TFF2, a MUC6-binding lectin stabilizing the gastric mucus barrier and more (Review)

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Abstract. The peptide TFF2 (formerly 'spasmolytic polypeptide'), a member of the trefoil factor family (TFF) containing two TFF domains, is mainly expressed together with the mucin MUC6 in the gastric epithelium and duodenal Brunner's glands. Pathologically, TFF2 expression is observed ectopically during stone diseases, chronic inflammatory conditions and in several metaplastic and neoplastic epithelia; most prominent being the 'spasmolytic polypeptide-expressing metaplasia' (SPEM), which is an established gastric precancerous lesion. TFF2 plays a critical role in maintaining gastric mucosal integrity and appears to restrain tumorigenesis in the stomach. Recently, porcine TFF2 has been shown to interact with the gastric mucin MUC6 and thus stabilize the gastric mucus barrier. On the one hand, TFF2 binds to MUC6 via non-covalent lectin interactions with the glycotope GlcNAcα1→4Galβ1→R. On the other hand, TFF2 is probably also covalently bound to MUC6 via disulfide bridges. Thus, implications for the complex multimeric assembly, cross-linking, and packaging of MUC6 as well as the rheology of gastric mucus are discussed in detail in this review. Furthermore, TFF2 is also expressed in minor amounts in the immune and nervous systems. Thus, similar to galectins, its lectin activity would perfectly enable TFF2 to form multivalent complexes and cross-linked lattices with a plethora of transmembrane glycoproteins and thus modulate different signal transduction processes. This could explain the multiple and diverse biological effects of TFF2 [e.g., motogenic, (anti)apoptotic, and angiogenic effects]. Finally, a function during fertilization is also possible for TFF domains because they occur as shuffled modules in certain *zona pellucida* proteins.

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Contents

1. Introduction

Mucous epithelia cover the inner surfaces of our body and are essential for interaction with the outside world (e.g., respiration, uptake and digestion of food, excretion, reproduction, visual and auditory systems). These delicate epithelia are exposed to a myriad of noxious agents and thus rely on multiple mucosal protection and defense mechanisms (1), including the: i) mucosal barrier (i.e., the extracellular mucous gel, the apical glycocalyx, and the polarized epithelium connected by tight junctions), ii) secretion of antimicrobial peptides, iii) rapid repair by cell migration (restitution), iv) continuous self-renewal by differentiation from stem and precursor cells, and v) an acute inflammatory response. For the structure and function of the extracellular viscoelastic mucous gel, i.e., the mucus, both secretory mucins (MUC2, MUC5AC, MUC5B, MUC6, MUC19) as well as trefoil factor family (TFF) peptides play key roles (2-5). Protection of the stomach epithelium is a unique problem because of the acidic pH of the gastric juice. Thus, the mucous gels covering the gastric surface mucosa and its glands, respectively, have a special composition designed to optimally fulfill their physiological function(s). Here, the function of TFF2 for the gastric mucus barrier is discussed.

TFF2, a gastroduodenal mucus constituent and more. TFF2 (originally termed 'pancreatic spasmolytic peptide') is a 106 amino acids containing secretory peptide conserved from birds to human containing two TFF domains (reviewed in refs 4-6). Of special note, the TFF domain (7), in the past often also referred to as 'trefoil domain', is not related to the 'β-trefoil fold' found in certain lectins. Each of the two TFF domains contains one conserved tryptophan and 6 conserved cysteine residues forming 3 disulfide bridges. Furthermore, a seventh disulfide bridge links Cys^{6} and Cys^{104} (Fig. 1) (8,9). This is probably the reason why TFF2 is stable in the gastric juice (4). Human gastric TFF2 is N-glycosylated containing a fucosylated N,N'-diacetyllactosediamine (LacdiNAc) oligo-

saccharide (10); in contrast, the porcine, murine, and rat TFF2 lack N-glycosylation sites. Thus far, the functional significance of the fucosylated LacdiNAc structure in human TFF2 is not known but it might influence microbial colonization (e.g., by *Helicobacter pylori*) particularly of the antrum (10). Neither is it known currently, if the fucosylated LacdiNAc structure is recognized by galectins, in particular by galectin-3. Interestingly, porcine pancreatic TFF2 forms non-covalently linked dimers in the solid phase (9,11) as well as in solution (12); the latter being unusually resistant even to boiling SDS (12).

TFF2 is typically co-secreted with the mucin MUC6 from gastric fundic and antral glands (mucous neck and antral gland cells, respectively) as well as duodenal Brunner's glands (13-17) and represents a characteristic constituent of the gastroduodenal mucus (18). Of special note, in the gastric fundic glands TFF2 mRNA and protein do not co-localize. TFF2 transcripts are detectable in mucous neck cell progenitors only. This has been shown for human (19), mouse (20), and rat (21). TFF2 also appears as a constituent of the gastric juice with dramatic diurnal variations (22). In the pig, in contrast to human, TFF2 is also abundantly secreted from exocrine pancreatic glands (acinar cells) (14). Furthermore, TFF2 is expressed in salivary glands (21,23).

Other than in mucous epithelial glands, TFF2 is also expressed in the immune and the central nervous systems (CNS). For example, TFF2 is found in macrophages and lymphocytes (24,25) as well as in the anterior pituitary and the developing brain (26).

