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Research Article

The trefoil protein TFF1 is bound to MUC5AC in human gastric mucosa

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Abstract. The trefoil protein TFF1 is expressed principally in the superficial cells of the gastric mucosa. It is a small protein and forms homo- and hetero-dimers via a disulphide bond through Cys58 which is located three amino acids from the C terminus. TFF1 is co-expressed with the secreted mucin MUC5AC in superficial cells of the gastric mucosa suggesting that it could be involved in the packaging or function of gastric mucus. We have previously shown that TFF1 co-sediments with mucin glycoproteins on caesium chloride gradients. To extend this observation we have now used gel filtration under physiological conditions, immunoprecipitation and Western transfer analysis to characterise the interaction of TFF1 with gastric mucin glycoproteins. We show that TFF1 co-elutes with MUC5AC but not MUC6 on gel filtration and that immunoprecipitation and Western transfer analysis confirms that TFF1 interacts with MUC5AC. We also demonstrate that the TFF1 dimer is the predominant molecular form bound to MUC5AC. Salt and chelators of divalent cations such as EDTA and EGTA disrupted the TFF1- MUC5AC interaction and increased the degradation of MUC5AC, whereas calcium increased the amount of TFF1 bound to MUC5AC. These data support the contention that TFF1 is pivotal in the packaging and function of human gastric mucusa.

Key words. Trefoil peptide; TFF1; MUC5AC; MUC6; gastric mucosa; mucus; soluble mucin; mucosal protection.

The gastric mucosa is protected from damage on the apical surface by a glycocalyx formed from insoluble mucins, and by a continuous adherent mucus gel layer formed from soluble mucins secreted by the superficial epithelial cells [1–5]. Mucins are a family of large glycoproteins that contain up to 80% O-linked oligosaccharides [6]. In the stomach, the two major gel-forming mucins are MUC5AC, which is produced by the surface epithelium [7], and MUC6, which is produced in the gastric glands [8]. The membrane-associated mucin in the glycocalyx is MUC1 [9].

The trefoil factor family (TFF) are small secreted proteins which share homology within a conserved trefoil domain of 42–43 amino acids [10, 11]. There are three human trefoil proteins TFF1, TFF2 and TFF3. TFF1 and TFF3 each contain one trefoil domain and are 60 and 59 amino acids long, respectively, while TFF2 contains two trefoil domains in a single chain of 106 amino acids [10, 11]. The trefoil motif contains several well-conserved features including six cysteine residues with essentially conserved spacings.

Trefoil proteins are expressed almost exclusively by cells that synthesise and secrete mucins and have been suggested to be important for the formation and function of mucin polymers. They are expressed at highest levels in normal tissues in the gastrointestinal tract. TFF1 is expressed in the superficial epithelium of the gastric fundus and antrum [12, 13]. TFF2 is expressed in the mucus neck

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cells in the fundus, in the basal glands of the antrum and pylorus and in Brunner's glands of the duodenum. TFF3 is expressed principally in the goblet cells of the small and large intestine. TFF1-null mice do not produce mucus in the gastric antrum or pylorus [14], clearly reinforcing the hypothesis that TFF1 has a critical role in the biosynthesis, stability and function of gastric mucus. Yeast two-hybrid analysis has shown that murine TFF1 interacts with MUC5AC and MUC2. However, the yeast two-hybrid system is artificial in that human secreted proteins may be incorrectly folded in yeast cells and protein-protein interactions identified in yeast may not occur in normal human tissues because the proteins are not expressed in the same cells.

The increased expression of trefoil proteins in the mucosa at sites of naturally occurring [15] and experimentally induced [16] damage suggest that they play an important role in the repair of the epithelium of the gastrointestinal mucosa. They are motogenic, are thought to be involved in the process of restitution, and show a protective effect in animal models of gastrointestinal damage [17–19]. Despite all this evidence for a crucial role for trefoil proteins in healing of the epithelium after damage, how they function remains unclear.

The extra-trefoil domain cysteine residues of trefoil proteins are potential mediators of covalent intermolecular interactions [11, 20]. Both mammalian single-domain trefoil proteins contain one conserved extra-trefoil domain cysteine residue, three amino acids from the carboxy terminus. The conservation of this free cysteine residue implies that it is of functional significance. Studies with recombinant TFF1 have shown that the C-terminal cysteine forms intermolecular disulphide bonds to produce TFF1 homo-dimers [20]. The TFF1 dimer has greater activity that the TFF1 monomer both in vitro and in an animal model [19, 21]. In normal human gastric mucosa, TFF1 is present in three molecular forms: as TFF1 monomer, TFF1 homo-dimer and as an ~25-kDa complex that is stabilised by a disulphide bond, with another as yet unidentified protein [22]. The most prominent form present in the gastric mucosa and mucus gel layer is the TFF1 complex.

