The C-terminus of the transmembrane MUC17 mucin binds to the scaffold protein PDZK1 that stably localizes it to the enterocyte apical membrane in the small intestine

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Running title: PDZK1 localizes the MUC17 mucin

Abbreviations used: BSA, bovine serum albumin; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane regulator; ERM, Ezrin, Radixin, Moesin domain; GFP, green fluorescent protein; mAb, monoclonal antibody; MUC, mucin; NHERF, Na^+/H^+ exchanger regulatory factor, PDZ, PSD-95/Dlg/ZO-1 domain; PBS, phosphate buffered saline; PMSF, phenyl methane sulphonyl fluoride; SEA, sea urchin sperm protein, enterokinase and agrin domain; SLC26A6, solute carrier family 26 member 6; YFP, yellow fluorescent protein.

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Summary

The membrane bound mucins have a heavily *O*-glycosylated extracellular domain, a single pass membrane domain and a short cytoplasmic tail. Three of the membrane bound mucins, MUC3, MUC12 and MUC17, are clustered on chromosome 7 and found in the gastrointestinal tract. These mucins have C-terminal sequences typical for PDZ domain binding proteins. To identify PDZ proteins able to interact with the mucins, we screened PDZ domain arrays using YFP-tagged proteins. MUC17 exhibited a strong binding to PDZK1 whereas the binding to NHERF1 was weak. Furthermore, we showed weak binding of MUC12 to PDZK1, NHERF1 and NHERF2. GST pull-down experiments confirmed that the C-terminal tail of MUC17 co-precipitates with the scaffold protein PDZK1 as identified by mass spectrometry. This was mediated through the C-terminal PDZ-interaction site in MUC17 which was capable of binding to three of the four PDZ domains in PDZK1. Immunostaining of wild-type or Pdzk1-/- mouse jejunum with an antiserum against Muc3(17), the mouse orthologue of human MUC17, revealed strong brush border membrane staining in the wild-type mice compared to an intracellular Muc3(17) staining in the Pdzk1-/- mice. This suggests that Pdzk1 plays a specific role in stabilizing Muc3(17) in the apical membrane of small intestinal enterocytes.

Keywords: PDZ, CFTR, Muc3, proteomics, mass spectrometry

INTRODUCTION

Mucins are found on epithelial surfaces where they are the dominating protein components in the mucus that lines and protects the surface of luminal organs [1]. Mucins are characterized by the mucin or PTS domains, which comprises serine, threonine and proline rich, often repeated sequences that carry a high number of *O*-linked glycans. The glycan content often reaches 80% of the molecular mass and contributes to the gel-like properties of the mucus. Based on their structure, mucins are subdivided into membrane bound and secreted, gel-forming mucins. While the gel-forming mucins constitute the free floating mucus network, the transmembrane mucins with their membrane domain are tightly anchored in the epithelial cells [2]. Typical for the transmembrane mucins are their heterodimeric nature with a large and a heavily *O*-glycosylated extracellular domain that extends far from the cell surface. The transmembrane mucins either contain a SEA (sea urchin sperm protein, enterokinase and agrin) or von Willebrand D domain (only MUC4) close to the extracellular membrane surface. Both these domains are cleaved in the endoplasmic reticulum, the SEA domain by an autocatalytic cleavage during folding [3]. After cleavage, the two parts remain tightly associated by non-covalent bonds. Although not proven, it has been suggested that the SEA domain can act as a sensor for mechanical forces [3]. The cytoplasmic tails of the transmembrane mucins are usually short, but specific interactions and phosphorylation might result in cell signaling events [4]. It is thus likely that the transmembrane mucins have important functions in the control of the mucosal surface environment.

The genes encoding the three transmembrane mucins *MUC3*, *MUC12* and *MUC17* are clustered on chromosome 7q22. These proteins all have an N-terminal large mucin domain, a SEA, a transmembrane and a cytoplasmic domain. The total size of MUC3 is still not known, but MUC12 and MUC17 encode proteins comprising 5478 and 4493 amino acids, respectively. They are all expressed in the gastrointestinal tract with highest levels of MUC3 in the small intestine and colon [5], MUC12 in the colon [6] and MUC17 throughout the entire intestinal tract with highest expression in the duodenum [7]. It was previously observed that mouse Muc3 is the orthologue of human MUC17 as they show highest sequence similarities and are located close to the same gene [7, 8]. We have chosen to call this mucin Muc3(17) (MUC designates the human mucin while Muc is the murine form) [7, 8].

Except for the MUC1 mucin, there is limited knowledge about how expression and targeting of transmembrane mucins to the apical surface of epithelial cells is regulated. The

physiologically relevant levels of cell surface localized mucins are likely regulated by a set of molecules that promote insertion in and removal of the mucin from the plasma membrane. The MUC1 mucin is known to recycle at the apical membrane, a process that is linked to an increased sialylation [9]. This membrane cycling is dependent on palmitoylation of the cysteines in the MUC1 transmembrane domain [10]. Such cysteines are absent in the MUC3, 12, 17 mucin sequences and suggests other mechanisms for apical targeting and potential recycling.

