Microbial-induced meprin β cleavage in MUC2 mucin and a functional CFTR channel are required to release anchored small intestinal mucus

André Schütte[a], Anna Ermund[a], Christoph Becker-Pauly[b], Malin E. V. Johansson[a], Ana M. Rodriguez-Pineiro[a], Fredrik Bäckhed[c], Stefan Müller[d], Daniel Lottaz[e], Judith S. Bond[f], and Gunnar C. Hansson[a,1]

Departments of [a]Medical Biochemistry and [c]Molecular and Clinical Medicine, University of Gothenburg, 40530 Gothenburg, Sweden; [b]Unit for Degradomics of the Protease Web, Institute of Biochemistry, Christian Albrechts University, 24118 Kiel, Germany; [d]FACSlab, Institute of Pathology, and [e]Department of Clinical Research, University of Bern, CH-3010 Bern, Switzerland; and [f]Department of Biochemistry and Molecular Biology, Pennsylvania State University, Hershey, PA 17033

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The mucus that covers and protects the epithelium of the intestine is built around its major structural component, the gel-forming MUC2 mucin. The gel-forming mucins have traditionally been assumed to be secreted as nonattached. The colon has a two-layered mucus system where the inner mucus is attached to the epithelium, whereas the small intestine normally has a nonattached mucus. However, the mucus of the small intestine of meprin β-deficient mice was now found to be attached. Meprin β is an endogenous zinc-dependent metalloprotease now shown to cleave the N-terminal region of the MUC2 mucin at two specific sites. When recombinant meprin β was added to the attached mucus of meprin β-deficient mice, the mucus was detached from the epithelium. Similar to meprin β-deficient mice, germ-free mice have attached mucus as they did not shed the membrane-anchored meprin β into the luminal mucus. The ileal mucus of cystic fibrosis (CF) mice with a nonfunctional cystic fibrosis transmembrane conductance regulator (CFTR) channel was recently shown to be attached to the epithelium. Addition of recombinant meprin β to CF mucus did not release the mucus, but further addition of bicarbonate rendered the CF mucus normal, suggesting that MUC2 unfolding exposed the meprin β cleavage sites. Mucus is thus secreted attached to the goblet cells and requires an enzyme, meprin β in the small intestine, to be detached and released into the intestinal lumen. This process regulates mucus properties, can be triggered by bacterial contact, and is nonfunctional in CF due to poor mucin unfolding.

protease | von Willebrand factor | gastrointestinal tract

The gastrointestinal (GI) tract is protected from self-digestion and microbiota by mucus (1). This mucus is differently organized throughout the GI tract: the stomach and colon have a two-layered mucus system with an inner mucus layer attached to the epithelium and formed by stratified mucin sheets (2). This layer is 50–200 μm thick and impenetrable for bacteria, is constantly renewed by secreted mucins from the goblet cells, and further toward the lumen proteolytically converted into a nonattached and less dense outer mucus layer. This outer layer is the habitat and nutritional source for the commensal bacteria (2). In contrast, the small intestine has only one single mucus type that is not attached to the epithelium (3).

The main structural component of the intestinal mucus is the heavily glycosylated polymeric MUC2 mucin which is densely packed inside the goblet cell and after secretion and a 1,000-fold expansion forms flat, net-like structures stacked into stratified mucin sheets in the colon (4). The same MUC2 mucin is processed differently in the small intestine where it appeared less organized but still filled the space between villi (3). This mucus was easily aspirated and penetrable to beads the size of bacteria (3). Bacteria could penetrate, but still the space between the villi was kept free of bacteria in the small intestine due to effective intestinal peristalsis, fast mucus renewal, and a high concentration of antibacterial peptides and proteins (3, 5). In fact, the structure of the small intestinal mucus as a nonattached and less organized layer mimics the situation for the outer colon mucus layer that has been shown to be generated from the inner mucus layer by proteolytic processing of the MUC2 mucin (2).

Meprins are zinc-dependent metalloendopeptidases and belong to the astacin family (6). They comprise two homologous enzymes, meprin α and meprin β, where meprin α is the secreted and meprin β the membrane-tethered variant. They can form heterodimers (meprin α and β), homodimers (meprin β), and large oligomers (meprin α), forming one of the largest secreted protease complexes known. Although both enzymes share a common domain structure, they exhibit distinct features in substrate recognition and cleavage specificities. The enzymes can hydrolyze a broad variety of substrates, such as growth factors, peptide hormones, or compounds of the extracellular matrix like procollagen III, fibronectin, and tenascin-C (7–9). The meprin β enzyme is highly expressed in the enterocytes of the small intestine and is thereby localized close to the mucin networks (10).