The three-dimensional structures of human TFF1 monomer and dimer [23, 24], porcine TFF2 [25–27] and human TFF3 monomer [28] have been determined. Within the trefoil domains, the cysteine residues are paired 1-5, 2-4 and 3-6 to form the compact trefoil motif which comprises three closely packed loops with the third loop positioned between the first and second. Several conserved surface residues are juxtaposed to form a small hydrophobic patch comprising adjacent parts of the second and third loops. This area has been proposed to represent a receptor- or ligand-binding site capable of interacting with an oligosaccharide or an aromatic amino acid [23–26]. The TFF1 dimer comprises two TFF1 monomer units folded in the characteristic trefoil motif connected by a flexible linker consisting of the two C-terminal regions with an intermolecular disulphide bond between the Cys58 residues [24].

We have previously provided evidence that TFF1 interacts with human gastric mucins [22]. Fractionation using CsCl density gradients showed that the majority of the TFF1 dimer and some TFF1 monomer are bound firmly but non-covalently to soluble gastric mucins [22]. It is possible, that the TFF1 complex co-localises with mucus, but is not physically associated with mucins. Alternatively, there may be an association between the TFF1 complex and mucins which is disrupted by caesium chloride. To address this and to characterise the interaction between the TFF1 dimer and specific soluble mucins, studies were undertaken under physiological conditions. Individual mucins have been identified and evidence sought for a direct interaction between different molecular forms of TFF1 and mucins.

Materials and methods

Preparation and characterisation of monoclonal antibody against TFF1

Recombinant TFF1 was produced in *Escherichia coli* as described previously [20]. The expression construct produces a fusion protein that is secreted into the periplasmic space of *E. coli*. The fusion protein was released from the periplasmic space of the bacteria by osmotic shock, purified by affinity chromatography on IgG-Sepharose, digested with factor Xa, the fusion partner removed by affinity chromatography on IgG Sepharose and the TFF1 protein purified to homogeneity by ion exchange and size exclusion chromatography. The TFF1 dimer was produced by treatment of recombinant TFF1 Cys 58 with cysteine, purification of the correctly folded monomeric form of the protein and then incubation to allow formation of the dimer. The dimer was purified from monomer by size exclusion chromatography [20, 24]. Mice were immunised with TFF1 dimer. Hybridoma cell lines were produced by fusion of mouse spleen cells to NS-1 cells. Screening for production of antibodies was by ELISA. Assays in which microtitre plates were coated with the three human trefoil proteins TFF1, TFF2 and TFF3 demonstrated that the antibody only reacts with TFF1. Western transfer analysis confirmed that the antibody reacted with all three molecular forms of TFF1 present in gastric mucosa: monomer, dimer and complex [22].

Gel filtration

The Superose 6HR column, which separates proteins between 5 kDa and 50×10^5 kDa, was run on an AKTA prime (Amersham Pharmacia Biotech) chromatography

system and calibrated using thyroglobulin (669 kDa), ferritin (440 kDa), BSA (68 kDa), RNAse A (13.7 kDa) and Gly-Tyr-Ala (0.3 kDa) peptide. The elution volumes of recombinant TFF1 dimer (13.3 kDa) and TFF1 monomer (6.65 kDa) were 18 and 19 ml. Human gastric mucosa was homogenised in a glass:glass homogeniser in 1 ml of buffer (67 mM sodium phosphate, pH 6.5, 1 mM iodoacetamide, 10 mM EDTA, 100 mM α-aminocaproic acid, 5 mM benzamidine, 10 mM N-ethyl maleimide and 4 mM PMSF) per 100 mg tissue and centrifuged at 10,000 g for 10 min.

Gel filtration was performed with 2 mg of gastric protein, at a flow rate of 0.3 ml/min in 150 mM NaCl, 67 mM sodium phosphate, pH 6.5. Fractions (1 ml) were collected and analysed immediately by ELISA for TFF1, MUC5AC and MUC6.

ELISA

Forty-five microliters of each fraction collected after gel filtration was coated overnight at 4°C onto 96-well M29AR microtitre plates (Dynex Technologies) in bicarbonate buffer pH 9.5. The plates were blocked for 30 min in PBS-0.1% Tween 20 and then incubated with monoclonal antibodies against TFF1, MUC5AC or MUC6 (NCL-MUC-5AC and NCL-MUC-6 from Novocastra Laboratories) in PBS-3% BSA for 2 h at 37°C. The TFF1 antibody was raised against recombinant human TFF1 as described above. The anti-MUC5AC and anti-MUC6 antibodies were raised against the tandem repeated sequences of MUC5AC and MUC6. The plates were washed with PBS-0.1% Tween 20 and incubated with alkaline-phosphatase-conjugated anti-mouse immunoglobulins for a further 2 h at 37°C. The plates were washed with PBS-0.1% Tween 20 and developed using para nitro phenyl phosphate (1 mg/ml in 100 mM diethanolamine, 5 mM $MgCl_2$). After 3 h at 37°C, the absorbance at 405 nm was read using a DYNEX MRX plate reader (Dynex Technologies).