Common moderators of polarized expression and function of membrane proteins in epithelial cells are the PSD-95/Dlg/ZO-1 (PDZ) domain proteins (commonly called PDZ proteins). PDZ domains are the most abundant protein-protein interaction domains in metazoans. By specific binding to short C-terminal sequences of their ligand proteins, they assemble protein networks and signaling complexes and assist in targeting and retention of interacting proteins at specialized compartments of the cell. To help coordinate this, PDZ proteins often contain multiple PDZ domains in tandem and sometimes a range of other protein interaction domains (for example SH3, PH, LIM and GUK domains) [11, 12].

The Na^+/H^+ exchanger regulatory factor (NHERF) family of PDZ domain proteins consists of four proteins, NHERF1, NHERF2, PDZ domain containing 1 (PDZK1) and PDZ domain containing 3 (IKEPP). They are present at the brush border of for instance mammalian intestine and renal proximal tubules [13]. These proteins have two or four PDZ domains and the NHERF1 and -2 also comprise ERM (Ezrin, Radixin, Moesin) domains with which they attach to the cytoskeleton. PDZK1 (also called CAP 70, Na-Pi-CapI, or CLAMP) is expressed at the apical border of epithelial cells in the kidney, liver, gastrointestinal tract and pancreas [14]. Due to its multiple PDZ domains, it can simultaneously bind to several ligand proteins as shown for transporters and ion channels of the apical membrane. These include the Cystic Fibrosis Transmembrane conductance Regulator (CFTR), Multidrug Resistance-associated Protein 2 (MRP2), Scavenger Receptor B (SR-BI) and the ClC-3B chloride channel, [15-18]. From studies of the apical membrane of epithelial cells in the kidney, it has been established that PDZK1, together with NHERF1, creates an extended network beneath the apical membrane to which membrane proteins and regulatory elements can attach [19, 20]

Utilizing PDZ domain arrays, we have screened PDZ domains for binding to the cytoplasmic tail of human MUC3, MUC12, and MUC17, identifying a major interaction between MUC17 and PDZK1. Pull-down experiments using the cytoplasmic tails of the MUC3, MUC12

and MUC17 mucins affirmed PDZK1 as a binding partner for the MUC17 mucin. The absence of Pdzk1 in mouse jejunum relocalized Muc3(17) from the apical membrane to cytoplasmic vesicular structures in the cytoplasm of enterocytes.

MATERIALS AND METHODS

Plasmids

Plasmids (pGST-MUC3CT, -12CT, -17CT) encoding the complete cytoplasmic parts of the human MUC3, MUC12 and MUC17 mucins fused with GST were generated by RT-PCR (Taq polymerase, Fermentas) using the primer pairs 5' GC GAATTC CGG GCG GTG CGC TCC GGA TGG TG – 5' CG GAATTC GGC CCC GCA GGG CTC ACA CTG AGG, 5' CG GAATTC TCC CAG AGA AAA CGG CAC AGG GAA C – 5' CG GAATTC GGC TCA CAC AGT GGA TGC TAC CAT C and 5' GC CAATTG CGC TCC AAG AGA GAG GTG AAA CGG C – 5' CG CAATTG ACT CCC AGA CTT CTC AGC TCC ATG CC respectively, and cloned into the pGEX-4T-1 vector (GE Healthcare). A human small intestinal cDNA library was used as template (Invitrogen). The GST-MUC17CT-ΔSF was generated by site directed mutagenesis (QuickChange, Stratagene) introducing a premature stop codon in pGST-MUC17-CT. The plasmids encoding FLAG-tagged PDZK1 and PDZK1 PDZ domains in the pGEX-5X3 vector were described previously [18]. The Yellow Fluorescent Protein (YFP)-tagged YFP-MUC17-CT plasmid was generated by removing the EcoRI site by mutagenesis and subcloning the MUC17-CT fragment into a pYFP vector (Clontech) where cysteine 606 had been deleted by mutagenesis to prevent dimerization of YFP. The pGST-Muc3(17)-CT plasmid, encoding the complete cytoplasmic tail of Muc3(17), was generated by cloning an annealed oligonucleotide 5' GAT CCG TGT ACA ACA CCT TCC AGC CTT CCC TAA ACC ACA TAA ACC CAG AAA GAA AGA TCC AGA TTC AGA GGC CCC AGG TAG TCA TGA CAT CGT TGT AAG – 5' AAT TCT TAC AAC GAT GTC ATG ACT ACC TGG GGC CTC TGA ATC TGG ATC TTT CTT TCT GGG TTT ATG TGG TTT AGG GAA GGC TGG AAG GTG TTG TAC ACG) (MWG Biotech) with *Bam*HI and *Eco*RI overhangs into the pGEX-4T-1 vector.

Antibodies

The Muc3(17) protein was detected using the pAb anti-Muc3(17)-S2 [8], the FLAG-tag by the mAb anti-FLAG M2 (Sigma) and YFP with the mAb anti-Green Fluorescent Protein (GFP) (BD

Biosciences). Antibody against Pdzk1 was raised by immunizing rabbits with the peptides CLRAGPEQKGQIIKDI and CQSQELPNGSVKEGPA, coupled to Keyhole Limpet Hemocyanin conujugate via the heterobifuctional linker m-Maleimido-Benzoyl -N-hydroxySuccinimidyl ester (MBS). Affinity putified antibody was made using solely the second peptide sequence.