Significance

Mucus with its major constituent, the gel-forming mucins, is important for protecting the host epithelium from bacteria. Under normal conditions, these mucin networks are constantly released into the small intestinal lumen. This release required a proteolytic cleavage in the mucin by the metalloprotease meprin β and was absent in germ-free animals but induced by bacteria. The small intestinal mucus in cystic fibrosis is also attached, not due to lack of enzyme, but rather that the mucin is not properly unfolded in the absence of a functional cystic fibrosis transmembrane conductance regulator channel and sufficient bicarbonate levels. Mucus can thus appear both attached and released as part of a system controlling bacterial removal. This new concept may lead to new ways for the treatment and therapy of cystic fibrosis.

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[1]To whom correspondence should be addressed. Email: gunnar.hansson@medkem.gu.se.

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Cystic fibrosis (CF) is a severe disease that affects most of the mucus-producing organs of the body (11). The disease is caused by a nonfunctional cystic fibrosis transmembrane conductance regulator (CFTR) channel that normally mediates passive transport of chloride and bicarbonate ions (12, 13). Although a majority of the clinical CF symptoms can be attributed to stagnant mucus, the more precise link between the lack of CFTR function and mucus properties has been difficult to understand. We recently showed that the small intestinal mucus of CF mice, in contrast to the WT, was attached to the epithelium and impossible to aspirate (14). Although CF mice have only minor lung problems, their intestinal phenotype is similar to the human disease characterized by meconium ileus and distal intestinal obstruction syndrome (DIOS). When the CF mucus was secreted into a solution of ∼100 mM bicarbonate, the mucus was released from its attachment. Mucins are densely packed in the goblet cell granulae due to Ca^{2+} ions and low pH, and the role of bicarbonate is to remove the Ca^{2+} ions and increase the pH to allow for the >1,000-fold mucin expansion (4). When already formed mucus was treated with bicarbonate, the mucus was normalized and possible to aspirate, although its increased mucin density remained largely unaltered (14). This suggested that mucin attachment and expansion might be different phenomena and made us analyze this further.

We have now found that meprin β is able to cleave the MUC2 mucin N terminus and that this is involved in the detachment of the mucus of the small intestine in a process controlled by bacteria and a functional CFTR channel. We thus describe a fundamental constitutive mechanism which involves an endogenous protease acting on the mucus network to alter its attached properties.

Results

Meprin β-Deficient Mice Exhibit a Distinct Mucus Phenotype in the Small Intestine. In contrast to the attached and dense mucus in the small intestine of CF mice (CftrΔF508), WT mice exhibit a loosely organized, nonattached layer of mucus. This can be observed in immunostainings with an anti-Muc2 antibody (Fig. 1 *A* and *C*). Searching for potential enzyme candidates releasing CF mucus and with an expected tissue distribution focused our attention on the zinc-dependent metalloprotease meprin β (MEP1B), as this membrane-bound enzyme is highly expressed in the brush border of the small intestinal enterocytes and thus localized close to the secreted MUC2 mucins (Fig. 1*G*) (10, 15). The ileal mucus of mice lacking the metalloprotease meprin β ($Mep1b^{-/-}$) partially resembled the CF phenotype, and these animals exhibit a denser mucus (Fig. 1*B*). This was even more visible by Alcian blue/periodic acid-Schiff (PAS) staining showing mucus that seemed to be more adhesive and attached to the goblet cells, just as for the CF mice (Fig. 1 *E* and *F*). As observed before, the small intestinal mucus of WT mice, but not the CF mucus, can be aspirated under normal physiologic conditions by simply pipetting off the mucus formed on tissue explants (Fig. 1 *J* and *L*) (14). The meprin β-deficient mice (Fig. 1*H*) had a mucus that resembled the CF mice and was not possible to aspirate (Fig. 1*K*), supporting the visual impressions from tissue staining. The inner mucus layer of the colon is not penetrable by bacteria or fluorescent beads the size of small bacteria (0.5 to 1 μm), in contrast to the outer colon mucus layer and the WT small intestine (Fig. 1*M*) (3, 16). As observed previously, the small intestinal mucus of CF mice was not penetrable to these fluorescent beads (Fig. 1*O*). When the $Mep1b^{-/-}$ mice were analyzed, the beads were able to penetrate the mucus, but still some beads were stuck at the mucus surface (Fig. 1*N*).

Lack of meprin β cannot alone explain why the CF mucus was attached, as meprin β was stained similarly in CF and WT mice (Fig. 1*I*). Furthermore, the mucus properties differed in $Mep1b^{-/-}$ and CF mice as the CF mucus was not permeable to fluorescent beads the size of bacteria, whereas the $Mep1b^{-/-}$ mice had a more penetrable mucus closer to WT mice (Fig. 1 *N* and *O*). This led us to assume that proteolytic activity by the meprin β could be responsible for detaching and altering the organization of the small intestinal mucus but that meprin β- and Cftr-deficient mice were not identical.