Western transfer

Acrylamide gels for the analysis of TFF1 were prepared using the acrylamide/bisacrylamide ratios described by Giulian et al. [29]. The separating gels contained 20% (w/v) and the stacking gels 10% (w/v) acrylamide, both contained 10% (v/v) glycerol and 0.1% SDS. The samples were boiled for 10 min in a non-reducing buffer [62.5 mM Tris-HCl, pH 6.8, 12.5 mM EDTA, 10% (v/v) glycerol, 0.005% bromophenol blue and 1% SDS] before loading. The separated proteins were transferred for 13 min at 100 mA to a 0.2-µm polyvinylidene fluoride (PVDF) membrane (Schleicher & Schuell) using a semi-dry transfer chamber (Schleicher & Schuell) with anode buffers containing both 20% methanol and respectively 300 and 25 mM Tris, pH 10.4, and the cathode buffer containing 25 mM Tris, pH 9.4, 40 mM 6-amino-n-hexanoic acid and 20% methanol. The membrane was fixed in 0.2% glutaraldehyde for 1 h at room temperature, blocked in 5% BSA, PBS-0.1% Tween 20 for an hour at room temperature and then incubated with TFF1 monoclonal antibody at 4°C overnight. After washes in PBS-0.1% Tween 20, the membrane was incubated with horseradish-peroxidase-conjugated rabbit anti-mouse IgG in 5% milk, PBS-0.1% Tween 20 for 1 h at room temperature. It was then washed and developed using the 'Super Signal West Dura' chemiluminescence kit (Pierce).

For MUC5AC detection, gels were prepared with the acrylamide/bisacrylamide ratios of 1:200 for the separating gel and 1:20 for the stacking gel. The separating gels contained 6% (w/v) and the stacking gels 3% (w/v) acrylamide and both contained 0.1% SDS. The samples were boiled for 10 min in reducing buffer (same buffer as used for the analysis of TFF1 but supplemented with 2.5% β-mercaptoethanol). Separated proteins were transferred for 1 h to 0.45-µm nitrocellulose (Schleicher & Schuell) as described above but with 0.02% SDS in the transfer buffers. The membranes were processed in the same way as for TFF1 but using MUC5AC monoclonal antibody as the primary antibody.

Immunohistochemisty

Sections of formalin-fixed, paraffin-embedded gastric mucosa were processed as described previously [30], except that heat retrieval was for 1 min in a pressure cooker in 10 mM sodium citrate, pH 6.0. The sections were incubated with the primary antibodies for 1 h, with the secondary antibody for 30 min and with avidin-biotin immunoperoxidase complex (Vector Laboratories) for 30 min at room temperature, developed with diaminobenzidine and counterstained with haematoxylin.

Immunoprecipitation

Gastric cytosol (50 µg protein) was mixed with 20 µl of 1:1 slurry of protein G-Sepharose 4B and incubated end-over-end for an hour at 4°C in 100 mM Tris HCl, pH 8, 0.2% Nonidet-P40, 300 mM NaCl, 1 mM PMSF, 5 mM benzamidine, 1 mM iodoacetamide and 100 mM α-aminocaproic acid. Following centrifugation at 5000 g for 5 min, the cytosol was incubated with TFF1 or MUC5AC monoclonal antibodies or murine IgG for 4 h at 4°C in the same buffer. The antibodies were precipitated by addition of 50 µl of 1:1 slurry of protein G-Sepharose 4B (Sigma) and end-over-end incubation overnight at 4°C. The Sepharose beads were washed three times with the same buffer used for the immunoprecipitation and the immunoprecipitated proteins were eluted and analysed by Western transfer.

Results

TFF1 is co-packaged with MUC5AC in normal human stomach

The presence of high concentrations of TFF1 in the adherent mucus gel layer of the human stomach suggests that it is co-secreted with gel-forming gastric mucins [22]. The association between TFF1 and the secreted gastric mucins, MUC5AC and MUC6 was investigated in the present study using monoclonal antibodies specific for the three proteins. The distribution of the three proteins in normal human gastric mucosa detected with these monoclonal antibodies by immunohistochemistry is shown in figure 1.

Haematoxylin and eosin staining of a serial section showed the foveolar and surface eptithelium and antral glands (fig. 1A). PAS-positive mucus is present in epithelial cells throughout the mucosa but the reaction was strongest in the mucus granules of the foveolar and surface epithelium (fig. 1B). High levels of MUC5AC expression were detected throughout the surface epithelial cells, both in cells on the apical surface of the mucosa and in cells lining the foveoli (fig. 1C). In contrast, MUC6 expression in the mucosa was restricted to cells of the basal glands (fig. 1D). TFF1 expression was detected in the surface epithelial cells that contain MUC5AC but was absent in the basal glands in which MUC6 is located (fig. 1E, F). Detection following antigen retrieval with trypsin revealed the TFF1 cellular localisation reported previously [12, 13, 15] with a strong reaction throughout the cytoplasm that was concentrated around the theca but absent from the mucus granule (fig. 1E). However, following antigen retrieval with heat, the TFF1 expression detected was strongest within the mucus granules of the PAS-positive mucus-secreting cells that express MUC5AC but not MUC6 (fig. 1F). At higher magnification, TFF1 was visualised in the secretory vesicles within the mucus granules, both in cells along the surface (fig. 1G) and lining the foveoli (fig. 1H). These results suggest strongly that TFF1 is packaged within the secretory vesicles of gastric mucus granules with MUC5AC and