TFF2 is an early response gene after gastric mucosal injury (27). During various chronic inflammatory conditions, TFF2 is expressed ectopically in the ulcer-associated cell lineage (28-30). Furthermore, pathological expression of TFF2 occurs in several metaplastic and neoplastic epithelia (compilations in refs. 4,17,30,31), most prominent being the spasmolytic polypeptide-expressing metaplasia (SPEM), which is found at the base of gastric fundic units as a consequence of dysregulated trans-differentiation of zymogenic cells as well as arrest of mucous neck cell trans-differentiation into zymogenic cells (32,33). SPEM gives rise to intestinal metaplasia and is even more strongly associated with gastric cancer than is intestinal metaplasia (34).

In the past, TFF2 was considered as a protective rapid response peptide (27,35) responsible for epithelial repair due to its relatively weak motogenic effects *in vitro* and moderate protective or healing effects *in vivo* (compilations in refs. 4,31). Detailed comparative studies demonstrated that only luminal, but not parenteral TFF2 was protective in one out of two colitis models *in vivo* (36). This is in line with a report that delivery of TFF2 by genetically modified *Lactococcus lactis* prevents and heals acute colitis in mice (37). The weak motogenic effect is a chemotactic effect and is dependent on the ERK1/2 pathway (38,39). Furthermore, also (anti)apoptotic and angiogenic effects have been reported for TFF2 (4,5,31). However, all attempts have so far failed to convincingly demonstrate a typical transmembrane receptor for TFF2. Currently, TFF2 is considered a low affinity ligand for the chemokine receptor CXCR4 (40,41). Furthermore, integrin β1 as well as a large transmembrane glycoprotein with similarity to CRP-Ductin/DMBT1/gp-340/hensin have been identified as TFF2 binding proteins in the porcine stomach (42). Interestingly, intravenously administered TFF2 was taken up by mucous neck cells, parietal cells, and pyloric gland cells and subsequently appeared in the mucus layer (43). This could be an indication for receptor-mediated transcytosis.

In contrast to the abundant synthesis of TFF2 in the stomach, Tff2-deficient ($Tff2^{KO}$) mice show surprisingly moderate gastric phenotypes. $Tff2^{KO}$ mice had increased susceptibility to *Helicobacter felis*-induced gastritis (25), they showed accelerated progression of gastritis to dysplasia in the antrum (44), and laser-induced photodamage of the gastric surface epithelium resulted in an attenuated alkalization of the surface pH in these animals (45). Furthermore, $Tff2^{KO}$ mice showed an altered expression of genes implicated in the immune system (46), they were hyperresponsive to interleukin-1β stimulation (25), exhibited increased susceptibility to *Yersinia enterocolitica* infection (47), and displayed reduced gut immunopathology after oral infection with *Toxoplasma gondii* (48). Taken together, $TFF2^{KO}$ mice show both gastric and immunological phenotypes.

The extracellular gastric mucus barrier. The gastric mucus-bicarbonate-phospholipid barrier is a viscoelastic gel acting as the first line of defense against chemical, physical, and biological insults (1,49,50). The secretory gel-forming mucins MUC5AC and MUC6 are major constituents of the gastric cell surface mucus barrier (3). These heavily O-glycosylated, expanded glycoconjugates confer viscous properties due to the enormous length of mainly linear mucin homo-oligomers and their extreme hydration (2). MUC5AC is secreted by surface mucous and pit cells of the gastric mucosa (Fig. 2) (51-53). Of special note, MUC5AC in the mid and lower pit regions is sulfated (Fig. 2) (54). In contrast, MUC6 is typically secreted by the gastric glands, i.e., mucous neck cells in fundic glands as well as antral gland cells (Fig. 2) (52,53). Interestingly, MUC6 is aberrantly expressed in surface mucous cells in *H. pylori*-infected patients (55). Gastric MUC6 is characterized by the specific carbohydrate moiety GlcNAcα1→4Galβ1→R at non-reducing terminals (56). A key enzyme for formation of this epitope is the α1,4-N-acetylglucosaminyltransferase (α4GnT) (57). This enzyme plays an essential role for gastric protection as mice lacking this enzyme ($A4gnt^{KO}$) typically show gastric mucosal inflammation and spontaneously develop adenocarcinoma exclusively in the gastric antrum through a hyperplasia-dysplasia-carcinoma sequence in the absence of *H. pylori* infection (58). The GlcNAcα1→4Galβ1→R moiety is recognized by the lectin GSA-II binding to the terminal GlcNAc (59), the monoclonal antibody HIK1083 (56), and paradoxical concanavalin A staining (60). Of note, GSA-II prevented the subsequent binding of HIK1083 (59).

Gastric mucins MUC5AC and MUC6 also differently regulate colonization with H. *pylori* (61). For example, *H. pylori* colonizes the MUC5AC layers of the gastric mucus (62-64). In contrast, the MUC6 layers are barely colonized because the terminal α1,4-GlcNAc of the MUC6 carbohydrate moiety has an antimicrobial activity against *H. pylori* by inhibiting the biosynthesis of cholesteryl-α-D-glucopyranoside, an essential cell wall constituent of *H. pylori* (58,65).

Furthermore, the membrane-tethered mucin MUC1, which is also capable of intracellular signaling, is found on the apical

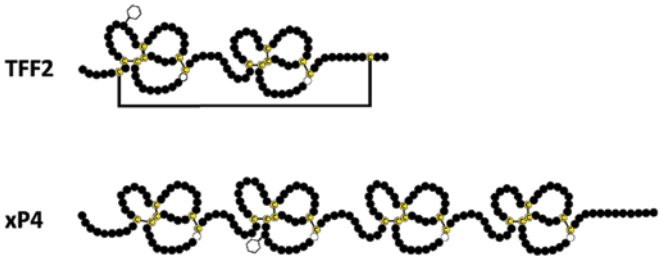

Figure 1. Schematic representation of the structure of mammalian TFF2 and amphibian xP4 consisting of 106 and 207 amino acids, respectively. Shown are the conserved cysteine residues (yellow) including their disulfide bridges as well as the conserved tryptophan residues (white) characteristic of each TFF domain. The N-glycosylation sites in human TFF2 and in xP4.1 from *X. laevis* are indicated by hexagons.

