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ARTICLE *in* JOURNAL OF PROTEOME RESEARCH · JUNE 2010

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Human Intestinal TFF3 Forms Disulfide-Linked Heteromers with the Mucus-Associated FCGBP Protein and Is Released by Hydrogen Sulfide

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Received January 11, 2010

TFF3 is a secretory peptide belonging to the trefoil factor family with a predicted size of 59 amino acid residues containing seven cysteine residues. It is predominantly expressed in intestinal goblet cells where it plays a key role in mucosal regeneration and repair processes. In the course of these studies, human colonic TFF3 was shown to exist mainly as a high molecular weight heteromer. Purification of this heteromer and characterization by LC−ESI−MS/MS analysis identified the IgG Fc binding protein (FCGBP) as the disulfide-linked partner protein of TFF3. FCGBP is a constituent of intestinal mucus secreted by goblet cells. Furthermore, low amounts of TFF3/monomer and only little TFF3/dimer were detected in human colonic extracts. Here, we show that these TFF3 forms can be released from the purified TFF3-FCGBP heteromer complex *in vitro* by reduction with hydrogen sulfide (H_2S). Such a mechanism would be in line with the high H_2S concentrations reported to occur in the lumen of the colon. Of special note, this points to intestinal mucus as a reservoir for a biologically active peptide. Also proteolytic processing of FCGBP was observed which is in line with multiple autocatalytic cleavages as proposed earlier by Johansson et al. (*J. Proteome Res.* 2009, *8*, 3549−3557).

Keywords: TFF3 • trefoil factor • IgGFc binding protein • mucin • hydrogen sulfide • restitution • mucosal repair • mucus • MUC2 • CLCA1

Introduction

TFF3, previously called "intestinal trefoil factor",[1] is a prominent member of the trefoil factor family (TFF) (reviewed in refs 2−5). Human TFF3 (formerly also termed hP1.B) is a cysteine-rich secretory peptide with a predicted size of 59 amino acid residues.[6] Besides its major expression site in intestinal goblet cells,[6,7] TFF3 is synthesized also in other human organs such as the salivary glands,[8] the esophagus,[9] the gastric antrum and cardia,[9,10] the endocrine pancreas,[11] the Vater's ampulla,[12] the respiratory tract,[13] the uterus,[14] the vagina,[15] the urinary tract,[16] the conjunctiva,[17] the efferent tear ducts,[18] and the hypothalamus.[19] Generally, TFF3, like the other TFF peptides, is predominantly expressed by various mucin secreting cells (reviewed in ref 4) and it is, together with the mucin MUC2, also a constituent of the intestinal mucus layer.[7] However, TFF3 and MUC2 expression are not coordinately regulated in intestinal goblet cells.[20] Pathological expression of TFF3 occurs during inflammatory processes of mucous epithelia, particularly in the ulcer-associated cell lineage,[6] as well as in cartilage during osteoarthritis.[21] TFF3 expression is also subject to changes during multistep colon carcinogenesis.[22]

TFF3 is implicated in multiple mucosal protection and repair processes where it plays a key role for the integrity of mucous epithelia (reviewed in refs 2, 5, 23). For example, TFF3 is known to promote rapid repair by cell migration, a process called "restitution" (reviewed in ref 24). TFF3 stimulates cell migration principally by chemotaxis.[25] The motogenic effect has been demonstrated in various *in vitro* wound healing models (reviewed in ref 4). Furthermore, protective or healing effects have also been demonstrated in various *in vivo* studies (reviewed in ref 4). It has clearly been documented that only luminal application of recombinant TFF3 is protective in colitis models but not systemic delivery.[26] Also ectopic expression of TFF3 in the jejunum stabilized the mucosa against noxious agents.[27] In contrast, TFF3-deficient mice show a decreased resistance to colonic injury.[28] Of special note, TFF3 has been reported to have both pro- as well as antiapoptotic effects.[21,29]

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10.1021/pr100020c

Published on Web 04/28/2010

TFF3 also enhanced the tumor necrosis factor-α (TNF-α) induced interleukin-6 (IL-6) and IL-8 secretion.[30] Thus far, specific TFF3 receptors have not been characterized. However, chemokine receptors could be promising candidates.[31]

TFF3 contains seven cysteine residues. On the basis of investigations of recombinant TFF3, the formation of disulfide-linked TFF3/homodimers via cysteine-57 has been observed.[32−35] Also, the 3D-structures of recombinant TFF3/monomer and TFF3/dimer were analyzed.[36,37] Interestingly, only the TFF3/dimer has been reported to be protective in experimental colitis when applied luminally, whereas TFF3/monomer had no protective effect.[26] In contrast, when administered systemically, both the TFF3/monomer and the TFF3/dimer aggravated the colitis score with a particular pronounced effect observed for the TFF3/monomer.[26] However, both the TFF3/monomer and the TFF3/dimer showed motogenic effects *in vitro*.[32,38] In contrast, protection against apoptosis *in vitro* has been reported to require TFF3/dimer.[32]

There are no reports concerning purification and detailed proteomic analysis of TFF3 isolated from tissue extracts thus far. All protein data reported are based upon predicted cDNA sequences and anti-TFF3 antibodies against recombinant or synthetic peptides mainly using reducing SDS-PAGE. Only recombinant TFF3 produced in yeast has been characterized in detail by proteomic analysis.[34] However, there is a report on the possible existence of mixed TFF1-TFF3 heterodimers as well as TFF3/monomers in small intestinal specimens from a patient with Crohn's disease.[33] This is remarkable because gastric TFF1 has later been demonstrated to form disulfide-linked heterodimers with gastrokine-2/TFIZ1.[39] Thus, the aim of this study was to characterize TFF3 isolated from human colonic extracts and to clarify how TFF3 associates with the mucus.

Experimental Procedure

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 of the University of Magdeburg. Human colonic tissue (sigmoid part) was investigated from 4 patients undergoing colectomy. Samples were included in the study only when the formal histopathological review of the specimens excluded neoplastic changes.

