

PAPERS

The human trefoil peptide, TFF1, is present in different molecular forms that are intimately associated with mucus in normal stomach

J L Newton, A Allen, B R Westley, F E B May

Abstract

Background—TFF1 is a 6.5 kDa secreted protein that is expressed predominantly in normal gastric mucosa. It is coexpressed with mucins and it can form dimers via a free carboxy terminal cysteine residue.

Aims—To investigate the molecular forms of TFF1 that are present in normal human stomach and the association of the different molecular forms with mucus.

Subjects—All subjects had macroscopically normal stomachs at gastroscopy. None had a significant past medical history.

Methods—TFF1 was detected in normal gastric mucosa and adherent mucus by western transfer analysis after electrophoresis on reducing and non-reducing polyacrylamide gels. In some instances, proteins were fractionated by caesium chloride density gradient centrifugation prior to detection of TFF1. The location of TFF1 in gastric mucosa with an intact adherent mucus layer was assessed by immunohistochemistry.

Results—Three different molecular forms of TFF1 were detected: TFF1 monomer, TFF1 dimer, and a TFF1 complex with an apparent molecular mass of about 25 kDa. TFF1 was present at higher concentrations than realised previously. The TFF1 complex was present in the adherent mucus gel layer but while its interaction with mucin was destabilised by caesium chloride, the interaction between mucin and the TFF1 dimer was resistant to caesium chloride.

Conclusions—Most of TFF1 in normal human gastric mucosa is present in a complex that is stabilised by a disulphide bond. TFF1 is intimately associated with mucus. The high concentration, colocalisation, and binding of TFF1 to gastric mucus strongly implicate TFF1 in gastric mucus function.

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TFF1 is a small secreted protein of 60 amino acids.¹⁻³ Its function is uncertain, although like other members of the trefoil family of proteins, it is thought to have motogenic activity and a role in mucosal protection.⁴ Trefoil peptides share homology within a 42-43 amino acid sequence called the trefoil domain.⁵⁻⁷ Each protein contains between one and six copies of this trefoil domain. In humans, three trefoil proteins have been identified: TFF1 and TFF3 contain one trefoil domain while TFF2 contains two copies of the domain. These proteins were formerly called pNR-2/pS2,^{1,3} intestinal trefoil factor or ITF,^{8,9} and spasmolytic polypeptide or SP,¹⁰ respectively. The predominant site of expression of the three proteins in normal tissues is in the gastrointestinal tract: TFF1 is expressed mainly in the stomach, TFF3 in the intestine, and TFF2 in the stomach and duodenum.⁵⁻⁷

Mammalian trefoil proteins contain a number of highly conserved and semiconserved amino acids. These include six cysteine residues within the trefoil domain that form three intramolecular disulphide bonds and assist the formation of a characteristic three loop protein motif. The structures of the two domain porcine TFF2 purified from natural sources^{11,12} and of the single domain recombinant human TFF1¹³ have been solved. The three loops of each trefoil domain lie stacked together, with the third loop positioned between the first and second loops. In TFF1, the carboxy and amino termini are juxtaposed and form a short stretch of β sheet.¹³ Four highly conserved amino acids, Phe¹⁹, Pro²⁰, Pro⁴², and Trp⁴³, form a contiguous hydrophobic patch on the surface of the protein. Several of the residues with solvent accessible side chains that surround this patch are semiconserved. There is a 6 Å wide cleft in between loops two and three that contains the conserved hydrophobic patch.¹³ It has been suggested that this could provide a binding site for an oligosaccharide chain of the type found in mucin glycoproteins.¹¹ Alternatively, it could accommodate a hydrophobic interaction with amino

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Abbreviations used in this paper: ITF, intestinal trefoil factor; TFF, trefoil factor family.

acid side chains such as Phe or Trp and thus form part of a protein binding site.¹³

The single domain mammalian trefoil proteins all contain a seventh conserved extra trefoil domain cysteine, located three amino acids from the carboxy terminus. The conservation of this residue implies that it is of functional significance. We have produced recombinant TFF1 Cys⁵⁸ and the variant protein TFF1 Ser⁵⁸ in which Cys⁵⁸ has been mutated to the isosteric amino acid serine.¹⁴ Using these proteins, we have shown that TFF1 forms homodimers via an intermolecular disulphide bond between Cys⁵⁸ residues.¹⁵ Furthermore, we have shown that TFF1 secreted from breast cancer cells also forms homodimers and that it can interact with another molecule to form a hetero-oligomer.¹⁵

In the human stomach, TFF1 protein is expressed predominantly in the superficial foveolar epithelium of the gastric body and antrum.^{13 16 17} The major site of expression of rat TFF1 mRNA is also the stomach¹⁸ and in mice, TFF1 mRNA is expressed by the mucus secreting cells of the surface epithelium and pits of the fundus, antrum, and antrum-pyloric regions.¹⁹ TFF1 protein has been shown by immunohistochemistry in the antrum of normal mice.²⁰ In TFF1 null mice, the antral and pyloric gastric mucosa exhibit severe hyperplasia and are almost entirely devoid of mucus.²⁰ All TFF1 null mice developed antropyloric adenoma, and 30% developed multifocal intraepithelial or intramucosal carcinomas.²⁰

Although the function of trefoil peptides remains unclear, early clues were provided by the pattern of expression in a variety of pathological conditions. All three peptides are expressed at high levels in metaplasia of the human gastrointestinal tract, adjacent to ulceration.²¹ Their expression is induced in animal models of ulceration,²² and there is a transient ectopic expression of rat TFF1 in acetic acid induced colitis.¹⁸ These observations suggested that trefoil peptides might have a role in repair following ulceration and that their normal function could be to enhance restitution of the mucosal layer. Protection by trefoil peptides against damage has been shown using TFF1 transgenic mice²³ and by administration of recombinant proteins to both normal rats^{4 24 25} and to TFF3 null mice.²⁶ In addition, TFF3 null mice have decreased resistance to gastrointestinal damaging agents.²⁶ The possibility that trefoil peptides might enhance restitution and exert their protective effects by stimulating the migration of epithelial cells of the gastrointestinal tract has been investigated, and it has been shown that they stimulate the migration of a variety of cell lines.^{4 24 27}