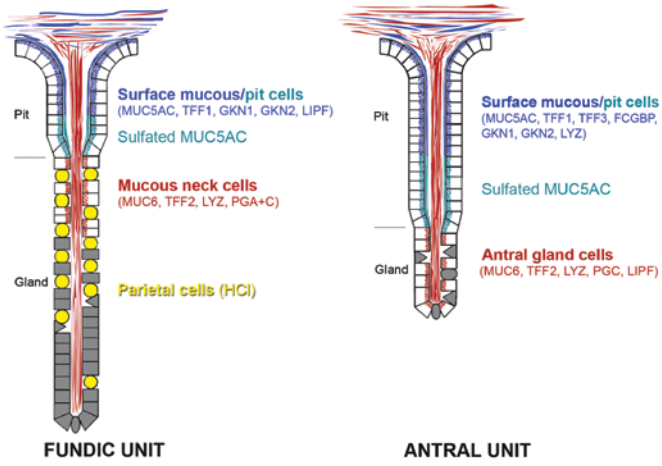

Figure 2. Schematic representation of human fundic and antral units and the excretory flow of gastric mucus. The layered mucus structure at the gastric mucosal surface consists of an alternating array of MUC5AC (blue; secreted from both surface mucous and pit cells) and MUC6/TFF2 (red; both originating from mucous neck and antral gland cells, respectively). Of note, MUC5AC in the mid and low pit regions is sulfated (54). Also shown are the major secretory products of the mucous cells such as mucins (MUC), TFF peptides, IgG Fc binding protein (FCGBP), gastrokines (GKN), gastric lipase (LIPF), lysozyme (LYZ), and pepsinogens (PGA, PGC). The hydrochloric acid (HCl) producing parietal cells are colored yellow.

side of surface mucous cells and in the mucous neck cell zone (66). In addition to tethering the mucus to the epithelial surface, MUC1 is a sensor of the environment including microorganisms and it can be shed from the cell surface (67). Of note, $Muc1^{KO}$ mice have thinner gastric mucus layers (68). MUC13 is another membrane-tethered mucin expressed in the surface epithelium as well as in gastric glands (69).

In addition to the gel-forming mucins MUC5AC and MUC6 as well as a variety of ions (e.g., H^+, Na^+, Ca^{2+}, Mn^{2+}, Cu^{2+}, Cl^-, HCO_3^-), gastric mucus also contains a complex mixture of additional proteins such as sIgA, IgG, IgM (67), TFF peptides (5), gastrokines (70), IgG-Fc-binding protein (FCGBP) (19), DMBT1/gp-340 (71), galectins (72), β-defensins (73), cathelicidin LL-37/hCAP18 (74), lysozyme (19), and the extremophilic gastric lipase (75), which binds optimally to lipid-water interphases at low pH. On top, the luminal surface of the gastric mucus seems to be coated with a hydrophobic film of surfactant phospholipids (1,76,77). We are only at the beginning in the understanding of how these proteins interact and form a viscous gel matrix. Certainly, a complex interplay of different transient and non-transient interactions is involved to build up a complex network (78).

The viscous gastric mucus barrier has multiple physiological functions: this biofilm lubricates the passage of undigested food, protects the epithelium from mechanical damage and pepsin digestion, it is essential for maintaining a pH gradient towards the acidic gastric juice, and it supports and also restricts the adhesion and colonization of microorganisms (such as H. *pylori*) (49).

In rats, the mucus covering the gastric mucosa has been described as being composed of two layers, i.e., a loosely adherent (outer) layer, which can be removed by gentle suction, and a firmly adherent (inner) layer attached to the epithelium (68,79). The thickness of the latter has been reported to be approximately 80 and 154 μm in the corpus and antrum, respectively (79). The loosely adherent layer had a similar thickness and is not present in all rats (79). One possibility is that it may be rubbed off after food intake. Mice have a significantly thinner firmly adherent mucus layer compared with rats (68). This inner mucus layer can only be removed after application of a strong force and it is penetrable to beads the size of bacteria (80). In contrast to the colon, only small amounts of the murine gastric outer mucus layer could be removed by gentle aspiration (80). Muc5ac is a major component in both the firmly and loosely adherent mucus layers in mice (68).

In human as well as in the rat (54), the firmly adherent, water-insoluble gastric mucus layer (mean thickness: 44 μm) is composed of an alternating laminated array of two types of mucin, i.e., MUC5AC and MUC6 (18,62,81,82). Furthermore, there is also soluble mucus mixed with the luminal gastric juice (49,83).

The firmly adherent gastric mucus layer, and not the loosely adherent layer, is important for the maintenance of a pH gradient across the mucus layer (84). There are numerous reports of a pH gradient across the gastric mucus barrier with near neutral pH at the mucosal surface (50,84,85). It is generally accepted that the buffering of the H^+ from the gastric juice occurs in the mucus via HCO_3^-, which is actively released from gastric surface mucous cells via an apical Cl^-/HCO_3^- exchanger (50).