Extraction of Proteins from Human Intestinal Tissue. Human colonic specimens lacking the muscularis mucosae (0.8−1.5 g tissue stored at −80 °C) were cut into small pieces and homogenized by grinding with a pestle under liquid nitrogen. The fine powder was dissolved in a 4-fold amount (w/v) of homogenization buffer (30 mM NaCl, 20 mM Tris/HCl pH 8.0) including a protease inhibitor mix (Complete, EDTA-free; Roche, Mannheim, Germany). After centrifugation (16 000× *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 (extract) was subject of Western blot analysis and then applied on a gel filtration column or stored at −20 °C.

Protein Purification by Gel Filtration. The colonic extract (2 mL) was fractioned by chromatography on a Sephadex G-200 (Fluka, Taufkirchen, Germany) column (270 × 15 mm) as described previously.[40] The flow rate was 0.15 mL/min. The elution profile was monitored by constantly measuring the absorbance at 280 nm. Sixty fractions were collected (1.35 mL each).

Protein Purification by FPLC. Selected fractions obtained after gel filtration were purified in a second step via the ÄKTA-FPLC system (Amersham Biosciences, Freiburg, Germany) using a Resource Q6 column (Amersham Biosciences) and eluted with a salt gradient from 20 mM Tris/HCl pH 8.0 to 20 mM Tris/HCl pH 8.0 + 1 M NaCl. The flow rate was 6 mL/min. The elution profile was monitored by constantly measuring the absorbance at 280 nm. 70 fractions were collected (1.0 mL each).

SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE), SDS-Agarose Gel Electrophoresis (SDS-AgGE), and Western Blot Analysis. Polyacrylamide gels were used under reducing or nonreducing conditions according to Laemmli with a 7.5% or a 15% separation gel and a 6% stacking gel, respectively. Protein staining was with Bio-Safe Coomassie Stain (Bio-Rad Laboratories GmbH, Munich, Germany). Silver staining was according to an established protocol.[41] As molecular weight markers, peqGOLD Protein-Marker I (Peqlab Biotechnologie GmbH, Erlangen, Germany) or All Blue (Bio-Rad Laboratories, Munich, Germany) was used.

Separation of high molecular weight proteins under nonreducing conditions was also accomplished by electrophoresis in horizontal 1.0% (w/v) agarose gels (20.5 × 20.5 cm) prepared in 40 mM Tris/acetic acid pH 8.0, 1 mM EDTA, 0.1% SDS (w/v) as previously described.[42] All Blue (Bio-Rad Laboratories) was used as a molecular weight marker.

Western blot analysis after SDS-PAGE, electrophoretic transfer, and fixation with 0.2% glutaraldehyde has been described previously.[40,43] When Western blot analysis after SDS-AgGE was performed, proteins were transferred to nitrocellulose membranes (PROTRAN; Whatman, Dassel, Germany) by capillary blotting with 0.6 M NaCl, 0.06 M trisodium citrate. Bands were visualized with the ECL detection system and semiquantitative analysis of the bands with the Gene Tools Gel analysis software (Syngene, Cambridge, UK) was performed as described in detail.[40] Periodic acid-Schiff (PAS) staining of mucins was performed according to a previous report.[44]

Antisera, Recombinant TFF3. An affinity-purified polyclonal antiserum (anti-hTFF3−3) was generated against the peptide FKPLQEAECTF representing the C-terminus of human TFF3[6] coupled to keyhole limpet hemocyanin (KLH) with glutaraldehyde as described previously.[13] Furthermore, a commercial polyclonal antihuman FCGBP antiserum (HPA003564, Sigma-Aldrich Chemie, Taufkirchen, Germany) against amino acids 289−417 of the FCGBP sequence was used. The secondary antibody was a horseradish peroxidase labeled antirabbit IgG(H+L) (PI-1000, Vector Laboratories, Burlingame, U.S.A.). Recombinant human TFF3/dimer produced in *Saccharomyces cerevisiae*[34] was kindly provided by Dr. L. Thim (Novo Nordisk A/S, Maaloev, Denmark).

LC-ESI-MS/MS Analysis of In-Gel Digested Proteins. Coomassie stained protein bands were excised from the gel, chopped into cubes and washed three times with acetonitrile (ACN)-water (1:1). The gel pieces were shrunk with pure ACN, rehydrated in 50 mM NH_4HCO_3 and dried in a speedvac. 10 mM dithiothreitol in 50 mM NH_4HCO_3 were added to the dried gel pieces and proteins were reduced for 45 min at 56 °C. In order to alkylate reduced cysteine residues, the remaining liquid was removed and an equal volume of 50 mM iodoacetamide in 50 mM NH_4HCO_3 was added and the reaction was allowed to proceed for 30 min in the dark. The gel pieces were washed and dried as above prior to in gel digestion. The gel pieces were rehydrated in an ice cold solution of 10 ng/μL trypsin (se-

quencing grade, Promega GmbH, Mannheim, Germany) in 10 mM NH_4HCO_3. After 45 min on ice, excessive trypsin solution was replaced by 20 μL of buffer without enzyme and proteins were digested at 37 °C overnight. The digest was stopped by the addition of 20 μL of 10% formic acid (FA) and peptides were extracted for 30 min at 37 °C.

Liquid chromatography (LC)-MS data were acquired on a HCT ETD II iontrap mass spectrometer (Bruker Daltoniks, Bremen, Germany) equipped with a nano ESI source (Bruker Daltonics, Bremen, Germany). Samples were introduced by an EASY nano-LC system (Proxeon, Odense, Dennmark) using a vented column setup comprising a 0.1 × 20 mm trapping column and a 0.075 × 100 mm analytical column, both self-packed with ReproSil-Pur C18-AQ, 5 μm (Dr. Maisch, Ammerbuch, Germany). Five to 18 μL of the samples were aspirated into the sample loop and a total volume of 25 μL was loaded onto the trap column using a flow rate of 6 μL/min. Loading pump buffer was 0.1% FA. Peptides were eluted with a gradient of 0% to 35% ACN in 0.1% FA over 20 min and a column flow rate of 300 nL/min. Subsequently the ACN content was raised to 100% over 2 min and the column was regenerated in 100% ACN for additional 8 min.

