Biosynthesis of Gastroke-2 in the Human Gastric Mucosa: Restricted Spatial Expression along the Antral Gland Axis and Differential Interaction with TFF1, TFF2 and Mucins

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Key Words

Gastroke-2 • TFIZ1 • GDDR • TFF-domain • Mucin • Restitution • Stomach • Gastric regeneration • Gastric stem cells • Laser microdissection

Abstract

Gastroke-2 (GKN2) is a secretory peptide of human gastric surface mucous cells (SMCs). It forms disulfide-linked heterodimers with the trefoil factor family (TFF) peptide TFF1. Binding with TFF2 was also reported. Antral SMCs differ from those of the corpus by their TFF3 expression. The aim of this study was to localize GKN2 expression along the antral gland axis, to characterize the continuous regeneration of antral glands, and to investigate the interactions of GKN2 with TFF1, TFF2 and mucins. Methods: The spatial expression of GKN1, GKN2, TFF1-3, MUC5AC and MUC6 was determined using laser

microdissection and RT-PCR analysis. Furthermore, antral extracts were separated by gel chromatography and the association of GKN2 with TFF1, TFF2, and mucins was investigated. Results: Differential GKN2 expression was localized along the rostro-caudal axis of the stomach. Laser microdissection revealed characteristic differential expression profiles of GKN1, GKN2, TFF1-3, MUC5AC and MUC6 along the antral gland axis. Both GKN2 and TFF1 were expressed in superficial SMCs. Surprisingly, the TFF1-GKN2 heterodimer did not associate with the mucin fraction; whereas TFF2 showed exclusive association with mucins. Conclusions: Maturation of antral SMCs occurs stepwise via trans-differentiation of TFF3 expressing progenitor cells. The TFF1-GKN2 heterodimer and TFF2 differ characteristically by their binding to gastric mucins. This points to different physiological functions of TFF1 and TFF2, the latter maybe acting as a "link peptide" for stabilization of the gastric mucus.

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Introduction

Gastrophilin-1 (GKN1; formerly CA11, AMP-18 or foveolin; M_r: 18269) was identified in gastric antral surface mucous cells (SMCs) by expression profiling due to its down-regulation in gastric cancer [1-3]. GKN1 and even fragments of this secretory peptide were shown to be both mitogenic and motogenic [2, 4]. Later, a gene related to GKN1 was discovered (originally named GDDR) which is also down-regulated in gastric cancer [5]. The corresponding protein, termed now GKN2, was identified and characterized independently by Westley et al. [6] where it was described as TFIZ1 due to its dimerization with TFF1. At that time, a mouse homologue of GKN2 was characterized (formerly termed blottin) and non-covalent binding to a TFF2 fusion protein was reported [7,8]. GKN2 is one of the most up-regulated genes after *Helicobacter pylori* eradication [9] and it shows a similar regulation pattern with respect to cytokines as the TFF genes [10].

GKN1 and GKN2 show pronounced similarity (26% of the amino acid residues in the mature proteins are identical and 49% are semi-conserved; [6]). Both peptides are synthesized via precursors typical of secretory proteins. GKN1 and GKN2 differ remarkably by an additional cysteine residue in GKN2 at position 38 which is linked via a disulfide bond to Cys-58 of TFF1 [6]. Thus, mature GKN2 (consisting of 164 amino acid residues; M_r: 18318) and TFF1 form a disulfide-linked heterodimer with a M_r of 24990; whereas GKN1 does not form such heterodimers. As a hallmark, both GKN1 and GKN2 contain the conserved BRICHOS domain. This domain contains a pair of conserved cysteine residues and was proposed to have a function for folding and intracellular transport or secretion [11].

The heterodimerization partner of GKN2 is TFF1, a secretory peptide consisting of 60 amino acid residues (M_r: 6674) [12] which belongs to the trefoil factor family (TFF; for reviews, see [12-14]). TFF1 is synthesized in gastric surface mucous cells (SMCs) together with the mucin MUC5AC and also binding to MUC5AC was observed in the presence of Ca²⁺ [15-17]. Using the yeast 2-hybrid system, a direct interaction between TFF1 and the vWF C-domain of mouse mucins Muc2 and Muc5ac was proposed [18]. Also *Helicobacter pylori* was reported to interact with TFF1 [19]. TFF1-deficient mice develop obligatory antropyloric adenoma and about 30 percent develop carcinomas [20]. These animals also show changes in the gastric differentiation from pre-pit to pre-parietal cells [21] which might be due to the anti-

apoptotic effect of TFF1 as well as its effect on cell proliferation (by delaying G1-S phase transition; [22]).

