The Relationship between Transmembrane Mucins, Ion Channels and PDZ Adaptor Proteins in the Small Intestine

Thaher Pelaseyed

Department of Medical Biochemistry
Institute of Biomedicine
Sahlgrenska Academy
University of Gothenburg
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Department of Medical Biochemistry
Institute of Biomedicine
Sahlgrenska Academy
University of Gothenburg
SWEDEN

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To my beloved family.

ABSTRACT

Thaher Pelaseyed
Department of Medical Biochemistry, Institute of Biomedicine
Sahlgrenska Academy at University of Gothenburg
Göteborg, Sweden

The human body is continuously exposed to challenges from the surrounding world. In analogy with the skin, mucus is a well-organized and highly regulated barrier composed of polymeric and *O*-glycosylated mucins that protects luminal organs such as the gastrointestinal tracts from the outer milieu. As part of a first defensive barrier, the secreted mucins entrap pathogens and act as a network for antiseptic enzymes and proteins. A second barrier is the dense glycocalyx which is composed of transmembrane mucins anchored to the apical membrane of cells.

The focus of this thesis was to identify novel interactions between transmembrane mucins and cytoplasmic PDZ adaptor proteins and to determine the role of these interactions in mucin expression and regulation. Furthermore, the interplay between transmembrane mucins and ion channels expressed in the small intestine was explored. Finally, the stability of the SEA domain in transmembrane mucins was assessed.

Using different techniques in molecular biology and confocal imaging, this thesis proves that the transmembrane mucins MUC3 and MUC17 bind to PDZ adaptor proteins that are interaction partners for two intestinal ion channels, namely CFTR and NHE3. Specifically, MUC17 is retained in the apical surface of enterocytes by PDZK1. In analogy with acute Ca^{2+}-mediated regulation of NHE3 and CFTR, the cholinergic agonist carbachol induces endocytosis of MUC17, concomitant with NHE3 internalization and CFTR recruitment to the cell surface. This thesis also demonstrates that the expression of MUC3 is counter-regulated by CFTR via a *trans*-Golgi-resident PDZ adaptor protein called GOPC. Finally, using atomic force microscopy, it is demonstrated that the SEA domain of transmembrane mucins protects the apical cell membrane by acting as a breaking point upon mechanical stress.

In summary, the results from this thesis deliver new evidence regarding the relationship between transmembrane mucins and ion channels. These novel networks cast light on important cellular processes, involving the formation of physical barriers coupled to fluid and mucin secretion, that occur in response to native and foreign provocations.

Keywords: intestine, mucin, MUC3, MUC17, SEA, PDZK1, GOPC, CFTR, NHE3

List of papers

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Malmberg, E. K., Pelaseyed, T., Petersson, A. C., Seidler, U. E., De Jonge, H., Riordan, J. R. and Hansson, G. C. (2008) **The C-terminus of the transmembrane mucin MUC17 binds to the scaffold protein PDZK1 that stably localizes it to the enterocyte apical membrane in the small intestine**. Biochem. J. 410, 283-289

II. Pelaseyed, T. and Hansson, G. C. (2011) **CFTR anion channel modulates expression of human transmembrane mucin MUC3 through the PDZ protein GOPC**. J. Cell. Sci. 124, 3074-3083

III. Pelaseyed, T., Zäch, M., Petersson, Å. C., Svensson, F., Johansson, D.G.A., Hansson, G. C. **Unfolding dynamics of the mucin SEA domain probed by force spectroscopy suggest that it acts as a cell protective device**
Submitted.

IV. Pelaseyed, T., Gustafsson, J. K., Gustafsson, I. J., Hansson, G. C **Carbachol-induced internalization of human transmembrane MUC17 mucin is concomitant with NHE3 internalization and CFTR externalization in enterocytes**
Manuscript.

Contents

Chapter 1: Introduction **9**

The Mucus Barrier 9

PDZ Adapter Proteins 16

The ion channels of the small intestine 18

Carbachol, a cholinergic agonist 22

The brush border of enterocytes 24

Vesicle trafficking in polarized epithelial cells 25

Trafficking of membrane-tethered mucins 28

Trafficking of CFTR and NHE3 29

Aims of thesis 31

Chapter 2: Methods **32**

Animals (Papers I and IV) 32

Antibodies (Papers I, II, and IV) 32

Atomic force microscopy (Paper III) 33

Labeling of surface glycoproteins (Paper IV) 33

Chapter 3: Results **34**

Transmembrane mucins are ligands for PDZ adaptor proteins (Paper I and II) 34

Transmembrane mucins and ion channels are co-regulated (Paper II and IV) 36

SEA-type transmembrane mucins act as protective devices (Paper III) 42

Chapter 4: Discussion **45**

Transmembrane mucins display specificity towards PDZ proteins 45

PDZ proteins regulate mucin expression and subcellular localization 46

Membrane mucins and ion channels are co- and counter-regulated 47

The SEA domain acts as a protective device 49

Future perspectives 51

Populärvetenskaplig sammanfattning **53**

Acknowledgements **55**

References **57**

Abbreviations

AFM	atomic force microscopy
AMOP	adhesion-associated domain in MUC4 and other proteins
AP	adaptor protein
BHK	baby hamster kidney
Ca^{2+}	calcium ion
CaCC	calcium-activated chloride channels
cAMP	cyclic adenosine monophosphate
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator
cGMP	cyclic guanosine monophosphate
CK	cysteine knot domain
ClC	chloride channel type
CME	clathrin-mediated endocytosis
CT	cytoplasmic tail
EE	early endosome
EEA1	early endosome antigen 1
ER	endoplasmic reticulum
GOPC	golgi-associated PDZ and coiled-coil motif-containing protein
GSK	glycogen synthase kinase
HA	hemagglutinin
I_{sc}	short circuit current
LE	late endosomes
mAb	monoclonal antibody
MDa	megadalton
MTOC	microtubule organizing centers
MUC	mucin
NHE	Na^+/H^+ exchanger
NHERF	Na^+/H^+ exchanger regulatory factor
NIDO	nidogen homology region
NMDA	N-methyl d-aspartate
NTA	nitriloacetate
pAb	polyclonal antibody
PDZ	PSD-95/Discs large/ZO-1
PKC	protein kinase C
PTS	proline-, threonine-, and serine-rich
RE	recycling endosomes
SEA	sea urchin sperm protein, enterokinase, and agrin
TGN	*trans*-Golgi network
vWC	von Willebrand C domain
vWD	von Willebrand D domain
Wt	wildtype

1 Introduction

The Mucus Barrier

The human body is continuously exposed to chemical and mechanical stress from the surrounding world. The skin is a specialized keratinous barrier which protects the human body from these challenges as well as from pathogens. In analogy with the keratinized skin, luminal organs such as the gastrointestinal, respiratory, and urovaginal tracts are protected from the outer milieu. In these cavities the keratinized skin is substituted for a well-organized and highly regulated mucus barrier with protective, lubricative and biological functions [1]. As part of a first protective barrier, the mucus layer which covers surfaces of luminal organs is an important component of the innate immunity. Apart from constituting a physical barrier, the mucus plays a part as an extensive network for antimicrobial peptides and proteins.

Mucins

Mucins are the major constituents of the mucus layer covering epithelial surfaces. Mucins are glycoproteins of high molecular weight, harboring large numbers of *O*-glycans on long domains composed of proline (Pro), serine (Ser) and threonine (Thr) amino acid residues, also called PTS domains [2]. The glycosylated PTS domain, called the mucin domain, accounts for approximately 80% of the molecular mass of a mucin glycoprotein. Initially, the amino acid sequences of the mucin domain were shown to be long repetitive sequences, thus they

were classified as tandem repeat regions [3, 4]. Tandem repeat regions varied in both length and sequence dependent on species and splice variants of individual mucins. However, more recent discoveries indicate that, as a result of a high frequency of irregularly scattered Ser and Thr residues in the PTS domain, not all mucin domains can be classified as tandem repeats [5]. Therefore, we use PTS domain as a more general terminology for *O*-glycosylated amino acid sequences in mucins. Due to the high density of the anchored *O*-glycans, mucins adopt a rigid extended, bottle-brush, conformation. Based on their structure, mucins are categorized into two distinct groups [6]. The gel-forming mucins are a category of mucins discharged from goblet cells or glands, giving rise to a luminal matrix of mucus [7, 8]. The second category of mucins consists of the membrane-tethered mucins which are characterized by a single-span transmembrane domain, anchoring the glycoprotein to the plasma membrane of epithelial cell, as summarized in Fig. 1 [9].

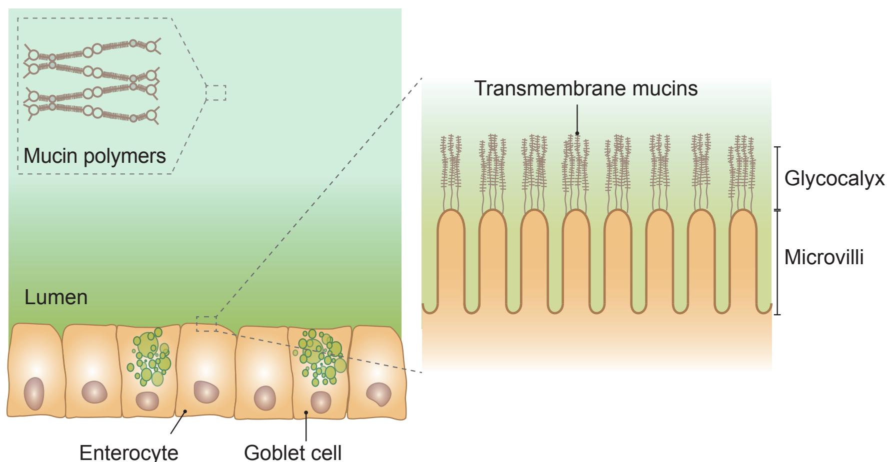


Fig. 1. Schematic drawing of the epithelial cells lining the small intestinal villi, covered with secreted mucins. The magnified view (right) displays microvilli protruding from the apical membrane of the enterocyte. The dense glycocalyx of transmembrane mucins has been suggested to act as a protective barrier.