Data-dependent acquisition of MS and tandem MS (MS/MS) spectra was controlled by the Compass 3.0 software. MS1 scans were acquired in standard enhanced mode. Five single scans in the mass range from m/z 400 to m/z 1400 were combined for one survey scan. Up to three doubly and triply charged ions rising above a given threshold were selected for MS/MS experiments. Ultrascan mode was used for the acquisition of MS2 scans in the mass range from m/z 100 to m/z 1600 and three single scans were added up. The ion charge control value was set to 250 000 for all scan types. Peaklists in Mascot generic format (mgf) were generated from the raw data by using the Data Analysis software module (Bruker Daltoniks, Bremen, Germany).

Database Searching. Proteins were identified by searching the SwissProt 56.9 database using a local installation of MASCOT 2.2 (Matrix Science Ltd., London, U.K.). Searches were submitted via Proteinscape 2.0 (Bruker Daltoniks, Bremen, Germany) with the following parameter settings: enzyme "semi-trypsin", species "human", fixed modifications "carbamidomethyl", optional modifications "Methionine oxidation" and missed cleavages "1". The mass tolerance was set to 0.4 Da for peptide and fragment spectra.

Treatment of the TFF3-FCGBP Heteromer with Hydrogen Sulfide (H_2S). FPLC-purified TFF3-FCGBP heteromer (2.0 mL) was treated via a capillary with freshly generated H_2S for 10 min at room temperature. Then, the reaction mixture was incubated for 10, 30, 60, and 120 min at 37 °C. The reaction was stopped by freezing in liquid nitrogen and the samples were stored at −80 °C until analysis by nonreducing SDS-PAGE was performed (without prior boiling).

Results

Purification of Human Intestinal TFF3 Immunoreactive Proteins. Extracts from human colonic specimens (sigmoid part) were subject to gel filtration on Sephadex G-200 column under nonreducing conditions. The extracts consisted of a high molecular fraction containing also mucins followed by two peaks of low molecular weight material (Figure 1A). The fractions were semiquantitatively analyzed for their TFF3 content by SDS-PAGE under reducing conditions followed by Western blots (Figure 1B). The major amount of TFF3 immu-

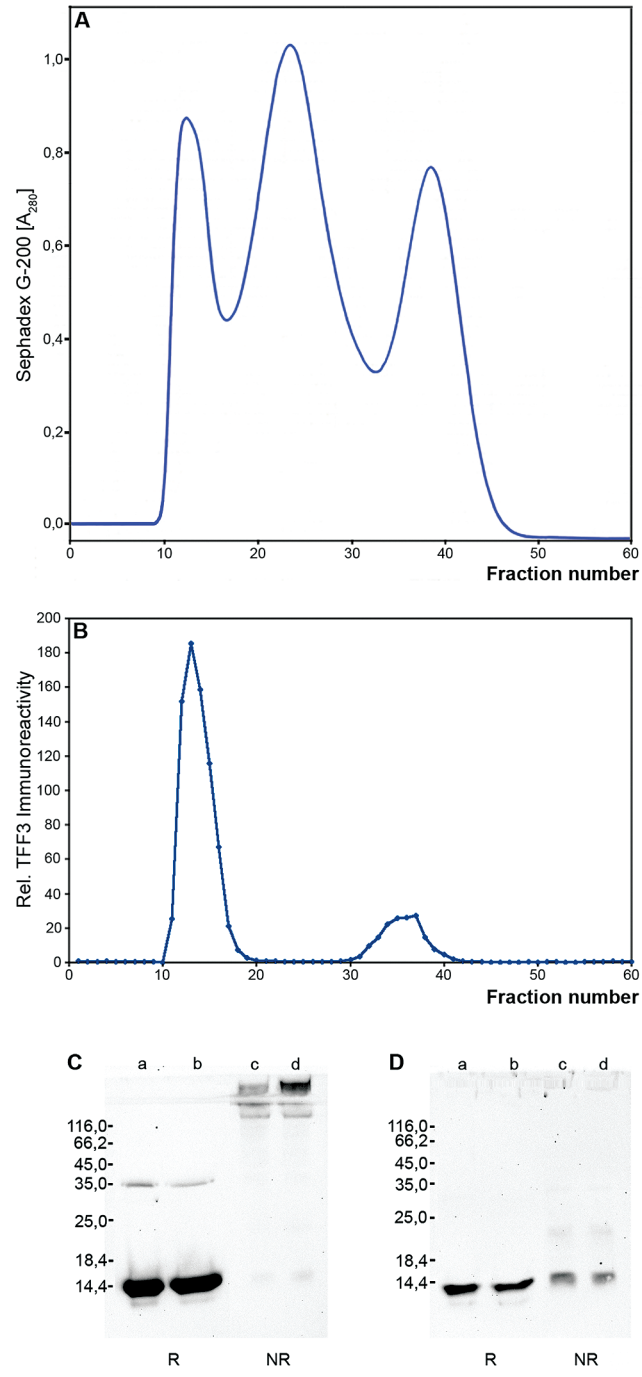

Figure 1. Gel filtration of human colonic extract and Western blot analysis. (A) Total elution profile after separation of colonic extract on Sephadex G-200 as determined by absorbance at 280 nm. (B) Distribution of the TFF3 immunoreactivity (affinity-purified anti-hTFF3−3 antiserum) as determined by Western blot analysis of the Sepadex G-200 fractions (separation on 15% SDS-PAGE under reducing conditions) and semiquantitative analysis of the band intensities. (C) 15% SDS-PAGE and subsequent Western blot analysis of the high molecular weight fractions No. 13 (lanes a and c) and 14 (lanes b and d) under reducing (R) or nonreducing (NR) conditions, respectively, using the affinity-purified anti-hTFF3−3 antiserum. The molecular size standard is indicated on the left. (D) 15% SDS-PAGE and subsequent Western blot analysis of the low weight fractions No. 35 (lanes a and c) and 36 (lanes b and d) under reducing (R) or nonreducing (NR) conditions, respectively, using the affinity-purified anti-hTFF3−3 antiserum. The molecular size standard is indicated on the left.

noreactive material was associated with the high molecular weight fraction (fractions 11−16) and only a minor part appeared as low molecular weight material (fractions 31−37). SDS-PAGE under nonreducing conditions and subsequent Western blot analysis of the high molecular weight fraction revealed that the TFF3 immunoreactive material has an unusually large M_r of much more than 100 000 (Figure 1C). After reduction, TFF3/monomer could be released (Figure 1C). Thus, TFF3 mainly forms a disulfide-linked heteromer with a high molecular weight partner protein. In contrast, the TFF3 immunoreactive material in the low molecular weight fraction mainly represents TFF3/monomer and only very little TFF3/dimer (Figure 1D).