Here, we analyzed gastric GKN2 expression along the rostro-caudal axis and along the antral gland axis using laser microdissection (LM). Furthermore, antral extracts were separated by gel chromatography and the association of GKN2 with TFF1, TFF2, and mucins was investigated. Surprisingly, the TFF1-GKN2 heterodimer did not associate with the mucin fraction; whereas TFF2 associated with the mucin fraction.

Materials and Methods

Human tissue

All investigations followed the tenets of the Declaration of Helsinki and the investigations were approved by the Ethic Committee of the Medical Faculty at the University of Magdeburg.

Human esophageal, gastric or duodenal tissue was investigated from the same patients undergoing gastrectomy as a result of carcinoma as described earlier in detail [23, 24]. Furthermore, the same standardized biopsy specimens from the gastric cardia (samples Z or Z-0.5), the fundic region (samples Z-4) or the gastric corpus (samples C) were analyzed as described in detail previously [24]. Generally, samples were included in the study only when the formal histopathological review of the specimens excluded neoplastic changes as well as intestinal metaplasia. The minimal distance to the margins of a pathological lesion, e.g. carcinomas or mucosal defects, was 2 cm.

Additionally, antral or duodenal specimens from non-neoplastic regions of additional patients were investigated. The gastric juice samples investigated were also described previously [23].

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR) analysis

The RNA extraction of human tissue, including purification via CsCl ultracentrifugation and RT-PCR analysis of TFF1, TFF2, TFF3, MUC2, MUC5AC, or MUC6 expression were performed as previously described [23, 24]. As a control for the integrity of the cDNA preparations, β-actin transcripts were amplified in parallel. The primer pairs used are outlined in Table 1.

Laser microdissection

Human gastric specimens were embedded in O.C.T. Compound (Plano W. Plannet GmbH, Wetzlar, Germany) at room temperature and stored at -80°C. 7 μm serial sections were obtained with a cryomicrotome (Leica JUNG CM3000, Bensheim, Germany). The sections were transferred onto membrane slides (1.4 μm PET-membrane in an aluminium frame, Art.No. 50102; Molecular Machines & Industries, Glattbrugg, Switzerland). The sample was mounted on the top of the membrane and then the membrane slide was placed up side down

Genes (Acc.No.)	Primer pairs (nucleotide position)	RT-PCR	Size (bp)
GKN1 (AY139182)	MB364 (6746) GCCTGATGTACTCAGTCAACC (6766) MB365 (7618) TAGTTCTCCACCGTGTCTCC (7599)	57°C, 28 cycles	205
GKN2 (NM_182536)	MB521 (80) TTAAGTCCAGCACCTTCTCTCTCC (101) MB522 (797) ATAAATACAGCATTTATATTTCTGAC (771)	57°C, 35 cycles	718
TFF1 (NM_003225)	MD11 (21) TTTGGAGCAGAGAGGAGG (38) MD12 (458) TTGAGTAGTCAAAGTCAGAGCAG (436)	57°C, 30 cycles	438
TFF2 (NM_005423)	MD13 (108) AGTGAGAAACCTTCCCCC (125) MD14 (473) AACACCCGGTGAGCCAG (457)	57°C, 30 cycles	366
TFF3 (NM_003226)	MD9 (322) GTGCCAGCCAAGGACAG (338) MD10 (623) CGTTAAGACATCAGGCTCCAG (603)	57°C, 30 cycles	302
MUC2 (M94132)	MB322 (2471) ACTGCACATTCTTCAGCTGC (2490) MB323 (2703) ATTCATGAGGACGGTCTTGG (2684)	57°C, 30 cycles	233
MUC5AC (AJ001403)	MB295 (8798) CCAAGGAGAACCTCCCATAT (8817) MB296 (9083) CCAAGCGTCATTCCTGAG (9066)	57°C, 30 cycles	286
MUC6 (U97698)	MB328 (1006) CAGCAGGAGGAGATCAGTTCAAG (1029) MB329 (1329) GTGGGTGTTTCTCTGTCTGTCATC (1306)	57°C, 30 cycles	324
β-Actin (BC013380)	MB344 (871) TTCCTGGGCATGGAGTCCT (889) MB345 (1074) AGGAGGAGCAATGATCTTGATC (1053)	57°C, 30 cycles	204