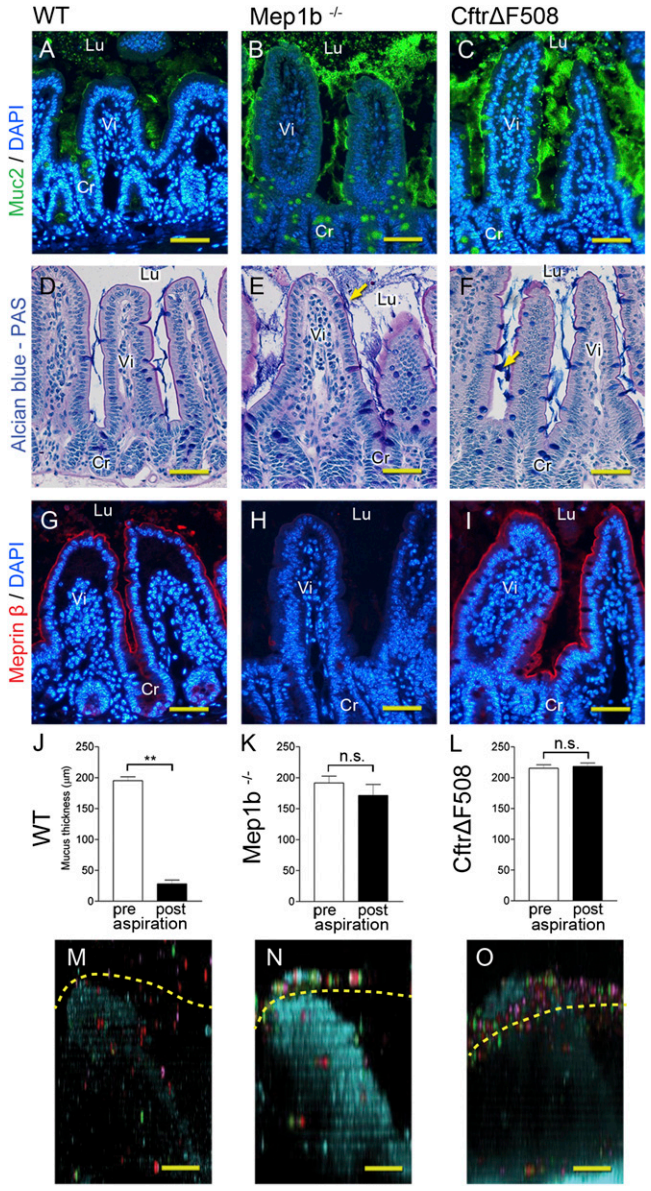

Fig. 1. The Muc2 mucin organization is dependent on the metalloprotease meprin β and the presence of a functional Cftr channel. (*A–C*) Muc2 stained (green) in the small intestine (ileum) in WT, $Mep1b^{-/-}$, and CftrΔF508 (CF) mice. (*D–F*) Alcian blue–PAS-stained. Yellow arrows highlight goblet cell-anchored mucus. (*G–I*) Meprin β stained red. DNA was visualized by DAPI. (*J–L*) The thickness of mucus formed on ileal explants was measured before (pre) and after (post) aspiration (**$P < 0.01$, $n = 5$). In WT (*J*) the mucus can be aspirated, but not in $Mep1b^{-/-}$ (*K*) and CftrΔF508 (*L*) (n.s., nonsignificant, $n = 6$). (*M–O*) Fluorescent beads (green 2 μm, purple 1 μm, red 0.5 μm) were applied to mucus formed on ileal explants, and their penetration was analyzed by confocal z-stacks. Villi stained in blue. The mucus surface is marked by yellow lines. Cr, crypt; Go, goblet cell; Lu, Lumen; Vi, villi. (Scale bars, 50 μm.)

Meprin β Cleaves the MUC2 Mucin, Releasing the D1–D2 Domain Assemblies. To further illustrate the effect of meprin β, we applied the meprin inhibitor actinonin apically to the mounted tissue explants from WT mice (17). The mucus formed with

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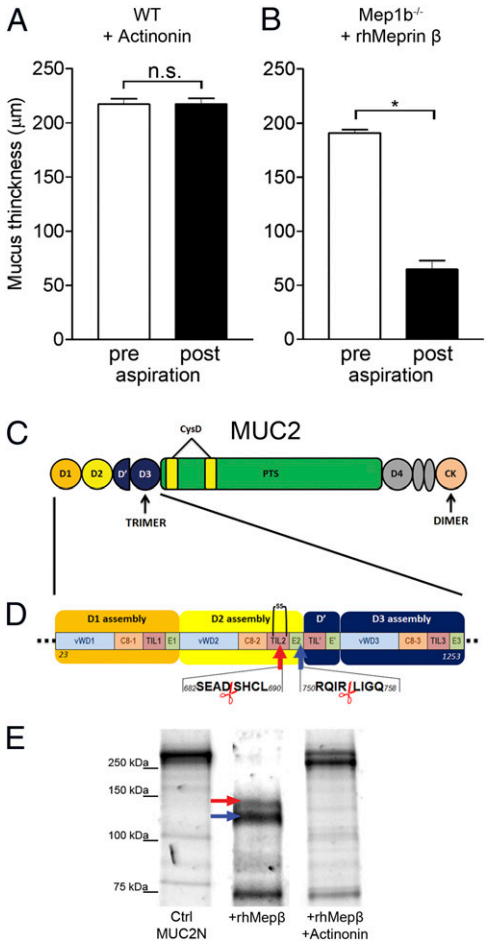