Trefoil proteins are expressed almost exclusively by cells which synthesise and secrete mucins; TFF1 is expressed by gastric mucus secretory cells^{3 16 17} whereas TFF3 is synthesised by colonic goblet cells.^{8 9} In the skin of *Xenopus laevis*, integumentary mucins are synthesised that contain trefoil domains.²⁸ These observations have focused attention on the possibility that trefoil peptides may have a role in the production, stability, or function of

mucus. This view has been reinforced by the absence of gastric antral mucus in TFF1 null mice.²⁰ Presecreted mucus is stored in intracellular vesicles and secreted mucus is present either as an insoluble adherent mucus gel layer or as freely mobile, partially degraded luminal mucus.²⁹ The adherent mucus covers the gastric mucosa and provides a protective barrier and a stable unstirred layer between the mucosa and the lumen. The mucus gel is formed by non-covalent interactions between high molecular weight mucus glycoproteins or mucins.³⁰ Stimulation of cell migration by human TFF2 and protection afforded by human TFF2 and TFF3 to the gastrointestinal mucosa from damage by a variety of agents are both enhanced by the addition of mucin glycoproteins.^{27 31} Furthermore, rat TFF2 is concentrated in adherent mucus³² and in a rat model, addition of human TFF2 inhibited proton permeation through mucus.³³

There have been numerous immunohistochemical studies of the expression and cellular location of TFF1 in the normal and diseased human stomach.^{3 16 17 21} However, this technique is not quantitative and provides no information on the molecular forms in which the protein is present. In this study, we have quantified the amount of TFF1 in gastric mucosa and report for the first time on the molecular forms of the protein that are present in the human stomach. This is also the first demonstration and characterisation of the association of the different forms of TFF1 with human gastric mucus.

Methods

COLLECTION AND PREPARATION OF HUMAN SAMPLES

Ethical approval for the removal of the normal human gastric samples was obtained. Written consent for the removal of tissue was given by all subjects prior to endoscopy.

All patients were undergoing diagnostic endoscopy for investigation of anaemia, dysphagia, or abdominal pain. All patients had a macroscopically normal stomach at gastroscopy. None had a significant past medical history or were taking acid suppressive or non-steroidal anti-inflammatory medication. Gastric antral mucosa was taken with standard endoscopic biopsy forceps from within 2 cm of the pylorus.

Four antral biopsy specimens from each subject were homogenised in a glass:glass homogeniser in 1 ml of inhibitor buffer: 1 mM iodoacetamide, 4 mM PMSF, 5 mM benzamidine HCl, 10 mM EDTA, 100 mM α -aminocaproic acid, 10 mM N-ethyl maleimide, 67 mM sodium phosphate pH 6.5, sample 1; or 300 μ l of inhibitor buffer, samples 2–5. After centrifugation for one hour at 100 000 g, the cytosol was stored at -70°C . The protein concentrations of the cytosols were measured using the bicinchoninic acid protein assay.³⁴

Adherent gastric mucus was obtained at routine endoscopy with a standard cytology brush. Samples of the adherent mucus gel layer were collected from three individuals by gently passing a 3 mm bristle endoscopic cytology

brush four times over the antrum. The mucus was dislodged from the brush by vortexing intermittently over 15 minutes in 200 μ l inhibitor buffer. The mucus suspension was then clarified by centrifugation at 3000 *g* for five minutes.

Adherent gastric mucus was also obtained from a gastrectomy specimen by laying a 4 cm² piece of nitrocellulose onto the luminal surface of the antrum. A second sheet of nitrocellulose was laid onto the antral surface to remove residual mucus. The underlying mucosal layer was then scraped from the surface.

CAESIUM CHLORIDE EQUILIBRIUM DENSITY CENTRIFUGATION

The density of gastric mucin was determined using gastric mucus prepared from gastric biopsy specimens. The specimens were pooled and homogenised in 10 ml of inhibitor buffer. Following centrifugation at 8000 *g* for one hour at 4°C, the supernatant was mixed with caesium chloride to give a starting density of 1.42 g/ml. The gradients were centrifuged for 24 hours at 100 000 *g* in a vertical rotor at 4°C. The resultant density gradient was separated into nine fractions of equal volume and assayed for glycoprotein content using the PAS reaction.³⁵ PAS positive gastric mucin banded in a density range of 1.41–1.48 g/ml. This is in keeping with previous work on human gastric mucin.^{36 37}

For TFF1 analysis, 12 gastric antral biopsy specimens, four from each of three subjects, were pooled and homogenised for one minute in 10 ml of inhibitor buffer. Following centrifugation at 8000 *g* for one hour at 4°C, the supernatant was mixed with caesium chloride to give a starting density of 1.42 g/ml. The gradients were centrifuged for 24 hours at 100 000 *g* in a vertical rotor at 4°C. The resultant gradient was fractionated and individual fractions were dialysed against distilled water and concentrated by lyophilisation. To evaluate the chaotropic effect of 6 M guanidine hydrochloride, 24 gastric antral biopsy specimens were processed as described above and the supernatant was divided in two. Caesium chloride was added to both to a starting density of 1.42 g/ml, and guanidine hydrochloride was added to one to a concentration of 6 M. They were then centrifuged, and analysed as described above.

The association between TFF1 and mucus in adherent gastric mucus was evaluated in mucus obtained from five patients with a 3 mm bristle standard cytology brush. The mucus was dispersed in inhibitor buffer, caesium chloride was added to the mucus, and the resultant mixture was centrifuged, fractionated, and analysed as described above.

WESTERN TRANSFER ANALYSIS

Samples were electrophoresed on polyacrylamide gels that contained 0.1% sodium dodecyl sulphate (SDS) as described previously.¹⁵ For the experiments shown in figs 1A, 1C, 3A, 4A, and 4B, the stacking gels contained 5% (wt/vol) acrylamide and the separating gels contained a gradient of 10:35% (wt/vol)

acrylamide. For the experiments shown in figs 1B, 6A, and 6B, the stacking gels contained 10% (wt/vol) acrylamide, the separating gels contained 20% (wt/vol) acrylamide, and both contained 10% glycerol. The separated proteins were transferred from the gels to 0.2 μ m PVDF membrane (Schleicher and Schuell) using a semidry transfer apparatus (Schleicher and Schuell) for 10 or 15 minutes at 100 mA, and then left uncovered at room temperature overnight. They were fixed in 0.2% glutaraldehyde for 45 minutes, blocked with 3% (wt/vol) bovine serum albumin (BSA) in phosphate buffered saline (PBS) for one hour at 37°C, and then incubated with a 1/2000 dilution of rabbit polyclonal anti-TFF1 antiserum in 3% (wt/vol) BSA and PBS for two hours at 37°C. They were incubated for a further two hours at 37°C with alkaline phosphatase conjugated pig antirabbit IgG in PBS containing 0.1% Tween 20, and then developed as described previously.^{3 15}

IMMUNOHISTOCHEMISTRY

Gastric antral biopsy specimens were wrapped in a 1 cm block of liver that acted as a support medium, snap frozen, and stored at -20°C.³⁸ Cryostat sections (18 μ m thick) were cut onto APES coated slides for immunohistochemical staining. Sections were air dried for 20 minutes, fixed in 100% ethanol for 10 minutes, and stained for cytokeratin or TFF1 using a diaminobenzidine peroxidase-antiperoxidase technique, essentially as described previously.³⁹ Endogenous peroxidase was blocked by incubation of the sections in 5% phenyl hydrazine for 45 minutes at room temperature after the incubation with the primary antiserum.