Gel-forming mucins

The secreted mucins are MUC2, MUC5AC, MUC5B, MUC6, and MUC7. All of these secreted glycoproteins form large and complex gel-like structures with the exception of the small non-gel-forming MUC7 found in saliva [10]. Four gel-forming mucin genes, *MUC2*, *MUC5AC*, *MUC5B* and *MUC6,* are clustered on human chromosome 11p15 [11]. These gel-forming mucins form polymers via their N- and C-termini that contain conserved von Willebrand D (vWD) domains [12]. Gel-forming mucins harbor long mucin domains that vary in length, amino acid sequence and glycosylation patterns. Cysteine-rich domains, also designated CysD domains, are dispersed along the mucin domains and are suggested to mediate non-covalent crosslinking between mucin molecules [13]. MUC2 is the major secreted mucin in the small intestine and colon. It is a 2.5 MDa large glycoprotein with *O*-linked glycans attached to hydroxyl amino acids in its PTS domain [2]. MUC2 dimerizes via its C-terminus in the endoplasmic reticulum (ER), followed by heavy *O*-glycosylation in the Golgi apparatus. Further polymerization of the glycoprotein takes place when trimers, held together via N-terminal disulfide bonds, polymerize in acidic secretory vesicles [14]. Besides two CysD domains in its mucin domain which is further flanked by N- and C-terminal vWD domains, MUC2 harbors a von Willebrand C domain (vWC) and a cysteine knot domain (CK) on its C-terminal end [15, 16].

Membrane-tethered mucins

The membrane-tethered mucins MUC1, MUC3, MUC4, MUC12, MUC13, MUC16, MUC17, and MUC20, are type I transmembrane proteins and share common features such as mucin and cytoplasmic domains, while they differ in length, sequence and glycosylation patterns. They all have an N-terminal signal sequence,

allowing targeting of the membrane-tethered mucin to the secretory pathway. The signal sequence is followed by a PTS domain which is heavily decorated with *O*-glycans. The membrane-tethered mucins MUC1, MUC3, MUC12, MUC16, and MUC17 harbor EGF-like domains and a SEA (sea urchin sperm protein, enterokinase, and agrin) domain C-terminal to the PTS domain, thus they are classified as SEA-type mucins [17-26]. The 110-residue SEA domain is autocatalytically cleaved in an intramolecular strain-dependent process taking place in the endoplasmic reticulum [27-29]. The site of the autocatalytic cleavage is N-terminal to the serine residue in a G↓SVVV consensus motif found in the SEA domain (Fig. 2). The SEA cleavage of the single precursor protein results in a heterodimer which is held together via non-covalent interactions. Thus, SEA-type transmembrane mucins are presented as cleaved but yet associated heterodimers on the surface of cells.

A comparison between known SEA domain sequences reveals amino acid sequence differences between SEA domains in different SEA-type transmembrane mucins. MUC16, a transmembrane mucin originally identified as an ovarian cancer antigen, is the only transmembrane mucin with multiple SEA domains in tandem. All SEA domains on MUC16, with exception of possibly one, are incapable of strain-dependent cleavage due to the absence of the GSVVV consensus motif and the presence of a conserved pair of cysteines that stabilize an intramolecular disulfide bond between the β2 and β3 strands of the domain [30, 31]. Other SEA domains, e.g. domains found in MUC1, MUC3, MUC12, and MUC17, lack the particular cysteine pair in MUC16. Interestingly, the lack of a stabilizing cysteine pair correlates to the ability to undergo strain-dependent SEA cleavage. It has been suggested that transmembrane mucins harboring cleavable SEA domains are biologically destined to dissociate at the plasma membrane [28].

Unlike the SEA-type transmembrane mucins, MUC4 harbors two unique domains among membrane-tethered mucins; a nidogen homology region (NIDO) and the adhesion-associated domain in MUC4 and other proteins (AMOP) domain [21, 32]. In addition, MUC4 has a vWD domain. Consequently, MUC4 has been classified as a NIDO-AMOP-vWD-type mucin [26]. The vWD domain harbors a GD↓PH (glycine, asparagine, proline, and histidine) motif which has been proposed to be cleaved in the ER by a serine protease [33]. The GDPH motif is a feature shared with the secreted mucins MUC2 and MUC5AC, which instead undergo a pH-dependent autocatalytic cleavage in their GDPH motifs [34, 35].

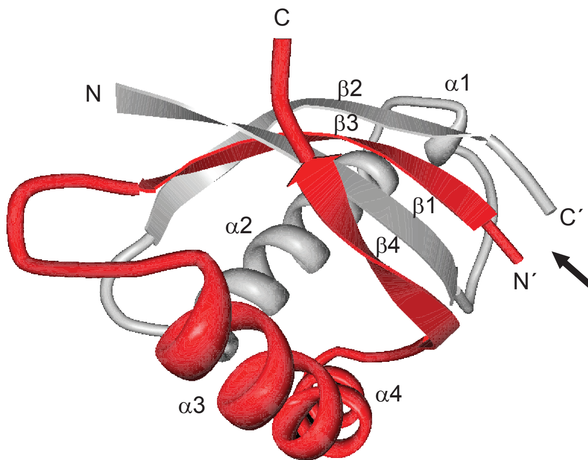


Fig. 2. A schematic presentation of the SEA domain with its α-helicec and β-sheets. The SEA cleavage (G↓SVVV) site is marked with a black arrow.

The transmembrane mucin MUC1

MUC1 is a extensively studied membrane-tethered mucin. It is assumed to be involved in tumor progression and immune activation. Along with MUC4 and MUC16, MUC1 is expressed on the surface of epithelial cells in the normal lung [36]. In addition to epithelial cells in the respiratory tract, MUC1 is expressed on apical surfaces of secretory epithelia and in a range of hematopoietic cells [3, 37]. The human *MUC1* gene is comprised of 7 exons which can be alternatively spliced to form transcripts from 3.7 to 6.4 kb, coding for a number of protein products. MUC1/SEC, lacking the type I transmembrane region,

is a secreted form of MUC1 [38]. The splice variants MUC1-CT80 and MUC1-CT58 differ from wild type MUC1 with regards to the length of their cytoplasmic tails (CTs) [39] and a fourth splice variant, MUC1/Y, lacks the extracellular glycosylated mucin domain [40].

The CT domain of MUC1 harbors several intracellular interaction and sorting motifs. MUC1CT interacts with various kinases, such as c-Src , members of the epidermal growth factor receptor (EGFR or ErbB) family, protein kinase C δ (PKCδ) and glycogen synthase kinase 3β (GSK3β) [41-45]. Other binding partners of MUC1CT are β-catenin and the tumor repressor p53, regulating transcriptional events [46-48].

The intestinal transmembrane mucins MUC3, MUC12, and MUC17

The membrane-tethered mucins MUC3, MUC12, and MUC17 are mainly found in the gastrointestinal tract. MUC3 is found in the intestine, colon and the gall bladder [49-51], MUC12 is expressed in the colon [20], while MUC17 is expressed throughout the intestinal tract, reaching an expression maximum in the duodenum [24]. The intestinal membrane-tethered mucins are genomically clustered on chromosome 7q22. Each of them is coded by 10 exons and they share similarities regarding exon-intron positions [2]. The signal sequence is coded by exon 1 and the first EGF-like domain is encoded by exon3 after exon 2 coding for the PTS domain. The SEA domain is coded by the third, fourth and fifth exons, while the second EGF-like domain is encoded by exons 6 and 7. The transmembrane domain lays in exon 7 followed by the cytoplasmic tail encoded by exons 7 to 10. It has been suggested that two forms of MUC3 coded by two distinct genes, *MUC3A and*

MUC3B, exist as a result of a recent gene duplication [50]. MUC3B displays amino acid changes in the end of the PTS domain. However, it is likely that the discovery of two forms of MUC3 is attributed to single nucleotide polymorphism in the single *MUC3* gene.

The corresponding genomic region of intestinal membrane-tethered mucins expressed in the mouse is chromosome 5, locus qG2. The first SEA domain identified in the mouse genome was mapped to this chromosome region in the vicinity of the *Trim56* gene, a feature that is shared with the human *MUC17* gene which is found near the human *TRIM56* [2]. Despite the fact that the mouse mucin in this region has been annotated as *Muc3*, it is more likely that this protein is the mouse *MUC17* orthologue and therefore designated murine *Muc3(17)* [52]. The murine orthologues of the remaining two human transmembrane mucins expressed in the intestine, Muc3 and Muc12, are not fully sequenced, thus rendering difficulties when studying the features and functions of theses mucins in the mouse.

The major function of the intestinal transmembrane mucins is still an enigma. There are reports of increased amounts of Muc3(17) in a cystic fibrosis model in mice [39, 52, 53]. Endogenous human MUC17 has been shown to promote cell migration and suppress apoptosis in colonic cell cultures. The underlying mechanism is dependent on ERK phosphorylation stimulated by EGF-like domains on MUC17 [54]. Other studies indicate that suppression of MUC17 in intestinal cell lines resulted in increased permeability and increased susceptibility to invasion by enteroinvasive *E. coli* [55]. These reports suggest that transmembrane mucins own cell-protective properties.

PDZ adapter proteins

PDZ domains, originally identified in Post synaptic density protein 95, Drosophila disc large tumor suppressor, and Zonula occludens-1 protein, are the most common protein interaction domains in the metazoan [56]. In the human genome, over 1,100 PDZ domains have been identified in over 500 proteins, whereas the mouse genome contains over 700 PDZ domains belonging to more than 300 proteins [57, 58]. PDZ domains consist of 80-100 amino acids long single or repeated sequences sharing sequence homology across multiple species [59]. Their key function is the ability to bind ligands harboring C-terminal PDZ binding motifs (Table I). The first identified interaction between a PDZ protein and its ligand was binding of two N-terminal PDZ domains of PSD-95 to the specific C-terminal peptide motif (-E-T/S-D/E-V) of Shaker-type K^+ channels [60] and the NR2

Table I. A list of known ligands for three important Class I PDZ adaptor proteins.

Ligand protein	PDZ adaptor protien	Effect of interaction	Reference
BCR	PDZK1	Stabilization	72
NHE3		Signaling, activation	73
CFTR		Stabilization	74, 75
MRP2		Sorting and/or stabilization	65
DRA		Signaling	76
SR-BI		Signaling	77
OAT4		Sorting and/or stabilization, activation	78
β_2- AR	NHERF1	Sorting and/or stabilization	79
NHE3		Stabilization, activation	80
CFTR		Sorting and/or stabilization, activation	81, 82, 83
iNOS		Sorting, activation	84
PDZK1		Stabilization, activation	85, 86
CFTR	GOPC	Sorting	87

subunit of the NMDA receptor [61, 62]. These findings revealed that PDZ domains function as modular units and their C-terminal binding ability can be acquired when transferred to other heterologous proteins.

