmark of real mucins (151). FCGBP is expressed in mucus producing goblet cells along the gastrointestinal tract, but is also found in other mucosal surfaces such as the lung, oral cavity and reproductive organs (152-154). The sequence is highly repetitive and contains 13 vWD domains interspersed by TIL, TIL-associated and C8 domains. Eleven of these vWD domains have a GD/PH cleavage site. This sequence is autocatalytically cleaved in the MUC2 and MUC5AC mucins which each contain one vWD domain with GDPH (57, 155). Furthermore, FCGBP is co-purified with intestinal MUC2 in the chaotropic agent, guanidium hydrochloride, which suggests covalent bonds between the two molecules (156). This is probably mediated through the reactive anhydride in the new C-terminal end that is formed within the aspartic acid after the GDPH cleavage. An anhydride is able to covalently react and bind to both N- and OH-groups. This could be a mechanism to further crosslink the MUC2 polymers in the mucus, but needs to be proven. The recombinant expression of full length FCGBP and truncated recombinant proteins enabled us to study the GDPH cleavage and reactive anhydride formation in more detail.

FCGBP is autocatalytically cleaved

One prominent feature of FCGBP is the GDPH sequence found in 11 out of 13 vWD domains. Western blot analyses after reducing SDS-PAGE of human colon lysate with a FCGBP specific antibody against the N-terminal region of the protein detect a 50 kDa fragment corresponding to the theoretical mass of the N-terminal down to the first GDPH cleavage site, indicating that this site is cleaved. Cleavage products could also be detected using mass spectrometry methods on human colonic mucus samples (Table 1, paper III). The high sequence homology of the vWD domains made it however difficult to determine exactly which sites had been cleaved. The C-terminal peptides formed by GDPH cleavage could however not be identified, arguing that these peptides were involved in cross-links and could thus not be recognized as normal peptides. The GDPH cleavage is expected due to the occurrence in other proteins that harbor the vWD domain motif (67, 155, 157). Recombinant expression in CHO-K1 cell of the full length protein of 5,405 amino acids tagged in the C-terminal with myc- and Flag-tags acids turned out to be difficult. However, a protein band around 600 kDa corresponding to the theoretical mass of FCGBP could sometimes be detected after non-reducing SDS-PAGE. Due to the irreproducibility

of the expression of the full length plasmid, possibly by the complexity of the protein, we decided to construct a truncated version of FCGBP. The shorter construct consists of the N-terminal and the two first vWD domains and is C-terminal tagged with myc- and Flag-tags. The expression plasmid of the truncated version of FCGBP coded for a protein with a theoretical mass of 130 kDa named FCGBP-ND1D2-MH. After CHO-K1 transfection, bands at 130, 80 and 50 kDa using an anti-myc antibody was detected after non-reducing conditions, suggest that the protein is partly cleaved or fully cleaved and partly held together at the two GDPH sequences (Fig. 2B, paper III). Under reducing conditions only a single band at 55 kDa is detected with the anti-myc antibody showing that at least the second vWD domain was fully cleaved. Also, in cell lysates the 55 kDa band was the major band suggesting that processing is an early event in the cell. Furthermore, by labeling of purified recombinant FCGBP-ND1D2-MH produced in CHO-K1 cells at its primary amines using dimethyl followed by mass spectrometry analyses, showed that the cleavage occurred prior to sample handling.

To investigate the behavior of the truncated version in a cell model that resembles the natural FCGBP expressing cell we used the colonic epithelial cell line LS174T expressing an inducible dominant negative TCF4 (158). After induction this cell line cease to divide and differentiate into a goblet cell-like cell with endogenous MUC2 stored in granules (159). When the truncated protein was expressed in these cells the same 130, 80 and 50 kDa bands were detected with the anti-myc antibody (Fig. 2C, paper III). A minor difference when using the CHO-K1 cells was a more prominent 50 kDa protein band in non-reduced gels indicating a lower degree of disulfide bonding between the cleaved fragments. Also the FCGBP-ND1D2-MH protein was mainly detected in the lysate and only minor secretion was observed. Preliminary results show that FCGBP-ND1D2-MH is sorted and stored in MUC2 containing vesicles inside the cell. As other overexpressed proteins are secreted from this cell line, the effect observed is probably a result of correct protein sorting into MUC2 containing vesicles.