Table 1. Oligodeoxynucleotides used for RT-PCR analysis and calculated size of the products.

on a glass slide; this oriented the sensitive sample between the membrane and the glass slide and protected it from degradation and contamination. Microdissection was performed with the SmartCut Microdissection System (Olympus Deutschland GmbH, Hamburg, Germany) using special isolation tubes with an adhesive material inside the cap (Art.No. 50202; Molecular Machines & Industries). The cap was placed onto the membrane above the region of interest and this area was cut out with the laser. Then, the cap was lifted and the microdissected cells adhered to the cap of the tube. The samples were lysed in the inverted tube with lysis buffer at room temperature using the Absolutely RNA Nanoprep Kit (Stratagene, Amsterdam, The Netherlands). Then, the tubes were spun gently and RNA was isolated according to the manufacturer's instructions.

Parallel sections were used as "navigation slides" after immunofluorescence staining with the Ki-67 antibody.

Antisera

The various anti-TFF and anti-MUC5AC antisera (anti-hTFF1-1, anti-hTFF2-1, anti-hMUC5AC-2) employed in this study were described previously [23-25]. An anti-GKN1 antiserum from sheep [3] was kindly provided by Dr. K. Oien (Glasgow). A monoclonal mouse antibody against human Ki-67 (clone MIB1, No. M7240) was purchased from DakoCytomation (Hamburg, Germany).

For the generation of a GKN2-specific polyclonal antiserum, two antigenic peptides, CSSTTIFDYKHGYIASRVLSRRA (58-80) and CKHIPLYKGEVVENTHNVGA (143-162) were selected from the protein sequence using the laser gene

software (DNASTAR, Madison, WI, USA). They were synthesized as a single peptide and one as a multiple peptide antigen (MAP) peptide (CKHIPLYKGEVVENTHNVGA)₈-(Lys)₄-(Lys)₂-Lys-Gly-OH using standard Fmoc/tBu chemistry on a multiple peptide synthesizer Syro II (MultiSynTech, Witten, Germany). The single peptides were purified using RP-HPLC and their identity was confirmed using MALDI-MS. Peptide purities were determined via analytical RP-HPLC and proved to be higher than 90%.

Each of the single peptides was specifically coupled to keyhole limpet haemocyanin (KLH) using the maleimide-method via the N-terminal cysteine. The MAP conjugate was purified using a PD-10 gel filtration column in PBS (pH 7.2). A rabbit was immunized (Charles River Laboratories, Sulzfeld, Germany) with a 1:1:1 mixture of the two KLH-peptide conjugates and the MAP conjugate. The corresponding antiserum was termed anti-hGKN2-1. Affinity purification was performed as described previously [26].

Immunofluorescence

OCT-embedded human specimens stored at -80°C were cut into 7 µm sections with a cryomicrotome (Leica JUNG CM3000). The sections were transferred to regular glass slides and fixed in 70% ethanol for 2 min. For immunofluorescence, the fixed sections were equilibrated in phosphate-buffered saline (PBS), blocked with 1% bovine serum albumin (BSA) at room temperature for 20 min, incubated with the monoclonal Ki-67 antibody (1:100 dilution, clone MIB1; DakoCytomation) in 0.5% BSA at room temperature for 3 h and then washed with

Fig. 1. RT-PCR analysis. GKN1, GKN2, TFF1 or TFF3 expression was monitored in surgical specimens from the esophagus (E), the gastric cardia (Ca), the gastric corpus (C), the gastric antrum (A), the pyloric region (P), the proximal or the distal duodenum (D_{pr}, D). Each set of specimens was investigated from three different individuals. Specimens from the *H. pylori*-positive individual are marked (*). The integrity of the cDNAs was tested by monitoring the β -actin transcripts.