Results

DETECTION OF TFF1 PROTEIN IN NORMAL HUMAN GASTRIC MUCOSA.

It has been established by *in situ* hybridisation that TFF1 mRNA is expressed at high levels in the foveolar and surface cells of the gastric mucosa.¹⁶ Immunohistochemical studies have shown that the protein is present in the same cells.^{3 16 17} To determine whether we could detect TFF1 by western transfer analysis in gastric mucosa, samples of macroscopically normal gastric antrum were collected during endoscopy. Cytosol was prepared and TFF1 protein measured as described in Methods. Proteins were separated by polyacrylamide gel electrophoresis, transferred to PVDF membranes, and reacted with TFF1 antiserum. As fig 1A shows, anti-TFF1 antiserum³⁹ reacted with a protein band of about 6.5 kDa which comigrated with recombinant TFF1. It was detected at varying levels in all five samples of gastric mucosa.

We have shown recently that TFF1 can dimerise via the formation of a disulphide bond at cysteine 58.¹⁵ There have been no studies on the molecular forms of TFF1 in normal human stomach mucosa. In the experiment shown in fig 1A, the proteins were treated prior to electrophoresis with the thiol agent β -mercaptoethanol which reduces disulphide bonds. The experiment was repeated without

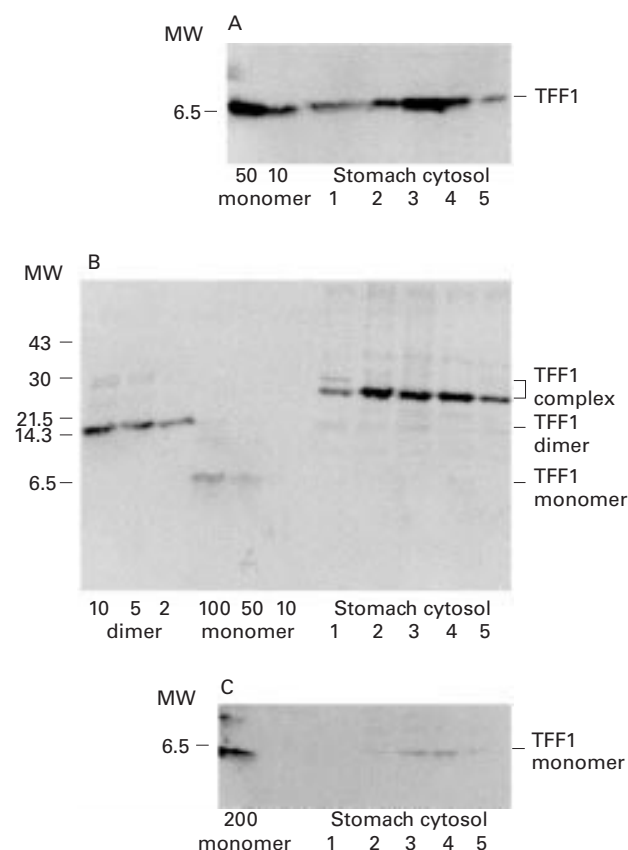


Figure 1 Different molecular forms of TFF1 present on normal human gastric mucosa. Cytosol was prepared from small biopsy specimens of gastric mucosa. Aliquots of 5 μ l, or 10 μ l for sample 1 in A and C, were electrophoresed on 10–35% (A and C) or 20% (B) polyacrylamide gels and the separated proteins transferred to PVDF membrane for 15 min (A and B) or 10 min (C) and then incubated with anti-TFF1 antisera. Prior to electrophoresis, the samples were boiled in sample buffer that did (A) or did not contain β -mercaptoethanol (B and C). Known amounts of recombinant TFF1 monomer or TFF1 dimer were included in the gels as indicated below in ng. The positions of molecular mass markers are shown on the left, and of mature TFF1 (A), TFF1 complex, TFF1 dimer, TFF1 monomer (B), and TFF1 monomer (C) on the right.

treatment with β -mercaptoethanol to investigate whether the peptide is present as a monomer or dimer (fig 1B). In all five samples of cytosol prepared from normal human gastric antrum, the most intensely reactive band has an apparent molecular mass of about 25 kDa. A second slightly larger reactive band was also detected in sample 1 (fig 1B). These reactive protein bands presumably contain a complex between TFF1 and another molecule(s) that is stabilised by a disulphide bond as all of the immunoreactivity is present within the TFF1 6.5 kDa protein band after treatment with β -mercaptoethanol. TFF1 dimer was present in very small amounts, less than 1 ng per sample, and the TFF1 monomer was not detected. To determine whether small amounts of monomeric TFF1 are present, the experiment shown in fig 1B was repeated under conditions which favour detection of the monomer. As

Table 1 TFF1 in cytosol from normal human gastric mucosa (GM)

Sample	Total TFF1 (μ g/mg protein)	TFF1 monomer (μ g/mg protein)	TFF1 dimer (μ g/mg protein)	TFF1 complex (μ g/mg protein)
GM1	4.7	0.6	0.16	3.9
GM2	7.7	1.3	0.20	6.3
GM3	12.9	3.9	0.25	8.8
GM4	10.9	4.1	0.06	6.8
GM5	2.8	1.1	0.09	1.6

shown in fig 1C, some monomer is present, but at relatively low levels compared with the total amount of TFF1 protein present (fig 1A).

The intensities of the reactive bands suggest that the majority of cytosolic gastric TFF1 is in the TFF1 complex. This was confirmed by quantitation using image analysis and comparison with the reaction of known amounts of recombinant TFF1. Preliminary experiments were performed to confirm that the intensity of the protein band detected is proportional to the amount of protein loaded onto the gel (data not shown). The amounts of TFF1 detected were corrected for the amount of cellular protein loaded onto the gel and are listed in table 1. There was a fivefold variation in total TFF1 concentration. The concentrations of TFF1 monomer and dimer were estimated as described above and are shown in table 1. The amount of TFF1 in the TFF1 complex was calculated by subtraction of the amount of TFF1 monomer and dimer from the total amount of TFF1 and is shown in table 1. These results suggest that in normal human gastric antral mucosa, approximately 12–40% of TFF1 in the cytosol is present as monomer and that the majority of the remainder is bound to another molecule(s) to form a complex of about 25 kDa that is stabilised by a disulphide bond.