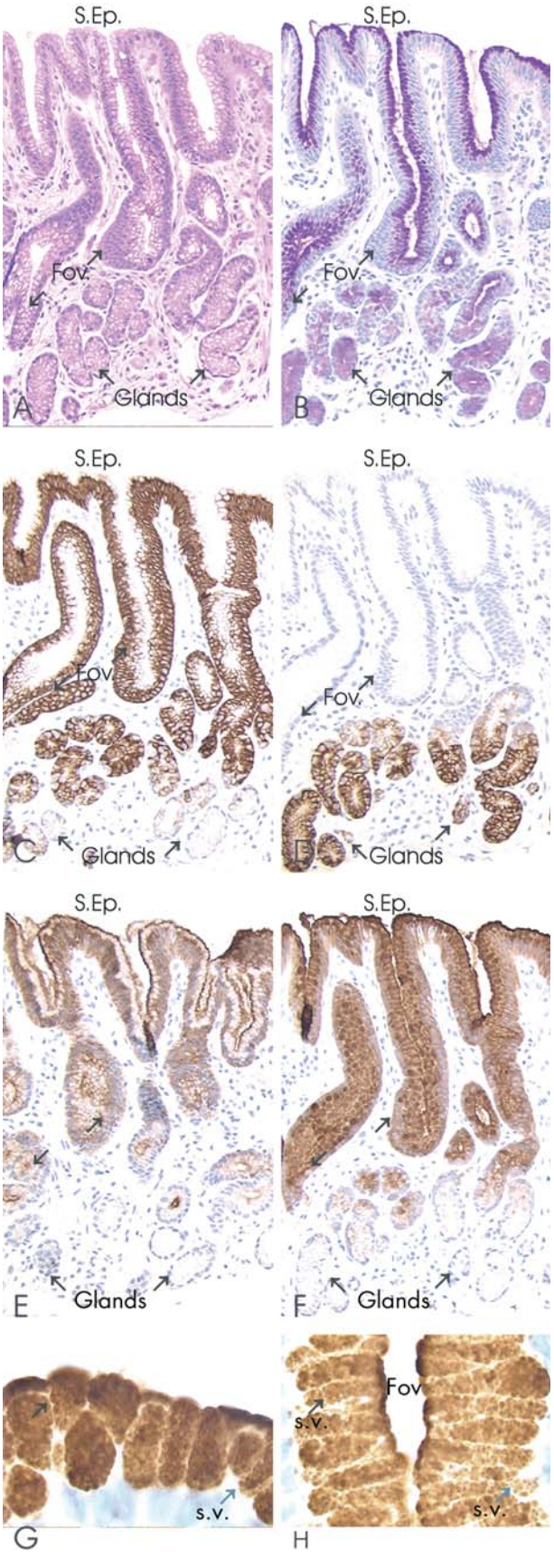

Figure 1. Distribution of MUC5AC, MUC6 and TFF1 in human gastric mucosa. Sections of gastric mucosa were processed for immunohistochemistry to locate MUC5AC, MUC6 and TFF1. (*A*) Haematoxylin and eosin staining of a serial section of the gastric antrum that shows foveolar (Fov.) and surface epithelium (S. Ep.) and the basal glands (Glands). (*B*) Alcian blue/PAS staining is visible throughout the epithelial cells but is strongest in the mucus granules of the foveolar and surface epithelium. (*C–I*) Immunoreaction for MUC5AC (*C*), MUC6 (*D*) and TFF1 (*E–H*). Antigen retrieval was with heat in a pressure cooker (*C*, *D*, *F–H*) or with a 10-min incubation with trypsin (*E*). Higher-magnification photomicrographs are shown for the TFF1 immunoreaction within the mucus granules of surface cells (*G*) and cells lining the foveoli (*H*). The secretory vesicles are highlighted (s.v.).

that they are released together from the vesicles into the adherent mucus gel layer.