A single PDZ domain consists of six antiparallel β-strands (βA-F) organized in a sandwich and flanked by two α-helices (αA and αB). The βB strand and the αB helix are arranged into a groove which acts as a binding pocket for internal or C-terminal peptide motifs by entrapment of a free carboxylate group at the end of the ligand peptide. The loop responsible for binding of the carboxylate group lies between the βA and βB strands and the interaction between the carboxylate-binding loop and the carboxylate oxygen is necessary for the PDZ recognition of C-terminal peptides of the ligand. [43, 63]. The amino acid sequence for the carboxylate-binding loop (R/K-XXX-GLGF where X represents any amino acid) is therefore highly conserved among PDZ domains [64, 65].

The specificity and affinity of PDZ domains for corresponding C-terminal PDZ ligands has been readily studied. C-terminal amino acid motifs of PDZ ligands can be categorized into three distinct classes based on amino acid sequences from position 0 to position -2 on the ligand protein [66]. The first class of C-terminal PDZ motifs, designated Class I PDZ motifs, involves a hydrophobic amino acid residue on position 0 and serine or a threonine amino acid residue on position -2. The side chain hydroxyl group on the Ser or Thr amino acids forms a hydrogen bond with N-3 nitrogen present on a highly conserved histidine in position αB1 [63]. The second class of PDZ motifs, Class II PDZ motifs, have hydrophobic residues at both position -2 of the peptide ligand and the αB1 position of the PDZ domain [66]. Finally, a third class of PDZ motifs, Class III PDZ motifs, requires a negatively charged amino acid residue at position -2 on the peptide

ligand and a tyrosine at αB1 position on the PDZ domain [67]. Despite a rigid classification of C-terminal PDZ motifs, several reports indicate that while residues at the positions 0 and -2 are important for PDZ interactions, more N-terminal sequences in the peptide ligands also contribute to specificity of the binding. For example, the amino acid at position -3 has also been reported to be in contact with the peptide-binding groove of the PDZ domain [63, 68, 69]. In fact, residues as far as position -8 have been reported to modulate the specificity of PDZ interactions [66, 70, 71].

The ion channels of the small intestine

The small intestine is a highly specialized organ dedicated to digestion and absorption. It is lined with goblet cells that produce, store and discharge mucus, and with epithelial cells, that participate in absorption, ion exchange and hormonal activities [88, 89]. The absorptive enterocytes are polarized, columnar cells with distinct apical and basolateral domains that are separated

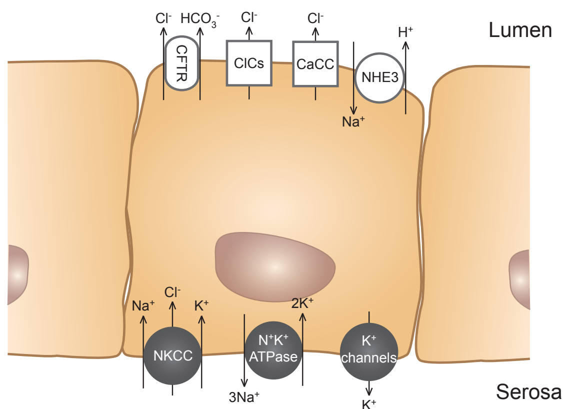


Fig. 3. A summary of secretory and absorptive ion channels present on the apical and basolateral membranes of an enterocyte in the small intestine.

by tight junctions. Each membrane domain possesses separate populations of proteins that require specific protein sorting machineries [86].

The small intestine coordinates transport of the major ions, Na^+, Cl^-, K^+ and HCO_3^-, and combines ion transport with solute transport in order to move fluid across membranes (Fig. 3) [90]. Three channels are the major players involved in Cl^- secretion; the Cystic fibrosis transmembrane conductance regulator (CFTR), calcium-activated chloride channels (CaCC); and chloride channel type-2 (ClC-2) channels. A requirement for apical chloride secretion is the basolateral Na^+-K^+-ATPase. Driven by ATP, this channel transports three Na^+ ions out of the cell while two K^+ ions are carried into the cell [91, 92]. Chloride secretion is also dependent on the basolateral Na^+-K^+-$2Cl^-$ cotransporter 1 (NKCC1) which mediates Cl^- uptake at the basolateral membranes of enterocytes [93, 94]. In addition to NKCC1, the basolateral membrane is the site of expression for potassium channels which through K^+ efflux maintain intracellular electroneutrality and enable apical Cl^- secretion [95].

Cystic Fibrosis and the CFTR anion channel

The *CFTR* gene, coding a protein belonging to the ATP-binding cassette (ABC) sub-family C of transporters, was identified in 1989 [96, 97]. ABC transporters constitute the largest family of transporters identified [98]. 48 ABC transporters have been identified in the human genome, all sharing the presence of two homologous nucleotide-binding domains as a common feature [99]. The CFTR ion channel consists of two six membrane-spanning domains (MSDs) and two nucleotide-binding domains (NBD) of which the first is flanked by the transmembrane domains and a regulatory (R) domain. The second NBD is located intracellularly C-terminal of the second transmembrane

domain. Finally, an intracellular C-terminal end domain contains a Class I PDZ binding motif [100].

CFTR differs from other members of the ABC transporter family by acting as an ion channel. Its function can be regulated by conformational changes in the two NBDs and modulation of the R domain which upon phosphorylation by protein kinase A (PKA) or C (PKC) increases channel opening. The probability of an actively gating or a quiet channel is therefore dependent on, and regulated by, the balance between kinases and phosphatases acting on multiple sites on the R domain [101].

CFTR mediates movement of chloride ions in order to enable the secretion and absorption of salts across the epithelial barrier [102]. Furthermore, it has long been known that CFTR is important for secretion of HCO_3^- and stimulation of Cl^-/HCO_3^- exchangers [103, 104]. It has also been postulated that CFTR regulates other ion channels such as epithelial sodium channel (ENaC) and the outwardly rectifying chloride channel (ORCC) [105, 106].

Mutations in the *CFTR* gene cause cystic fibrosis (CF), an autosomal recessive genetic disorder affecting organs such as the lung, pancreas, small intestine and sweat glands. The disease is characterized by impaired chloride and bicarbonate conductance across the apical surface of epithelial cells in the affected organs, resulting in a dehydrated mucus barrier which serves as habitat for pathogens that cause infections [107]. Up to date nearly 1.500 different mutations have been mapped to the *CFTR* gene. Although the majority of these mutations are associated with disease, ΔF508, a single codon deletion of the amino acid phenylalanine at position 508, is the most common mutation on the *CFTR* gene. This mutation accounts for approximately 90% of CF cases in certain populations. The ΔF508 mutation results in incorrect folding of the CFTR protein, followed by retention

of the defective protein in the endoplasmic reticulum [108, 109]. The immature CFTR protein is then trapped by chaperones and degraded by cytosolic proteasomes [110, 111]. In the context of CF, the impaired efflux of HCO_3^- in response to either forskolin or carbachol results in decreased alkalinization of the lumen, followed by hyperviscosity of secreted mucins due to altered processing [41].

Na^+/H^+ Exchanger 3, an absorptive ion channel

Na^+/H^+ exchange is the means by which Na^+ is absorbed across epithelia. The first evidence of sodium and hydrogen transport across brush border membranes of epithelial cells in the intestine was presented nearly forty years ago [112]. The discovery proved that protons were moved out across brush border membranes when sodium was added to the luminal side of cells, thus acidifying the extracellular medium, and that the Na^+ influx and H^+ efflux with the stoichiometric ratio 1:1, was mediated through an antiport mechanism. A gene encoding the first member of the Na^+/H^+ exchanger was cloned in 1989 [113]. Na^+/H^+ exchangers are expressed in every cell type in eukaryotes and their primary function is to regulate intracellular pH, cell volume and proliferation, and to mediate transepithelial absorption of Na^+ [114, 115]. In the human, Na^+/H^+ exchanger 3 (NHE3) is expressed primarily in kidneys, small intestine and colon [8]. It is expressed in the brush border membrane of renal proximal tubule cells and in the brush border of epithelial cells lining intestinal villi and to a lesser extent in epithelial cells of the crypts [116, 117]. Similar to other members of Na^+/H^+ exchangers, NHE3 has a N-terminal part, consisting of 11 membrane-spanning α-helices [118]. This N-terminal part is necessary for Na^+/H^+ exchange, whereas the C-terminal domain mediates calmodulin-dependent inhibition of basal activity and

regulatory inhibition via PKC phosphorylation [119, 120]. In addition, the C-terminal domain harbors growth factor-mediated stimulatory sites [119]. Membrane-tethered NHE3 is linked to the underlying cytoskeleton via a direct interaction mediated by an internal ezrin-binding site on the C-terminus of the transporter. The C-terminus contains an additional internal site for PDZ-binding to NHERF1 and NHERF2 [13, 15, 16]. Finally, NHE3 has a Class I PDZ binding motif for PDZK1 on its far C-terminus [121].

Carbachol, a cholinergic agonist

The human intestinal tract secretes approximately 8 liters of fluid during a 24-hour period. In normal conditions the major part of this volume is absorbed during transition through the small and large intestine, reaching maximum fluid absorption in distal parts [93]. Chloride secretion from enterocytes is induced by secondary messengers, i.e. cAMP and cGMP and $[Ca2^{+}]_i$ [93]. Moreover, hormones such as Prostaglandin E_1 (PGE_1) and Vasoactive intestinal polypeptide (VIP) are involved in Cl^- secretion by increasing levels of cAMP [122]. Specifically, cAMP induces PKA-mediated phosphorylation of CFTR, thus generating Cl^- secretion. Increased levels of either cAMP or cGMP result in inhibited Na^+ and Cl^- absorption [123, 124].