INTERACTION OF DIFFERENT FORMS OF TFF1 WITH MUCIN IN HUMAN GASTRIC MUCOSA

There has been considerable interest in the association between trefoil peptides and mucus, and the possibility that they may be involved in the production, stability, or function of mucus. Mucin can be separated from other cellular proteins by caesium chloride density gradient centrifugation. Before proceeding, the buoyant density of normal human gastric mucus obtained from small antral biopsy specimens was determined as described in Methods. The starting density of the gradient was 1.42 g/ml, and after centrifugation and fractionation the densities of the frac-

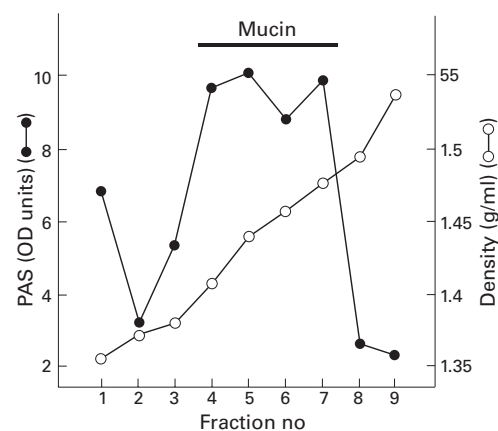


Figure 2 Fractionation of human gastric mucus by equilibrium density centrifugation in a caesium chloride gradient. Normal gastric mucus was prepared from 2 mm³ antral biopsy specimens, and CsCl was added to give a density of 1.42 g/ml. After centrifugation the gradient was divided into nine fractions of equal volume and the density of the fractions measured. The glycoprotein content of the fractions was measured as described previously.³⁵

tions ranged from 1.35 g/ml to 1.54 g/ml (fig 2). Cellular proteins band with a buoyant density of between 1.29 and 1.37 g/ml.³⁶ The mucin was identified by the PAS reaction,³⁵ and was in fractions 4 to 7. The buoyant density at which human gastric mucin bands was thus shown to be between 1.41 and 1.48 g/ml. These densities were used to define the mucin containing fractions in future gradients. This density range is in agreement with previous work that showed that human gastric mucin is

well separated from protein containing fractions.^{36, 37}

To investigate the association between different forms of the TFF1 protein and mucin, cytosol from 12 gastric biopsy specimens was fractionated by caesium chloride density centrifugation. The gradient was divided into nine fractions of equal volume which were then processed to detect TFF1. After electrophoresis of the proteins under non-reducing conditions, two higher molecular weight protein bands corresponding to the TFF1 complexes were detected, mainly in fractions 1 to 4 which are of lower density (fig 3A). TFF1 dimer was also detected but it was concentrated in fractions 4 to 7. After electrophoresis of the proteins under reducing conditions, all of the TFF1 immunoreactivity was with a protein of 6.5 kDa, confirming that the protein bands are detected because they contain TFF1, and that the TFF1 complex is stabilised by a disulphide bond (data not shown).

Collection of samples for analysis of trefoil peptide was limited to 12 biopsy specimens which yield microgram quantities of mucin that are on the borderline for quantitative analysis by the PAS reaction. Mucin containing fractions were identified according to density. The PAS estimation of the fractions always showed a small peak above background in the CsCl gradient at the same density as mucin. For the gradient shown in fig 3, fractions 5 to 7 encompass the density range 1.41–1.47 g/ml at which gastric mucin bands. Quantitation of the TFF1 immunoreactivity with the TFF1 complex (fig 3B) or TFF1 dimer (fig 3C) confirms that the former are in the protein rich and the latter in the mucin rich fractions of the gradient. This suggests either that the TFF1 complex is not associated with mucin or that the association is disrupted by caesium chloride. The TFF1 dimer is strongly associated with mucin and the interaction between the two is not disrupted by caesium chloride.

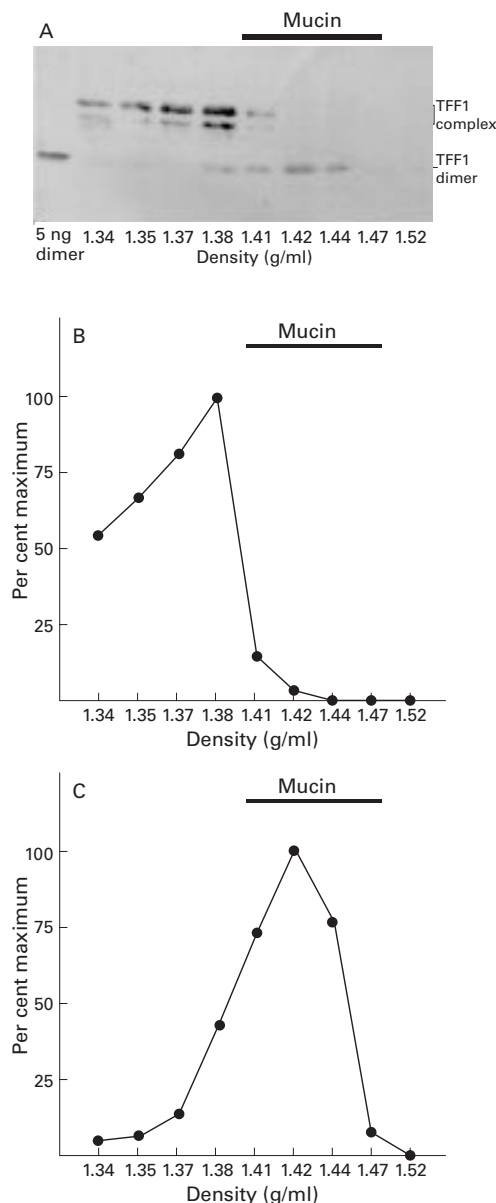


Figure 3 Association of different molecular forms of TFF1 with gastric mucin. Twelve 2 mm² biopsy specimens of normal gastric mucosa were homogenised and centrifuged; caesium chloride was added to the supernatant to give a density of 1.42 g/ml. After centrifugation to equilibrium, the gradient was divided into nine equal fractions which were analysed for the presence of TFF1 under non-reducing conditions by western transfer. The resultant membrane is shown (A) with the positions of the TFF1 complex and TFF1 dimer indicated on the right hand side. Recombinant TFF1 dimer 5 ng was included in the gel. The intensity of the reaction with the different protein bands was quantified by image analysis and is expressed as a percentage of the maximum intensity for either the TFF1 complex (B) or the TFF1 dimer (C).