Reactivity after autocatalytic cleavage

The mechanism for the formation of the internal anhydride after cleavages at Asp–Pro bonds between the two carboxy groups of the generated C-terminal aspartic residue have been reported (160). The formed anhydride is fairly reactive and can react with primary amines and hydroxyl groups. To investigate if GDPH cleavages in FCGBP generate reactive C-terminals CHO-K1 produced FCGBP-ND1D2-MH was allowed to react with biotinylated ethylenediamine (B-EDA). Reacted material was detected with streptavidin and show both the disulfide-linked full length 130 kDa protein as well as two of the 50 kDa fragments after reduction (Fig. 4, paper III). Newly formed C-terminals after cleavage can react with primary amines as shown for other proteins harboring the same domain (67, 155, 161). However, the occurrence of the reaction in vivo and to which substrate, amines or hydroxyl groups, remains unknown. The

most likely candidate as a donor is, however, the MUC2 mucin with its array of available hydroxyl groups on the glycans as previously proposed (127).

Normal mucus phenotype in Fcgbp$^{-/-}$ mice

For additional insights into FCGBP protein function *in vivo* we turned to the Fcgbp$^{-/-}$ mice. Intestinal tissue explants were mounted in a horizontal perfusion chamber developed for mucus studies (162). First, the thickness of the mucus layer was measured and compared to WT control mice. Fcgbp$^{-/-}$ mice display a mucus thickness that did not differ from the control animals. When fluorescent bacterial sized beads were applied and allowed to sediment on the mucus, penetrability of the beads in the mucus monitored by confocal microscopy. From these experiments it was evident that also Fcgbp$^{-/-}$ animals display a normal mucus phenotype that is able to separate the beads from the epithelia. These results contradict the hypothesis of FCGBP as a cross-linker of the mucus gel, at least in the sense of reducing the pore size of the mucus network to hinder bacteria. Also that FCGBP was largely found as small fragments also argue against a cross-linking function. It is however possible that eventual cross-linking is important in other aspects such as stability. The penetrability data is in accordance with immunostainings of Carnoy fixed distal colon sections where the inner layer was shown to be devoid of bacteria (Fig. 5A, paper III). That no bacteria were in close proximity to the epithelium is also in line with the observation that the mice did not show any signs of inflammation. Finally, the Fcgbp$^{-/-}$ mice was not more susceptible to DSS colitis than the control group.

In conclusion, FCGBP is one of the most abundant protein in proteomic analysis of the mucus and proposed to act as a cross linker of the barrier (127). The results show that FCGBP protein is auto catalytically cleaved early on during biosynthesis, and the newly formed C-terminals are reactive at least *in vitro*. The lack of Fcgbp in a mouse model does not alter the mucus thickness or bead penetration allowing the mucus to separate the luminal bacteria from the host cells.

Paper IV

Mucus associated protein ZG16

Zymogen granule protein 16 (ZG16) was first identified in zymogen granules from rat pancreas (136). Zymogen granules are specific organelles found in acinar cells responsible for digestive enzyme storage and secretion. These granules resemble the mucin granules found in the goblet cells of the intestine. The function of ZG16 in these organelles is not yet fully understood, but it was postulated that ZG16 mediated binding of zymogens to the granule membrane or the sub-membrane network of glycopeptides, stabilizing the membrane (163). ZG16 is also expressed in goblet cells throughout the gastro intestinal tract (Paper I) (136, 164). The protein is secreted and highly abundant in the mucus layer and shows an expression profile similar to the Muc2 mucin (Paper I). Overall, the role of ZG16 in the context of mucosal barrier function is poorly understood. By the generation of recombinant ZG16 expressed in CHO-K1 cells as well as a ZG16 specific antiserum enabled us to study ZG16 function in more detail.