Fig. 2. Meprin β cleaves the MUC2 mucin at two distinct sites. (*A*) Meprin β protease inhibitor actinonin (1 μM) was added to WT small intestinal explants, and mucus secretion was stimulated ($n = 5$). (*B*) Recombinant human meprin β (200 nM) was added to the mucus of ileal Mep1b$^{-/-}$ explants leading to detached mucus (*$P < 0.05$, $n = 4$). (*C*) Domain structure of the human MUC2 mucin. Green (PTS) refers to the PTS domain that becomes O-glycosylated to form the mucin domain. (*D*) Domain-annotated N-terminal part of MUC2 with the cleavage sites determined by Edman sequencing indicated. (*E*) Recombinant human MUC2 N-terminal protein (MUC2N, Ctrl, 20 μg) was incubated with recombinant human meprin β (+rhMepβ, 500 nM) and analyzed by SDS/PAGE gel. Incubation with actinonin inhibited the cleavage of MUC2N by meprin β. Colored arrows point to the bands subjected to Edman sequencing, and the corresponding cleavage sites are shown in *D*.

inhibitor present was no longer possible to remove by aspiration (Fig. 2*A*), again suggesting that meprin β is involved in the release of the secreted mucus network. Adding recombinant meprin β to the explant tissue from Mep1b$^{-/-}$ made the mucus removable by aspiration (Fig. 2*B*). Meprin β thus reestablished a normal mucus phenotype in meprin β-deficient explants, confirming its importance as a proteolytic regulator of small intestinal mucus properties.

As the MUC2 mucin is the major and structure-forming molecule in mucus and thus the most likely target, we asked if meprin β was able to cleave the MUC2 protein. As native MUC2 is insoluble, we used recombinant parts of the MUC2 mucins for in vitro studies (Fig. 2 *C* and *D*). The C terminus and CysD2 domain were unaffected, but the N-terminal part (MUC2N) was cleaved by recombinant meprin β, a cleavage inhibited by the protease inhibitor actinonin (Fig. 2 *D* and *E*). Two new bands with an approximate mass of 130 and 110 kDa appeared. These bands were subjected to Edman sequencing that identified the N-terminal amino acid sequences $_{686}$SHCLE$_{690}$ and $_{754}$LIGQS$_{758}$ where the domains of the MUC2 N terminus were assigned according to a recent annotation of the von Willebrand factor (VWF) (Fig. 2*D* and Figs. S1 and S2) (18). The cleavage at SHCLE followed the published predicted specificities, whereas the other did not (19). The cleavage sites are located in the TIL2 and between the E2 and TIL′ domains of the MUC2 N terminus as illustrated in Fig. 2*D*. According to this annotation, the first cleavage is expected to be held together by a disulfide bond, whereas the second is not (Fig. 2*D*). This suggests that the N-terminal end of MUC2 containing the von Willebrand D1 and D2 assemblies will be separated from the rest of the MUC2 mucin by the cleavage in the sequence QIR/LIG. This cleavage site is similarly located to the furin cleavage site in the von Willebrand factor (20). In that case, the cleavage takes place intracellularly, and the N-terminal part containing the D1 and D2 assemblies is separated from the von Willebrand factor polymer after secretion. Although the mechanism for anchoring MUC2 to the goblet cells is not known, release of the N-terminal part of MUC2 will not affect the polymeric net-like nature of the mature MUC2. It can thus be suggested that the MUC2 N terminus is the anchor point for MUC2, and its separation from the rest of MUC2 would release the attached mucin.

Meprin β Is Not Shed from the Epithelial Surface Under Germ-Free Conditions. The colonic mucus system in germ-free (GF) animals is similar to that of WT animals (2). In the small intestine, the mucus was less developed and sparser, also reflected in lower Muc2 mRNA levels (Fig. 3 *A* and *B*). Surprisingly, the mucus of GF mice was more attached than in colonized mice (Figs. 1*J* and 3*C*). The level of meprin β mRNA was not significantly lower in GF than in conventionally raised (CONVR) mice (Fig. 3*B*). When GF mice were colonized with CONVR ceacal flora, it was possible to aspirate the ileal mucus after 6 wk (Fig. 3*D*). Interestingly, when small intestinal tissues were stained for meprin β, this enzyme was more abundant on the epithelial cell surface in GF, compared with conventionally raised mice (CONVR, Fig. 3*E*). When ileal whole-tissue lysates were collected and analyzed by immunoblots for meprin β, specific bands whose identity was also confirmed by proteomics were observed at 140 kDa in both CONVR and GF tissues (Fig. 3*F*). However, the cleaved and thus soluble meprin β was only found in the mucus of CONVR mice. That meprin β was secreted into the mucus was confirmed by proteomic studies of aspirated mucus that showed meprin β in the mucus from the CONVR mice, but not from the GF mice (Fig. 3*G*). Thus, release of the attached GF mucus was dependent on meprin β being secreted from the enterocytes out into the mucus. The metalloproteases ADAM10 and ADAM17 have been shown to be involved in shedding meprin β from the cell surface (21, 22). Consequently, we analyzed ADAM10 and 17 mRNA expression levels in whole tissue, but neither of these showed significantly lower levels in GF and are probably therefore not sufficiently altered to explain the lack of meprin β in the GF mucus (Fig. S3). Nevertheless, the lack of meprin β shedding in GF compared with CONVR mice suggests that the shedding of this enzyme is likely triggered by bacterial challenges or microbial signaling. Consequently, mucus processing can be activated and stimulated by bacteria, causing bacteria trapped by the mucus to be washed away. In this manner, proteolysis may contribute to the fast distal transport of mucus and bacteria in the small intestine.