STABILITY OF THE INTERACTION BETWEEN TFF1 DIMERS AND MUCIN

To investigate the nature of the interaction between TFF1 dimer and mucin further, the caesium chloride gradients were repeated in the absence and presence of 6 M guanidine hydrochloride. The gradients were fractionated and processed as described above to detect the different forms of TFF1. In the absence of guanidine hydrochloride, fractions 5 to 7 had the buoyant density of mucins. The TFF1 complexes were concentrated in the protein rich fractions of the gradient (fig 4A,C). The TFF1 dimer was only detected in the mucin rich fractions, confirming its association with mucin (fig 4A,E). In this experiment, TFF1 monomer was detected in fractions 1 to 8; with a slight rise in concentration coincident with the mucin. This suggests that there may be some specific interaction between the two although diffusion of the 6.5 kDa TFF1 monomer through the gradient could account for some of that present in the mucin containing fractions.

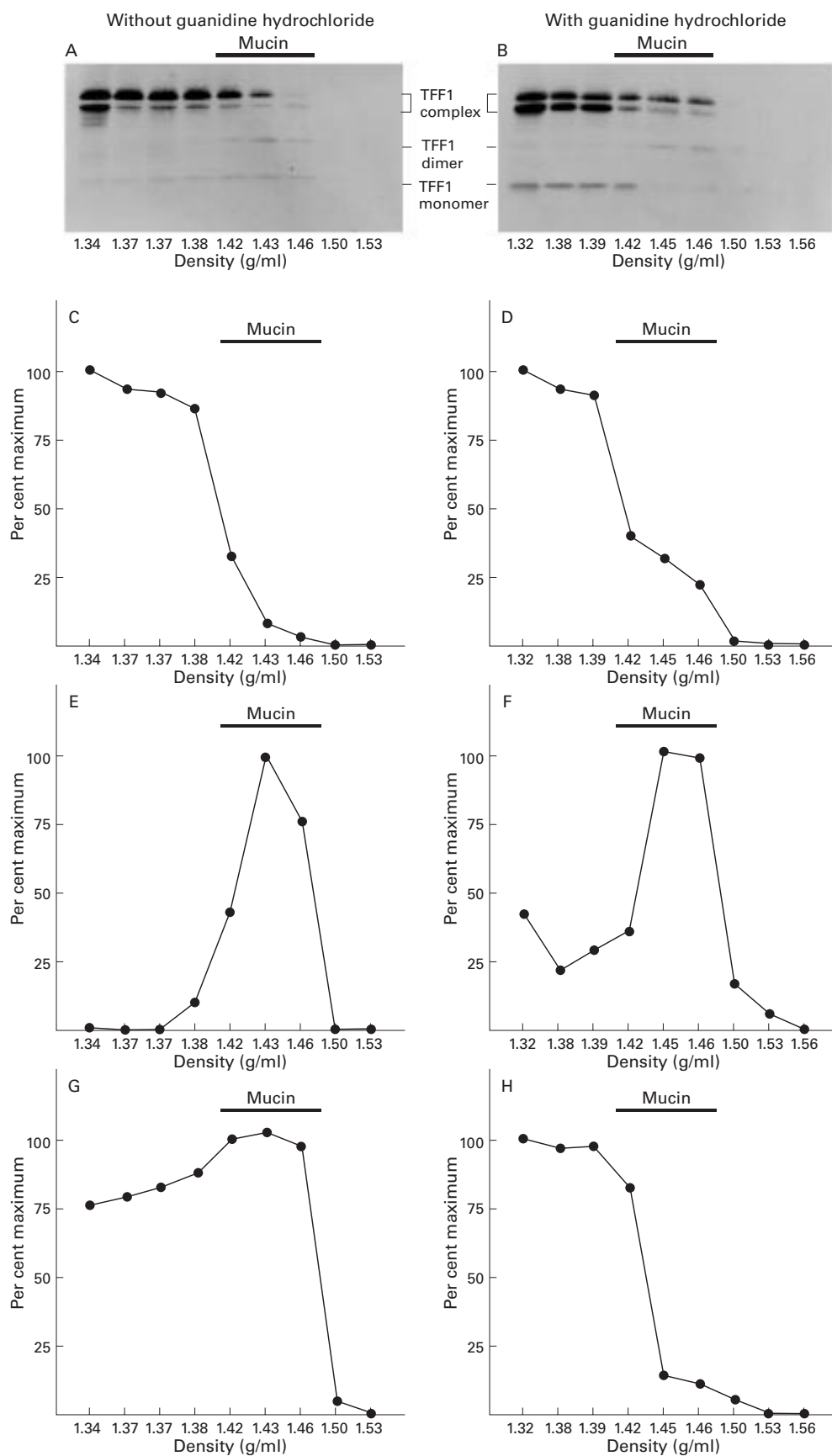


Figure 4 Stability of the interaction between TFF dimer and gastric mucin. Twenty four 2 mm³ biopsy specimens of normal antral mucosa were homogenised and centrifuged and the supernatant was divided into two halves. Caesium chloride was added to both to a final concentration of 1.42 g/ml, and guanidine hydrochloride to one half to a final concentration of 6 M. After centrifugation to equilibrium, the gradients were each divided into nine fractions of equal volume which were analysed for the presence of TFF1 by western transfer after electrophoresis under non-reducing conditions. The resultant membranes are shown (A and B) with the positions of the TFF1 complexes, TFF1 dimer, and TFF1 monomer indicated on the right. The intensity of the reaction with the TFF1 complex (C and D), TFF1 dimer (E and F), and TFF1 monomer (G and H) were quantified by image analysis and expressed as a percentage of the maximum intensity for each form.

In the presence of 6 M guanidine hydrochloride, the buoyant density of the fractions shifted so that fractions 4 to 6 had the buoyant density of mucins. The position of the TFF1 complex was not affected by the inclusion of guanidine hydrochloride in the gradient (fig 4B,D). Most of the TFF1 dimer remained associated with mucin (fig 4B,F). There appeared to be a shift in the proportion of monomer in the different fractions, with an increase in the lower density fractions (fig 4B,H). Although exposure to 6 M guanidine hydrochloride caused some disruption of the association between TFF1 dimer and mucin, the majority of the TFF1 dimer remained associated with mucin.

PRESENCE OF TFF1 IMMUNOREACTIVITY WITHIN THE MUCUS LAYER

The experiments described above show an association between TFF1 dimer and gastric mucin. The gastric mucosal biopsy specimens contain both adherent mucus and the underlying mucosa. To determine where the TFF1 is localised, immunohistochemistry was performed on normal gastric tissue samples similar to those used in the biochemical studies described above. Although routine histological processing disrupts the adherent mucus gel layer, it can be preserved by snap freezing of the tissue and a subsequent brief fixation of the cryosections in 100% ethanol.³⁸ Parallel sections were stained with anticytokeratin and anti-TFF1 antibodies. There was no specific reaction in a control section incubated without primary antibody (fig 5A). The anticytokeratin reacted strongly with all the epithelial cells (fig 5B). The anti-TFF1 reacted strongly with all cells of the foveolar and surface epithelium (fig 5C). In contrast to the staining pattern with anticytokeratin, a strong reaction was also obtained within the adherent mucus gel layer, which colocalises with the region stained using Alcian blue/PAS (data not shown). This suggests that the TFF1 protein is not only colocalised with mucin within the cells that produce mucus, but is secreted with the mucus, and that the association is maintained within the protective mucus layer of the gastric antrum.