The major aim of this study was to purify and identify the high molecular weight partner protein of TFF3. Thus, the next step was separation of the high molecular weight fraction by FPLC using a Resource Q column under nonreducing conditions. Figure 2A represents a typical elution profile obtained. Fractions containing the TFF3 heteromer were identified by SDS-PAGE under reducing conditions and subsequent semiquantitative Western blot analysis (Figure 2B). The TFF3 containing peak fractions obtained after FPLC were then separated under nonreducing conditions on a 7.5% SDS-PAGE. Two high molecular weight bands were observed after Coomassie blue staining (designated as B>500A and B>500B, respectively; Figure 2C) and then excised and subjected to proteomic analysis. Furthermore, the TFF3 containing peak fractions obtained after FPLC were also separated under reducing conditions on a 7.5% or a 15% SDS-PAGE in order the release TFF3 from its partner protein. Multiple bands were observed with a molecular weight range between 50 000 and 150 000 after 7.5% SDS-PAGE and Coomassie blue staining (Figure 2C). Predominant bands appeared with an estimated M_r of 123 000, 73 000, 61 000, 55 000, and 51 000, respectively. Furthermore, a band was detected with a M_r below 14 000 after separation by 15% SDS-PAGE (Figure 2D). These 6 protein bands (designated as B123, B73, B61, B55, B51, and B<14, respectively) released from the TFF3 heteromer by reduction were also excised from the gel and subjected to LC−ESI−MS/MS analysis.

Characterization of the TFF3 Heteromer by LC−ESI−MS/MS Analysis. The 2 bands excised from the nonreducing gel (i.e., B>500A and B>500B) as well as the 6 bands excised from the reducing gel (i.e., B123, B73, B61, B55, B51, and B<14) were subjected to in-gel tryptic digestion followed by LC−ESI−MS/MS for protein identification. Searches were performed against the Swiss-Prot 56.9 database. The major results are summarized in Table 1. A complete list of all proteins identified in the 8 bands including all peptide fragments is presented in Table S1 (Supporting Information).

The protein identified with the highest score in the 2 nonreduced samples (i.e., B>500A and B>500B) was IgG Fc binding protein (FCGBP) with a nominal mass of ~600 000[45] which is a secretory product of intestinal goblet cells as TFF3 is. This is the first indication that FCGBP could be the high molecular weight partner protein of TFF3. Furthermore, the high molecular mass mucin MUC2 presenting the predominant intestinal mucin secreted by goblet cells was found with relative low scores and a small number of matching peptides. Also α-2-macroglobulin (A2MG) was identified, a large plasma protein synthesized in the liver.

Analysis of the 6 bands isolated after reducing SDS-PAGE revealed that only the smallest protein band B<14 contained TFF3 sequences (score 137.13). In contrast, FCGBP was clearly identified with the highest scores in all other bands (i.e., B123, B73, B61, B55, and B51). This again points to FCGBP as the high molecular weight partner of TFF3. However, the bands