Another interesting point discussed repeatedly is, how can hydrochloric acid, i.e., H^+ and Cl^- ions, be secreted across the mucus layer in order to reach the gastric juice (50). In the past, temporary canals have been reported in the mucous layer (86); there are also studies which failed to show these channels (50). However, ultrastructural studies of the excretory flow of gastric glands clearly described the merging of zymogenic contents with MUC6 and the development of laminated mucus structures at the surface (54). Based on the penetrability using beads (80) as well as on *in vivo* studies (50), there is clear evidence that the firmly adherent mucus layer is freely permeable to ions and small molecules.

2. TFF2 and its function in the gastric mucus barrier

TFF2 interacts with the mucin MUC6. TFF2 is an integral part of the laminated gastric mucus barrier. This has been clearly demonstrated by immunohistochemistry where TFF2 is asso-

ciated with the gland mucin layers, i.e., MUC6, and not with layers representing the surface cell mucin MUC5AC (18). The association of TFF2 with mucins was also observed after size exclusion chromatography of both human and porcine gastric mucosa extracts. Here, TFF2 exclusively appeared in the high-molecular mass mucus fraction (10,12,87). Furthermore, after anion exchange chromatography and non-reducing non-denaturing agarose gel electrophoresis TFF2 was predominantly associated with the mucin MUC6 in the porcine stomach forming an ultra-high molecular mass complex hardly entering the gel (12). Of special note, the porcine TFF2-MUC6 complex had an even higher M_r than MUC5AC (12), which is in line with a previous report that also native human MUC6 appeared to be of larger size than MUC5AC (52).

In contrast, gel electrophoresis under reducing denaturing conditions easily released monomeric TFF2 from MUC6 (12). However, after non-reducing denaturing gel electrophoresis both a high molecular mass TFF2 heteromer as well as monomeric TFF2 were observed (12). This indicates that in the porcine stomach TFF2 is bound to MUC6 in part covalently by disulfide bridges and in part non-covalently. Thus, a lectin activity of TFF2 was predicted to be responsible for its non-covalent binding to MUC6, particularly to its characteristic structure GlcNAcα1→4Galβ1→R (12). Of note, based on its crystal structure a lectin activity cross-linking mucins have already been discussed for TFF2 (9). In particular, two hydrophobic clefts have been identified, one within each TFF domain, and all conserved residues are localized in their vicinity; two monosaccharides have been suggested to fit into each groove with the highly conserved Trp^{45} and Trp^{94} (Fig. 1) being very important residues in these binding pockets (11).

Non-covalent lectin interactions of TFF2 and MUC6. Based on these results, the sugar epitope responsible for TFF2 binding was characterized in more detail. With the help of TFF2 fusion proteins, the carbohydrate specificity was narrowed down in a porcine gastric mucin preparation to GlcNAcα1→4Galβ1→4GlcNAcβ with only minor cross-reactivity to GlcNAcα1→4Galβ, which points to an extended glycotope that comprises more than a common disaccharide (88). The GlcNAcα1→4Galβ1→4GlcNAcβ glycotope has been described previously as part of core 2 mucin structures from human and porcine stomach (56,89) as well as from duodenal Brunner's glands, which also secrete MUC6 (90).

Lectin binding using TFF2 fusion proteins has been reported as being Ca^{2+}-independent (88). This would be unusual as many lectins are calcium-dependent (91). However, studies with TFF2 purified from porcine pancreas suggest that its lectin activity is modulated by Ca^{2+}. For example, binding of ^{125}I-labeled TFF2 to FPLC-purified gastric mucus preparations from $Tff2^{KO}$ mice immobilized on a CNBr-activated Sepharose™ 4B column depends on Ca^{2+} (Stürmer and Hoffmann, unpublished data). Furthermore, binding of ^{125}I-labeled TFF2 to FPLC-purified gastric mucus preparations after gel electrophoresis and western blotting is reduced in the absence of Ca^{2+} (Richter, Stürmer and Hoffmann, unpublished data).

Possible covalent interactions of TFF2 and MUC6. Other than as a lectin, TFF2 is probably also covalently bound via disulfide bridges to the cysteine-rich domains of MUC6 at least in the porcine gastric mucus (12). The latter would be reminiscent to the formation of TFF1-GKN2 and TFF3-FCGBP heteromers (87,92,93) and could particularly cross-link MUC6 dimers via an inter-molecular TFF2 bridge. However, TFF2 contains an even number of cysteine residues forming 7 disulfide bridges; thus, at least one disulfide bridge must be opened in order to enable formation of a heteromer. Of special note, the linkage between Cys^6 and Cys^{104} (Fig. 1) has been reported to be particular sensitive to reduction (94) making this disulfide bridge the most likely candidate. Thus, the question arises, which cysteine residues of MUC6 could form a heteromer with TFF2.

Human MUC6 is a 2439 amino acid residue long protein (pre-pro numbering, Fig. 3) with a mosaic structure typical of the human secretory mucins MUC2, MUC5AC, MUC5B, and MUC19 and the frog integumentary mucin FIM-B.1 (2,95-97). The cysteine-rich domains have similarities to von Willebrand factor (vWF). MUC6 consists of a cysteine-rich N-terminal domain (D1D2D'D3), a highly O-glycosylated domain rich in proline, threonine and serine residues (PTS), and a C-terminal cystine knot (CTCK) domain (Fig. 3) (95). Native MUC6 is known to form large oligomeric complexes (52) and both the N-terminal as well as the C-terminal cysteine-rich domains are essential for its oligomerization (98).