Gel filtration of gastric cytosol, co-elution of TFF1 and MUC5AC

Gastric cytosol was fractionated by gel filtration on a Superose 6HR column. No protein was detected by absorption at 280 nm in the excluded volume, whereas two protein peaks were detected in the included volume. The first minor peak was in fractions 9 and 10 and the second, which typically had 15- to 30-fold greater absorbance was in peaks 17 and 18. MUC5AC eluted as a single peak with a maximum at 9 ml and a shoulder at 11 ml (fig. 2A). This corresponds to a molecular mass of approximately 500 kDa and is at the upper end of the fractionation range of the column. No higher-molecular-mass MUC5AC immunoreactive material was detected in the excluded volume nor was there any reaction with proteins below ~200 kDa. In contrast, MUC6 appeared more heterogeneous, with a maximum at 12 ml corresponding to a molecular mass of ~200 kDa, and immunoreactivity detectable in material of considerably smaller molecular mass with a shoulder at 14 ml and a minor peak at 17 ml. TFF1 immunoreactivity comprised two peaks: the first was at 9 ml, which is close to the exclusion volume of the column and indicates that TFF1 elutes with material of high molecular mass. The second peak was at 18 ml and occurs in the fraction range of proteins of less than ~50 kDa. It coincided with the elution peaks of recombinant TFF1 dimer and monomer (data not shown). This experiment showed that a proportion of TFF1 eluted in the high-molecular-mass range and that it co-eluted with MUC5AC but not MUC6. This is consistent with a physical interaction between TFF1 and MUC5AC.

The molecular forms of MUC5AC and TFF1 present in the gel filtration fractions were analysed by Western transfer analysis. Three major forms of MUC5AC were detected after electrophoresis under reducing conditions (fig. 2B). The two larger forms, which have apparent molecular masses of around 500 and 300 kDa, predominated in fractions 9–11 and the third form, which has an apparent molecular mass of ~250 kDa, was more prominent in fractions 12–14.

TFF1 was analysed after electrophoresis under non-reducing conditions to determine the relative amounts of TFF1 monomer, which has a molecular mass of 6.65 kDa, TFF1 dimer, which has a molecular mass of 13.3 kDa [20] and TFF1 complex, which has an apparent molecular mass of 25 kDa [22]. Both the TFF1 complex and TFF1 dimer were present in fractions 9–11 (fig. 2C). The TFF1 dimer was the more prominent form and it co-eluted with the ~500-kDa and ~300-kDa forms of MUC5AC. The TFF1 complex was the only form of TFF1 detected in the second peak of TFF1 immunoreactivity.

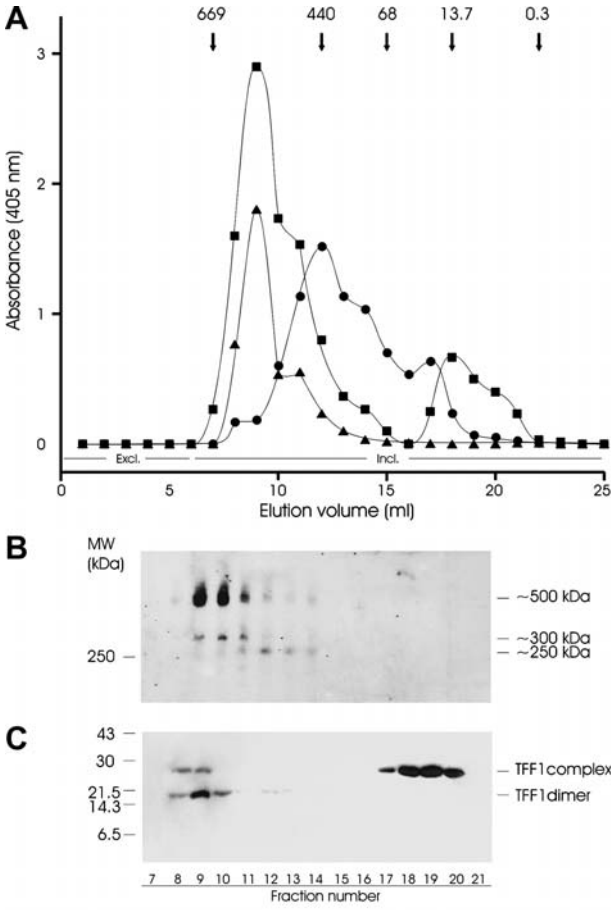

Figure 2. Association of MUC5AC with TFF1 in human gastric mucosa. (*A*) Human gastric mucosa cytosol (2 mg of protein) was fractionated by gel filtration in 150 mM NaCl, sodium phosphate pH 6.5 and the fractions were analysed for MUC5AC (triangles), MUC6 (circles) and TFF1 (squares) by ELISA. Arrows show the position of elution of proteins used for the column calibration as described in Materials and methods. Excl. and Incl. indicate the excluded and included volumes of the column. (*B*) Immunodetection of MUC5AC subunits in fractions collected during gel filtration of human gastric mucosa cytosol. From each 1-ml fraction, 16 µl was electrophoresed on a 6% polyacrylamide gel under reducing conditions. Separated proteins were transferred onto nitrocellulose and then processed as described in Materials and methods. (*C*) Immunodetection of TFF1 in fractions collected during gel filtration of human gastric mucosa cytosol. From each 1-ml fraction, 16 µl was electrophoresed on a 20% poylacrylamide gel under non-reducing conditions. Separated proteins were transferred onto PVDF and then processed as described in Materials and methods. The positions of the molecular-mass markers are shown on the left and of the different forms of the proteins on the right.