Cell culture

Syrian hamster kidney BHK-21 and human colon adenocarcinoma HT-29 cells lines (ATCC) were cultured at 37°C in 5% CO_2. The BHK-21 cells were transiently transfected using LipofectAMINE PLUS or LipofectAMINE 2000 reagents (Invitrogen).

PDZ domain array

Cytoplasmic tail of MUC17 tagged with Yellow Fluorescent Protein (YFP) was expressed in BHK-21 cells. TranSignal™ PDZ Domain Array II and IV (Panomics, Redwood City, CA) were used as instructed by the manufacturer. Bound cytoplasmic tail of MUC17 was detected with the mAb anti-Green Fluorescent Protein (Clontech).

Immunoprecipitation and pull-down

Immunoprecipitations and GST pull-down experiments were performed as described previously [21]. Briefly, binding was performed in 0.09% or 1% Nonidet P-40 lysis buffer (Nonidet P-40, 150 mM NaCl, 50 mM Tris-HCl pH 7.4, 20 mM $NaMoO_4$) containing Complete protease inhibitor cocktail (Roche) over-night at 4ºC. Glutathione Sepharose™ 4B beads (GE Healthcare) were washed four times in 0.09% Nonidet P-40 buffer and bound proteins eluted for 30 min with SDS-PAGE sample buffer containing 1,4-ditriotheitol and loaded onto SDS-PAGE gels.

In-gel trypsin digestion and mass spectrometry

The gels were stained by Imperial Blue Protein Stain (Pierce). The bands to be analyzed were cut out from the gel and digested with trypsin as described [21]. The peptides were analyzed by nanoLC-MS and MS/MS using a Thermo LTQ-FT instrument and 50 µm x 20 cm Kromasil C-18 column eluted with an acetonitrile gradient in water at a flow of 200 nl/min. The obtained

MS/MS spectra and accurate molecular masses of the peptides were searched against the Swiss-Prot protein database using the Mascot software.

Breeding of knock-out mice

The Pdzk1-deficient mouse strain was generated in the Dept. of Pathology, Beth Israel Deaconess Medical Center as described before [22]. These mice have normal body electrolyte concentration and normal intestinal mucosal histology. Pdzk1 -/- and +/+ mice were bred on a 129SvEv background in the animal facility of the Medical School of Hannover under standardized light and climate conditions and had access to water and chow ad libitum. For the experiments in this project, age- and sex matched littermates of 2-3 months of age were used. NHERF1-/- mice backcrossed for 8 generations into the FVB/n background were produced from heterozygotes that were generated at the Duke University Medical Center as described before [23]. NHERF2-/- mice showing complete absences of NHERF-2 protein in all tissues studied were produced from heterozygotes generated at the Erasmus University Medical Center and were backcrossed for 14 generations into the FVB/n background. C57/BL6 mice were used as wild-type controls. Experiments followed protocols at the Medical School of Hannover and at the Erasmus Medical Center Rotterdam, approved by the local authorities for the regulation of animal welfare (Regierungspräsidium) and by the Dutch Animal Welfare Committee (DEC), respectively.

Mouse tissue preparation and lysis

Small intestine of wild-type mice (C57/BL6) was excised and washed gently with ice-cold phosphate-buffered saline containing Complete EDTA-free Protease Inhibitor Cocktail (20X) (Roche), 10 mM EDTA and 1mM PMSF (phenyl methane sulphonyl fluoride). The small intestine was cut in smaller pieces and homogenized in a Dounce homogenizer (pestle B) in 0.09% Nonidet P-40 lysis buffer (Nonidet P-40, 150 mM NaCl, 50 mM Tris, pH 7.4, 10 mM $NaMoO_4$) containing protease inhibitor cocktail (Roche), EDTA and PMSF.

Immunohistochemistry

Intestinal tissue from mouse jejunum and colon of wild-type, Pdzk1-/- [22] , Nherf1-/-, Nherf2-/- and Nherf1+2-/- mice were fixed, paraffin imbedded and sectioned (10 µm). The tissue sections were deparaffinized and rehydrated in xylene (6 min), 100% ethanol (4 min), 70% ethanol (2

min), and washed in H_2O. Antigen retrieval was performed by boiling twice for 2 and 7 min in 0.01 M citric acid, pH 6.0 and washed in PBS (phosphate buffered saline). Tissues were enclosed with a PAP pen and blocked with 1% BSA (bovine serum albumin) in PBS. Primary and secondary antibodies were added in dilution buffer (0.1% bovine serum albumin, 0.05% Triton-X-100). Cover slips were mounted using Prolong antifade Gold (Invitrogen) and polymerized for 24 h. Samples were examined using an immunofluorescence microscope (Axiolab, Zeiss) or an LSM Meta 510 confocal microscope (Zeiss).

RESULTS

MUC17 is a PDZ domain binding protein

Analysis of the cytoplasmic sequences of the MUC3, MUC12 and MUC17 mucins revealed that all had sequences in their extreme C-termini resembling PDZ domain binding ligands of class I (X-S/T-X- Φ, where X represents any amino acid and Φ represents a hydrophobic amino acid) (Figure 1A). In order to investigate if MUC3, MUC12, and MUC17 C-terminal tails were able to bind scaffolding PDZ proteins, we expressed the YFP-tagged cytoplasmic tails of the mentioned membrane-associated mucins in BHK-21 cells and assessed their ability to bind to PDZ domains immobilized on four protein PDZ domain arrays. Interestingly, we observed a strong binding of YFP-MUC17-CT to the second PDZ domain of PDZK1, the third member of the Na^+/H^+ exchanger regulatory factor (NHERF) family (Figure 1B). YFP-MUC17-CT also displayed weak binding to PDZK1-D1 and NHERF1-D1. Faint interactions were also observed between YFP-MUC12-CT and NHERF1-D1, NHERF2-D1, and PDZK1-D2.