ZG16 binds to enterocytes

Immunostaining of human colonic tissue with a ZG16 specific antiserum verified the localization to mucin granules in goblet cells (Fig. 1, paper IV). A closer look also revealed a faint staining along the apical border of enterocytes, a cell type with no ZG16 production. We asked if the enterocyte staining was an effect of ZG16 binding. To answer this, we used the enterocyte-like cell line Caco-2 and incubated the cells after detachment with recombinant ZG16. Bound proteins on the cell surface were detected by flow cytometry and showed concentration dependent binding. The binding was dramatically reduced if the cells were treated with trypsin before incubation with ZG16. The reduction of binding indicated that ZG16 interacted with a protein based ligand. To identify the potential receptor on the surface of Caco-2 cells, immunoprecipitated recombinant ZG16 was used to fish out potential ligands from Caco-2 cell lysates separated by SDS-PAGE. The identities of the proteins in the gel were determined by a proteomics approach. The most slow migrating band on the gel showed high scores for the low-density lipoprotein receptor 1 and 2 (LRP1 and 2). LRPs are large transmembrane proteins known to be endocytotic receptors for a large variety of ligands (165). A recent proteomic study of the membrane bound proteins in the human colon showed that LRP1 is expressed but not LRP2 making LRP1 a more likely receptor candidate for ZG16 (166). Co-staining of Caco-2 cells after incubation with recombinant ZG16 detecting LRP1 and bound ZG16 indicating that LRP1 is expressed on Caco-2 cells and co-localized with bound ZG16.

ZG16 is internalized

ZG16 binding to Caco-2 cells could also be shown using a microscopy based

method. Recombinant ZG16 was added to the growth media of Caco-2 cells grown on cover slips and incubated before fixation of the cells. At short time points ZG16 was localized to the plasma membrane of the cells and at longer incubations ZG16 was found to be internalized as observed in small vesicles inside the cell (Fig. 3A, paper IV). Furthermore, the addition of Dynasore, a dynamin inhibitor, blocked the uptake process and ZG16 vesicles did not leave the plasma membrane. This was in agreement with LRP1 mediated endocytosis a process previously shown to be dynamin dependent (167). As ZG16 interact with the bacterial cell wall component peptidoglycan (PGN), we tested if ZG16 could mediate uptake of PGN and whole bacteria. Preincubation with labeled solubilized peptidoglycan (sPGN) together with recombinant ZG16 prior to the addition to the cells yielded intracellular vesicles containing the two molecules. This process of internalization was however not totally ZG16 dependent, only enhanced as incubation with sPGN alone yielded some intracellular sPGN. ZG16 did not mediate whole bacteria uptake into the cells.

ZG16 is transported towards the basal side of the cell

To investigate the internalization processing of ZG16, Caco-2 cells incubated with recombinant ZG16 were co-stained with intracellular markers for different compartments. No co-staining could be observed with the lysosome marker LAMP1 but ZG16 was observed in vesicles positive for the early endosome marker EEA1 (Fig. 5, paper IV). As we could not localize ZG16 to any compartments distally to early endosomes all that could be concluded was that ZG16 was not further directed to the lysosomes. Furthermore, recombinant ZG16 was incubated with confluent Caco-2 cells and imaged by confocal microscopy after fixation and staining and showed ZG16 binding to the apical side of the cell, internalized and transported towards the basal part of the cell. In mice lacking $Muc2^{-/-}$, immunostaining of ZG16 showed a diffuse punctuated staining in both the apical and basal part of several enterocytes (Fig.7, paper IV). This suggests that internalization of ZG16 also occurs *in vivo* under specific conditions.

In conclusion, ZG16 is produced by goblet cells of the intestine and found in ample amounts in the mucus. ZG16 is able to bind the endocytic receptor LRP1 on the surface of enterocytes. After binding, ZG16 is internalized and able to bring peptidoglycan as cargo. The internalization process seems to be enhanced during inflammation and it is intriguing to speculate that internalization of ZG16 is a mechanism for the host to sample the intestinal content.