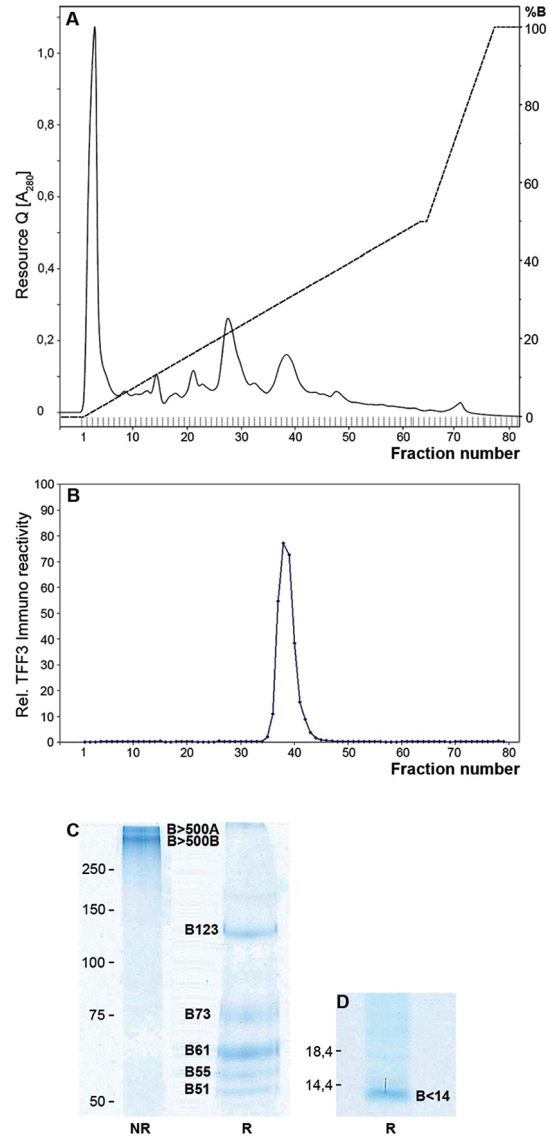

Figure 2. Purification of the TFF3 heteromer by FPLC and SDS-PAGE. (A) Total elution profile of the TFF3 heteromer (high molecular weight fraction as obtained by gel filtration on Sephadex G-200) after FPLC separation on a Resource Q6 column as determined by absorbance at 280 nm. Elution was with a salt gradient (dashed line) from 20 mM Tris/HCl pH 8.0 (buffer A) to 20 mM Tris/HCl pH 8.0 + 1 M NaCl (buffer B). The percentage of buffer B is shown on the right. (B) Distribution of the TFF3 immunoreactivity (affinity-purified anti-hTFF3−3 antiserum) as determined by Western blot analysis of the Resource Q6 fractions (separation on 15% SDS-PAGE under reducing conditions) and semiquantitative analysis of the band intensities. (C) Separation of the FPLC-purified TFF3 heteromer by 7.5% SDS-PAGE under nonreducing (NR) or reducing (R) conditions followed by Coomassie staining. Marked are the bands excised and subjected to LC−ESI−MS/MS analysis later on (B>500A, B>500B, B123, B73, B61, B55, and B51, respectively). The molecular size standard is indicated on the left. (D) Separation of the FPLC fractions containing the TFF3 heteromer by 15% SDS-PAGE under reducing (R) conditions followed by Coomassie staining. Marked is the band excised and subjected to peptide mass fingerprint analysis later on (B<14). The molecular size standard is indicated on the left.

Table 1. Proteins Identified by LC−ESI−MS/MS Analysis of In-Gel Digests[a]

gel band	proteins identified	Swiss-Prot accession	nom. mass (kDa)	score	peptides matched
B>500A	FCGBP	Q9Y6R7	571.64	3125.79	58
	A2MG	P01023	163.19	923.22	18
	MUC2	Q02817	539.96	497.78	13
B>500B	FCGBP	Q9Y6R7	571.64	3570.85	69
	A2MG	P01023	163.19	611.12	13
	MUC2	Q02817	539.96	115.58	3
B123	FCGBP	Q9Y6R7	571.64	1794.45	35
	K2C1	P04264	66.00	165.04	4
B73	FCGBP	Q9Y6R7	571.64	1103.64	21
	CLCA1	A8K714	100.11	309.54	8
B61	FCGBP	Q9Y6R7	571.64	1050.36	22
	IGHA1	P01876	37.63	351.70	7
B55	FCGBP	Q9Y6R7	571.64	1922.25	35
	K1C10	P13645	58.79	257.27	5
B51	FCGBP	Q9Y6R7	571.64	1551.10	30
	IGHG1	P01857	36.08	148.67	5
B<14	TFF3	Q07654	8.64	137.13	3

[a] Significance threshold $P \leq 0.05$.

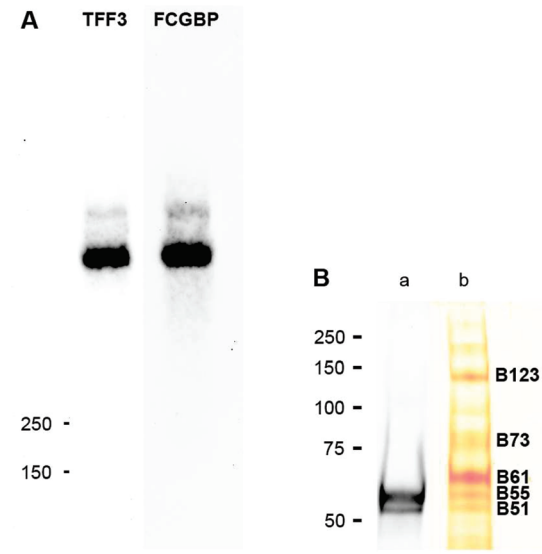

Figure 3. Characterization of the FPLC-purified TFF3 heteromer. (A) Separation of the TFF3-FCGBP heteromer by nonreducing 1% SDS-agarose gel electrophoresis and subsequent Western blot analysis using either the affinity-purified anti-hTFF3-3 antiserum or the commercial anti-FCGBP antiserum (HPA003564). The molecular size standard is shown on the left. (B) Lane a: Separation of the TFF3-FCGBP heteromer by 7.5% SDS-PAGE under reducing conditions and subsequent Western blot analysis using the commercial anti-FCGBP antiserum (HPA003564). Lane b: Silver staining of parallel lane. Marked are the bands previously characterized by peptide mass fingerprint analysis (B123, B73, B61, B55, and B51). The molecular size standard is shown on the left.

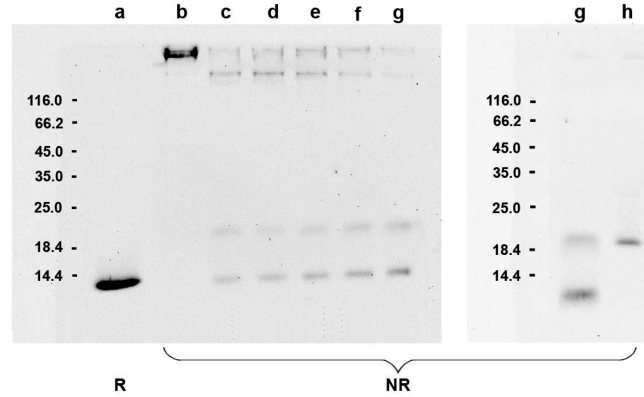

Figure 4. Reduction of the FPLC-purified TFF3-FCGBP heteromer by H_2S. Separation of the H_2S treated TFF3-FCGBP heteromer by 15% nonreducing (NR) SDS-PAGE and subsequent Western blot analysis using the affinity-purified anti-hTFF3-3 antiserum. Shown are samples not treated with H_2S at all (lane b) or treated with H_2S for 10 min at room temperature (lane c) followed by incubation at 37 °C for 10 min (lane d), 30 min (lane e), 60 min (lane f), or 120 min (lane g). As a control, a sample not treated with H_2S is shown under reducing conditions (R) releasing TFF3/monomer (lane a). Furthermore, 20 ng recombinant human TFF3/dimer was loaded for comparison (lane h). The molecular size standard is shown on the left.

B123, B73, B61, B55, and B51 are much smaller than full length FCGBP and thus represent only fragments of FCGBP. Thus, a detailed analysis of the peptides assigned to the complete FCGBP sequence was performed. The results are presented in Figure S1 (Supporting Information) and revealed the following: (i) B123 contains mainly C-terminal FCGBP sequences, (ii) B73 and B61 consist of internal FCGBP sequences, and (iii) B55 and B51 mainly contain N-terminal FCGBP sequences. This is a clear indication that fragmentation of FCGBP occurred. Furthermore, in B73 also CLCA1 was identified (score: 309.54) which represents probably a secretory metal-dependent hydrolase typically found in mucin granules of intestinal goblet cells.[46,47] Most bands also contained different isoforms of cytokeratins or fragments thereof which were regarded as typical contaminants.