The cytoplasmic tails of the MUC3, MUC12 and MUC17 mucins were fused to GST and the fusion proteins were used for pull-down experiments in lysates from the intestinal epithelial colon cancer cell line HT29. Nine bands were excised and analyzed using mass spectrometry after in-gel trypsination. A unique 70 kDa Coomassie stained band was present in the co-precipitate with the MUC17 C-terminal tail, while the band was absent in coprecipitates with GST and MUC3, and MUC12 C-terminal tails (Figure 1C). Mass spectrometry identified 20 peptides covering 37% of the human PDZK1 protein sequence (Figure 1 D). No other precipitated bands for the three mucin tails were identified as PDZ proteins and further explored (data not shown).

PDZK1 interacts with MUC17

To further prove the interaction between MUC17 and PDZK1, FLAG-tagged PDZK1 was transiently expressed in BHK-21 cells. Lysates from these cells were exposed to immobilized GST-MUC3, -MUC12 and -MUC17 C-terminal fusion proteins. The bound products were separated on SDS-PAGE and analyzed by western blot using the mAb anti-FLAG M2. As expected, the precipitate from the GST-MUC17-CT pull-down contained PDZK1 (Figure 2A). GST alone and both the other two mucins were negative for PDZK1 binding. This suggests high binding specificity between MUC17 and PDZK1, as the MUC3 and MUC12 mucins were negative despite their typical PDZ ligand sequences of the same subclass. Consequently, the novel PDZK1 binding to MUC17-CT became our main point of interest.

It has been shown that the amino acids in the -0 and -2 positions from the COOH-terminus are critical for most PDZ interactions that are mediated through C-terminal ligands [11, 12, 24]. To study if this was true also for MUC17 and PDZK1, the two last amino acids of the extreme C-terminus of MUC17 in the GST-MUC17-CT fusion protein were deleted and GST pull-down experiments were performed. MUC17 lacking the serine and phenylalanine, failed to interact with PDZK1 (Figure 2B), corroborating that the interaction is PDZ mediated.

To pin-point the specific PDZ domain(s) in PDZK1 that mediates the interaction with MUC17, all four domains, separate or in tandem were fused to GST [18]. These fusion proteins were used to perform pull-down experiments in lysates prepared from BHK-21 cells transiently expressing an YFP tagged C-terminal tail of MUC17. SDS-PAGE separation of co-precipitated material and subsequent western blot analysis using an anti-GFP mAb revealed that PDZ domains one, two and four of PDZK1 were able to interact with the C-terminus of MUC17 (Figure 2C). PDZ domain three potentially also binds to MUC17, but with lower affinity. Alignment of amino acid sequences for the four PDZ domains does not reveal any specific variations in the known conserved regions of the PDZ domain (not shown) that correlated with the observed differences in affinity for the MUC17 cytoplasmic tail.

Pdzk1 stabilizes Muc3(17) at the enterocyte apical membrane in the mouse small intestine

To address if PDZK1 had any influence on the localization of MUC17, we turned to mice with ablation of Pdzk1 [22]. The extreme C-terminal tail of the human MUC17 mucin has the sequence -TTSF and its mouse orthologue, Muc3(17) –MTSL (Figure 1A). Thus, the Muc3(17) sequence is different, but still represents a class I PDZ ligand. To determine if the mouse Pdzk1 and Muc3(17) were able to interact, a GST fusion protein with the C-terminal tail of Muc3(17) was generated. This was used to perform GST pull-down experiments in lysates prepared from wild-type mouse jejunum. Both the human GST-MUC17-CT as well as the mouse GST-Muc3(17)-CT fusion proteins were able to interact with the mouse Pdzk1 (Figure 3). This suggests a conservation of the interaction between these two molecules despite the sequence differences.

Jejunum from wild-type or Pdzk1-/- mice was sectioned and studied by immunofluorescence using the pAb anti-Muc3(17)-S2 directed against a juxtamembrane sequence in the extracellular part of mouse Muc3(17) [8]. The confocal fluorescence microscope images revealed a strong apical brush border staining in the wild-type animals with an increase in intensity towards the tip of the villi (Figure 4A). In contrast, the epithelial cells of the Pdzk1-/- mouse jejunum showed a prominent, particulate cytoplasmic staining with an accumulation in vesicular-like structures subapical as well as perinuclear (Figure 4B and C). The apical membrane was also stained, although relatively weaker as compared to the wild-type animals.

To investigate the role of Pdzk1 on Muc3(17) localization in the colon and whether absence of Pdzk1 caused the same relocalization of Muc3(17) as in the small intestine, colon sections from wild-type and Pdzk1-/- mice were stained with the pAb anti-Muc3(17)-S2. No difference could be observed in the subcellular localization of Muc3(17) in the colon of any of the knock out mice compared to wild-type controls (Figure 4D).