PBS. The secondary antibody, Cy3-labeled sheep anti-mouse IgG (H+L) (1:100 dilution; dianova, Hamburg, Germany), was incubated at room temperature for 30 min. DNA within the nuclei was stained with 4',6-diamidino-2-phenolindole (DAPI; Sigma-Aldrich Chemie, Taufkirchen, Germany). After being washed with PBS and water, the sections were covered with fluorescent mounting medium (DakoCytomation).

Photographs were taken on an Axiophot microscope (Carl Zeiss Jena, Jena, Germany) similar as described previously [23].

Gel filtration of human antral extracts and Western blot analysis

Human gastric tissue (0.8-1.5 g tissue stored at -80°C) was cut into small pieces and homogenized by grinding with a pestil under liquid nitrogen. The fine powder was dissolved in a 4-fold amount (w/v) of homogenization buffer (144 mM NaCl, 1 mM MOPS, 1 mM HEPES, pH 7.4) including a protease inhibitor mix (Complete, EDTA-free; Roche, Mannheim, Germany). After centrifugation (21,000 $\times$ g, 10 min, 4°C) the supernatant was extracted with an equal amount of chloroform and centrifuged again under the same conditions. The aqueous phase was analyzed by SDS-PAGE and Western blot and then applied on a gel filtration column or stored at -20°C .

The extract was fractionated by chromatography on a Sephadex G-200 (Fluka, Taufkirchen, Germany) column (27 $\times$ 1.5 cm) at 4°C using 20 mM Tris/HCl pH 8.0, 30 mM NaCl, 0.5 mM benzamidine hydrochloride hydrate (Sigma-Aldrich Chemie), 0.1 mM Pefabloc SC and 1 $\mu\text{g}/\text{ml}$ Leupeptin (both from biomol, Hamburg, Germany) as elution buffer. The elution profile was monitored by constantly measuring the absorption at 280 nm.

Western blot analysis was performed as reported previously [23, 27]. In brief, portions (37.5 μl) of each fraction (1.35 ml) were treated with 4x electrophoresis sample buffer, separated by SDS-PAGE (15% gel for TFFs and GKN2, 7.5% gel for MUC5AC and PAS staining). Proteins were transferred to nitrocellulose membranes (PROTRAN; Whatman, Dassel, Germany), fixed with 0.1% glutaraldehyde in PBS and incubated with affinity-purified antibodies to TFF1, TFF2, GKN2, and MUC5AC, respectively. Bands were visualized with the ECL detection system (GE Healthcare, Freiburg, Germany) and recorded with GeneGnome (Syngene, Cambridge, UK). Semi-quantitative analysis of the bands was performed with the Gene Tools Gel analysis software (Syngene, Cambridge, UK). PAS staining was performed according to a previous report [28].

Fig. 2. RT-PCR analysis. GKN1, GKN2, TFF1 or TFF3 expression was monitored in endoscopic specimens from the Z-line (Z), the gastric cardia (Z-0.5), the gastric fundus (Z-4) or the gastric corpus (C). Each region was analyzed from the same three individuals. Specimens from the *H. pylori*-positive individual are marked (*). The integrity of the cDNAs was tested by monitoring the β -actin transcripts.

Results

RT-PCR analysis of the esophageal, gastric and duodenal mucosa

Surgical specimens from the esophagus (regions 1-4 according to [23]), gastric cardia (regions 5-8 according to [23]), corpus (regions 9-12 according to [23]), antrum (regions 15-18 according to [23]), pylorus (regions 19-22 according to [23]), proximal (regions 23-26 according to [23]) and distal duodenum were investigated for their GKN1, GKN2 mRNA content (Fig. 1). For comparison, the TFF1 and TFF3 mRNA content was measured. All gastric samples lacked MUC2 expression (data not shown), confirming the results of the histopathological review, i.e., the absence of intestinal

Fig. 3. RT-PCR analysis after laser microdissection. Cells were isolated from three different regions along the gastric antral gland axis. Left panel: immunofluorescence of „navigation slide“ with monoclonal anti-Ki-67 antibody and Cy3 label (red). Nuclei are counterstained with DAPI (blue). Region I contains superficial surface mucous cells, region II the Ki-67 positive cells and region III the antral gland cells. Right panel: RT-PCR analysis. GKN1, GKN2, TFF1, TFF2, TFF3, MUC5AC or MUC6 expression was monitored. Each region was analyzed from the same three individuals (+ specimens from an individual positive for *H. pylori*). The integrity of the cDNAs was tested by monitoring the β -actin transcripts.