Characterization of the TFF3 Heteromer by Western Blot Analysis. SDS-AgGE under nonreducing conditions and subsequent Western blot analyses of the FPLC-purified TFF3 heteromer were performed in order to test whether FCGBP really represents the disulfide-linked partner protein of TFF3. As shown in Figure 3A, both the anti-TFF3 as well as the anti-FCGBP antisera clearly detected identical protein bands with a M_r of much more than 250 000 indicating that both TFF3 and FCGBP form indeed a heteromer.

Furthermore, the FPLC-purified TFF3 heteromer was subjected to reducing SDS-PAGE and subsequent Western blot analysis using a commercial anti-FCGBP antiserum against the N-terminal portion of FCGBP (amino acids 289−417). Two bands were strongly recognized by this anti-FCGBP antiserum, which could be identified as B51 and B55 by comparison with a silver stained sample in a parallel lane (Figure 3B).

Reduction of the TFF3-FCGBP Heteromer by H_2S. High H_2S concentrations have been reported to occur in the lumen of the colon. Thus, FPLC-purified TFF3-FCGBP heteromer was treated with H_2S for various times in order to test if TFF3 can be liberated in this way. After separation of the reaction mixture by SDS-PAGE and subsequent Western blot analysis, mainly TFF3/monomer was detectable increasingly with time but also low amounts of TFF3/dimer were generated (Figure 4). In contrast, the TFF3-FCGBP heteromer was continuously degraded with time. As a control, the purified TFF3-FCGBP heteromer was incubated for various times without prior H_2S treatment. Here, TFF3 release was not detectable (data not shown).

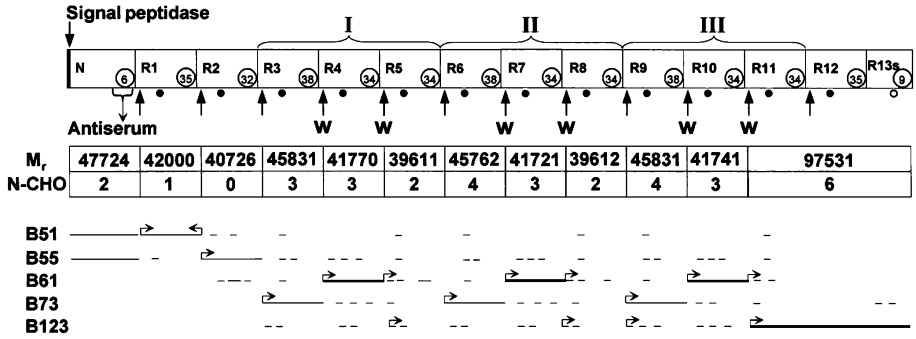

Figure 5. FCGBP and its expected proteolytic processing. Shown is the schematic structure of pre-FCGBP (signal sequence, N-terminal domain, 13 repetitive units R1-R12 and R13s; based on NCBI Accession Number NP_003881.2). The number of cysteine residues in each domain is indicated (encircled). The repetitive units R3-R5, R6-R8, and R9-R11 are part of three larger repeats (I, II, and III). Arrows denote predicted cleavage sites either by signal peptidase or autocatalytic processing sites in the sequence GD/PHY. W indicates autocatalytic processing sites formed by the sequence WGD/PHY. Knobs indicate the C*XX*C motifs typical of thioredoxin and thioredoxin-like folds. Also shown is the sequence recognized by the commercial anti-FCGBP antiserum (HPA003564). Additionally, the M_r of various FCGBP fragments is given as well as the number of potential N-glycosylation sites. Furthermore, results from the peptide mass fingerprint analysis of the bands B51, B55, B61, B73, and B123 (for details, see Figure S1, Supporting Information) are presented schematically. Regions with the highest coverage are indicated by solid lines (results concerning the predominant bands B61 and B123 are shown in bold). Little horizontal arrows denote peptides identified which contained either the amino- or the carboxyterminal portion of the cleavage site GD/PHY.

Discussion

The major result of our studies has been to show that intestinal TFF3 exists mainly as a high molecular weight heteromer. FCGBP has been identified as the disulfide-linked partner protein of intestinal TFF3. Of special note, various fragments of FCGBP were released from the heteromer after reduction. Furthermore, it cannot be excluded to date that CLCA1 is also part of the intestinal TFF3-FCGBP complex. However, this is not subject of the investigations presented here.

TFF3 and FCGBP Form Disulfide-Linked Heteromers. FCGBP, which is entirely different from Fcγ receptors, is most highly synthesized in intestinal goblet cells, is a constituent particularly of the colonic mucus and is also found in the murine stool, and probably N-terminal parts are covalently attached to the mucin MUC2.[48−51] Thus, FCGBP is clearly expected to be a typical secretory protein with a N-terminal cleavable signal sequence. This would also be in line with the merocrine secretion mode reported at least for the rat FCGBP.[52] However, in the past no such sequence has been described for FCGBP.[45] Based upon a computer analysis using the SignalP 3.0 Server (CBS, Technical University of Denmark; www.cbs.dtu.dk) cleavage by signal peptidase can be predicted after G-19, T-21 or A-24 (see Figure 5). Cleavage after T-21 could result in formation of an N-terminal pyroglutamic acid residue of mature FCGBP due to cyclization of Q-22 similar as has been observed for many secretory proteins, for example, caerulein.[53] Pre-FCGBP comprises 5405 amino acid residues. The mature form binds specifically the Fc region of IgG[45] and thus also inhibits complement-mediated hemolysis of red blood cells *in vitro*.[49] As a hallmark, FCGBP contains 12 complete and one shortened cysteine-rich repetitive units arranged in tandem (Figure 5; for details, see Figure S2, Supporting Information). Each of the complete units consists of somewhat less than 400 amino acid residues (8% cysteine residues). All repetitive units contain the C*XX*C motif (CGLCGN or CGACGN) typical of thioredoxin and thioredoxin-like folds of the a-type such as the protein disulfide isomerase family.[45,54] Furthermore, the repetitive units 1−11 contain the sequence GDPHY which is an expected processing site due to autocatalytic cleavage between the aspartate and the proline residue similar as has been found in the mucins MUC2 and MUC5AC.[51,55,56] FCGBP is also highly glycosylated. For example, it contains an unusual high content of serine/threonine residues (12.3%) typical of O-glycosylation[45] and 33 potential N-glycosylation sites (Figure 5).