IDENTIFICATION OF THE FORMS OF TFF1 IN ADHERENT GASTRIC MUCUS

To determine which molecular forms of TFF1 are present in the adherent mucus layer, the adherent mucus layer and the underlying mucosa were collected separately from a gastrectomy specimen. Western transfer analysis showed that there is abundant TFF1 protein in both the adherent mucus and in the underlying mucosa. In both, by far the majority of the protein was present as the ~25 kDa TFF1 complex (fig 6A). The molecular forms of TFF1 present in mucosal brushings collected at endoscopy from patients with macroscopically normal gastric mucosa were also investigated. As shown in fig 6B, the only molecular forms of TFF1 detected in all three samples of adherent gastric mucus were the TFF1 complexes.

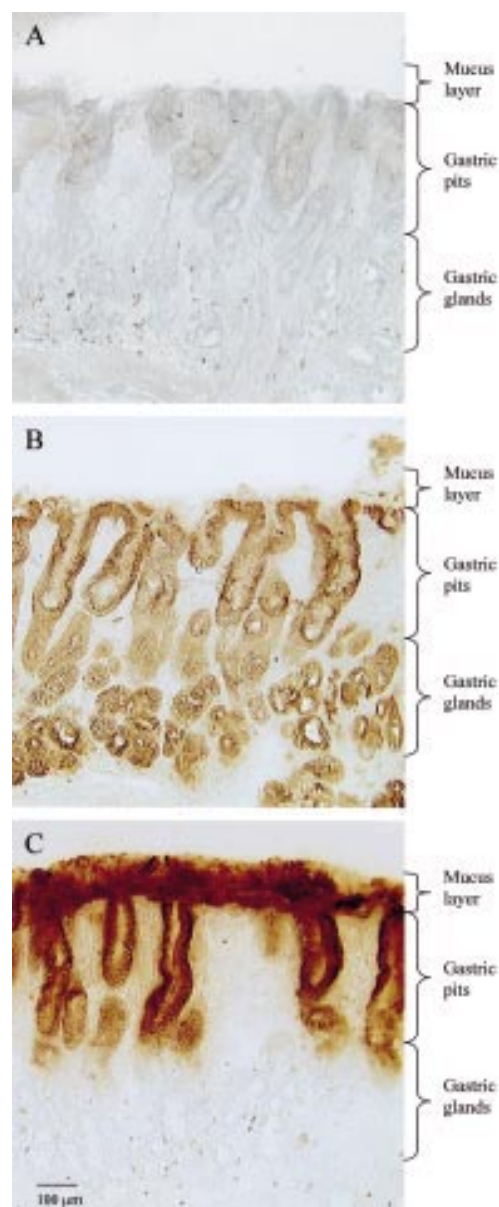


Figure 5 Distribution of TFF1 in situ in cryostat sections of normal human gastric antral mucosa. Sections were processed by immunohistochemistry to locate cytokeratins and TFF1. (A) No specific immunoreaction was obtained in the absence of primary antibodies. (B) There was an intense cytoplasmic immunoreaction of epithelial cells throughout the mucosa with antibodies to cytokeratins. (C) With anti-TFF1 antibodies, there was an intense immunoreaction in the cytoplasm and mucus granules of the epithelial cells of the foveolus and of the mucus layer.

The possibility that the TFF1 complex in the adherent mucus layer might be more strongly associated with mucin than in the mucosal samples analysed previously was investigated. Gastric antral mucus was obtained from five individuals at endoscopy as described above, processed, and fractionated by caesium chloride density centrifugation as described in Methods. As found previously, the majority of the TFF1 immunoreactivity with the TFF1 complex banded in the low density protein rich part of the gradient (fig 7A). The proportion of TFF1 dimer compared with TFF1 complex was much lower in the adherent mucus than in the whole

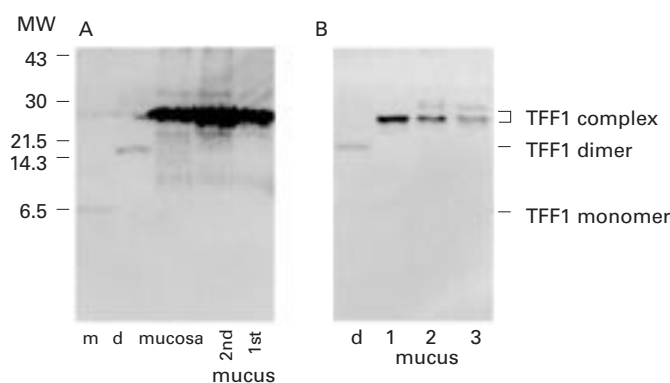


Figure 6 TFF1 complex in adherent gastric mucus. Adherent gastric mucus (1st mucus), residual mucus (2nd mucus), and the underlying mucosal layer (mucosa) were obtained from the antrum of a gastrectomy specimen. (A) The proteins were solubilised with non-reducing gel electrophoresis sample buffer. (B) Gastric mucus was obtained from antral brushings taken from three individuals at endoscopy by suspension in inhibitor buffer. Each brushing was suspended in 200 μ l and 2 μ l aliquots were electrophoresed. Proteins were separated on 20% polyacrylamide gels, transferred to PVDF membrane, and reacted with anti-TFF1 antisera. The resultant membranes are shown (A and B) with the positions of the TFF1 complexes, TFF1 dimer, and TFF1 monomer indicated on the right.

gastric mucosa. The small amounts of TFF1 dimer that were detected were as before in the mucin rich fractions (fig 7B). Thus it appears that the interactions between TFF1 complex and mucin in adherent mucus and in intracellular presecreted mucus are similar.

Discussion

Three molecular forms of TFF1 were detected in normal human gastric mucosa. The majority of the TFF1 protein is present as a complex with an apparent molecular mass of about 25 kDa that is stabilised by a disulphide bond. The next most abundant form of TFF1 comigrates with recombinant TFF1 monomer with an apparent molecular mass of 6.5 kDa. Small amounts of TFF1 dimer were also detected. Both the total amount of TFF1 and the proportions of the different molecular forms varied between the individual samples of mucosa.