Acetylcholine, an organic neurotransmitter, has been shown to induce Cl^- secretion by elevating levels of $[Ca2^{+}]_i$. Studies on cultures of colonic or intestinal-like cells lines lacking neuronal features, have revealed that the action of analogues of acetylcholine, i.e. carbachol, on the intestinal epithelium is direct [125]. In cell cultures, carbachol acts in a dose-dependent manner and generates a transient change in the short circuit current (I_{sc}), lasting approximately 10 min. This is accompanied by an equally transient increase in Cl^- secretion. However, isolated

intestinal tissue contains other secretagogues, such as PGE_1 and VIP, resulting in a more sustained change in I_{sc} [125]. Mitochondria, ER, calmodulin and basolateral Ca^{2+} and Na^+ channels are all involved in mobilization of $[Ca^{2+}]_i$ [126].

In the duodenum, secretion of HCO_3^- via CFTR is stimulated by elevated cAMP as a result of forskolin. Carbachol stimulation, i.e. elevated $[Ca^{2+}]_i$, also generates HCO_3^- secretion [127, 128]. In fact, it has been shown that carbachol stimulates Cftr exocytosis at the brush border membrane (BBM) in the small intestine of wild-type rats. NHE3 regulation occurs in both physiological and pathological conditions. For example, extensive loss of water via secretion and impaired NHE3-mediated absorption during diarrhea in response to bacterial toxins, are caused by elevation of second messengers such as cAMP, cGMP, and $[Ca^{2+}]_i$ [129]. In fact, elevation of $[Ca^{2+}]_i$ via carbachol or ionomycin, inhibits NHE3 due to increasing endocytosis, followed by formation of intracellular multiprotein complexes containing NHE3 [21, 130-134]. It has been shown that acute $[Ca^{2+}]_i$-mediated regulation of NHE3 in the plasma membrane is dependent on PDZ adaptor proteins belonging to the NHERF family [129, 133]. However tissue-dependent, generally NHERF1 is involved in cAMP-mediated inhibition of NHE3, whereas $[Ca^{2+}]_i$-mediated inhibition of NHE3 is dependent on NHERF2 [135]. PDZK1 (NHERF3) mediates both cAMP- and $[Ca^{2+}]_i$-dependent inhibition of NHE3 [136, 137]. While NHERF4 facilitates exocytosis and increased NHE3 activity in response to elevated $[Ca^{2+}]_i$ [138].

Several studies have revealed that CFTR possesses the ability to influence NHE3 and several mechanisms have been suggested. One report has shown that CFTR mediates forskolin-induced elevation of $[Ca^{2+}]_i$, thus potentially affecting NHE3 distribution and activity

[139]. In another study, it was shown that forskolin-induced activation of CFTR amplifies inhibition of NHE3 via cAMP. Data from mouse pancreas and fibroblast expressing both CFTR and NHE3 heterologously reveal that NHERF1 crosslinks CFTR and NHE3 [140].

The brush border of enterocytes

The apical domain of enterocytes is characterized by a brush border consisting of a dense array of microvilli protruding from the cell surface. Approximately 1,000 microvilli extend out from each cell, generating a 30-fold increase in cell surface area and allowing efficient exchange with the extracellular milieu [141, 142]. The brush border consists of a BBM covered by a dense glycocalyx and an underlying cytoskeletal structure. The glycocalyx is a thick filamentous coat covering

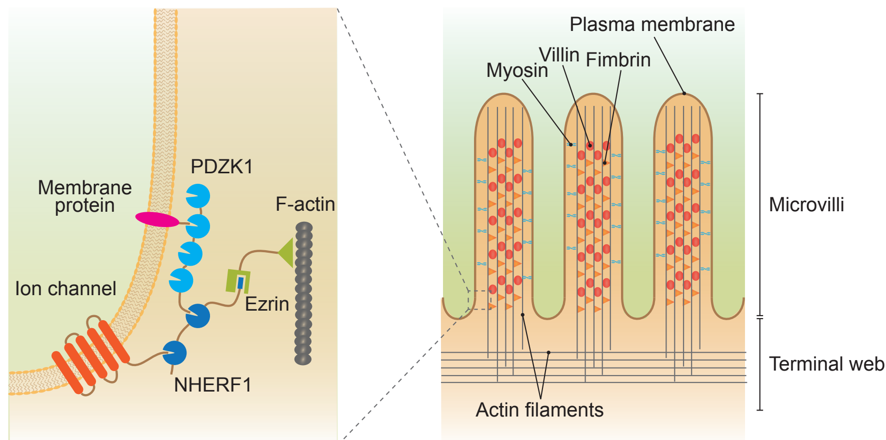


Fig. 4. A cartoon showing the arrangement of the apical brush border of epithelial cells. The right panel displays the organization of the terminal web and the microvilli. The left panel shows the connections between a membrane-tethered ion channel and F-actin via the PDZ protein NHERF1 and the F-actin-binding protein Ezrin. NHERF1 is also able to anchor additional PDZ adaptor proteins, e.g. PDZK1, which in turn can bind membrane-anchored proteins.

the microvilli of enterocytes (see Fig. 1) [44]. Initially termed as the fuzz, this thick layer reacts with periodic acid-Schiff stain, demonstrating the existence of glycoproteins and glycolipids. Moreover, positive staining with Alcian blue points toward the presence of sulfated and carboxylated glycoproteins in the glycocalyx [58]. Long stretched transmembrane mucins reaching approximately 1 μm in length, are the only known proteins expressed on apical surfaces which could explain the characteristic filamentous appearance of the glycocalyx. Although existing data indicate that heavily glycosylated transmembrane mucins make up the major part of the glycocalyx covering intestinal cells, tangible proof is still lacking.

The underlying cytoskeletal structure that is required for the ordered arrangement and dynamics of the BB is composed of vertical filamentous actin (F-actin) along the length of the microvillar region and horizontal F-actin in the terminal web region situated under the base of microvilli (Fig. 4). Other protein components are responsible for crosslinking of actin filaments in both regions. Briefly, F-actin serves as a core bundle in the microvilli. In turn, F-actin is cross-linked by fimbrin and villin [143, 144]. The cross-linked F-actin core bundle is radially attached to the adjacent microvillar membrane through ATP- and calcium-sensitive myosin1A-calmodulin-complexes [145, 146]. This complex mediates apical membrane migration along actin filaments towards the tip of microvilli and has been suggested to promote shedding of vesicles from BBM [147].

Vesicle trafficking in polarized epithelial cells

Polarized epithelial cells display conserved features in different organs. They are characterized by their organization into tight monolayers of cells, acting as boundaries between internal compartments and

the extracellular milieu. The specialized functions of epithelial cells require: 1) separated apical and basolateral membrane domains, 2) distinct populations of membrane proteins at each membrane domain, and 3) a polarized sorting machinery that targets different membrane proteins to the apical and basolateral domains [148]. The major site for intracellular sorting of various membrane proteins is the outermost cisterna of the Golgi apparatus, namely the *trans*-Golgi network (TGN) [101]. Here, at least two sorting pathways for exocytosis, a lipid raft-dependent pathway and a galectin-dependent pathway, are at play [149-153]. Furthermore, sorting signals on cargo proteins destined to be presented on apical or basolateral membrane surfaces are responsible for polarized transport from the TGN to the two distinct plasma membrane domains. Apical sorting signals include ectodomain signals such as *N*- and *O*-glycans, glycosylphosphatidylinositol (GPI) anchors, and cytoplasmic signals [154-157]. Basolateral sorting signals, mainly cytoplasmic tyrosine togethter with hydrophobic residues, or dileucine motifs, are identified by adaptor complexes (APs) that together with clathrin form clathrin-coated vesicles, that enclose the cargo during transport from the TGN to basolateral membranes and endosomal compartments [158-162].

Another trafficking route characteristic for specialized secretory cells, such as goblet cells, is the regulated secretory pathway. This pathway involves synthesis, modification, sorting, storage, and controlled release of secretory proteins such as secreted mucins from the cell. In detail, translated secretory mucins undergo folding, assembly and glycosylation in the ER [163, 164]. Proteins are then transported to the Golgi apparatus, where they undergo further processing and maturation [59]. Synthesized mucins are then sorted in the *trans*-Golgi network and stored in regulated secretory vesicles that await secretory stimuli prior to discharge from the cell

[165]. The packing of mucins in theses vesicles requires acidic pH as well as high concentrations of calcium [166-168]. These conditions trigger the aggregation of the N-terminal end of the mucin and a well-organized packing which allows release and expansion without entanglement [169]. Both microtubules and actin filaments are involved in the transport of secretory granules to the plasma membrane, where cargo is emptied in the lumen upon membrane-tethering, docking and fusion [170, 171].

In analogy with exocytosis, endocytosis in epithelial cells occurs in a polarized fashion, involving functionally and physically specialized compartments. Two major types of endocytic routes have been described: 1) clathrin-mediated endocytosis (CME) involving clathrin, AP-2 and dynamin, and 2) lipid raft-dependent endocytosis facilitated by caveolae, which are vesicles rich in cholesterol and caveolin 1[172-175]. Membrane proteins are targeted for CME when cytoplasmic conserved motifs, containing tyrosines and amino acids with large hydrophobic side chains, are identified by AP2 [176, 177]. AP2 also provides binding sites for clathrin, thus mobilizing clathrin which in turn induces membrane curvature and vesicle budding mediated by the GTPase dynamin [178, 179]. Endocytosed vesicles carrying cargo membrane proteins are then sorted in endosomes. Firstly, incoming endocytosed cargo reaches slightly acidic early endosomes (EE), where sorting occurs [180, 181]. Secondly, the cargo reaches recycling endosomes (RE) via a geometric transition of EEs. REs containing cargo are either recycled to the plasma membrane or translocated to the perinuclear cytoplasm, where they congregate in close association with microtubule organizing centers (MTOC) [182-184]. Perinuclear REs, which contain an intracellular pool of recycling cargo, can in turn be remobilized to the plasma membrane to release their cargo. For example, up to 75%

of expressed transferrin receptors reside in perinuclear REs, awaiting recycling to the plasma membrane [185]. Another transport passage for cargo residing in EEs is along microtubules destined for late endosomes (LEs). LEs contain lysosomal enzymes, e.g. hydrolase, which degrade internalized content. Finally, internalized cargo is accumulated in lysosomes.