Direct interaction between TFF1 and MUC5AC

The co-elution of the TFF1 dimer and a proportion of the TFF1 complex with MUC5AC is consistent with a direct physical interaction between TFF1 and MUC5AC. This was investigated by immunoprecipitation of TFF1 or MUC5AC from gastric cytosol followed by Western transfer analysis using antibodies against both proteins. Multiple subunits/forms of MUC5AC were detected in

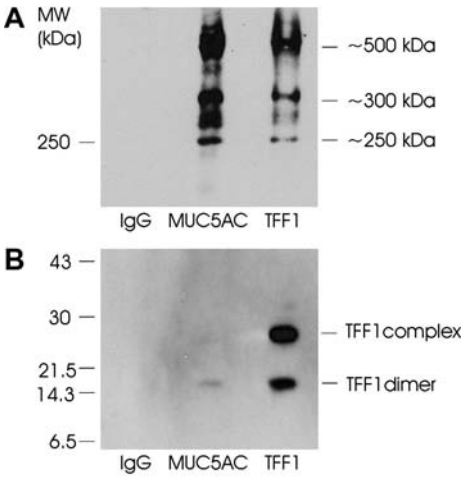

Figure 3. Co-immunoprecipitation of TFF1 and MUC5AC. Human gastric mucosa cytosol (50 µg protein) was immunoprecipitated with MUC5AC or TFF1 monoclonal antibodies or murine immunoglobulins and electrophoresed on a 6% SDS-PAGE gel for MUC5AC immunodetection (*A*) or on a 20% SDS-PAGE gel for TFF1 immunodetection (*B*), as described in Materials and methods. The positions of the molecular-mass markers are shown on the left and of the different forms of the proteins on the right.

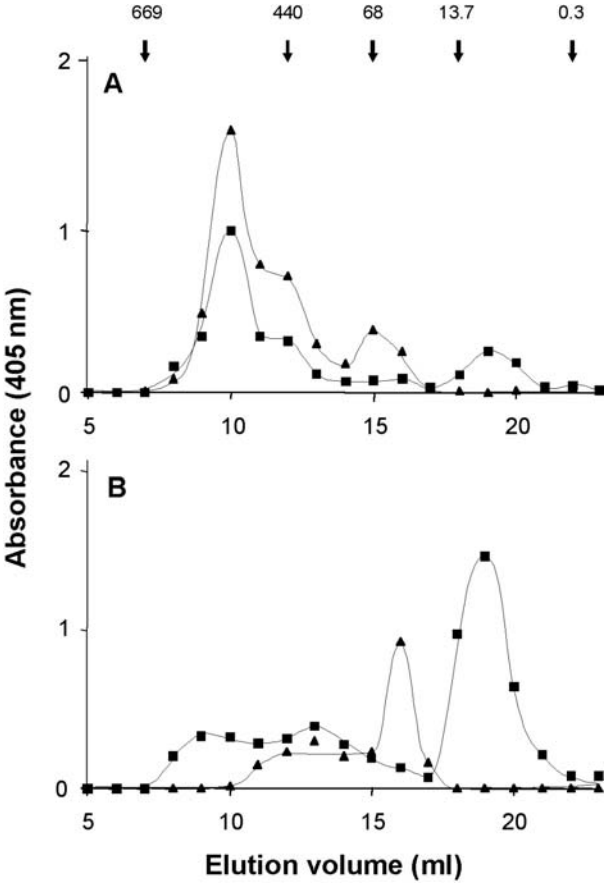

Figure 4. Effect of salt on the association between TFF1 and MUC5AC. Human gastric mucosa cytosol (2 mg of protein) was fractionated by gel filtration in sodium phosphate pH 6.5 in the absence (*A*) and presence (*B*) of 500 mM NaCl and the fractions were analysed for MUC5AC (triangles) and TFF1 (squares) by ELISA. Arrows show the elution positions of proteins used for the column calibration as described in Materials and methods.

the immunoprecipitate of MUC5AC (fig. 3A) and the same subunits/forms, albeit with slightly lower intensity, were detected in the immunoprecipitate of TFF1, whereas no MUC5AC immunoreactivity was present in the control immunoprecipitation with murine IgG. This shows that there is a direct interaction between MUC5AC and TFF1 and that TFF1 is associated directly with all fragments of MUC5AC detected in figure 2B. In the reciprocal experiment, TFF1 dimer was detected preferentially in the MUC5AC immunoprecipitate, whereas the TFF1 complex was the more prominent molecular form in the TFF1 immunoprecipitate (fig. 3B). The co-immunoprecipitation of TFF1 and MUC5AC demonstrates that there is a physical interaction between TFF1 and MUC5AC, and confirms that MUC5AC binds preferentially to TFF1 dimer.