Paper V

ZG16 bind Gram-positive bacteria via peptidoglycan

ZG16 sequences similarities between different species are high and all orthologs contain an N-terminal signal sequence and a large lectin domain, classified as a Jacalin like carbohydrate recognition domain (168). Jacalin is a lectin from jackfruit seed and specifically binds the Thomsen-Friedenreich antigen (Galβ1-3GalNAcα1-O-S/T), generally known as the T-antigen (169). The binding specificity of ZG16 has been tested by us and others on carbohydrate arrays, something that suggested binding to polymannose structures (164). Also binding to heparin and heparin sulfate has been shown in the literature (170). ZG16 is located on chromosome 16p13.3 a region that has been classified as an IBD susceptibility locus and transcript levels are down regulated in patients with ulcerative colitis (171).We wanted to further investigate the binding specificity of ZG16, as the reported results are not conclusive. Another lectin expressed in the intestinal tract, RegIIIγ, binds to one of the most abundant glycan structures found in the lumen, peptidoglycan (104). Peptidoglycan is the major bacterial cell wall component composed of repeated *N*-acetylmuramic acid (MurNAc) and *N*-acetylglycosamine (GlcNAc) residues cross-linked with a short peptide chain (172). As no bacterial derived glycans were included in the glycan array we developed a microtiter based binding assay. ZG16 was found to bind insoluble peptidoglycan as well as Gram-positive bacteria that have peptidoglycan exposed. The recently published structures of ZG16 (173) and peptidoglycan (174) allowed *in silico* docking of the two molecules, showing that the *N*-acetylmuramic can bind in the ZG16 binding pocket and that the peptidoglycan peptide cross-link can further stabilize such a binding (Fig. 1D-F, paper V).

ZG16 cause bacterial aggregation

We wanted then explore the effect of the binding to bacteria. To our surprise, the addition of ZG16 did not affect bacterial viability of any of the strains tested. Since ZG16 contains two cysteines in the far C-terminal proposed to form an intramolecular disulfide bond (175), we wanted to explore if antimicrobial activity were unmasked if the disulfide bonds were reduced as for the human β-defensin 1 (176). This was investigated by adding ZG16 to agar plates with an increasing concentration of dithiothreitol (DTT) together with the Gram-positive bacteria *Bacillus subtilis*. As no increased clear zone was observed on these radial diffusion assays, the results suggest that ZG16 does not have any bactericidal activity, at least by itself. However, ZG16 binding did affect bacterial aggregation as Gram-positive bacteria incubated with ZG16 and spread on microscopy slides showed significantly larger aggregates than the untreated control. Also in an assay with a low concentration of agar, in an attempt to resemble the polymeric mucus network treatment of ZG16, caused bacterial aggregation and thus limited bacterial motility within the plates (Fig. 2D, paper V).

$Zg16^{-/-}$ mice have a mucus layer with increased penetrability

The next step was to address ZG16 function in the mucus layer, an important step was the generation of a ZG16 knockout mouse. First the mucus thicknesses were monitored in these mice, by mounting colonic tissue explant in a perfusion chamber and monitoring the mucus growth. Since no differences were observed between the two groups, $Zg16^{-/-}$ and WT, we accessed the mucus quality by adding fluorescent bacterial sized beads to the explants. Bead penetration in the mucus layer was monitored by acquiring multiple z-stacks in a confocal microscope (Fig.3B, paper V). The spatial separation in the WT animals was at least 100µm between the epithelium and the closest beads. In the $Zg16^{-/-}$ mice, however, more variation was observed and several animals had beads that penetrated down in to the mucus. The increased permeability could also be observed in Carnoy fixed distal colon sections from $Zg16^{-/-}$ mice. Firstly by staining the sections with the histological Gram stain, Brown-Breen, bacteria were found close to the epithelium. Secondly, by immunostaining the sections using a Muc2 specific antiserum and an antibody that reacts with lipoteichoic acid (LTA) present on Gram-positive bacteria showed a less organized inner mucus layer containing more Gram-positive bacteria than the WT controls (Fig. 3D, paper V).