The N-terminal D1D2D'D3 domain of MUC6 (Fig. 3) contains an odd number of Cys residues (i.e., 97), triggers MUC6 oligomerization (98) and shows striking similarity to the assembly domain of vWF (particularly conserved Cys and Trp residues). In vWF, this domain is responsible for dimeric N-to-N assembly between juxtaposed D3 domains via two homophilic inter-molecular disulfide bridges, i.e., Cys^{1142}-Cys^{1142} and Cys^{1099}-Cys^{1099} (99,100). In contrast to the standard formation of disulfide bridges, which is restricted to the endoplasmic reticulum, D3 assembly occurs via a rearrangement of disulfide bonds in the acidic environment of the trans-Golgi network. This process depends on an intrinsic oxidoreductase activity of vWF (CxxC motifs in D1, D2, and D3) (99,101). However, the D1D2D'D3 assembly domain of MUC6 shows some characteristic features, which distinguish it from vWF (Fig. 3). For example, the sub-domain C8-2 in MUC6 lacks two conserved Cys residues, but contains an additional Cys residue at position 587, which is not conserved in vWF and the secretory mucins MUC2, MUC5AC, MUC5B, and MUC19. Thus, C8-2 contains an odd number of Cys residues (i.e., 9), so that one can expect that this domain is capable of forming an inter-molecular disulfide bridge at Cys^{587} characteristic of MUC6 (Fig. 3). Furthermore, Cys^{1100} in C8-3 of MUC6 corresponds to the homologous Cys^{1099} of vWF, which forms a homophilic inter-molecular disulfide bridge essential for assembly of vWF (99). However, C8-3 in MUC6 (and also MUC2, MUC5AC, MUC5B, and MUC19) contains an additional Cys residue (Cys^{1118}) when compared with vWF resulting in an even number (i.e., 12). Thus, one could conclude, that the C8-3 MUC6 is capable of forming either two (if Cys^{1100} forms an inter-molecular disulfide bridge) or no inter-molecular disulfide bridge (if Cys^{1100} does not form an inter-chain bridge). Additionally, the TIL-3 domain is somewhat changed in MUC6. However, Cys^{1153} of MUC6 is homologous to Cys^{1142} in vWF, Cys^{1130} in MUC2, and Cys^{1199} in porcine submaxillary mucin (PSM)/Muc19; the latter three cysteine residues have been shown to form a homophilic

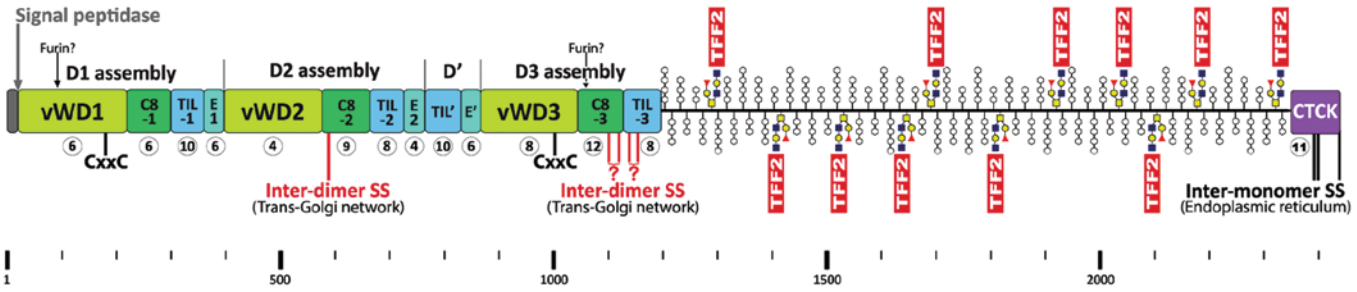

Figure 3. Schematic representation of the human mucin MUC6 and its interactions with TFF2. The mosaic domain structure of MUC6 (cysteine-rich N-terminal D1D2D'D3 and C-terminal CTCK domains) is depicted by its striking similarity to human von Willebrand factor (vWF) (100). Domains are scaled to length and residues are shown with pre-pro numbering. The number of cysteine residues in each domain is indicated (encircled). Also shown are the predicted inter-molecular disulfide bridges involved in C-terminal dimerization (inter-monomer SS, black) and N-to-N multimerization (inter-dimer SS, red) as well as the CxxC disulfide isomerase motifs and potential furin cleavage sites. The oligosaccharides in the central highly O-glycosylated PTS domain are indicated by multiple hexagons, whereas the structure GlcNAcα1-4Galβ1-4GlcNAcβ1-6(Fucα1-2Galβ1-3)GalNAc is shown coloured. The non-covalent lectin binding of TFF2 (containing two TFF domains) to GlcNAcα1-4Galβ1-4GlcNAcβ (88) would be perfectly designed to crosslink MUC6-monomers as well as MUC6-dimers. Furthermore, TFF2 could also covalently crosslink MUC6-dimers by forming heterophilic disulfide bridges between cysteine residues involved in N-to-N multimerization (red).

inter-molecular disulfide bridge essential for assembly of vWF (99), MUC2 (102), and PSM/Muc19 (103), respectively. Because TIL-3 in MUC6 contains an even number (i.e., 8) of Cys residues it is not clear if TIL-3 is capable of forming inter-molecular disulfide bridges in MUC6 (similar situation as in C8-3). Taken together, Cys^{587} in C8-2 of MUC6 (Fig. 3) is a prime candidate for an inter-chain disulfide bridge, either homophilic or heterophilic with TFF2 as a cross-linker (Fig. 3). Further potential inter-molecular disulfide bridges could originate from C8-3 and TIL-3 (either homophilic or heterophilic with TFF2; Fig. 3).