Attached CF Mucus Is Only Released After Unfolding. The mucus of the small intestine in CftrΔF508 mice was attached to the epithelium (Fig. 1*L*), but can be almost totally normalized by allowing the Muc2 mucin to be secreted into a luminal ∼100 mM $NaHCO_3$ buffer (Fig. 4*A*) (14). This detachment could be totally

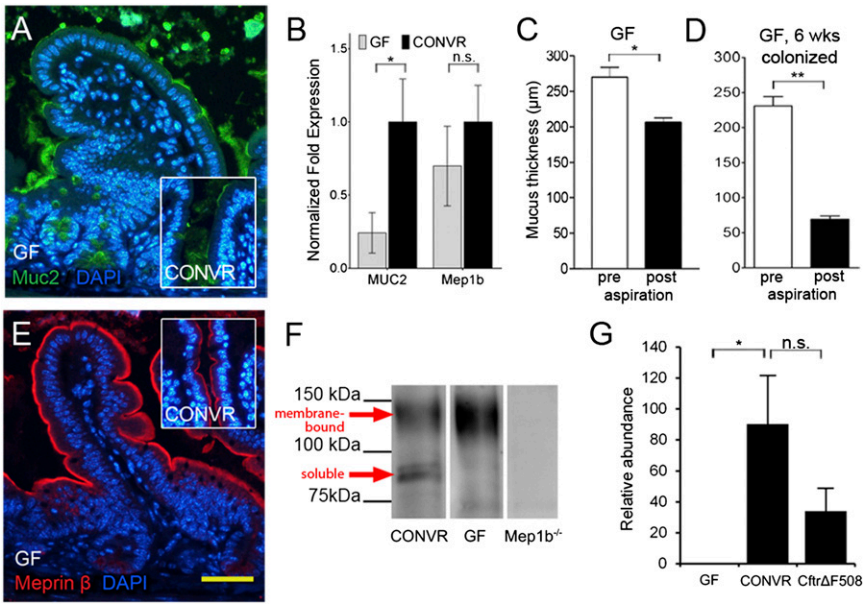

Fig. 3. Germ-free mice have attached mucus, as meprin β is not shed from the enterocytes. (*A*) Immunostaining of Muc2 in GF mice. (*B*) mRNA expression of meprin β and Muc2 is reduced in GF compared with CONVR. (*C*) It was not possible to fully remove the mucus on GF ileal explants ($*P < 0.05$, $n = 4$). (*D*) GF mice colonized with CONVR ceacal flora rendered the mucus normal; thus it was possible to aspirate after 6 wk ($**P < 0.01$, $n = 5$). (*E*) Immunostaining of GF ileum shows a stronger meprin β staining in GF than CONVR mice. (*F*) Immunoblots of ileal lysates for meprin β show more full-length membrane-anchored meprin β in GF than CONVR and the appearance of the cleaved secreted (soluble) meprin in CONVR. (*G*) Proteomic analyses of mucus aspirated from ileal explants show meprin β peptides in the CONVR and CftrΔF508, but not in GF mucus ($*P = 0.03$, $n = 4$). (Scale bars, 50 μm.)

inhibited by the addition of the meprin inhibitor actinonin showing that the CF mucus has similar release mechanisms as the Mep1b$^{-/-}$ mice (Fig. 4*B*). In contrast, addition of recombinant meprin β did not normalize the CF mucus as it was still impossible to aspirate (Fig. 4*C*). Secreted meprin β was found in aspirated CF mucus by proteomics (Fig. 3*G*) together implying that the attached Muc2 mucin in the CftrΔ508 mice was not due to low levels of meprin β or a failure to secrete the enzyme.