ZG16 translocate bacteria away from the epithelium

We wanted to explore the penetrability of the mucus layer further, since the fluorescent beads only mimic the size of the bacteria and not the surface properties and hence the direct effect of ZG16 binding. First, the WT colonic tissue was mounted and then the Gram-positive bacteria *Enterococcus faecalis* added to the tissue together with fluorescent beads. The fluorescent beads and the bacteria settled at the interface of the impermeable layer. If instead, the bacteria were treated with recombinant ZG16 before the addition to the explant, a shift in bacterial distribution away from the epithelium was observed. Second, the same methods were applied on tissue from $Zg16^{-/-}$ animals, in the control experiments the mucus were penetrable to both beads and bacteria. However if the bacteria were first treated with recombinant ZG16, the bacteria were unable to penetrate the impermeable mucus. Since the fluorescent beads still did penetrate after ZG16 treatment, the mode of action is not an alteration in the mucus structure, but rather a direct effect on the bacteria whose clumping excluded them from the mucus network. By mounting colonic tissue explants and staining mucus associated microbiota *in situ*, we observed that only a few bacteria were able to penetrate the impermeable layer in the WT mouse. In the $ZG16^{-/-}$ mouse, the mucus was heavily penetrated by the microbiota and bacteria were observed close to the epithelium surface (Fig. 5B and 5C, paper V). By DNA extraction from the mucus of WT and $Zg16^{-/-}$ followed by bacterial 16S quantification by qPCR a 50-fold increase in the 16S load was observed in the knockout animals. ZG16 did not only limit bacterial penetration of the mucus gel, the bacterial motility inside the gel was reduced also in the presence of ZG16.

Effect of a more penetrable mucus

With bacteria penetrating the mucus layer we hypothesized that more bacteria would translocate from the intestine into the systemic circulation. Samples taken from two systemic tissues, mesenteric lymph nodes (MLN) and spleen, indeed showed a significantly higher 16S load in MLN and spleen tissues recovered from ZG16$^{-/-}$ compared to WT controls. In these tissues the dominant taxonomic group was the Gram-positive phylum Firmicutes in the ZG16 knockout animals. The increased systemic bacteria load was also accompanied by immune system activity and three pro-inflammatory cytokines, IL-6, KC/GRO (murine IL-8 homologue) and TNFα titer were increased in whole blood serum samples. Finally, low grade inflammation is known to correlate with obesity and insulin resistance (177). Body mass comparison of age matched WT and Zg16$^{-/-}$ mice demonstrated a 17% and 20% increased mass in male and female knockout mice, respectively.

In conclusion, ZG16 is an important component for the generation of a functional mucus layer in the distal colon. Through the interaction with Gram-positive bacteria, the luminal microbiota is kept at a safe distance from the epithelium and by this limit systemic bacterial infiltration.

General conclusion

The intestinal mucus barrier along the murine gastro intestinal tract was sampled and analyzed by advanced mass spectrometry in order to reveal the mucus proteome along the intestinal axes. It could be concluded that the major mucins that build up the mucus is Muc5ac in the stomach and Muc2 in the small and large intestine. This data also allowed us to follow the expression profile of the proteins at the sampled sites and to pick out proteins with similar expression pattern as the Muc2 mucin. Related expression patterns indicate that the associated proteins are important for proper mucus formation or function.

AGR2 is an important molecule for proper mucus formation. The high levels of AGR2 in the mucus argue for extracellular effects of the molecule. The secretion is dependent on the modification of an internal cysteine residue.

FCGBP is autocatalytically cleaved early on during biosynthesis, and the newly formed C-terminals are reactive upon secretion. In regards to mucus formation, the Fcgbp$^{-/-}$ mice have a normal mucus thickness that is not penetrable to bacteria.

ZG16, with its carbohydrate binding domain, is able to bind Gram-positive bacteria via interactions with the cell wall component peptidoglycan. Binding of bacteria mediates aggregation and by this reduces the mobility of the bacteria in the mucus and translocates them further away from the epithelium. Mice deficient in Zg16 display a mucus layer that is penetrable to bacteria with a 10-fold higher bacterial load compared to control animals. The increased bacterial load in the mucus is also reflected in higher bacteria counts in systemic tissues, increased inflammatory cytokines and increased body mass. The binding and uptake of ZG16 into enterocytes implies that the protein is involved in a mechanism for the host to sample the intestinal content.