The weak interaction between MUC17 and NHERF1, but not NHERF2, as found on the PDZ protein arrays (Figure 1) prompted us to analyze also the localization of Muc3(17) in Nherf1, Nherf2, or Nherf1+2 knock-out mice. Intestinal sections were stained with the pAb anti-Muc3(17)-S2. Immunostaining of jejunum (Figure 4E) and colon (Figure 4F) revealed apical membrane staining of Muc3(17) in enterocytes, and no alteration in the localization of Muc3(17) in the Nherf1, Nherf2 and combined knock-out mice. Thus we could not verify any functional effect of Nherf1 and 2 on the Muc3(17) localization.

DISCUSSION

To maintain an intact barrier of mucus on epithelial surfaces exposed to detrimental environments, in for instance the respiratory and gastrointestinal tracts, it is crucial that the mucus favors this. The highly coordinated regulatory mechanisms involved in the regulation of mucus are poorly understood and important to elucidate. Emerging knowledge points at multiple pathologic conditions, such as various types of cancer and inflammatory diseases as associated with mucin aberrations. To further understand this, it is important to understand how the transmembrane mucins are involved in regulatory networks at mucous surfaces. Numerous ion channels bind to PDZ proteins, something that is known to regulate their activity [25]. The present study for the first time suggests a link between ion channels and the apical molecules that reaches furthest away from the epithelial cell membrane, the mucins. This supports the idea that the transmembrane mucins can act as apical sensors that can be part in the regulation of the epithelial cell apical surface liquid and ion homeostasis.

Transmembrane mucins are likely to play a pivotal role in maintaining homeostasis on mucosal surfaces but apart for MUC1, not much is known about the mechanisms that regulate the localization and expression of other transmembrane mucins. Here we demonstrate that the three gastrointestinal membrane-bound mucins, MUC3, MUC12, and MUC17, all have C-terminal sequences resembling PDZ ligands of class I. MUC17 was shown to interact with the multi-PDZ domain protein PDZK1 and this interaction appears important for proper localization of this mucin.

Most of the studies on PDZK1 function are performed in renal and hepatic tissues. There it plays a prominent role in the proper localization and function of a number of ion transporters in the plasma membrane. The Cl^- anion exchanger SLC26A6 (solute carrier family 26, member 6, also known as CFEX) is expressed in the brush border of the proximal tubules. SLC26A6 was demonstrated to interact with PDZK1 via a C-terminal PDZ motif. In Pdzk1 -/- mice, both the protein levels and the ability of this transporter to mediate anion exchange across the plasma membrane were profoundly decreased [26]. Similarly, cell surface expression and transport activities of the urate-anion exchanger URAT1, the proton-coupled peptide transporter PEPT2 as well as organic anion transporter 4 (OAT4) was shown to depend on PDZK1 expression [27-29]. Our results show that Pdzk1 interacts with, and plays an essential role in maintaining the mouse orthologue of MUC17, Muc3(17), at the apical membrane of enterocytes in the small intestine.

Immunofluorescence staining of Pdzk1-/- jejunum showed that loss of Pdzk1 results in a pronounced intracellular staining of Muc3(17) compared to wild-type mouse jejunum where most of Muc3(17) is present at the brush border membrane. This discrepancy in subcellular localization of Muc3(17), caused by lack of Pdzk1, can have at least two plausible explanations. Either Pdzk1 affects the biosynthesis and maturation of Muc3(17) which results in improper targeting to the apical membrane and redistribution of the mucin inside the cell. Alternatively, the Muc3(17) mucin is properly synthesized and delivered to the plasma membrane, but is not retained and rapidly internalized resulting in a decreased half-life at the apical surface. Given the absence of Muc3(17) in the basolateral membrane in the Pdzk1-/- mice, it is more likely that Muc3(17) is properly targeted to the apical membrane, but fails to anchor there and is thereby readily internalized.

A disease where mucin synthesis and secretion is markedly deregulated is Cystic Fibrosis (CF). It is characterized by heavy mucus accumulation in luminal organs, most prevalent in the airways causing chronic bacterial infections followed by inflammation and severe lung tissue damage. No direct functional connection between mucus and Cystic Fibrosis Transmembrane conductance Regulator (CFTR) is known so far. Although the airway symptoms are dominating the disease, the small intestine is commonly affected, with 10-15 % of infants suffering from meconium ileus and a comparable number of CF adults from distal intestinal obstruction syndrome. These are both severe conditions where the distal small intestine becomes obstructed by dehydrated mucus [30]. We previously showed increased protein levels of the Muc3(17) mucin in mucus from the small intestine of Cftr-/- mice compared to wild-type mice [8]. NHERF1 is a well known interactor and regulator of CFTR [31]. The first PDZ domain of NHERF1, PDZ1, binds CFTR with a greater affinity, enabling PDZ2 to interact with additional apical proteins [32]. Although NHERF1 could be a potential molecular connection between CFTR and the transmembrane mucin MUC17, we could not substantiate any interaction between NHERF1 and MUC17 in pull-downs. Neither were there any alternations in the apical expression of Muc3(17) in Nherf1, Nherf2 or Nherf1+2 knock outs, providing additional proof that NHERF1 or NHERF2 does not affect MUC17.