Trafficking of membrane-tethered mucins

While the mechanism behind trafficking of intestinal membrane-tethered mucins remains to be fully elucidated, data from studies on the membrane mucin MUC1 reveal a complex endocytic machinery and regulated sorting in different endosomal vesicle populations. It has been shown that *N*-glycans in the vicinity of the MUC1 transmembrane domain are necessary for apical targeting of the membrane mucin [186]. Also, endocytosis of MUC1 depends on the extent of *O*-glycosylation of the mucin domain, as mucin with less *O*-glycosylation undergoes faster endocytosis [187]. Others studies have shown that EGF-stimulation induces internalization of MUC1CT via CME followed by fusion of endocytic vesicles with Rab5-positive vesicles in the early endosome [188]. The complex mechanism behind MUC1 internalization becomes evident when taking into account the requirement of cytoplasmic tyrosine residues for endocytosis via binding to AP-2 and growth factor receptor-bound protein 2 (Grb2) [189]. The subsequent recycling of MUC1 is regulated by post-translational transmembrane *S*-palmitoylation. Blocked *S*-palmitoylation results in decreased recycling of MUC1 to the cell surface and accumulation in recycling endosomes [190].

Trafficking of CFTR and NHE3

Biosynthesis, exocytosis, and endocytosis of CFTR are subjected to extensive regulation. In the ER, CFTR undergoes folding and a quality control machinery targets misfolded proteins for ubiquitination and degradation via ER-associated degradation (ERAD) [191, 192]. Further processing of correctly folded protein takes place in the Golgi apparatus where mannose sugars, present on the fourth extracellular loop of CFTR, are converted to complex oligosaccharides [17, 191]. From the Golgi, CFTR is transported in clathrin-coated vesicles to the apical surface of the cell, where the ion channel undertakes anion secretion. CFTR displays rapid turnover due to extensive endocytosis and recycling [17, 193]. It has been shown that endocytosis of CFTR is clathrin-dependent and that the internalized anion channel travels via EEs to REs while some fractions continue to the lysosomes for degradation [19, 108]. Interestingly, a major fractions of mature CFTR undergoes degradation in lysosomes via LEs. In fact, only 5 to 15% of mature and functional CFTR needs to be apically expressed for proper intestinal anion secretion [25, 194].

In contrast to CFTR, basal endocytosis of a fraction of NHE3 is dependent on formation of discrete lipid raft membrane domains [195]. However, reports of CME of NHE3 suggest different endocytic mechanisms involved in basal and stimulated endocytosis of the exchanger [196]. The localization of NHE3 in BBM of polarized cells is regulated by alterations in $[Ca^{2+}]_i$ [21, 138]. Stimulation of intestinal enterocytes with carbachol, that leads to elevated $[Ca^{2+}]_i$, has been reported to induce internalization of NHE3 into early endosomes [197].

In the context of ion channel trafficking, the role of PDZ adaptor proteins belonging to the NHERF family must be mentioned. Members of the NHERF family are not only involved in regulation of CFTR and NHE3 but

they also regulate targeting and retention of ion channels in different subcellular compartments. In Nherf2 KO mice, the localization of Nhe3 was shifted from the apical surface to intracellular pools, thus resulting in decreased Nhe3 activity [135]. NHERF1 has been shown to link CFTR to Ezrin, thereby tethering the anion channel to the plasma membrane. Furthermore, it has been proven that the C-terminal PDZ motif of CFTR plays a role in recycling of endocytosed CFTR to the plasma membrane [198]. Golgi-associated PDZ and coiled-coil motif-containing protein (GOPC), a TGN-resident PDZ protein, is able to target CFTR to lysosomes for degradation, a mechanism that is bypassed by NHERF1 that redirects CFTR to apical membranes [87, 199, 200]. In summary, the distribution and activity of ion channels and exchangers is closely monitored and regulated by adaptor and scaffolding proteins associated with various subcellular compartments and organelles.

Aims of thesis

The overall aim of the thesis work was to provide a deeper understanding of the function and regulation of intestinal SEA-type transmembrane mucins.

Specific aims

1. To identify novel interactions between C-termini of transmembrane mucins and PDZ adaptor proteins.

2. To study the specific action of PDZ adaptor proteins on expression and subcellular localization of transmembrane mucins.

3. To study the relationship between transmembrane mucins and ion channels expressed in the small intestine.

4. To assess the magnitude of forces stabilizing the SEA domain of MUC1.

Methods

2

Animals (Papers I and IV)

All animal experiments were approved by the animal ethics committee at the University of Gothenburg. The Pdzk1$^{-/-}$ mice were generated in the Department of Pathology, Beth Israel Deaconess Medical Center [201]. NHERF1$^{-/-}$ mice, backcrossed for 8 generations into the FVB/n background, were generated from heterozygotes produced at the Duke University Medical Center [202]. NHERF2$^{-/-}$ mice, backcrossed for 14 generations into the FVB/n background, were produced from heterozygotes generated at the Erasmus University Medical Center [203]. C57/BL6 mice were used as wild-type controls.

Antibodies (Papers I, II, and IV)

A number of raised antibodies against human and murine transmembrane mucins were used in the studies described in this thesis. Briefly, synthetic peptides against specific regions on the transmembrane mucin of interest were conjugated to KLH, purified and used for immunization of two rabbits. The polyclonal antibody (pAb) against murine Muc3(17) has previously been described [52]. An anti-MUC17S1 pAb was raised against the peptide LKNHSSQEFQEFKQTFTEQMNIC in a region N-terminal of the SEA cleavage site. Anti-MUC17S2 pAb was raised against the peptide KYTPEYKTVLDNATEVVKEKITKVC, targeting a region C-terminal of the SEA cleavage site. Anti-

MUC3C1 polyclonal antibody was raised against a CDTTMKVHIKRPEMT peptide on the C-terminal tail of human MUC3.

Atomic force microscopy (Paper III)

Atomic force microscopy was used for force measurements on a recombinant protein, containing three MUC1-derived SEA domains, at 23±0.5°C using PicoScan software (version 5.3.3; Molecular Imaging) together with a PicoSPM atomic force microscope (Molecular Imaging). The recombinant protein was immobilized on mica sheets coated with a 5 nm thick chromium adhesion layer and a 25 nm thick gold layer. Double-side gold-coated CSC38/Cr-Au cantilevers chips (kspec = 0.03 N/m) were coated with thiolated-NTA (HS(CH2)11-EG3-NTA in ethanol). The approach/retraction speed for all experiments was $v \approx 820$ nms^{-1} (loading rate $vk \approx 25$ nNs^{-1}).

Labeling of surface glycoproteins (Paper IV)

Surface glycoproteins, presented on the surface of polarized Caco-2 cells, were labeled using biotin-hydrazide after oxidation of carbohydrate moieties with sodium *meta*-periodate. The method was first described by O'Shannessy *et al.* [204]. Labeled surface glycoproteins where then extracted by subsequent clearance of cell lysates, removal of free biotin-hydrazide and affinity precipitation using streptavidin conjugated to magnetic beads.

Results

3

Transmembrane mucins are ligands for PDZ adaptor proteins (Paper I and II)

There is limited knowledge regarding the function of the three transmembrane mucins, MUC3, MUC12, and MUC17, expressed in the human small intestine. While the domain structure of these SEA mucins has been determined, as described in Chapter 1, the cytoplasmic tails of MUC3, MUC12, and MUC17 have not been assigned any specific features or functions. As a first measure, we set out to identify consensus sequences in the intracellular C-termini of each of the three transmembrane mucins. Using the Eukaryote Linear Motif resource, we identified a four amino acid residue consensus sequence classified as a Class I PDZ motif in the far C-termini of MUC3, MUC12, and MUC17 (see Fig. 5) [205]. A Class I PDZ motif is characterized by the consensus sequence X-[S/T]-X-Φ, where X represents any amino acid and Φ represents a hydrophobic amino acid.

Mucin	Position (-50 … -40 … -30 … -20 … -10 … -2 0)
MUC3CT	-QDRKWFETWDEEVVGTFSNWGFEDDGTDKDTNFHVALENVDTTMKVHIKRPEMT**SSSV**
MUC12CT	-KEGTPGIFQKTAIWEDQNLRESRFGLENAYNNFRPTLETVDSGTELHIQRPEMV**ASTV**
MUC17CT	-EDSGPAPGTFQNIGFDICQDDDSIHLESIYSNFQPSLRHIDPETKIRIQRPQVM**TTSF**

Fig. 5. Class I PDZ motifs (bold letters) in the far C-termini of transmembrane mucins MUC3, MUC12, and MUC17.

MUC3 C-terminus binds Golgi-resident GOPC

In order to screen the Class I PDZ motif of MUC3CT for binding to PDZ adaptor proteins, we used an array of 123 different immobilized human PDZ domains. MUC3CT was fused to Yellow Fluorescent protein (YFP) and analyzed with an overlay assay involving PDZ domain arrays. A strong binding to the Golgi-associated PDZ and coiled-coil motif containing protein, GOPC, was observed (Fig. 2A, Paper II). Interestingly, the YFP fusion protein of MUC12CT did not bind GOPC while YFP-MUC17CT displayed a weak interaction. Although the Class I PDZ motif is a shared feature amongst MUC3, MUC12, and MUC17, only MUC3 exhibited high binding specificity for GOPC (Fig. 2B, Paper II). In order to assure that the MUC3CT-GOPC-interaction was PDZ mediated, the Class I PDZ motif on MUC3CT was removed. This inhibited the binding to GOPC (Fig. 2C, Paper II).

MUC17 C-terminus binds scaffolding protein PDZK1

Similarly, MUC17CT was screened for binding to PDZ adaptor proteins. MUC17CT displayed a strong interaction for the second PDZ domain of PDZK1 (PDZK1-D2) (Fig. 1B, Paper I). Next, MUC17CT fused to Glutathione S-Transferase (GST) was used as a bait in lysates from the colonic adenocarcinoma cell line, HT29. Compared to GST, GST-MUC3CT, and GST-MUC12CT that did not bind any PDZ adaptor proteins, GST-MUC17CT interacted with endogenous PDZK1 in HT29 lysates (Fig. 1C, Paper I). The specificity of MUC17CT for PDZK1 was shown to be PDZ-dependent as truncated MUC17CT, lacking the Class I PDZ motif, did not bind PDZK1 (Fig. 2B, Paper I). Finally, we demonstrated that the first, second and fourth PDZ domains of PDZK1 exhibited the ability to bind the specific Class I PDZ motif present on MUC17CT (Fig. 2C, Paper I).