Fig. 4. Gel filtration of human antral extract and Western blot analysis. (A) Total elution profile after separation of antral extract on Sephadex G-200 as determined by absorbance at 280 nm. The hatched area represents the presence of mucins as determined by PAS staining of each fraction or by Western blot analysis concerning MUC5AC (both after separation on 7.5% SDS-PAGE under reducing conditions). (B) Distribution of GKN2 (red), TFF1 (green) and TFF2 (blue) as determined by Western blot analysis (separation on 15% SDS-PAGE under reducing conditions) and semi-quantitative analysis of the band intensities.

metaplasia. Generally, both GKN1 and GKN2 transcripts were restricted to the stomach. A similar profile was observed for TFF1 with additional low level expression also in the proximal duodenum. The expression levels were diminished in the corpus when compared with the cardia and antrum. In contrast, TFF3 transcripts were abundant in all duodenal samples and also detectable in the esophagus and the border regions of the stomach. However, the corpus was nearly devoid of TFF3 transcripts.

Furthermore, biopsy specimens of four defined regions including the Z-line (samples Z), the cardia

Fig. 5. SDS-PAGE (15%) and subsequent Western blot analysis. 20 μ g protein of fraction no. 29 after gel filtration of human antral extract (peak of TFF1 and GKN2 content, see Fig. 4) was analyzed under reducing (R) or non-reducing (NR) conditions for TFF1 and GKN2, respectively. The molecular weight standard is indicated *left*.

Fig. 6. SDS-PAGE (15%) and subsequent Western blot analysis. 5 μ l of human gastric juice from three patients each (indicated 1, 2, 3) were analyzed under non-reducing conditions for their TFF1 and GKN2 contents, respectively. The molecular weight standard is indicated *left*.

(samples Z-0.5, Z-4) and the gastric corpus (samples C) were analyzed (Fig. 2). Clearly, all samples contained GKN1, GKN2, and TFF1 transcripts. In contrast, TFF3 transcripts were only detectable in gastric specimens from the Z-line and the cardia (Z-0.5).

Laser microdissection and RT-PCR analysis of antral glands

Antral glands consist of different cell types, i.e., of SMCs at the gastric foveola, a population of dividing precursor cells and stem cells (characterized by the Ki-67 antigen) at the isthmus, and the deeper laying antral gland cells [29]. In the course of this study, cells were isolated from each of the three regions (termed regions I, II, and III) with the help of LM and analyzed for their GKN1, GKN2, TFF1, TFF2, TFF3, MUC5AC, and MUC6 mRNA content. The expression pattern of these genes was very different. In Fig. 3, the genes analyzed were arranged according to their distinct expression profiles in regions I-III. GKN1, GKN2, TFF1, and MUC5AC transcripts were mainly located in region I, and, to lesser extent, in region II. In contrast, TFF3 transcripts accumulated in region II and were also slightly detectable in region I; whereas TFF2 and MUC6 transcripts were heavily enriched in region III and a weak TFF2 signal was also visible in region II and sometimes also in region I.

Gel filtration of antral extracts

In the past, binding of TFF1 and mucins has been reported [16-18]. Also an interaction of TFF2 with mucins was observed [30, 31]. Thus, in the course of the study presented here human antral extracts were separated via gel filtration under non-reducing conditions. The extracts consisted of a high molecular mucin fraction (hatched in Fig. 4A) followed by two peaks of low molecular weight material (Fig. 4A). The fractions were analyzed via Western blots under reducing conditions for their GKN2, TFF1, TFF2 or mucin contents (Fig. 4B). GKN2 and TFF1 showed similar elution profiles and both were clearly not associated with mucins. Only a very small proportion ($<0.1\%$) of GKN2 and TFF1 appeared together with the mucin fraction (data not illustrated). In contrast, TFF2 was exclusively associated with the mucin fraction.