The CFTR channel is responsible for Cl^- and HCO_3^- transport, and CF mucus was released from its attachment after secretion into a high-concentration bicarbonate buffer (13), suggesting that bicarbonate is necessary for activating meprin β or to unfold the MUC2 mucin, allowing processing by meprin β. Meprin β is known to be active at acidic pH, but the additional influence of Ca^{2+} is unknown (23). Testing recombinant meprin β for activity revealed that Ca^{2+} was inhibitory at higher pH, but not at pH 5 (Fig. 4*D*), making it unlikely that the attached CF mucin was caused by inactive meprin β. The second alternative was tested by incubating the recombinant MUC2N at pH 5 and 6 with and without Ca^{2+} to cause packing and ring structure formation (4). The MUC2N was cleaved as before in Ca^{2+}-free buffer, but MUC2N was unaffected in the presence of Ca^{2+} ions where larger MUC2N complexes were also observed (Fig. 4*E*, arrow). Although we do not have any structural details on the packing of the D1–D3 assemblies of the MUC2 N terminus, it has been suggested, based on comparisons with the von Willebrand factor, that the D1–D2 assemblies are folded back on to the D3 assembly, as sketched in Fig. 4*F* (4). The meprin β cleavage sites (Fig. 2*D*) are likely not accessible in such a conformation until the mucin has been properly unfolded and the sites exposed. This may explain why meprin β did not release the attached CF mucus (Fig. 4*C*).

Discussion

Mucus protection of the intestine was long ignored and considered as passive and static. The small intestinal mucus covers all space between villi and also most of the villi tips (3). However, this mucus is normally not attached and is easily moved with the intestinal content and peristaltic waves, exposing the villus tips for luminal content and bacteria. The discovery that germ-free mice have their small intestinal mucus not movable and attached to the epithelium suggests that the small intestine also has a dynamic mucus protection system. One can envision that mobile mucus can easily transport bacteria trapped in the mucus distally and in this way efficiently keep the epithelium free of bacteria. As learnt from the attached mucus in CF mice, stagnant mucus will easily cause bacterial overgrowth (11, 24, 25).

Meprin β is not shed into the mucus in germ-free mice, but is shed after bacterial contact or microbial signaling to the enterocytes. The mechanism behind the coupling of bacteria and meprin β shedding is not known and has to be addressed separately. Consequently, mucus processing can be activated and stimulated by bacteria, thus trapping pathogens inside the mucus and keeping them away from the epithelium by a protease-controlled "flushing" system. Thereby, proteolysis contributes to the fast distal transit and efficient transport of mucus and bacteria within the small intestine.

The goblet cells and their main product, the MUC2 mucin, have recently been shown to be involved in antigen presentation to CD103$^+$ dendritic cells and are likely involved in tolerogenic processes (26, 27). The processing of the MUC2 mucin and its cleavage by meprin β into a nonattached form is likely to be important for these processes.

We identified two specific cleavage sites of meprin β in the recombinant MUC2N, located close to each other in the TIL2 and between the E2 and TIL′ domains of MUC2 N terminus as illustrated in Fig. 2*D*. The MUC2 QIR/LIG cleavage site will separate the von Willebrand D1–D2 domains from the rest of the MUC2 mucin. The molecular nature of the MUC2 anchor to the goblet cell is not known and will be difficult to reveal, as the MUC2 mucin is insoluble (28). Although the MUC2 anchor is not known, separation of the N-terminal part of MUC2 from the remaining MUC2 will not affect the polymeric net-like nature of the mature MUC2 mucin. It can thus be suggested that