PDZK1 anchors MUC17 to apical membranes of enterocytes (Paper I)

The murine orthologue of human MUC17 is designated Muc3(17). In analogy to MUC17, the C-terminus of Muc3(17) contains a Class I PDZ motif consisting of the amino acid sequence MTSL (L at position 0). We tested whether Muc3(17)CT was able to bind murine Pdzk1 in lysates from mouse jejunum. Indeed, GST-Muc3(17) CT bound Pdzk1. Interestingly, Pdzk1 was also able to bind human MUC17, suggesting a conserved functional interaction between PDZK1/Pdzk1 and MUC17/ Muc3(17) across the human and the mouse, despite the differences in their PDZ-binding motifs (Fig. 3, Paper I). Next, the expression and subapical localization of Muc3(17) was analyzed in jejunal sections of wild-type (Wt) and Pdzk1$^{-/-}$ mice. In Wt mice, Muc3(17) was localized to the outer part of the apical brush border of enterocytes while in Pdzk1$^{-/-}$, Muc3(17) was localized in intracellular vesicles, with a local accumulation in the subapical region below the apical plasma membrane (Fig. 4 A-C, Paper II). Thus, we concluded that Pdzk1 was important for membrane-tethering of Muc3(17).

Transmembrane mucins and ion channels are co-regulated (Paper II and IV)

Having identified PDZ proteins as binding partners for the transmembrane mucins MUC3 and MUC17, we turned our attention to the functional aspects of these novel PDZ-mediated interactions.

MUC3 interacts and colocalizes with GOPC

Our previous *in vitro* data suggesting a binding between MUC3CT and GOPC were tested in the BHK 21 cell line. A recombinant protein consisting of MUC3CT fused to the transmembrane and SEA domains of MUC17 (Myc-MUC17/MUC3CT) was stably expressed

in BHK 21 cells. Likewise, the same protein lacking the last six amino acid residues and thus the Class I PDZ motif of MUC3CT, was stably expressed in BHK 21 cells. GOPC was fused to a hemagglutinin (HA) tag and overexpressed in the two stable cell lines. Myc-MUC17/MUC3CT colocalized with GOPC whereas Myc-MUC17/MUC3CTΔ6 did not (Fig. 3 B-D, Paper II). Co-immunoprecipitation experiments validated these results as Myc-MUC17/MUC3CT precipitated HA-GOPC while Myc-MUC17/MUC3CTΔ6 only bound very weakly to HA-GOPC. These results were reproduced in a reciprocal co-immunoprecipitation experiment where HA-GOPC was used as bait (Fig 4, Paper II).

GOPC suppresses total levels of MUC3 but does not affect membrane-tethered MUC3

Published data suggest that GOPC entraps CFTR in the *trans*-Golgi network and targets the ion channel to the lysosomes for degradation [87, 199, 200, 206]. We tested whether GOPC possessed the ability to suppress total protein levels of MUC3. Indeed, overexpression of HA-GOPC resulted in decreased total levels of Myc-MUC17/MUC3CT, whereas HA-GOPC did not suppress Myc-MUC17/MUC3CTΔ6 (Fig. 5B, Paper II). Using fluorescent-associated cell sorting (FACS) the effect of HA-GOPC on total and membrane-anchored Myc-MUC17/MUC3CT was assessed. Similar to the results from western blot analysis, total levels of Myc-MUC17/MUC3CT were decreased in presence of HA-GOPC (Fig. 5D, Paper II). However, we did not observe any significant decrease in surface levels of Myc-MUC17/MUC3CT upon introduction of HA-GOPC (Fig. 5C, Paper II). We also observed increased total levels of Myc-MUC17/MUC3CTΔ6 in mock-transfected cells compared to Myc-MUC17/MUC3CT

in mock-transfected cells. A possible explanation for this is that recombinant MUC3 lacking the Class I PDZ motif bypasses GOPC-mediated lysosomal degradation, thus resulting in higher overall levels of the truncated protein in comparison to MUC3 with an intact Class I PDZ motif.

CFTR counteracts the down-regulatory action of GOPC on MUC3

At this point, we had identified GOPC as a shared PDZ binding protein for MUC3 and CFTR. We had also shown that GOPC suppressed the total protein levels of both the transmembrane mucin and the anion channel. Consequently, we postulated that MUC3 and CFTR coexisted in late Golgi and competed for binding to the single PDZ domain present on GOPC proteins. To test this hypothesis, increased amounts of CFTR were introduced in BHK 21 cells stably expressing Myc-MUC17/MUC3CT. With increasing amounts of CFTR, the total levels of Myc-MUC17/MUC3CT increased correspondingly. This effect was not observed in Myc-MUC17/MUC3CTΔ6 (Fig. 6, Paper II). Next, we turned to Caco-2 cells which express both endogenous MUC3 and CFTR. Firstly, YFP-MUC3CT or YFP-MUC3CTΔ6 was introduced into Caco-2 cells and the overall levels of CFTR were assessed using FACS. Overexpression of YFP-MUC3CT resulted in a 20% increase in total levels of endogenous CFTR (Fig. 7A, Paper II). In contrast, introduction of YFP-MUC3CTΔ6 did not alter CFTR levels. Secondly, increasing amounts of the cytoplasmic tail of CFTR (YFP-CFTR1440-80) were introduced into Caco-2 cells and total protein expression of endogenous MUC3 was analyzed. Overall MUC3 levels were augmented with increasing amounts of YFP-CFTR1440-80 (Fig. 7B, Paper II). These data suggested that CFTR can compete with MUC3 for binding to GOPC and that CFTR engaged free PDZ domains on

GOPC molecules, thus rescuing MUC3 from lysosomal degradation. Moreover, the reciprocal setup suggested that overexpression of MUC3 resulted in CFTR bypassing lysosome-mediated degradation, as summarized in our model (see Fig. 6).

Apical MUC17 dissociates from PDZK1 and resides in early endosomes upon carbachol-mediated endocytosis

The results obtained from our study of MUC3 indicated that transmembrane mucins can share cellular regulatory systems with ions channels that are important for cell homeostasis and mucus hydration. MUC17 is the major transmembrane mucin in the intestine and it reaches maximum transcript level in the duodenum, with a slight decrease in the ileum, followed by elevated

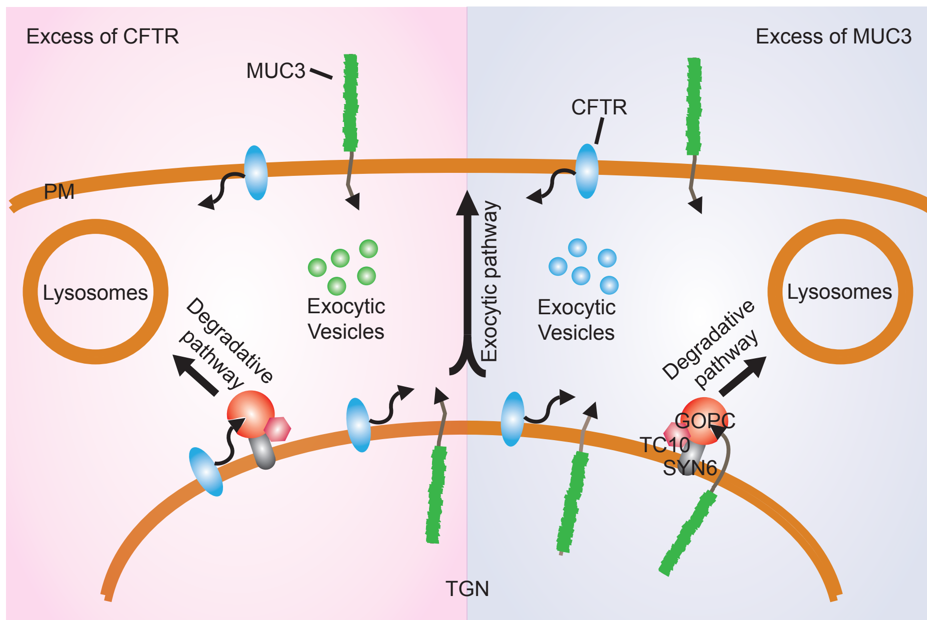


Fig. 6. A proposed model for the PDZ-mediated competition between MUC3 and CFTR for binding to GOPC in the *trans*-Golgi network.

numbers in the transverse colon and finally, slightly lower transcript levels in the descending colon and rectum [24]. Interestingly, the protein expression profile of MUC17 in the small and large intestine follows the distribution of CFTR and NHE3 ion channels [207, 208]. The scaffolding protein PDZK1 is involved in the correct subcellular localization of MUC17, CFTR, and NHE3 and moreover, PDZK1 is involved in the regulation of the two ion channels. As mentioned in Chapter 1, both CFTR and NHE3 are sensitive to carbachol (CCh) stimulation. In the case of NHE3, PDZK1-binding is abolished in response to CCh-induced elevation of $[Ca2^{+}]_i$ which results in NHE3 endocytosis. Consequently, we hypothesized that CCh could modulate MUC17 recruitment to the apical cell membrane of enterocytes. This hypothesis was tested in Caco-2 cells expressing endogenous MUC17 (Fig. 1A, Paper IV). Surface biotin-labeling of membrane-anchored MUC17 revealed that surface MUC17 was decreased upon CCh stimulation of Caco-2 cells (Fig. 1E-F, Paper IV). Interestingly, the dynamin-inhibitor Dynasore could inhibit the endocytosis of MUC17. This was in agreement with the existence of a putative AP-2 binding site on the MUC17CT. Confocal microscopy in untreated cells showed that MUC17 was expressed on the upper portion of the microvilli, separated from the dense F-actin staining defined as the boundary between the microvilli and the underlying terminal web. However, when cells were treated with CCh, MUC17 was internalized and redistributed to the F-actin stained region (Fig. 2A-B, Paper IV). A similar experiment performed on tissue from mouse duodenum, indicated that Muc3(17) was internalized upon CCh-treatment (Fig. 7, Paper IV). Colocalization studies showed that a majority of endocytosed MUC17 in CCh-stimulated cells was found in EEA1-positive early endosomes (Fig. 3, Paper IV). We also analyzed the expression and localization of

MUC17 in relation to the scaffolding protein, PDZK1. While MUC17 colocalized with PDZK1 under basal conditions, less colocalization was observed in CCh-treated cells, suggesting that CCh induces dissociation of MUC17 from PDZK1, and that this dissociation allows for endocytosis of the transmembrane mucin (Fig. 4, Paper IV).