The C-terminal cysteine-rich domain of MUC6 is much shorter than that of the other secretory mucins and vWF (104) and it has been shown to be responsible for dimerization (98). It consists only of a CTCK domain containing an odd number of Cys residues (i.e., 11) with striking similarity to vWF (105). It is known that three intra-chain disulfide bridges of the CTCK domain are responsible for C-to-C dimerization of vWF in the endoplasmic reticulum (106). A similar situation has been reported for PSM/Muc19 (107). Thus, it is expected that the homologous residues (i.e., Cys^{2393}, Cys^{2395}, and Cys^{2437}) in MUC6 are also involved in dimerization of MUC6 (Fig. 3). Whether cross-linking via TFF2 plays a role in this process is a matter of speculation.

Function of TFF2 for MUC6 assembly in the secretory pathway and for mucus rheology. Based on multiple and characteristic structural similarities, the biosynthesis of MUC6 is generally expected to share many typical features already observed in both vWF (100) and secretory mucins (2,96,108). Furthermore, TFF2 and MUC6 are co-expressed in the same cells, and it is expected that TFF2 and MUC6 share the same secretory pathway and end up in the same secretory vesicles.

The primary translation product of MUC6 is probably co-translationally cleaved by the signal peptidase and then dimerizes in the endoplasmic reticulum (ER; pH ~7.5) via disulfide bridges in the CTCK domain (Fig. 3) similar to vWF (100,106) as well as MUC2 and other gel-forming mucins (96,98). Generally, folding and initial polymerization of mucins occur in the ER, which requires the protein disulfide isomerase

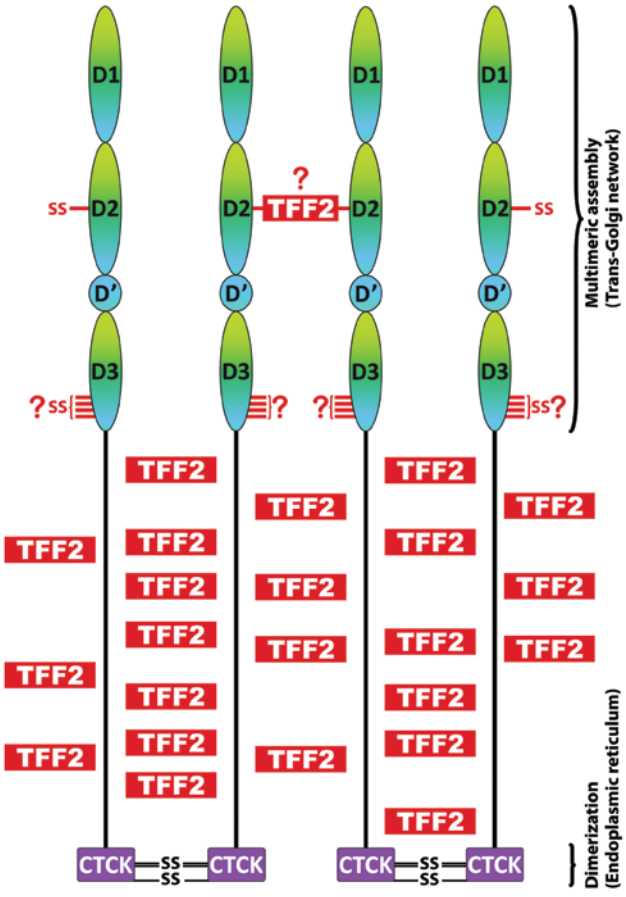

Figure 4. Suggested structure of the condensed and highly organized form of the MUC6/TFF2 complex in the secretory granules. On the one hand, non-covalent lectin interactions of MUC6 (via its PTS domain) and TFF2 are expected to result in dimeric bouquet structures, which could even show supra-structural organization. On the other hand, besides C-to-C dimerization, N-to-N multimeric assembly is expected to occur via the D2 (C8-2; see Fig. 3) and maybe also the D3 domains (C8-3 and TIL-3; see Fig. 3) probably resulting in a branched structure. Here, TFF2 could also covalently crosslink MUC6-dimers by forming heterophilic disulfide bridges.

AGR2 (109,110). AGR2 has been reported to be involved in ER stress and the unfolded protein response (111). Of note, AGR2

is expressed in the stomach specifically together with MUC6 and TFF2 in mucous neck and antral gland cells, respectively and $Agr2^{KO}$ mice develop severe glandular hyperplasia at the expense of chief but also of pit and parietal cells (112). If the protein disulfide isomerase ERp57 and the calnexin-calreticulin cycle play a role for correct folding of MUC6 and/or TFF2 is not known currently.