Future perspectives

Although the results in this thesis shed some light on the role of some of the mucus associated proteins found in the intestine, it also gives rise to new and interesting questions. The mucus barrier was for a long time neglected by most scientists that studied intestinal homeostasis. This is now changing and the mucus layer is attracting more and more attention. There are still however, many questions to be answered with respect to mucus formation, function and regulation. The huge amount of bacteria in the lumen and the effects they have on the host add another dimension of complexity. I strongly believe that many of these questions could be answered by increasing knowledge of the proteins found associated with MUC2. The vast majority of these proteins are currently poorly understood. The task of revealing novel functions of these proteins are however tedious and difficult. If knock out models are available, the use of tissue explants from these animals opens up the possibility to screen mucus properties. This will provide a starting point and models with an altered mucus phenotype can be further studied for a better understanding. One of the key questions is the penetrability of the mucus seen in the mouse colitis models as well as in human UC allowing bacteria close to the epithelium. Understanding this regulation opens up the possibility of developing tools to reinforce the barrier in a variety of debilitating disease states. Results shown in this thesis point to ZG16 as a candidate for the translocation of bacteria away from the epithelia and further out in the mucus therefore lower the antigen pressure on the tissue. Further experiments administering recombinant ZG16 to mouse models that spontaneous develop colitis or to chemical induced colitis should be conducted. This may to elucidate if ZG16 is a well suited candidate to be used as a protein based medicament in the treatment of intestinal disease.

Populärvetenskaplig sammanfattning

Kroppen använder barriärer för att förhindra skadliga angrepp från vår omgivning. Det mest uppenbara är skinnet på utsidan av kroppen som består av ett tjockt lager döda hudceller. Även kroppens håligheter så som andningsvägar och magtarmkanal behöver dock skyddas. Men på grund av kroppsliga funktioner så som gasutbyte, upptag av näringsämnen och vätska över epitelet, kan inte barriären bestå av döda celler. Istället är cellerna skyddade av ett finmaskigt slemlager där den viktigaste strukturella komponenten är stora polymerer bestående av glykosylerade proteiner, så kallade muciner. I magtarmkanalen har slemlagret förmågan att skydda de underliggande cellerna från syra, matspjälkningsenzym, gallsalter och stora mängder mikroorganismer medan normala funktioner tillåts. I distala kolon där mängden av bakterier är som störst är slemlagret organiserat i två distinkta skikt. Det yttre är uppluckrat och löst sittande medan det inre är kompakt och fastsittande på underliggande epitel. Ständig produktion och förnyelse underifrån hindrar bakterier att penetrera det inre lagret. Följden blir en rumslig separation av bakterier och värdens celler och därmed minskar risken för inflammation och bakterier som tar sig över epitelet.

Målet med denna avhandling har varit att identifiera vilka proteiner som tillsammans med det huvudsakliga mucinet bygger upp denna barriär, och förstå dess funktion i mukuslagret. Tre identifierade proteiner studerades närmare för att specifikt utreda deras roll i denna skyddsbarriär.

AGR2 har föreslagigts att tillhöra an grupp proteiner som hjälper till vid proteinveckning i det endoplasmatiska nätverket och har tidigare visats vara nödvändig för MUC2 produktion. Genom molekylär biologiska metoder och experiment med cellkulturer visar vi att AGR2 inte binder terminala MUC2 konstrukt och att utsöndring av molekylen är beroende av en centralt belägen cystin aminosyra.

Det stora och repeterade proteinet FCGBP innehåller 13 von Willebrand D domäner. 11 av dessa innehåller en sekvens som klyvs genom en autokatalytisk process. Klyvningsreaktionen resulterar i en reaktiv grupp på den nyformade C-terminalen. I cellkulturförsök sker denna klyvningsreaktion tidigt under biosyntesen av molekylen.

ZG16 har nu visats att binda molekylen som bygger upp bakteriers cellvägg, peptidoglykan, genom sin sockerbindande domän. Bindning till bakterier påverkar inte viabiliteten, däremot har proteinet en bakterieaggregerande effekt. Genom att använda en musmodell som saknar proteinet kan vi visa att ZG16 binder till Grampositiva bakterier och förflyttar dem ut i slemlagret. Genom att binda till entrocyter genom en protein receptor på cellens yta fungerar möjligen även ZG16 som en signalbärare.

Sammanfattningsvis, resultaten i den här avhandlingen visar att MUC2 associerade proteiner är viktiga för att bilda ett funktionellt skyddande mukuslager som hindrar bakterier att nå epitelet och inducera sjukdom.

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