FCGBP expression is up-regulated in ulcerative colitis,[57] which is in line with reports that FCGBP is induced by IL-13[58] and IL-9.[59] In contrast, FCGBP is down-regulated in the colon in the normal-adenoma-carcinoma sequence in human[60] as well as in a mouse model.[61] Generally, FCGBP was suggested to prevent injurious complement mediated reactions particularly in the intestinal wall, but also on other mucosal surfaces.[49]

In the course of the studies presented here, TFF3 has been shown to be linked to FCGBP via a disulfide bridge. The formation of a large, disulfide-linked TFF3-heteromer is in agreement with previous preliminary data.[62,63] FCGBP contains 435 cysteine residues. This uneven number is a strong indication that FCGBP forms at least one intermolecular disulfide bridge, for example with TFF3, because the oxidation machinery of the endoplasmic reticulum enforces disulfide bond formation in secretory proteins.[54] Interestingly, nearly all different domains of FCGBP contain an even number of cysteine residues, that is, the N-terminal domain and R2-R11 (Figure 5). Only R1 and R12 contain both an additional cysteine residue in the region homologous with the von Willebrand factor (vWF) D domain, that is, R1 and R12 contain 35 cysteine residues each; whereas in R13s one conserved cysteine residue is missing, another cysteine residue is newly introduced, and this domain is C-terminally shortened to an odd number of cysteine residues, that is, 9 (see Figure S2, Supporting Information). Thus, domains R1, R12, and particularly R13 represent the regions potentially designed to bind TFF3. Theoretically, also other protein(s) with an odd number of cysteine residues could additionally be linked to FCGBP. Formation of a TFF3-FCGBP heterodimer could occur, for example, via a disulfide bridge between C-57 of TFF3 and C-5403 of FCGBP. The hypothesis that only a single TFF3 molecule is linked to FCGBP is in agreement with a quantitative analysis of TFF3 released

from the TFF3-FCGBP heteromer (data not shown). Thus, the ability of TFF3 to form a disulfide-linked heteromer is comparable with that of TFF1 which is known to form a heterodimer with gastrokine-2/TFIZ1.[39] However, the TFF3-FCGBP heteromer is mucus associated; whereas the TFF1-GKN2 heterodimer is not associated with the gastric mucin fraction.[40]

Under reducing conditions, TFF3/monomer was easily released *in vitro* (Figure 1C). LC−ESI−MS/MS analysis of this band (B<14) detected a tryptic fragment representing the predicted N-terminus of TFF3 starting with a glutamic acid residue (see Table S1, Supporting Information). Thus, the N-terminus of TFF3 in the TFF3-FCGBP heteromer is not cyclized to form a pyroglutamic acid residue. This is in line with results from the amino acid sequence analysis of recombinant human TFF3[34] and is also analogous to TFF1.[6]

Generation of the TFF3/Monomer and TFF3/Dimer−Functional Implications for Intestinal Restitution. Besides as a TFF3-FCGBP heteromer, TFF3 also exists as a TFF3/monomer and only little TFF3/dimer *in vivo* (Figure 1D). This result was somewhat unexpected because the TFF3/dimer was in the past favored to be the predominant TFF3 form, based on the results with recombinant TFF3. Purification and LC-ESI-MS/MS analysis of these low molecular weight TFF3 forms clearly detected TFF3 sequences including the N-terminal glutamic acid residue (data not shown).

The TFF3/monomer could be released from the TFF3-FCGBP heteromer *in vivo* by reduction with exogenous hydrogen sulfide (H_2S) due to high H_2S concentrations particularly in the lumen of the colon.[64] This hypothesis is in agreement with *in vitro* results where treatment of FPLC-purified TFF3-FCGBP heteromer with H_2S liberated mainly TFF3/monomer and little TFF3/dimer (Figure 4). Interestingly, this process is rather slow at 37 °C allowing continuous release of minute amounts of TFF3. Of special note, TFF3 released in this manner could be post-translationally modified by H_2S, that is, by S-sulfhydration.[65]

Theoretically, TFF3 could also be generated from the TFF3-FCGBP heteromer by a disulfide isomerization reaction. Particularly the conserved C*XX*C motifs in R1-R13s of FCGBP (see Figure 5) might catalyze a disulfide bond rearrangement. For example, cross-linking of fibronectin in the extracellular matrix has been suggested by such a C*XX*C motif.[66] Of note, the C*XX*C motifs in R12 and R13 are expected to be more active because of the vicinal basic amino acid residues (see Figure S2, Supporting Information).

As an integral mucus constituent, the TFF3-FCGBP heteromer would be ideally suited to build a reservoir for TFF3 that could be liberated possibly by H_2S acting then as a motogen to support rapid mucosal repair processes. Interestingly, endogenous H_2S production is markedly increased during inflammation.[64] Thus far, all attempts failed to demonstrate a direct motogenic activity of the purified TFF3-FCGBP heteromer (Znalesniak, Albert and Hoffmann, data not shown). Consequently, the TFF3-FCGBP heteromer is expected to represent a biologically inactive storage form of TFF3 concerning restitution. Thus, most challenging for the future will be to test the biological activity of the TFF3/monomer released *in vivo*.