As the next steps in the secretory pathway, N- and O-glycosylations of MUC6 are completed in the Golgi and multimerization via the N-terminal D1D2D'D3 domain occurs in the trans-Golgi network (pH ~6.0). Multimerization of both vWF as well as the mucins MUC2 and PSM/Muc19 is known to require an acidic pH and Ca^{2+} as well as an intrinsic oxidoreductase activity (CxxC motifs) (103,113-115). Thus, the CxxC motifs in the vWD1 and vWD3 domains (Fig. 3) are expected to catalyze N-to-N multimerization of MUC6. However, multimerization of vWF occurs via N-terminal dimerization (100,114), whereas both MUC2 and PSM/Muc19 form N-terminal trimers (96,102,116). This fundamental difference in the multimerization between vWF and MUC2 leads to linear thread-like structures in vWF and two-dimensional net-like sheets in MUC2, respectively. It is not known currently, how MUC6 multimerizes. Of special note, the N-terminal assembly domain of human MUC6 differs characteristically by some cysteine residues when compared with conserved positions in both vWF and the secretory mucins MUC2, MUC5AC, MUC5B (Fig. 3): particularly Cys^{587} (C8-2) is present in MUC6 only, whereas some conserved cysteine residues in the C8-1 and vWD2 domains are lacking in MUC6. The latter also lacks the E3 and CysD domains and C-terminal cysteine-rich regions (96). Furthermore, in contrast to the human secretory mucins MUC2, MUC5AC, MUC5B, and MUC19, His^{395} essential for pH-dependent multimerization of vWF (117) is not conserved in MUC6. On the other hand, TFF2 is synthesized together with MUC6 connecting the PTS domains via its lectin activity (Fig. 3) and probably also forming disulfide bridge(s) with MUC6, e.g., at Cys^{587} (perhaps with the help of the CxxC motifs in vWD1 and vWD3; Fig. 3). Non-covalent cross-linking of the PTS domains via TFF2 could play a major role for zipping up a dimeric MUC6 bouquet (Fig. 4), a reaction with functional analogy to vWF (100). Taken together, the multimerization of MUC6 is expected to be different from both vWF and the mucins MUC2 and PSM/Muc19; for MUC6, a complex branched rather than a simple linear structure is expected. TFF2 probably plays a role here.

It should be mentioned that MUC6 as well as the secretory mucins MUC2, MUC5AC, and MUC5B contain 2 potential furin cleavage sites at conserved positions (in MUC6 after positions 88 and 1054, respectively; Fig. 3). It is not known currently whether MUC6 is processed by this Ca^{2+}-dependent protease.

The last steps in the secretory pathway of MUC6/TFF2 are storage in secretory granules (diameter of mucin granules: <1 μm) and mainly regulated exocytosis after extracellular stimulation with a secretagogue, such as ATP (110). Secretory granules from a variety of cells are typical acidic Ca^{2+} stores (pH ~5.5-5.9; total Ca^{2+} bound to a matrix, 20-150 mM; free Ca^{2+}, 10-100 μM) (118,119). In contrast, resting Ca^{2+} levels in the cytosol are only ~100 nM. Progressive acidification along the secretory pathway is maintained by a vesicular H^+-ATPase and Ca^{2+} uptake probably occurs via a SERCA-type Ca^{2+}-ATPase (119,120). Furthermore, there are probably also channels for K^+ and Cl^- import; whereas Ca^{2+} release is probably operated by IP3/ryanodine receptors (118,120). It has been demonstrated that both vWF as well as secretory mucins are stored in a highly condensed and well-organized packaging in the corresponding secretory granules in the presence of Ca^{2+} at pH <6.0. vWF assembles into helices forming the tubules of the Weibel-Palade bodies (100,114); whereas secretory mucins are tightly packed forming a condensed polyanionic matrix with Ca^{2+} as counter-ions embedded in a fluid phase (108,115,121,122). Particular for MUC2 a detailed mechanistic model has been proposed for packaging and unfolding (115). In the case of MUC6, non-covalent cross-linking of the PTS domains via the lectin activity of TFF2 would be a perfect design enforcing extremely dense packaging of MUC6 within the secretory granules (Fig. 4). Furthermore, the many acidic residues of TFF2, often clustered, could also serve as additional Ca^{2+} binding sites. During exocytosis, rapid unfolding of condensed mucins is triggered by an exchange of Ca^{2+} against Na^+ from the extracellular fluid thus doubling the number of counter-ions and causing the mucin polymer to expand up to 600-fold in its volume by osmotic swelling within 20-30 msec (108,122,123,124). Of special note, HCO_3^- plays a crucial role for the extremely rapid transition into the expanded hydrated phase because it efficiently competes for Ca^{2+} binding (125). In many organs, this process is linked with the CFTR-channel, which also transports HCO_3^-. However, in the human stomach the CFTR is expressed at low levels only and gastric surface epithelial cells secrete abundantly bicarbonate via an apical Cl^-/HCO_3^- exchanger (50) allowing expansion of MUC6. Gastric HCO_3^- secretion is stimulated by E-type prostaglandins (50), which is in agreement with their general protective function - here, in particular due to their role for proper MUC6 exocytosis.

Upon release and expansion of MUC6, TFF2 still stays attached also at lower Ca^{2+} concentrations allowing the formation of three-dimensional networks. Generally, the assembly and the structure of the TFF2-MUC6 network are expected to be quite different when compared with MUC2. TFF2 seems to be an important link peptide enabling a dense MUC6 network, which is typical of glands (fundic glands, antral glands, Brunner's glands). This would also explain why native MUC6 appeared to be of larger size than MUC5AC (12,52) despite the fact that the MUC6 monomer is much smaller than that of MUC5AC. The formation of large TFF2-MUC6 complexes probably supports the proper excretory flow from the gastric glands (54). Furthermore, the large TFF2-MUC6 complexes probably ensure that MUC5AC and MUC6 do not mix homogeneously, but rather form a layered mucus structure.

The proposed role of TFF2 for MUC6 assembly and rheology fits well with several experimental observations. For example, TFF2 increased dramatically the viscosity and elasticity of porcine gastric mucin solutions *in vitro* (126). These solutions showed non-Newtonian pseudo-plastic behavior, i.e., the viscosity decreased when increasing the applied shear rate (126). Such a behavior is typical of an entangled network. Of note, the viscous response was pH-dependent with highest viscosity at low pH (126). This is typical of gastric mucus (127) and is an indication for the involvement of hydrogen bonds.