The similar elution profiles of TFF1 and GKN2 would be in perfect agreement with the formation of TFF1-GKN2 heterodimers [6]. Thus, Western blot analysis of the peak fraction was performed under non-reducing or reducing conditions, respectively (Fig. 5). Clearly, both the anti-GKN2 as well as the anti-TFF1 antiserum detected the TFF1-GKN2 heterodimer under non-reducing conditions (calculated M_r : 24990); whereas under reducing conditions the TFF1 (calculated M_r : 6674) or the GKN2 (calculated M_r : 18318) monomer was detected, respectively. This also demonstrated that the antiserum

anti-hGKN2-1 is suitable of detecting both GKN2 as well as the TFF1-GKN2 heterodimer.

In contrast, Western blot analysis with the anti-GKN1 antiserum [3] detected an 18 k band under reducing as well as under non-reducing conditions indicating that GKN1 is, as expected, not capable of forming (hetero)dimers (data not shown).

Western blot analysis of gastric juice

Because both TFF1 as well as GKN2 are typical secretory proteins, it was tested whether the TFF1-GKN2 heterodimer is present also in gastric juice (Fig. 6). Both antisera detected the TFF1-GKN2 heterodimer in the gastric juice of three different patients.

Discussion

Molecular anatomy and continuous regeneration of the human antral gland

The antral mucosa is known for its continuous bi-directional renewal and the principles were described mainly in the mouse so far. Antral units consist of the pit (or foveola), the isthmus and the gland [29]. In the isthmus, stem cells give rise to progenitor cells which migrate outwards or inwards and differentiate into SMCs and antral gland cells, respectively [32-35]. Of note, the human antral mucosa has a much higher turnover rate than the body mucosa [36, 37]. Gastric epithelial progenitor cells were defined in the human oxyntic mucosa [38] and in the mouse gastric epithelial progenitor cells were characterized even on a molecular level using laser capture microdissection [39]. So far, characterization of the human antral mucosa is scarce. In the past, we could show by immunohistochemistry of human stomach samples that regeneration of SMCs in the corpus is different from the antrum and the cardia [23, 24]. In the latter, maturation of superficial SMCs occurs stepwise via trans-differentiation of TFF3 expressing progenitor cells. In the course of the studies presented here, the spatial expression of genes encoding typical secretory proteins such as GKN2 was determined along the antral gland axis using LM and RT-PCR analysis. From the spatial expression pattern obtained, a regeneration profile was deduced.

LM clearly revealed the differential expression patterns of the various secretory proteins investigated, i.e., GKN1, GKN2, TFFs, MUC5AC, and MUC6 (Fig. 3). GKN2 and TFF1 are co-expressed mainly at the SMCs at the foveolae. This is completely in line with results from localization studies using immunohistochem-

istry [8, 15, 16, 23, 40] and agrees with the report that TFF1 and GKN2 form heterodimers [6]. A similar expression profile was observed for GKN1 and MUC5AC which also confirms previous immunohistochemical studies [3, 41].

In contrast, TFF3 transcripts accumulate in the deeper laying region containing the progenitor and stem cells. This is in complete agreement with our previous report using immunohistochemistry [23] and confirms the assumption that antral SMCs differentiate from their progenitor cells in a stepwise manner, i.e., first TFF3-secreting SMCs emerge and only later on TFF1-secreting SMCs develop after migration to the pit [23, 24]. Thus, foveolar SMCs originate from trans-differentiation of deeper laying SMCs. The accumulation of TFF3 expression in the region of dividing progenitor cells might be an indication that TFF3 influences regeneration and differentiation of these cells.

The observation that TFF2 and MUC6 were co-expressed in the antral gland cells fits well with previous reports on peptide level [31, 40-42] and is the result of a downward migration of differentiating cells toward the bottom of the gland. Of note, TFF2 expression seems to start somewhat earlier than MUC6 expression because it can be detected also in the region of dividing progenitor cells.

Taken together, the expression profile of the GKNs, TFFs and MUCs investigated here is in agreement with the general model of a bi-directional regeneration of antral glands [35]. However, our results confirm our previous modification of this model by now presenting the first data on mRNA level. In this model a trans-differentiation step during maturation of foveolar SMCs was introduced [23, 24]. Of note, this trans-differentiation is also observed in the gastric cardia and is missing in the gastric corpus [23, 24].