High concentrations of salt disrupt the interaction between TFF1 and MUC5AC

The nature of the interaction between TFF1 and MUC5AC was investigated by gel filtration of human gastric mucosa cytosol in the absence and presence of NaCl. Fractions were collected and TFF1 and MUC5AC measured by ELISA. The elution profiles for TFF1 and MUC5AC in the absence of salt were similar to those shown in figure 1, with the major TFF1 peak at the same elution volume as the MUC5AC peak in the higher-molecular-mass fractionation range and a second TFF1 peak in the smaller-molecular-mass range (fig. 4A). The TFF1 elution profile was altered dramatically by the presence of 500 mM NaCl, resulting in loss of the mucin-bound TFF1 in the first peak, whereas the amount of unbound TFF1 in fractions 18–19 was augmented (fig. 4B). The inclusion of 500 mM NaCl decreased the MUC5AC high-molecular-mass peak with a corresponding appearance of lower-molecular-mass MUC5AC fragments. This suggests that either MUC5AC is degraded more rapidly in the presence of salt or, more probably, that the interactions between MUC5AC subunits are broken by high salt concentrations and that high salt concentrations disrupt the interactions between TFF1 and MUC5AC, which hold MUC5AC subunits together.

Calcium is involved in the interaction between TFF1 and MUC5AC

TFF1 and mucins are highly negatively charged and the possibility that divalent cations are involved in their interaction was investigated [31]. Gel filtration of gastric mucosa cytosol in the presence of 5 mM EDTA, a chela-

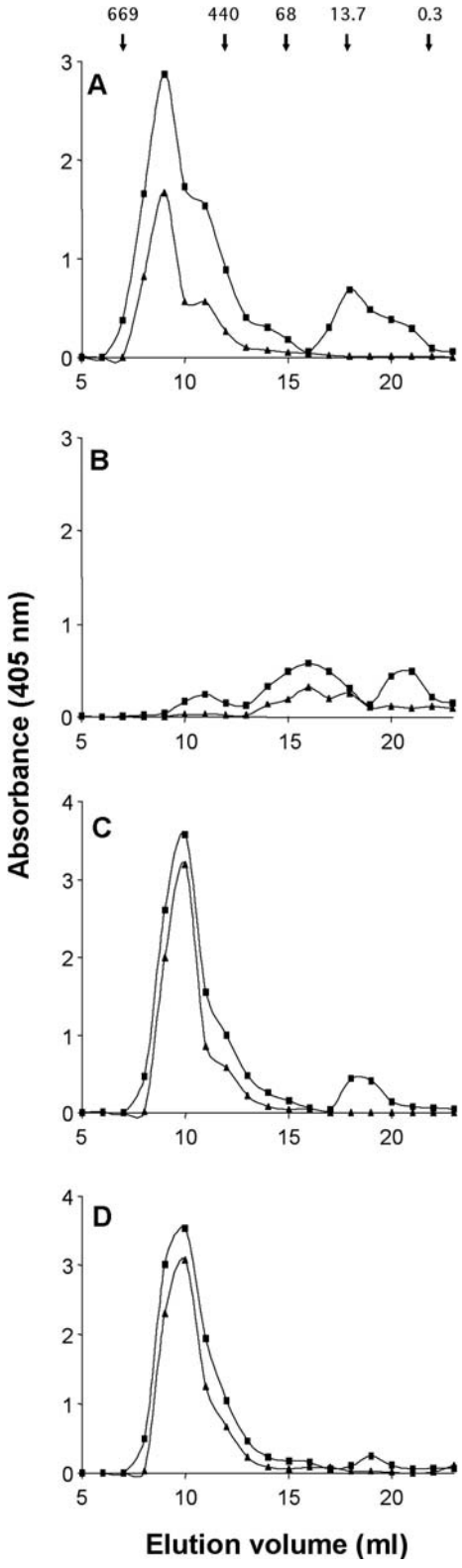

Figure 5. Effect of calcium ions on the association between TFF1 and MUC5AC. Human gastric mucosa cytosol (2 mg of protein) was fractionated by gel filtration in sodium phosphate, pH 6.5, in the absence (*A*) and presence (*B*) of 5 mM EGTA or in 50 mM Tris HCl, pH 6.5, in the absence (*C*) and presence (*D*) of 10 mM $CaCl_2$, and the fractions were analysed for MUC5AC (triangles) and TFF1 (squares) by ELISA. Arrows show the elution positions of proteins used for the column calibration as described in Materials and methods.

tor of divalent cations, led to a dramatic decrease in the amount of mucin-bound TFF1 detected in the first peak, whereas more TFF1 was detected in later fractions (data not shown). EDTA had a similar effect on MUC5AC. The possibility that Ca^{2+} is responsible for the interaction is suggested by the high concentrations of this divalent cation present in mucus granules [32]. This was investigated by gel filtration in the presence of EGTA, a calcium chelator, or Ca^{2+} ions. The effect of 5 mM EGTA was similar but more dramatic than that obtained in the presence of EDTA. TFF1 was not detected in fraction 9, where the mucin-bound TFF1 elutes when cytosol is run in phosphate buffer alone. TFF1 eluted as three broad peaks around fractions 11, 16 and 21 (fig. 5A, B). The MUC5AC elution profile was also dramatically affected by EGTA (fig. 6). The little MUC5AC that was detected eluted as a broad peak between fractions 14 and 20.