Internalization of apical MUC17 is concomitant with NHE3 endocytosis and recruitment of CFTR to the apical surface

Several studies point toward the role of CCh in distribution of NHE3 and CFTR in the apical brush border of enterocytes. Jakab *et al.* have reported that Nhe3 is endocytosed while Cftr is recruited to the apical surface when rat small intestine is stimulated with CCh [197] and Zachos *et al.* reported similar findings regarding NHE3 in Caco-2 cells [73]. Therefore, the distribution of MUC17 in the brush border of Caco-2 cells was studied in relation to NHE3. MUC17 and NHE3 were localized to the outer part of microvilli in untreated cells and both were endocytosed in presence of CCh (Fig. 5A-B, Paper IV). Similarly, we analyzed the distribution of MUC17 in relation to CFTR. Previous studies have shown that only a small fraction of CFTR is presented on the cell surface, while a major population is found intracellularly where CFTR undergoes quality control [25, 194]. Indeed, the strongest staining for CFTR was found below the terminal web region in Ctrl Caco-2 cells. Stimulation with CCh caused the redistribution of CFTR towards the apical surface (Fig 6, Paper IV). This suggested that CFTR is recruited to the lower part of the microvilli. We concluded that the internalization of MUC17 in response to CCh is concomitant with NHE3 endocytosis and CFTR recruitment to the apical surface of Caco-2 cells.

SEA-type transmembrane mucins act as protective devices (Paper III)

The folding dynamics of the SEA domain has been readily studied. Macao *et al.* showed that the MUC1 SEA domain undergoes an autocatalytic strain-dependent cleavage during early biosynthesis in the ER [28]. They also suggested that the dissociation of the processed SEA domain is most likely mediated by mechanical forces. Still, only speculations exist regarding the actual function of this highly conserved domain.

The heterodimeric SEA domain of MUC1 is instable at elevated temperatures

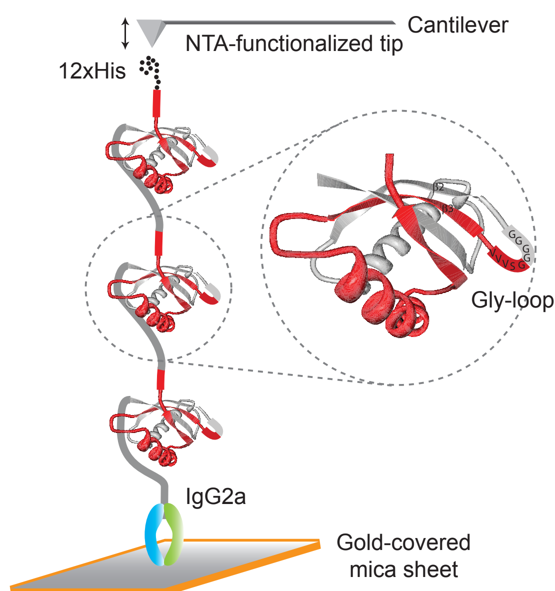
As a first measure, the stability of the MUC1 SEA domain was assessed. A recombinant protein harboring a SEA domain with a native cleavage site (LFRPG↓SVVV) and a recombinant protein with a non-cleavable SEA domain (LVPRGSVVV) were subjected to different temperatures. The cleavable SEA domain dissociated completely at 100°C and incomplete dissociation was observed at 80°C (Fig. 1C, Paper III). The native SEA domain was resistant to cleavage at 60°C. We concluded that the native SEA domain of MUC1 was stable at biologically relevant temperatures. So we turned our attention to mechanical forces that could dissociate the SEA domain.

A recombinant protein harboring three non-cleavable SEA domains in tandem unfolds in an ordered fashion

Atomic force microscopy (AFM) is a widely applied technique for high-resolution scanning of surfaces. The technique is also used for force spectroscopy defined as the measurement of tip-specimen interaction forces. We brought this technique into play aiming at measuring forces required for dissociation of a single SEA domain. A recombinant protein was designed, composed of three identical MUC1 SEA domains, flanked by an N-terminal

12×Histidine (12×His)-tag and a C-terminal IgG2a-tag (See Figure 7). The cleavage site in each SEA domain was looped with four additional glycines (GGGGGSVVV), rendering a non-cleavable site [28]. The AFM cantilever was functionalized with nitrilotriacetic acid (NTA), which forms coordination compounds with metal ions. In presence of Ni^{2+}, a His-NTA-complex connected the cantilever to the N-terminal 12×His-tag of the recombinant protein. The recombinant protein was immobilized on a gold surface via its C-terminus prior to binding to the cantilever. Pulling the cantilever at a loading rate of 25 nNs^{-1}, generated an average unfolding force of 168±73 pN for a single MUC1 SEA domain (Fig. 5, Paper III). A SEA domain consists of 104 amino acid residues, each with a persistence length of 0.4 nm. In other words, a fully stretched SEA domain measures approximately 42 nm. The obtained data indicated that the complete unfolding of a single SEA domain resulted in a contour length of 32±7 nm (Fig. 4, Paper III). When the distance between N- and C-terminal amino acids in the folded SEA domain (approximately 3.5 nm) is taken into account, the contour length obtained from our experiments is in good agreement with the theoretical length of a fully stretched out SEA domain.

Figure 7. A cartoon showing a recombinant protein, harboring three MUC1-derived SEA domains, flanked by a N-terminal 12×His-tag and a C-terminal IgG2a-tag.



4 Discussion

This thesis explores the relationship between transmembrane mucins and ion channels important for cell homeostasis and protection. The thesis presents evidence for a molecular interaction between transmembrane mucins and PDZ adaptor proteins, a relationship that further connects transmembrane mucins to ion channels in the intestine. The results also cast light on the stability of the SEA domain in exposed mucins on cell membranes and suggest that the SEA domain acts as a protective device against mechanical stress exerted on cell membranes.

Transmembrane mucins display specificity towards PDZ proteins

Our data show that the binding of certain transmembrane mucins to their corresponding PDZ binding partners is specific. Despite the presence of a class I PDZ motif in both MUC3 and MUC12, the binding specificity of PDZK1 is strictly limited to the class I PDZ motif of MUC17. Similarly, MUC3 binds GOPC while MUC12 and MUC17 display either no or very weak binding towards GOPC. This phenomenon can be explained by the fact that the selection specificity of PDZ domains depends on amino acid as far as at position -8 and is not solely restricted to the residues at positions 0 and -2 [66, 209]. The interaction between MUC3 and GOPC was in fact completely inhibited when MUC3CT was truncated at position -5. Published data indicate that consensus

sequences categorized as Class I PDZ motifs are not as rigid as previously reported but in reality much more flexible and discrete. All four amino acids, ranging from position 0 to -4, are involved in selectivity for the binding pocket of a PDZ domain [210]. These properties have been suggested to diminish cross-reactivity between PDZ domains and ligands with identical PDZ motif classifications. Besides specificity based on amino acid sequence, co-regulated gene expression and colocalization of PDZ domains and their corresponding ligands, render more complex PDZ-mediated networks. This explains the fact that human MUC17 is able to interact with murine Pdzk1 in the same extent as Muc3(17), thus proving a conserved functional relationship between MUC17 and PDZK1 across human and mouse species. Our findings are supported by the documented correlation between expansion of PDZ domains and increased organism complexity [209].

PDZ proteins regulate mucin expression and subcellular localization

Reports from Gentzsch *et al.* indicate that GOPC suppresses overall levels of CFTR and immunocytochemistry proved that GOPC also suppressed CFTR levels in the plasma membrane [206]. The fact that GOPC reduces CFTR Cl^- currents strengthens these conclusions [199]. Therefore, we were surprised when GOPC suppressed overall levels of MUC3 but did not affect plasma membrane levels of the mucin. There are possible explanations for this finding. First, the existence of MUC3-containing recycling endosomes could guarantee stable levels of MUC3 in the cell membrane even when total protein amounts decrease. Secondly, higher amounts of GOPC than used in our experiments could be required in order to suppress membrane levels of MUC3. Again, this explanation argues for a mechanism that upholds the half-life of

MUC3 in the cell membrane despite suppressed overall levels of the protein. Certainly, additional experiments are required to address this issue.

We also showed that Pdzk1 is necessary for membrane-tethering of Muc3(17) in enterocytes. Muc3(17) in Pdzk1$^{-/-}$ mice was localized in intracellular vesicles below the apical membrane. The question that arises is whether Pdzk1 is involved in trafficking of Muc3(17) to the plasma membrane or if Pdzk1 is involved in endocytosis of Muc3(17). Published data on the interaction of PDZK1 with NHE3, indicate that PDZK1 is important for retention of the exchanger in the BBM under basal conditions [73]. It has also been shown that the Class I PDZ motif of CFTR is an apical retention motif and that it is vital for CFTR recycling to the membrane but that it does not affect apical membrane endocytosis of CFTR [198]. Hence, data regarding NHE3 and CFTR trafficking and membrane-retention point towards the need for PDZK1 in recycling of MUC17 to the apical membrane. It is likely that MUC17 resides in recycling endosomes in absence of PDZK1, although this remains to be proven.

Membrane mucins and ion channels are co- and counter-regulated

This thesis reveals an intimate relationship between transmembrane mucins and the ion channels CFTR and NHE3. MUC3 expression can be regulated by CFTR and vice versa, via GOPC in the *trans*-Golgi network. Carbachol-induced endocytosis of MUC17 occurs simultaneously with internalization of NHE3 and recruitment of CFTR to the apical membrane. What do these results mean and what is the underlying purpose of the relationship between transmembrane mucins and ion channels in the small intestine?

Early studies of the intestinal phenotype of CF mice showed that CF pathology is mild in the duodenum and

increases distally, most likely due to a thickened mucus layer and increased bacterial load [211]. Recent data on mucus formation reveal that a functional CFTR ion channel and bicarbonate secretion are vital for mucus hydration and expansion [41]. In CF, a disease that is characterized by a non-functional CFTR and impaired secretion of HCO_3^-, the expelled mucus remains anchored to the epithelium. This phenotype can be rescued by addition of high concentration of HCO_3^- to the apical solution into which the mucus is released. In healthy tissue, NHE3 counteracts CFTR-mediated mucin hydration and expansion through absorption of Na^+ coupled to secretion of H^+ that neutralizes HCO_3^-. Therefore, in a scenario which requires CFTR recruitment and activation, NHE3 has to be removed from cell membranes and deactivated.