PDZK1 interacts with CFTR and was proposed to assemble two CFTR molecules in the plasma membrane, via simultaneous binding of two CFTR C-termini to domain three and four, and thereby stimulate chloride channel activity [15]. The facts that PDZK1 is a well known

interacting partner of CFTR and that Pdzk1 binds Muc3(17) and stabilizes it at the apical membrane may suggest, for the first time, a direct connection between CFTR and mucins. Whether transmembrane mucins via PDZ proteins can influence the surface expression of CFTR or the opposite is an open question. However, recent studies in the small intestine of Pdzk-/- mice reveal mild reduction in maximal Cftr activation and a large reduction in Na^+ absorption, suggesting that also Cftr is affected in these mice [33]. This is in line with the suggestion, although still controversial, that the PDZ interacting domain of CFTR has a role in its endocytic recycling. Deletion of this domain in CFTR did not affect the targeting to the apical membrane, but reduced the half-life of CFTR in the membrane [34]. Furthermore, Gage *et al.* reported that type I PDZ ligands promote rapid recycling of G protein coupled receptors, such as the adrenergic receptor (β_2-AR) [35]. It has recently, without proof, been suggested that the fully glycosylated MUC17 undergoes an endocytic recycling cycle similar to the MUC1 mucin [36]. It can thus be postulated that PDZK1 is involved in post-endocytic sorting of MUC17 and that interaction between these proteins facilitates endocytic recycling of the mucin back to the membrane. Absence of this interaction might then cause an accumulation of MUC17 in vesicles inside the cell, something that is consistent with our results.

In conclusion, PDZK1 was shown to interact with a classic PDZ interaction motif in the extreme C-terminal tail of the mucin MUC17. Immunofluorescence microscopy in the small intestine of Pdzk1-/- mice revealed that Pdzk1 plays an essential role in stabilizing the mouse form of this mucin, Muc3(17), at the apical enterocyte membrane. Other mechanisms or another PDZ protein is probably responsible for the proper retention of this mucin in the apical membrane of the epithelial cells in the colon, as absence of Pdzk1 had no discernable effect on Muc3(17) localization. However, as Muc3(17) has a substantially lower expression in colon, the absence of Pdzk1 might have a less pronounced effect not as easy to observe.

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Figure legends

Figure 1. The transmembrane mucin MUC17 binds the PDZ proteins PDZK1 and NHERF1.

A) The three transmembrane mucins MUC3, MUC12 and MUC17 contain sequences in their extreme C-termini that can be suggested to interact with PDZ proteins. Additionally, the MUC17 mouse orthologue Muc3(17) contains an extreme C-terminal tetrapeptide resembling a PDZ class I motif. Amino acids marked in bold letters represent consensus sequences resembling PDZ ligands of class I with the consensus sequence X-S/T-X- Φ, where X represents any amino acid and Φ represents a hydrophobic amino acid. B) The binding of YFP-tagged cytoplasmic tail of MUC3, MUC12, and MUC17 to PDZ domains immobilized on a membrane. Lysates of YFP-MUC3-CT-, YFP-MUC12-CT- and YFP-MUC17-CT-transfected BHK-21 cells were added to the membranes and bound tagged cytoplasmic tails visualized with an anti-GFP mAb. GST indicates Glutathione S-Transferase only and PDZ pos indicate PDZ domain positive control on the array. C) GST fusion proteins with the C-terminal tails of MUC3, MUC12 and MUC17 were immobilized on Glutathione-Sepharose beads and further incubated with lysate from HT29 cells. Bound products were separated using SDS-PAGE and the gel was stained with Coomassie blue. Bands pointed out by arrows were excised , in-gel trypsin digested and the peptides identified using nanoLC-mass spectrometry and mapped using the Mascot software. GST fusion proteins only and GST incubated with lysate from HT29 cells served as controls. D) The domain structure of PDZK1 and the peptides identified (marked by horizontal black bars) in the trypsinized band marked with * in C. The masses of the identified peptides are shown together wither theoretical masses.

Figure 2. The MUC17 mucin binds specific PDZ domains of PDZK1.

A) Immobilized GST-fusion proteins with the C-terminal tails of MUC3, MUC12 and MUC17 were incubated together with lysates from BHK-21 cells transiently expressing FLAG-tagged PDZK1. Bound products were separated on SDS-PAGE followed by western blotting using the anti-FLAG M2 mAb. Lysate input lane accounts for 5% of the protein amount used in the pull-down reactions. B) FLAG-tagged PDZK1 was transiently expressed in BHK-21 cells and lysates prepared from these cells were incubated with Glutathione-Sepharose beads with immobilized

GST-MUC17-CT, GST-MUC17-ΔSF fusion proteins or GST alone. Bound products were analyzed by SDS-PAGE with subsequent western blotting using the anti-FLAG M2 mAb. Lysate input lane accounts for 5% of the protein amount used in the pull-down reactions. C) GST fusion proteins with each of the four PDZ domains of PDZK1, either separately or in tandem, were incubated together with lysates from BHK-21 cells transiently expressing YFP-MUC17. Bound products were separated by SDS-PAGE and analyzed by western blot using an anti-GFP mAb. Lysate input lane accounts for 10% of the protein amount used in the pull-down reactions.

Figure 3. The mouse orthologue Muc3(17) interacts with mouse Pdzk1.