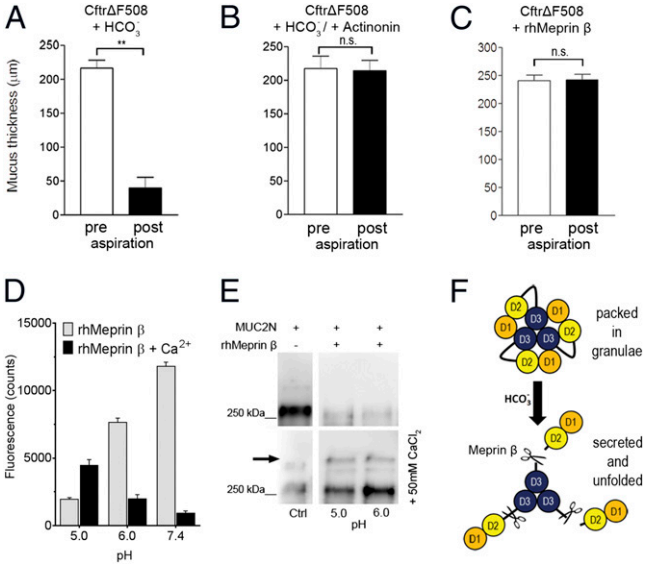

Fig. 4. Ileal attachment of CF mucus is dependent on bicarbonate and meprin β. (*A*) Mucus from CftrΔF508 mice were secreted into a buffer with 115 mM $NaHCO_3$ giving a detached mucus ($P < 0.008$, $n = 5$) (10). (*B*) Adding actinonin (1 μM) to mucus from CftrΔF508 mice secreted into a 115-mM $NaHCO_3$ buffer inhibited the mucus detachment ($n = 5$). (*C*) Adding recombinant human meprin β (200 nM) to ileal explants from CftrΔF508 mice did not detach the mucus ($n = 4$). (*D*) Meprin β activity under different buffer conditions (pH 5 to pH 7.4), with (black bars) and without (gray bars) 50 mM $CaCl_2$; $n = 4$. (*E*) The MUC2N is cleaved by meprin β at both pH 5 and pH 6 without $CaCl_2$ but is unaffected and forms large oligomers (black arrow) under the influence of 50 mM $CaCl_2$. (*F*) Schematic view of the MUC2 mucin N terminus and its unfolding in the presence of HCO_3^- upon secretion. Colors are as in Fig. 2*C*.

the MUC2 N terminus is the localization of the anchor point for MUC2 and that its separation from the rest of MUC2 would release the attached mucin. As MUC2 is secreted as enormous polymers, only single attachment sites are necessary on each polymer, making the problem of revealing the molecular nature of this anchor even more difficult (4). It has been suggested that the D1–D2 assemblies are folded back onto the D3 assembly, as outlined in Fig. 4*F*, making the meprin β cleavage sites inaccessible until the mucin has been properly unfolded. This may explain why meprin β did not release the attached CF mucus. It can thus be predicted that correcting the attached CF mucus phenotype has to address the unfolding process and not the proteolytic cleavage events.

Meprin β is thus a key player in regulating the ileal mucus system by proteolytic cleavages in the MUC2 mucin N terminus and thereby helping to reorganize the structure of the mucin network. It is not only the attachment that is affected, but also the mucus properties, as beads the size of bacteria penetrated the Mep1b$^{-/-}$ mucus less well than in WT. The observation that meprin β has to be released from the enterocytes suggests that the cleavages must take place in the secreting goblet cell or secreted mucus. Our previous and present observations (Fig. 1) suggest that the MUC2 mucin is attached to the goblet cells (14). The present results pinpoint the necessity of meprin β for mucin release from the goblet cells in the small intestine. In the colon the MUC2 mucin must also be released from the goblet cells when the mucus is transformed from the inner to the outer mucus layer, but this process is not affected in the Mep1b$^{-/-}$ mice. Thus, it may be suggested that the MUC2 mucin normally remains attached at secretion and requires meprin β in the small intestine and a yet unidentified protease in the colon to be detached. Similarly, the other three human gel-forming mucins (MUC5B, MUC5AC, and MUC6) might also remain attached after secretion and, in contrast to the general assumption, require protease activities to be released. The attached CF mucus not only causes the intestinal obstruction syndrome in CF (DIOS) but also the altered microbiota and bacterial overgrowth found in CF of both mice and man (11, 24, 25). This suggests that a nonattached small intestinal mucus is important for the normally relatively low bacterial counts in this part of the gastrointestinal tract and suggests a physiological role for the system for mucus release described here. That the attached small intestinal CF mucus cannot be normalized by the addition of the specific protease also shows that the CF phenotype has to be rescued by normalizing mucin unfolding by a sufficiently high concentration of bicarbonate. An explanation for the human CF lung phenotype and disease in relation to stagnant mucus should also be sought in using the principle of gel-forming mucins being attached to their secretory cells.

Materials and Methods

Animals. All animal procedures were approved by the local Laboratory Animal Ethics Committee, Gothenburg. C57BL/6, CftrΔF508 mice and Mep1b$^{-/-}$ deficient mice were kept in individually ventilated cages. Germ-free animals were kept in flexible film incubators. The homozygous CftrΔF508 mice on C57BL/6 background (back-crossed 13 generations) were kept as described, but given regular water 2–3 d before use (29). Mice used for experiments were 12–16 wk old. GF mice (8–10 wk) were gavaged with ceacal content of WT mice and studied after 6 wk.

Histology, Immunostainings, and Fluorescence Microscopy. Fixation and immunostaining of segments from ileum were performed without flushing the luminal content (2). Staining was performed with anti-meprin β (AF3300, R&D Systems).