An opposite effect was observed when gel filtration was performed in the presence of 10 mM $CaCl_2$. The first peak became broader and a higher proportion of TFF1 was mucin bound, whereas the second peak containing unbound TFF1 was less prominent (fig. 6C, D). These data suggest a critical role for Ca^{2+} ions in the interaction of TFF1 with MUC5AC and again show the importance of this association in the stabilisation of MUC5AC.

Discussion

In this report, we showed a direct association between TFF1 and MUC5AC. The immunohistochemical results confirm the co-localisation of TFF1 and MUC5AC in mucus secretory cells [33] and the presence of TFF1 within the secretory granules [22, 34]. They demonstrate further that TFF1 is concentrated in the secretory vesicles in which mucins are packaged prior to release into the adherent mucus gel layer. This is consistent with the conclusions of the TFF1-null mice study [14] that TFF1 is important for the packaging, secretion and function of mucus. Using gel filtration of gastric cytosol we have shown that a proportion of TFF1 co-elutes with MUC5AC under physiological conditions. The TFF1 dimer is the predominant form associated with MUC5AC but, under these conditions, some TFF1 complex was also mucin associated. Crucially, co-immunoprecipitation studies revealed a physical interaction between TFF1 and MUC5AC and preferential binding of MUC5AC to the dimer form of TFF1. This association is, at least partially, dependent on ionic interactions and the presence of divalent cations.

Complete solubilisation of mucins requires high concentrations of chaotropic salts, such as 6 M guanidinium HCl or 6 M urea [35]. These denaturing conditions are important to protect against degradation by proteolytic enzymes. Reduced subunits of mucins are particularly sus-

ceptible to cleavage by proteases [36]. In the present study, a cocktail of proteases inhibitors was used to prevent mucin degradation. Guanidinium HCl and caesium chloride density gradient centrifugation were not used in the present study because the presence of chaotropic solvents and high salt concentrations might interfere with interactions between TFF1 and mucins. We have now shown that high concentrations of salt disrupt TFF1-MUC5AC interactions.

The degree of oligomerisation of gastric MUC5AC and MUC6 has not been established fully. Their multimerisation may involve non-reducible bonds, as has been shown for MUC2 [35]. Nordman and co-workers [37] found that porcine mucins derived from gastric glands were larger than those from the surface epithelium and have since suggested that human MUC6 monomers are larger than MUC5AC monomers [38]. In our experiments, MUC6 appeared smaller than MUC5AC from human gastric mucosa (fig. 3 A). This could be because MUC6 is more susceptible to degradation than MUC5AC, which is localised at the epithelium surface and therefore routinely exposed to enzymes present in the gastric lumen.

In view of the strong negative charge of both mucin and TFF1 [31], their co-localisation in mucus vesicles (fig. 2) and the high concentration of calcium in mucus granules [32, 39], calcium might plausibly have a role in the association between TFF1 and MUC5AC. Our results support this hypothesis and suggest that Ca^{2+} ions promote binding of the TFF1 dimer to MUC5AC. No evidence for the induction of local conformational change by Ca^{2+} ions has been found for the TFF1 dimer, or for changes in the oligomerisation of TFF1 with Ca^{2+} ions, in the pH range 5.6–7.6 or at different protein concentrations [24]. The TFF1 complex comprises one molecule of TFF1 associated with another molecule via a disulphide bond. Until the TFF1 complex is characterised, the possibility that it is affected by Ca^{2+} ions cannot be investigated.

The binding surfaces between TFF1 and MUC5AC remain to be determined. The repetitive domains of the mucin in the core of the protein are highly O-glycosylated and these oligosaccharides have been proposed as potential binding sites for interaction with trefoil proteins via the conserved hydrophobic patch situated between loops two and three [23–25]. Recently, screening of a murine gastric and duodenal cDNA library for proteins that interact with murine TFF1 using the yeast two-hybrid system identified two mucins MUC5AC and MUC2. They were shown to interact with TFF1 through the von Willebrand factor C1 and 2 cysteine-rich domains in this system [40].

Trefoil proteins were shown recently to alter the physical properties of mucins in solution, resulting in an increase in viscosity and elasticity and the transformation of a mucin solution into a gel-like structure [41]. The viscosity of the mucus gel was also augmented at low pH, which the authors suggested was due to hydrogen bonding. The involvement of trefoil proteins in mucus gel formation and stabilisation could contribute to the protective effects of mucins on the epithelial surface.

In conclusion, we have shown that TFF1 is co-packaged with MUC5AC within secretory vesicles, that it is bound to MUC5AC, and that the TFF1 dimer is preferentially involved in the interaction. This interaction is influenced by ionic strength and by calcium ions. These results provide conclusive evidence that human TFF1 and MUC5AC interact in vivo. This underpins the accumulation of evidence from in vitro studies [41] and animal models [14] of the important role of TFF1 in the production and stabilisation of mucins which are critical for the maintenance of the integrity of the mucus gel within the stomach.

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