Protection from commensal and pathogenic bacteria is an important role of secreted mucins. Recent evidence shows that carbachol stimulates mucus discharge from goblet cells of the small intestine and colon, thus resulting in extensive flushing of the intestinal lumen and removal of bacteria entrapped in the mucus overlying the epithelium [8, 212]. Up to now, the fate of the transmembrane mucins during secretion has not been determined. Our results point towards a mechanism where increased secretion and decreased absorption in response to carbachol occurs as transmembrane mucin MUC17 is removed from the cell surface.

Plasma membrane-anchored MUC17 presents an array of *O*-glycans to the outer environment of the cell. These *O*-glycans are suitable attachment sites for bacteria that have penetrated the luminal floating mucus network. In a scenario where bacteria induce secretion, i.e. membrane recruitment and activation of CFTR hand in hand with NHE3 deactivation and endocytosis, the internalization of MUC17 could either act as a way for the cell to eliminate luminal bacteria in the vicinity of the epithelial surface or act as a mechanism that couples

cellular and immunological responses to the specific bacteria. Indeed, MUC1 which is expressed in lung epithelia possesses a signal transduction machinery that is activated upon interaction with *P. aeruginosa* [213]. So far, no signaling pathway associated with MUC17 has been identified but a possible signaling pathway could involve the attachment of bacteria to the mucin domain of MUC17, followed by conformational changes in the SEA domain that in turn induce intracellular signaling. However, this remains to be elucidated.

To summarize, further studies are required for a deeper understanding of the role of transmembrane mucin in cellular responses to bacterial insult in the small intestine.

The SEA domain acts as a protective device

SEA-type mucins, such as MUC1, MUC3, MUC12, and MUC17, are cleaved during biosynthesis in the endoplasmic reticulum. An autocatalytic strain-dependent cleavage renders a mucin heterodimer that remains associated via the SEA domain and once the mucin is presented on the cell surface. In this thesis, we determined the forces required for the dissociation of this heterodimer into its two distinct components; an extracellular subunit composed of a mucin domain and a C-terminal subunit that is anchored to the cell membrane through a type I transmembrane domain. The C-terminal subunit also contains a cytoplasmic tail that harbors binding motifs for various intracellular adaptor proteins and sequences which can be phosphorylated by kinases.

Our experiments showed that a force with the magnitude of approximately 170 pN at a loading rate of 25nNs^{-1} was able to break a single SEA domain. The dissociation force of the SEA domain should be understood in relation to forces exerted on membrane proteins in general and membrane proteins in epithelial cells in particular. A membrane protein is anchored to the plasma membrane via hydrophobic interactions between

the membrane-spanning amino acids of the protein and the surrounding lipids. Furthermore, a membrane protein is anchored to the intracellular cytoskeleton via scaffolding proteins. These two components contribute to a force of 400-600 pN that has to be overcome in order to extract a single membrane protein from the plasma membrane [214]. Thus, when mechanical forces are exerted on a SEA membrane protein, SEA dissociation occurs long before the plasma membrane is affected. This is an indication of the protective role of SEA mucins expressed on cell surfaces.

MUC1 is expressed in the lungs where vigorous and constant ciliary beating generates a force of approximately 60 pN [215]. Based on our data, the SEA domain of MUC1 is resistant to the forces rendered by beating cilia. A second case to take into consideration is binding of bacteria to SEA-typ mucins on enterocytes of the intestine. Due to the constant flow of fluid towards distal segments, the mass of a single *E. Coli* bacteria would assert a force of approximately 10^{-14} N on a single SEA mucin [216]. Consequently, over 15,000 bacteria are required to break a single SEA domain. Thus, we conclude that transmembrane mucins with a cleavable SEA domain are resistant to mechanical forces from bacteria. However, as previously mentioned, there is a possibility that bacterial binding generates conformational changes in the mucin that in turn results in intracellular signaling and a subsequent cellular response. Moreover, bacteria bound to transmembrane mucins could enzymatically break down these glycoproteins.

In summary, the SEA domain of transmembrane mucins is resistant to forces below 170 pN and can withstand both bacterial binding in the intestine and ciliary beating in the lungs. Thus, SEA mucins act as protective devices on cell surfaces.

Future perspectives

The results presented in this thesis have shed light on features and properties of transmembrane mucins that were previously not known. Still, several important questions remain to be addressed. One important issue is the dynamics of transmembrane mucins in the plasma membrane of polarized cells. The membrane trafficking of MUC1 has been extensively studied [188, 189]. However, the major transmembrane mucins of the small intestine, MUC3, MUC12, and MUC17, lack many binding motifs found in the cytoplasmic tail of MUC1, while MUC1 lacks the PDZ motif in the C-termini of MUC3, MUC12, and MUC17. Therefore, it is likely that trafficking of MUC3, MUC12, and MUC17 in cell membranes displays alternate dynamics as it is regulated by other intracellular adaptor proteins than those involved in MUC1 trafficking. This thesis has already presented evidence for clathrin-dependent endocytosis of MUC17, mediated through an AP-2 binding site on MUC17CT. Therefore, a systematic characterization of the cytoplasmic tails of MUC3, MUC12, and MUC17 could reveal novel interaction sites that are involved in trafficking of these mucins.

A second issue to address is the potential binding of commensal and pathogenic bacteria to the intestinal transmembrane mucins. As mentioned, transmembrane mucins harbor heavily *O*-glycosylated mucin domains that may act as binding sites for bacteria. Besides identifying species of bacteria with the ability to interact with these mucin domains, it is intriguing to consider bacterial binding to transmembrane mucins as a cellular monitoring system involving internalization of bound bacteria. Indeed, this thesis has presented proof for carbachol-induced MUC17 endocytosis. It remains to be shown if bacteria accompany MUC17 into the cell and if so, whether endocytosed bacteria are degraded

by the cell and/or presented to the immune system. The latter process has recently been shown to take place in goblet cells [217]. Based on the existing knowledge on cellular responses to commensal and pathogenic bacteria, an assessment of a sensory mechanism mediated by transmembrane mucins is important.

Galectin-3, an endogenous beta-galactoside-binding lectin, has been shown to interact and colocalize with MUC1 in human conjunctival epithelium [218]. It has been argued that the formation of an epithelial barrier is facilitated by binding of galectin-3 to MUC1. These findings should be exploited in the context of intestinal barrier function. The fact that enterocytes express galectins makes it likely that these endogenous lectins are involved in the organization of the glycocalyx by multivalent interactions with cell surface mucins. Again, assessment of the mechanisms behind transmembrane mucin organization at the cell surface is important for a deeper understanding of the role of epithelial cell in protection against and immune responses towards pathogens.

The highly conserved SEA domains of MUC1, MUC3, MUC12, and MUC17 have evolved to permit their own dissociation. The data presented in this thesis show that the SEA domain dissociates when mechanical forces are applied to it. The question that arises is if conformational changes in SEA domains, generated by mechanical forces from the extracellular environment, can be interpreted into intracellular signals and if these signals result in appropriate cell responses to mechanical stress. Also, an interesting feature of the MUC1 SEA domain is two hydrophobic patches on the otherwise polar surface of the folded domain [28]. These patches have been suggested as binding sites for other proteins. Thus, binding studies involving human and bacterial surface proteins could disclose novel extracellular interaction partners for SEA membrane mucins.

Populärvetenskaplig sammanfattning

Människokroppen täcks av ett hudlager som fungerar som en skyddsbarriär gentemot främmande och skadliga ämnen i vår omgivning. På liknande sätt, skyddas de inre organen som är i direkt kontakt med denna omgivning av ett tjockt slemlager som också kallas mukus. Detta slem, som hittas såväl i mag- och tarmkanalen som i våra lungor, är ett välordnat nätverk av proteiner som fångar upp goda och skadliga mikroorganismer för att transportera dessa vidare ut ur kroppen. Huvudkomponenten i slemmet är stora proteiner som kallas muciner. En undergrupp av muciner utsöndras från specialiserade slemproducerande celler och bildar ett skyddande slemlager över den underliggande cellytan. Den andra undergruppen består av membranbundna muciner som bildas i epitelceller och som förblir fast förankrade på ytan av dessa celler.

Målet med denna avhandling har varit att identifiera tidigare okända interaktioner mellan membranbundna muciner och s.k. PDZ-proteiner som organiserar de olika proteinerna på cellens yta. Vidare, har studier gjorts för att se om dessa identifierade PDZ-proteiner fungerar som gemensamma kopplingsstationer för membranbundna muciner och jonkanalerna som har visat sig viktiga för bildandet av ett välfungerande slemlager. En tredje frågeställning som har besvarats är om membranbundna muciner kan stå emot mekaniska krafter utifrån och därmed skydda de celler som de är förankrade i.

Resultaten som presenteras i denna avhandling visar att membranbundna muciner samspelar med två PDZ-proteiner som i sin tur kan interagera med viktiga jonkanaler i tunntarmen. Det membranbundna mucinet MUC17 binds upp av ett PDZ-protein som är nödvändigt för att mucinet ska förankras till cellytan. Resultatet visar dessutom att mängden av det membranbundna mucinet MUC3 regleras av jonkanalen CFTR och vice versa. Detta sker via ett annat PDZ-protein. Avhandlingen demonstrerar också att stimulans som leder till ökad tarmsekretion och minskad absorption resulterar i att MUC17 transporteras tillbaka in i cellen. Slutligen, presenterar vi bevis på att den svagasta länken i många transmembranösa muciner har förmågan att absorbera mekaniska krafter för att på så sätt skydda cellmembranet mot mekanisk stress från omgivningen.

Sammanfattningsvis åskådliggör denna avhandling ett intimt samspel mellan membranbundna muciner och jonkanaler i tunntarmen. Dessa tidigare okända interaktioner skapar en bättre förståelse för hur tunntarmens celler reagerar på goda och skadliga mikroorganismer som är i nära kontakt med dessa celler.

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