Immobilized fusion proteins human GST-MUC17-CT, mouse GST-Muc3(17)-CT or GST alone were incubated with lysate prepared from wild-type mouse jejunum. Bound products were analyzed by SDS-PAGE with subsequent western blotting using the anti-Pdzk1 pAb. Lysate input lane accounts for 5% of the protein amount used in the pull-down reactions.

Figure 4. The Muc3(17) mucin is relocalized in Pdzk1-/- mouse jejunum.

Sections (10 µm) from Pdzk1 -/-, Nherf1-/-, Nherf2-/-, Nherf1+2-/- or wild-type mice were immunostained using the pAb anti-Muc3(17)-S2. Alexa-488 conjugated anti–rabbit secondary antibody was used for visualization. A) Sections of jejunum from Pdzk1-/- mice were examined using a confocal microscope. Bar is 50 µm. Areas enclosed in squares were photographed at double magnification (right). B) Cross-section of the outermost tip of villus from either a Pdzk1-/- or wild-type mouse as indicated. Tissues were examined using a conventional fluorescence microscope. Bar is 10 µm. C) Further magnification of Pdzk1-/- jejunum shown in A. Muc3(17) accumulates in vesicles subapical as well as perinuclear (arrows). Bar is 5 µm. D) The Muc3(17) mucin is not relocalized in the colon of Pdzk1-/- mice. Bar is 10 µm. E) The Muc3(17) mucin is not relocalized in Nherf1 -/-, Nherf2 -/- or Nherf1+2 double -/- mouse jejunum. Bar is 10 µm. Sections from wild-type, Pdzk1 -/- mice, and Nherf1 -/-, Nherf2 -/- or Nherf1+2 -/-mice were stained using the anti-Muc3(17)-S2 antibody. Alexa-488 conjugated anti–rabbit secondary antibody was used for visualization and tissues examined using a confocal microscope. Bar is 10 µm.

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A

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MUC3     -NVDTTMKVHIKRPEMTSSSV
MUC12    -TVDSGTELHIQRPEMVASTV
MUC17    -HIDPETKIRIQRPQVMTTSF
Muc3(17)-HINPERKIQIQRPQVVMTSL
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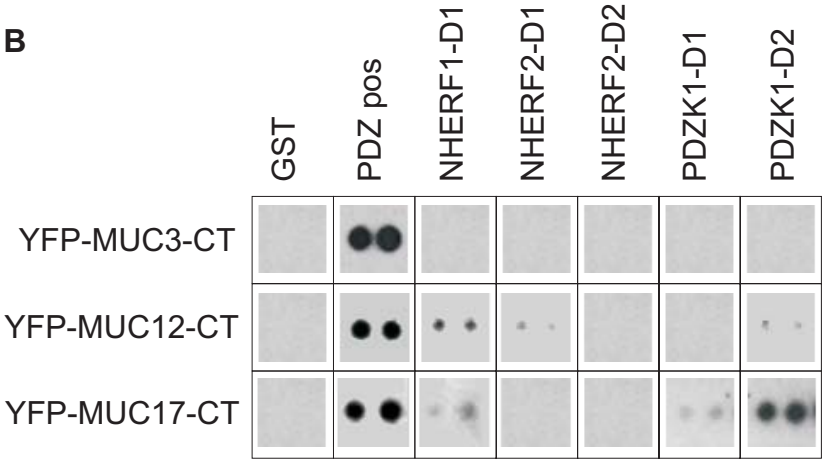
B



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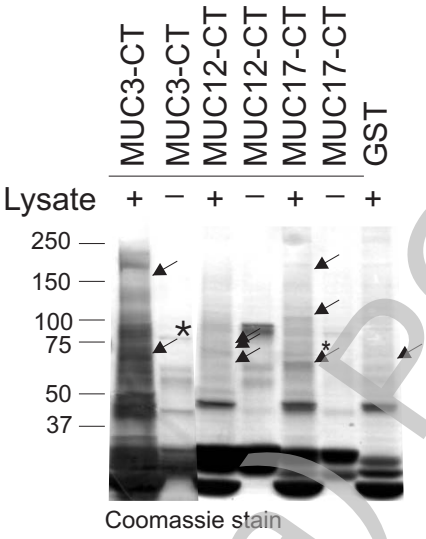

D

Start - End	Observed	Mr(calc)	Miss	Sequence
12 - 25	562.96	1685.85	1	K.LSKQEGQNYGFFLR.I
26 - 36	432.90	1295.68	1	R.IEKDTEGHLVR.V
29 - 36	463.74	925.46	0	K.DTEGHLVR.V
47 - 54	416.20	830.39	0	K.AGLQDGDR.V
47 - 57	400.55	1198.64	1	K.AGLQDGDRVLR.I
135 - 140	405.23	808.45	0	R.LCYLVK.E Propionamide (C)
141 - 150	522.75	1043.49	0	K.EGGSYGFSLK.T
156 - 171	600.30	1797.88	1	K.KGVYMTDITPQGVAMR.A
156 - 171	899.95	1797.88	1	K.KGVYMTDITPQGVAMR.A
157 - 171	835.90	1669.78	0	K.GVYMTDITPQGVAMR.A
236 - 242	430.76	859.50	0	K.LLPHQPR.I (Ions score 17)
270 - 283	694.83	1387.65	0	K.DIDSGSPAEEAGLK.N
311 - 329	719.68	2156.01	1	K.GGDQTSLLVVDKETDNMYR.L
351 - 377	953.80	2858.38	0	K.EAPAPTPTSLEVSSPPDTTEEVDHKPK.L
397 - 404	409.74	817.47	0	R.GLPGSFIK.E
439 - 448	544.81	1087.60	1	K.VVDRIQSSGK.N
458 - 466	567.29	1132.56	1	K.KAYDYFQAK.K
459 - 466	503.24	1004.46	0	K.AYDYFQAK.K
467 - 485	665.04	1992.08	1	K.KIPIVSSLADPLDTPPDSK.E
486 - 500	556.93	1667.76	0	K.EGIVVESNHDSHMAK.E