The TFF1-GKN2 heterodimer and TFF2 differ in their interaction with mucins

TFF1 and GKN2 were co-expressed in the same cells (foveolar SMCs, Fig. 3), TFF1 and GKN2 appeared in the same fractions after gel filtration (Fig. 4), and TFF1 and GKN2 formed disulfide-linked heterodimers (Fig. 5). Surprisingly, the majority of these TFF1-GKN2 heterodimers (>99.9%) was not associated with the mucin fraction (Fig. 4). Only a very small proportion (<0.1%) of the TFF1-GKN2 heterodimer was co-migrating with the mucin peak (data not shown).

This seems contradictory to previous results reporting on interactions of TFF1 and mucins [16-18]. How-

ever, the TFF1 binding described by Tomasetto et al. [18] was based on a yeast 2-hybrid system, where GKN2 was missing. Furthermore, Newton et al. [16] showed that only TFF1/dimer and TFF1/monomer, but not the TFF1-GKN2 heterodimer which represents the vast majority of TFF1, is associated with mucins after caesium chloride density gradient centrifugation. Finally, the statement concerning TFF1-MUC5AC binding by Ruchaud-Sparagano et al. [17] was based upon gel filtration and subsequent analysis of the fractions by ELISA. Of note, they even showed in this publication that the results obtained with ELISA and by regular Western blotting do not coincide and the results obtained with Western blotting are comparable to the results presented here, i.e., the majority of TFF1 present as TFF1-GKN2 heterodimer was not associated with MUC5AC. Thus, the previous reports [16, 17] are not in contrast to our observations. However, this does not exclude binding of the TFF1-GKN2 heterodimer and mucins *in vivo*. Association of TFF1 with the gastric mucus has been shown repeatedly [16, 23, 40] and our results only indicate that interaction of TFF1-GKN2 heterodimer with mucins (particularly MUC5AC) is rather weak and is not stable under the conditions used here. This weak interaction is also reflected by the presence of TFF1 [15] and of the TFF1-GKN2 heterodimer (Fig. 6) in the gastric juice.

The TFF1 and GKN2 peaks were not completely congruent showing a TFF1 shoulder at higher fraction numbers (Fig. 4). This was due to small amounts of TFF1 not forming a TFF1-GKN2 heterodimer, e.g., either forming a TFF1/homodimer or a TFF1/monomer (data not illustrated). This is in agreement with previous reports where the occurrence of similar molecular forms of TFF1 was described [16, 17, 43].

In contrast to the TFF1-GKN2 heterodimer, TFF2 was exclusively associated with the mucin peak. Thus, GKN2 and TFF2 do not bind to each other under the conditions used here. This is not in line with a previous report on interactions of gastric TFF2 and a mouse GKN2 fusion protein [8]. However, the specificity of this binding was not shown and duodenal TFF2 also did not bind

the GKN2 fusion protein. On the other hand, the association of TFF2 with gastric mucins, particularly MUC5AC (Fig. 4), is perfectly in line with previous reports [30, 31].

Taken together, we show here for the first time that TFF1, TFF2 as well as the TFF1-GKN2 heterodimers behave differently concerning their binding to gastric mucins. TFF2 is strongly associated with mucins; whereas TFF1 and TFF1-GKN2 heterodimer do not interact with mucins under the conditions described here. These fundamental differences between TFF1 and TFF2 concerning mucin binding are in perfect agreement with reports on dramatic changes of the viscoelastic properties of mucus by TFF2, but not by TFF1 or TFF3 [30, 44, 45]. Thus, the single domain TFF peptides TFF1 and TFF3 obviously differ characteristically in their binding characteristics from TFF2, which contains two TFF domains arranged in tandem. Consequently, TFF2 might fulfil its major physiological role as a “link peptide” in the gastric mucus; whereas TFF1 and TFF3 mainly act as “luminal surveillance peptides” [46] promoting gastric restitution [47] by modulating particularly cell migration patterns [48] as well as differentiation processes [21, 49, 50]. However, the function of GKN2 is still elusive but, by analogy to GKN1 [4], TFF1-GKN2 heterodimers would be perfectly designed to modulate cell migration processes.

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