This is the first report of the different molecular forms of TFF1 in normal human tissue and of the existence of a TFF1 complex in gastric tissue. The TFF1 complex reported here is significantly smaller than the hetero-oligomer detected in medium conditioned by breast cancer cells; the apparent molecular mass of the breast cell complex is between 60 and 70 kDa.¹⁵ In collaboration with Professor R Playford, we have shown previously that TFF1 dimers are significantly more biologically active than TFF1 monomers both *in vitro*, in cell migration assays, and *in vivo*, in protection against gastric damage.⁴ It is surprising that there are such low concentrations of the dimer in gastric mucosa. The role of the gastric TFF1 complex in the normal biology of TFF1 remains to be determined but it is unlikely that it is involved only in intracellular packaging of mucin and TFF1 as it is present in the adherent mucus layer.

The concentration of TFF1 in cytosol from normal human gastric antral mucosa ranged between 2.8 μ g/mg and 13 μ g/mg of total protein, which represents approximately 0.2–1% of total mucosal protein. Over 90% of mucus is

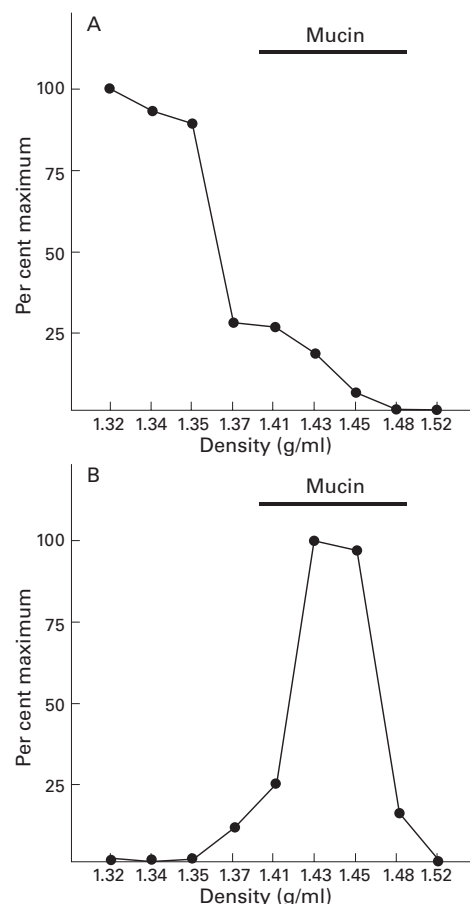


Figure 7 Association of different molecular forms of TFF1 with adherent gastric mucus. Adherent gastric mucus was obtained from five patients at routine endoscopy, dispersed in inhibitor buffer, and caesium chloride was added to a final concentration of 1.42 g/ml. After centrifugation to equilibrium, the gradient was divided into nine equal fractions which were analysed for the presence of TFF1 under non-reducing conditions by western transfer. The intensity of the reaction with the different protein bands was quantified by image analysis and expressed as a percentage of the maximum intensity for either the TFF1 complex (A) or the TFF1 dimer (B).

solubilised by homogenisation and the trefoil peptide measured would either have been associated with presecreted or adherent mucus, or have been soluble intracellular trefoil protein. There may be trefoil peptide in addition to that estimated here bound to insoluble material, including membrane glycoprotein, that is in the pellet. This is the first time that the concentration of the peptide has been estimated, but the levels are consistent with the strong reaction of anti-TFF1 antibodies in gastric tissue sections. TFF2 has been measured in rat antral mucosa and, from the results presented, appears to be at a similar concentration in rat gastric mucosa as TFF1 is in human gastric mucosa.³² The relatively high concentration of TFF1 in normal human tissue suggests that its function is dissimilar to that of growth factors such as EGFs and IGFs with which it has been compared, and that comparatively high concentrations may be required for it to elicit its normal biological functions. This is in agreement with previous work which has shown that concentrations of 6.25 μ g/ml and above are required to stimulate cell migration.⁴

The demonstration that TFF1 is found in adherent mucus in situ (fig 5) reinforces the notion that TFF1 has an important role in the structure and function of mucus. Both the TFF1 complex and TFF1 dimer were shown to be present in adherent mucus. Furthermore, following fractionation of homogenised biopsy specimens in a CsCl density gradient, the TFF1 dimer banded specifically with the mucin. Presumably the TFF1 dimer is not bound covalently to mucin via Cys⁵⁸ of TFF1, as this residue is responsible for TFF1 dimer formation.¹⁵ The TFF1 complex and TFF1 dimer appear to interact differently with mucus which suggests that they may have differing functions. There could be an ionic interaction between the TFF1 complex and mucin which is disrupted by caesium chloride. This would be in contrast to the non-covalent interaction between the TFF1 dimer and mucin which is resistant to caesium chloride and partially resistant to guanidine hydrochloride. It is tempting to speculate that the interaction involves the conserved hydrophobic patch on the surface of TFF1. It has been suggested that this region of the molecule could provide a binding site for a small hydrophobic structure, such as an oligosaccharide chain or protein side chain which could be provided by the glycosylated mucin protein.¹³

The exact function of TFF1 within the mucus layer remains to be determined. Both the observation that TFF1 and mucins are coexpressed, and the fact that TFF1 null mice are devoid of mucus in the gastric antrum promoted the view that trefoils are involved in mucus synthesis, secretion, or function. Our observations on the associations between the different molecular forms of TFF1 and mucus in normal human stomach reinforce the importance of this aspect of TFF1 function.