Meprin β Cleavage Analysis and Edman Sequencing. We used an N-terminal construct of MUC2N (SNMUC2-MG) with a length of 1,699 amino acids, comprising a signal peptide sequence, the first three von Willebrand D-assemblies, a fragment of the mucin-type proline/threonine/serine (PTS)-rich repetitive regions or mucin domains, and the cysteine-rich (CysD1) domain (30). The recombinant proteins CysD2 [SMCysD(2TR)-IgG2aH] and C-terminal end (SMG-MUC2C) of MUC2 were also tested (31, 32). As a negative control, Aprotinin 1 μM was added. For the cleavage analysis under different pH and calcium conditions, various buffers were tested (20 mM Mes pH5, 20 mM Mes pH6, 20 mM Tris/HCl pH7, 20 mM Hepes pH7.4, and 20 mM Tris/HCl pH8). To test if calcium affects the cleavage of MUC2N, 50 mM $CaCl_2$ was added to each buffer. The recombinant MUC2N (500 ng) was preincubated with the buffer for 1 h, and further incubated with meprin β (14 nM) for 2 h at room temperature. A Western blot of the 10% polyacrylamide gel with an antibody against a sequence inside the D3 domain (anti-MUC2N3) reveals the cleavage events by meprin β. Furthermore, the enzyme activity was tested under these specific conditions with the EnzChek Protease Assay Kit using the Bodipy-labeled casein as a substrate (Life Technologies). The protocol was modified as follows: The substrate was applied to the buffers directly from the stock solution to a final concentration of 10 μg/mL. Meprin β or trypsin (as a positive control) were added accordingly (20 nM). The plate was analyzed on a Victor II microplate reader using standard fluorescein filters (485/530 nm) (Perkin–Elmer).

For Edman sequencing, 20 μg of MUC2N were incubated with 2 μg (500 nM) recombinant meprin β for 16 h at 37 °C, then separated on a 10% (wt/vol) SDS gel and blotted on a PVDF membrane (1 h, 100 mA). After blotting, the membrane was stained by Coomassie R250 [0.1% Coomassie brilliant blue R250 in 40% (vol/vol) ethanol, 20% (vol/vol) acetic acid] for 1 h, then destained in 20% (vol/vol) ethanol until bands were clearly visible. Edman sequencing was performed at the core facility of the Karolinska Institutet, Stockholm.

Alignments and Sequence Analyses. Sequence alignments of the N-terminal parts of the human MUC2 (accession number Q02817), the murine Muc2 (accession number Q80Z19), and the according von Willebrand factor sequence (human VWF; accession number P04275) (18) were performed with ClustalX 2.1 and further manually reviewed and edited with the GeneDoc 2.7 software.

Real-Time Quantitative PCR. The mRNA of small intestinal lysates was extracted using the Qiagen RNeasy kit according to the manufacturer's instructions (Qiagen). After quantification with a Nanodrop spectrophotometer, 1 μg of mRNA was reverse-transcribed with the High Capacity cDNA Reverse Transcription Kit (Life Technologies). For quantitative PCR analysis, 20 ng of cDNA was added to the PCR mix (SsoFast EVAgreen supermix 2x, final concentration of primers: 200 nM, reaction volume: 20 μL, Bio-Rad). Housekeeping genes, GAPDH, and β-Actin were used for normalization. The PCR was performed on a Bio-Rad CFX 96 (Bio-Rad). The sequences used in the study were GAPDH: Forward: 5′-TGT TCC TAC CCC CAA TGT G-3, Reverse: 5′-CTC AGT GTA GCC CAA GAT GC-3; β-Actin: Forward: 5′-AAC GAG CGG TTC CGA TGC-3′, Reverse: 5′-GTA GT TCA TGG ATG CCA CAG G-3′; Meprin β: Forward: 5′-CAG GCA AGG AAC ACA ACT TC-3′, Reverse: 5′-TCT GTC CCG TTC TGG AAA G-3′; Muc2 Forward: 5′-GGC TCG GAA CTC CAG AAA G-3′, Reverse: 5′-CCA GGG AAT CGG TAG ACA TC-3′; ADAM10: Forward: 5′-GGG AGG TCA GTA TGG AAA TC-3′, Reverse: 5′-ATG TGA GAC TGC TCG TTT G-3′; ADAM17: Forward: 5′-AAA CCA GAA CAG ACC CAA CGA-3′, Reverse: 5′-GTA CGT CGA TGC AGA GCA AAA-3′. PCR cycle conditions were denaturation (94 °C for 30 s), annealing (57 °C for 30 s), and extension (72 °C for 45 s); number of cycles: 30.

Mucus Thickness and Penetrability Measurements in the Small Intestine. Mucus thickness measurements were performed on tissue explants of Mep1b$^{-/-}$, C57BL/6, and CftrΔF508 mice in horizontal Ussing chambers as described (33). In short, the mucus surface was visualized with charcoal, and the mucus thickness down to the crypt opening was measured. Mucus was aspirated by pipette as described, and mucus secretion was stimulated by the addition of serosal carbachol or prostaglandin E_2 (33). Penetrability measurements of explants were performed by adding fluorescent beads the size of bacteria as described (16).

Mass Spectrometry Protein Analysis. Aspirated mucus samples were processed through a modified filter-aided sample preparation (FASP) method (34). The obtained peptides were separated by nanoRPLC-ESI-MS/MS in an LTQ-Orbitrap XL mass spectrometer (Thermo Scientific) (34). MS data were analyzed with MaxQuant 1.2.2.5 (35) against the UniProt-SwissProt mouse database (April 2013 version), the MaxQuant contaminant database, and an in-house database (34). Protein quantities were calculated in relative amounts by the intensity-based absolute quantification (iBAQ) method (35), and results were averaged for four biological and two technical replicates.

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