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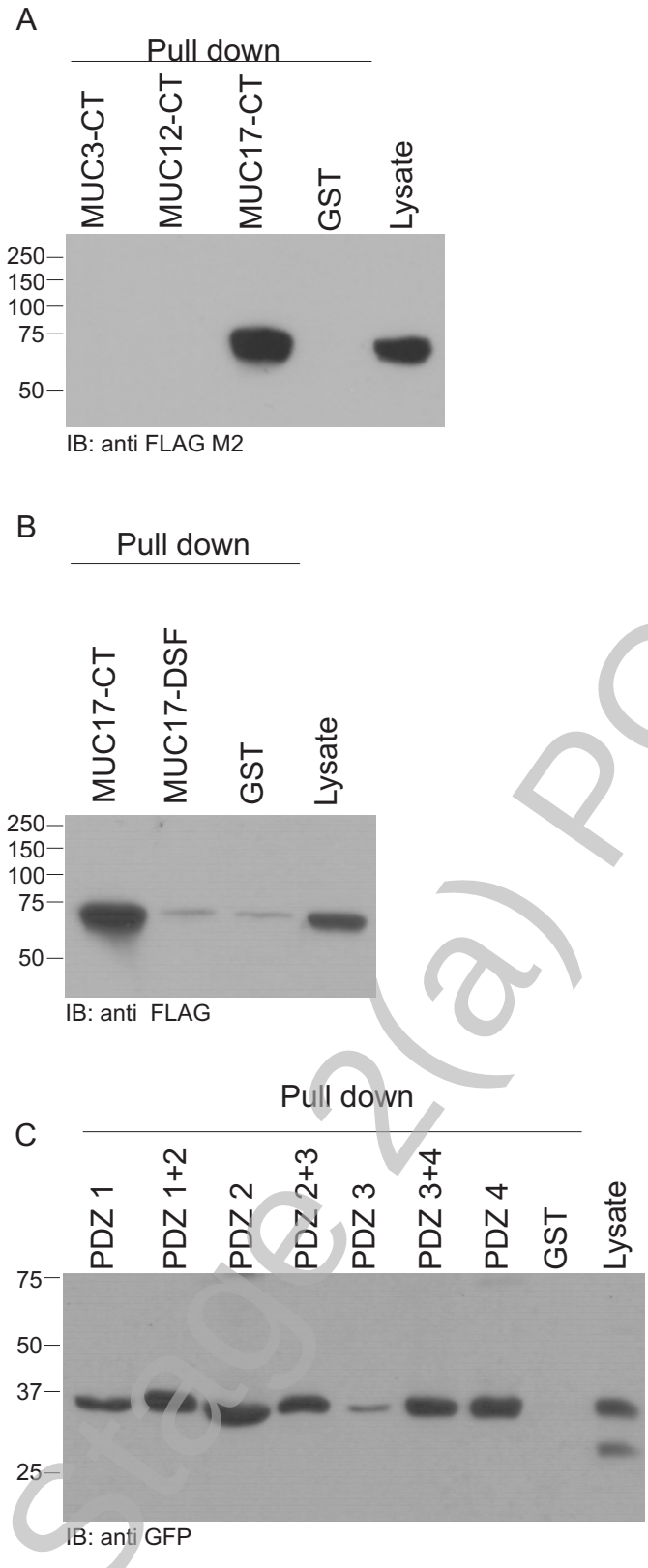

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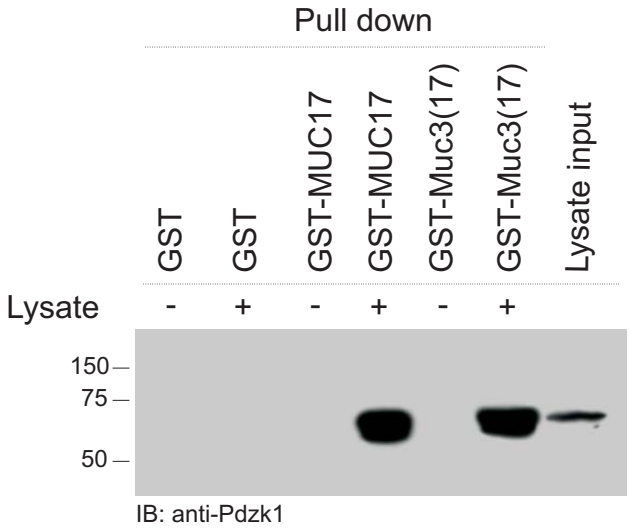

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