Furthermore, systemically administered TFF2 *in vivo* also increased the viscosity of stomach secretions after uptake and probably transcytosis to the mucosal surface (128). Also the effects on gastric pH regulation (45) and proton permeation through gastric mucus (129) fit well with a role of TFF2 for MUC6 assembly and stabilization of the mucus barrier.

TFF2 orthologs in the amphibian gastrointestinal tract. In the *Xenopus laevis* stomach epithelium, two peptides consisting of four TFF modules arranged in tandem are expressed in mucous neck and antral gland cells (xP4.1 and xP4.2) (130). They are considered to be the *X. laevis* functional homologs of mammalian TFF2 (31). Interestingly, xP4.1 is N-glycosylated (as is human TFF2) and expressed in all regions of the stomach; whereas xP4.2 lacks the N-glycosylation site (131) and is expressed with a decreasing gradient from the fundus to the antrum (31). Thus far, it is not known if xP4.1 contains the LacdiNAc oligosaccharide as human TFF2. However, both xP4.1 and xP4.2 contain 4x6 cysteine residues typical of the four TFF domains and, in contrast to TFF2, lack the additional cysteine residues at the N- and C-termini typical of TFF2 (Fig. 1). Thus, there is no additional disulfide bridge in xP4 equivalent to Cys^{6}-Cys^{104} of TFF2 (Fig. 1), which could be opened and used for a covalent cross-linking of a MUC6 homolog. As a consequence, the xP4 peptides are expected to only bind non-covalently via a lectin activity to the mucin.

3. Conclusions and future perspectives

TFF2 and the gastric barrier. Cross-linking of mucins, by both covalent and non-covalent interactions, plays a key role for the assembly of the laminated structure and the rheological properties of gastric mucus. Sugar-lectin interactions are known to stabilize mucin films in order to sustain their exceptional resistance to extreme salt conditions and to a broad pH range (132). The latter is particularly important for the gastric mucus, where the secreted hydrochloric acid is transported through temporary canals (86) and zymogenic secretions form initially droplet-like structures which finally merge with the gland mucus (54). Thus, TFF2 is expected to play a major role for cross-linking MUC6 and stabilizing particularly the mucus barrier of gastric glands as a 'link peptide'. However, many details still await experimental elucidation, particularly the N-to-N assembly of MUC6. Unfortunately, the data basis describing this process (98) is by far not comparable with that for MUC2, PSM/Muc19 or vWF.

As gastric mucus regulates the colonization with *H. pylori* (61), it is not to surprising that TFF2 is probably also involved in this process (25). TFF2 also has a protective function against the progression of premalignant lesions in *H. pylori*-infected mice (44). This is in line with the observation that epigenetic silencing of TFF2 by *H. pylori* infection leads to gastric tumor development (133). The human stomach is also the host for a variety of non-*H. pylori* microbiota (134) and it will be interesting in the future to determine whether TFF2 plays a role in the colonization of these microorganisms.

More roles for TFF2 and TFF modules as lectins in mucous epithelia, the immune system, the CNS, and during fertilization. TFF2 is also ectopically secreted from a variety of mucous epithelia during stone diseases, such as nephrolithiasis, hepatolithiasis, dacryolithiasis, and cholecystolithiasis (135,136). Thus, TFF2, together with MUC6, would be a prime candidate for initiating the complex process of stone formation. Furthermore, pH-dependent lectin activities have also been reported for TFF1 and TFF3 (137). Thus far, the carbohydrate specificities of TFF1 and TFF3 have not been determined. However, there are indications that the binding characteristics of these peptides are different. A general lectin activity of TFF domains could functionally explain the occurrence of such modules in various mosaic proteins, such as certain *zona pellucida* proteins (ZP1, ZPB), intestinal sugar-degrading enzymes (sucrase-isomaltase, α-glucosidase, maltase-glucoamylase), and some frog integumentary mucins (FIM-A.1, FIM-C.1) (compilations in refs. 31,138). Clearly, a function for the 3D-structure and rheology of mucus can easily be inferred for the TFF domains in frog integumentary mucins. However, a molecular function of TFF domains also beyond the structure of mucus and stone formation can be expected, e.g., during fertilization or for the extracellular degradation of saccharides. Interestingly, all TFF domains analyzed thus far are encoded by single exons establishing this domain as an evolutionary conserved shuffled lectin module.

Furthermore, the lectin activity could also explain the multiple and diverse biological effects of TFF2 [e.g., motogenic, proliferative, (anti)apoptotic, and angiogenic effects] (reviewed in refs. 4,5,31,41,104) by binding to a plethora of transmembrane glycoproteins, such as receptors, e.g., CXCR4 (40,41), integrins (42), and a CRP-Ductin/DMBT1/gp-340-like glycoprotein (42). Such transmembrane glycoproteins interacting with TFF2 (and possibly also with TFF1 and TFF3) are expected to occur particularly in the immune and central nervous systems, e.g., the body weight is regulated by hypothalamic TFF2 (139). In particular, the relatively high dissociation constants of lectin interactions explain now conclusively why relatively high TFF2 concentrations were necessary for biological activity (40,41). Furthermore, similar to galectins (140,141), TFF2 is also perfectly designed to form various two- and three-dimensional cross-linked lattices with transmembrane glycoproteins at the cell surface. Thus, TFF2, and probably also TFF1 and TFF3, are reminiscent to galectins in some ways, both occurring in the digestive tract as well as the immune and the nervous systems and regulating a variety of different biological processes not related to mucus.

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