Possible Function of the TFF3-FCGBP Heteromer for the Structure of Intestinal Mucus. The TFF3-FCGBP heteromer is an important structural component of the intestinal mucus. This secreted barrier contains also the mucin MUC2, immunoglobulins, antimicrobial peptides, phospholipids, galectins, and protease inhibitors.[67] Besides binding to immunoglobulins, the TFF3-FCGBP heteromer could particularly interact with the mucin MUC2 in a covalent[51] as well as in a noncovalent manner. The latter could originate from interactions between TFF3 and MUC2 giving rise to an entangled network. Noncovalent interactions of TFF domains with mucins have been proposed already in the past[68,69] because multiple TFF domains have been detected as cysteine-rich modules in frog integumentary mucins FIM-A.1 and FIM-C.1.[70−72] Such interactions could modulate the rheological properties of the intestinal mucus. This could also be of importance for protection of the fragile blebs (or "aposomes") which are observed in goblet cells and occur as a result of apocrine secretion.[73,74]

Additionally, the TFF3-FCGBP heteromer could also have a pivotal function in the complex folding process of MUC2. For example, the protein disulfide isomerase AGR2 is essential for production of intestinal mucus[75] and aberrant MUC2 assembly in mice causes endoplasmic reticulum stress and spontaneous colitis.[76] Such a function of the TFF3-FCGBP heteromer for the correct formation of disulfide bridges particularly in MUC2 could explain the observed phenotype of $Tff3^{-/-}$ mice which were more susceptible to experimental colitis.[28] This phenotype is comparable with that of $Agr2^{-/-}$ mice[75] and mice with a defect in the unfolded protein response (UPR).[77] Of special note, $Tff1^{-/-}$ mice have also been reported to accumulate misfolded proteins in the endoplasmic reticulum and show an activated UPR.[78]

Implications for the Proteolytic Processing of FCGBP. Human FCGBP has been reported to be cleaved into several polypeptides (>200k, 70−80k) still cross-linked by disulfide bonds.[45] Furthermore, four FCGBP fragments (estimated M_r: 150k, 85k, 75k, 65k) have been characterized in unexpectedly high abundance in the murine stool also indicating proteolytic processing.[50] Recently, 11 autocatalytic cleavages have been proposed to occur between the aspartate and the proline residues in the sequence GD/PHY present within the repetitive units R1−R11 (see Figure 5).[51] Similar sites in MUC2 and MUC5AC were shown to be cleaved at a pH below 6.0 or at neutral pH, respectively.[55,56] Interestingly, processing of FCGBP is changed in prostate secretion of $Atp12a^{-/-}$ mice, which lack colonic H−K-ATPase resulting in an increased pH in their prostate fluid.[79] Such an autocatalytic processing mechanism is also strongly supported by N-terminal sequencing of the 155k, 60k, and 55k fragments of an N-glycosylated IgG binding protein from the secretion of rat coagulating gland, which show high similarity to human FCGBP.[73] The hypothetical processing of human FCGBP at eleven GD/PHY sites should liberate an N-terminal fragment with a M_r of about 47.7k, 10 repetitive units with a calculated M_r between 39.6k and 45.8k, and a C-terminal fragment with a M_r of 97.6k (Figure 5). Furthermore, most of these fragments could also be modified by N- and O-glycosylation.

The results of the LC−ESI−MS/MS analysis of B123, B73, B61, B55, and B51 presented in Figure S1 (Supporting Information) are in complete agreement with the suggested autocatalytic processing of FCGBP at the 11 GD/PHY sites. The results are schematically illustrated in Figure 5. Proteomic analysis unambiguously identified the aminoterminal portion of the cleavage sites starting with the sequence PHY in R1-R11. Furthermore, also the carboxyterminal cleavage site was determined in one fragment, namely that one consisting mainly of R1. Taken together, B51 mainly consisted of the N-terminal FCGBP domain and R1, whereas B55 contained mainly the N-terminal FCGBP domain and R2, but also R4, R7, and R10

sequences were easily detectable. This is an indication that the N-terminal FCGBP domain appears in two different glycoforms, that is, B51 and B55, which is perfectly in line with the result of a Western blot analysis with an antiserum against the N-terminal FCGBP domain just recognizing these two bands (see Figures 3B and 5). B55 also contained sequences from R2. This FCGBP fragment is expected to be O-glycosylated in order to gain the observed M_r. B73 predominantly consisted of sequences liberated from the repetitive units R3, R6, and R9. In contrast, B61, which represented the major band after reducing SDS-PAGE (Figure 2C), mainly consisted of the repetitive units R4, R7, and R10, but also R2, R5, R8, and R11 sequences were detectable. The preponderance of B61 and B123 when compared with the other fragments (see Figure 2C) might be explained by a preferred processing at the boundaries of the repetitive units 4, 7, and 10, which are formed by the sequence WGD/PHY (see Figure 5 and Figure S2, Supporting Information). In contrast, all other GD/PHY sites in FCGBP are missing the characteristic tryptophan residue. Interestingly, the known processing sites in MUC2 and MUC5AC also contain the characteristic tryptophan residue.[55,56] Thus, one might speculate that this tryptophan residue accelerates the cleavage reaction when compared with the glutamine or serine residues, respectively, found at equivalent positions within R1, R2, R3, R6, and R9 (see Figure S2, Supporting Information).

Conclusions

The studies presented here identified TFF3 as part of a disulfide-linked heteromer with FCGBP, which is a known integral constituent of the intestinal mucus. To the best of our knowledge, this is the first report relating the mucus layer also to a defined function as a reservoir for a biologically active peptide, which can be released via reduction by H_2S.

Acknowledgment. We thank Prof. H. Lippert and Prof. A. Roessner for their continued support, J. Reising for excellent technical and E. Voss for valuable secretarial assistance, Dr. J. Lindquist for comments on the manuscript, and Dr. L. Thim for kindly providing recombinant TFF3. The early phase of this project was supported by grant 01ZZ0407/PFG5/TP4 from the Bundesministerium für Bildung, Wissenschaft, Forschung und Technologie (to W.H.). Analyses in the Central Bioanalytics of the Center for Molecular Medicine Cologne were supported by grant SFB 635 (to F.G.H.).

Supporting Information Available: Supplemental figures, table, and detailed protein report. This material is available free of charge via the Internet at http://pubs.acs.org.

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PR100020C