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- 1 Jakowlew SB, Breathnach J-MJ, Masiakowski P, et al. Sequence of the pS2 mRNA induced by estrogen in the human breast cancer cell line. *Nucleic Acids Res* 1984;6:2861-78.
- 2 Rio M-C, Lepage P, Diemunsch P, et al. Structure primaire de la protéine humaine pS2. *CR Acad Sci Paris* 1988;307:825-31.
- 3 Piggott NH, Henry JA, May FEB, et al. Antipeptide antibodies against the pNR-2 oestrogen-regulated protein of human breast cancer cells and detection of pNR-2 expression in normal tissues by immunohistochemistry. *J Pathol* 1991;63:95-104.
- 4 Marchbank T, Westley BR, May FEB, et al. Dimerization of human pS2 (TFF1) plays a key role in its protective/healing effects. *J Pathol* 1998;185:153-8.
- 5 Poulosom R, Wright NA. Trefoil peptides: a newly recognized family of mucin-associated molecules. *Am J Physiol* 1993;265:205-13.
- 6 May FEB, Westley BR. Trefoil proteins: their role in normal and malignant cells. *J Pathol* 1997;183:4-7.
- 7 Ribieras S, Tomasetto C, Rio M-C. The pS2/TFF1 trefoil factor, from basic research to clinical applications. *Biochim Biophys Acta* 1998;1378:F61-77.
- 8 Podolsky DK, Lynch-Devaney K, Stow JL, et al. Identification of human intestinal trefoil factor: goblet cell-specific expression of a peptide targeted for apical secretion. *J Biol Chem* 1993;268:6694-702.
- 9 Hauser F, Poulosom R, Chinery R, et al. hP1.B, a human P-domain peptide homologous with rat intestinal trefoil factor, is expressed also in the ulcer-associated cell lineage and the uterus. *Proc Natl Acad Sci USA* 1993;90:6961-5.
- 10 Tomasetto C, Rio M-C, Gautier C, et al. hSP, the domain duplicated homolog of pS2 protein, is co-expressed with pS2 in stomach but not in breast carcinoma. *EMBO J* 1990;9:407-14.
- 11 Gajhede M, Petersen TN, Henriksen A, et al. Pancreatic spasmolytic polypeptide (PSP): first three-dimensional structure of a member of the mammalian trefoil family of peptides. *Structure* 1993;1:253-62.
- 12 Carr MD, Bauer CJ, Gradwell MJ, et al. Solution structure of a trefoil-motif-containing cell growth factor, porcine spasmolytic protein. *Proc Natl Acad Sci USA* 1994;91:2206-10.
- 13 Polshakov VI, Williams MA, Gargaro AR, et al. High-resolution solution structure of human pNR-2/pS2: a single trefoil motif protein. *J Mol Biol* 1997;267:418-32.
- 14 Chadwick MP, May FEB, Westley BR. Production and comparison of mature single-domain "trefoil" peptides pNR-2/pS2 Cys⁵⁸ and pNR-2/pS2 Ser⁵⁸. *Biochem J* 1995;308:1001-7.
- 15 Chadwick MP, Westley BR, May FEB. Homodimerization and hetero-oligomerization of the single-domain trefoil protein pNR-2/pS2 through cysteine 58. *Biochem J* 1997;327:117-23.
- 16 Hanby AM, Poulosom R, Singh S, et al. Spasmolytic polypeptide is a major antral peptide: distribution of the trefoil peptides human spasmolytic polypeptide and pS2 in the stomach. *Gastroenterology* 1993;105:1110-16.
- 17 Rio MC, Bellocq JP, Daniel JY, et al. Breast cancer-associated pS2 protein: synthesis and secretion by normal stomach mucosa. *Science* 1988;241:705-8.
- 18 Itoh H, Tomita M, Uchino H, et al. cDNA cloning of rat pS2 peptide and expression of trefoil peptides in acetic acid-induced colitis. *Biochem J* 1996;318:939-44.
- 19 Lefebvre O, Wolf C, Kédinger M, et al. The mouse one P-domain (pS2) and two P-domain (mSP) genes exhibit distinct patterns of expression. *J Cell Biol* 1993;122:191-8.
- 20 Lefebvre O, Chenard M-P, Masson R, et al. Gastric mucosa abnormalities and tumorigenesis in mice lacking the pS2 trefoil protein. *Science* 1996;274:259-62.
- 21 Poulosom R. Trefoil peptides. *Baillieres Clin Gastroenterol* 1996;10:113-34.
- 22 Alison MR, Chinery R, Poulosom R, et al. Experimental ulceration leads to sequential expression of spasmolytic polypeptide, intestinal trefoil factor and transforming growth factor alpha mRNAs in rat stomach. *J Pathol* 1995;175:405-14.
- 23 Playford RJ, Marchbank T, Goodlad RA, et al. Transgenic mice that overexpress the human trefoil peptide pS2 have an increased resistance to intestinal damage. *Proc Natl Acad Sci USA* 1996;93:2137-42.
- 24 Playford RJ, Marchbank T, Chinery R, et al. Human spasmolytic polypeptide is a cytoprotective agent that stimulates cell migration. *Gastroenterology* 1995;108:108-16.
- 25 Babyatsky MW, deBeaumont M, Thim L, et al. Oral trefoil peptides protect against ethanol- and indomethacin-induced gastric injury in rats. *Gastroenterology* 1996;110:489-97.
- 26 Mashimo H, Wu D-C, Podolsky DK, et al. Impaired defense of intestinal mucosa in mice lacking intestinal trefoil factor. *Science* 1996;274:262-5.
- 27 Dignass A, Lynch-Devaney K, Kindon H, et al. Trefoil peptides promote epithelial migration through a transforming growth factor β -independent pathway. *J Clin Invest* 1994;94:376-83.
- 28 Hauser FW, Hoffman W. P-domains as shuffled cysteine-rich modules in integumentary mucin (FIM-C.1) from *Xenopus laevis*. *J Biol Chem* 1992;267:24620-4.
- 29 Allen A, Flemström G, Garner A, et al. Gastrointestinal mucosal protection. *Physiol Rev* 1993;73:823-57.
- 30 Selleres LA, Allen A, Morris ER, et al. Mucus glycoprotein gels role of glycoprotein polymeric structure and carbohydrate side-chains in gel-formation. *Carbohydrate Res* 1988;178:93-110.
- 31 Kindon H, Pothoulakis C, Thim L, et al. Trefoil peptide protection of intestinal epithelial barrier function: cooperative interaction with mucin glycoprotein. *Gastroenterology* 1995;109:516-23.
- 32 Jeffrey GP, Oates PS, Wang TC, et al. Spasmolytic polypeptide: a trefoil peptide secreted by rat gastric mucous cells. *Gastroenterology* 1994;106:336-45.
- 33 Tanaka S, Podolsky DK, Engel E, et al. Human spasmolytic polypeptide decreases proton permeation through gastric mucus in vivo and vitro. *Am J Physiol* 1997;35:G1473-80.
- 34 Smith PK, Krohn RL, Hermanson GT, et al. Measurement of protein using bicinchoninic acid. *Anal Biochem* 1985;150:76-85.
- 35 Mantle M, Allen A. A colorimetric assay for glycoproteins based on the periodic acid/Schiff stain. *Biochem Soc Trans* 1978;6:607-9.
- 36 Pearson J, Allen A, Venables C. Gastric mucus: isolation and polymeric structure of the undegraded glycoprotein: its breakdown by pepsin. *Gastroenterology* 1980;78:709-15.
- 37 Newton JL, Jordan N, Oliver L, et al. *Helicobacter pylori* in vivo causes structural changes in the adherent gastric mucus layer but barrier thickness is not compromised. *Gut* 1998;43:470-5.
- 38 Jordan N, Newton J, Pearson J, et al. A novel method for the visualisation of the in situ mucus layer in rat and man. *Clin Sci* 1998;95:97-106.
- 39 Henry JA, Piggott NH, Mallick UK, et al. pNR-2/pS2 immunohistochemical staining in breast cancer: correlation with prognostic factors and endocrine response. *Br J Cancer* 1991;63:615-22.