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Review

Molecular medicine of TFF-peptides: from gut to brain

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Summary. TFF-peptides (i.e. TFF1, TFF2, TFF3; formerly P-domain peptides, trefoil factors) have been established as secretory products typical of the gastrointestinal tract. Their synthesis has recently been recognized in a number of mucin-producing epithelial cells, for example, of the respiratory tract, the salivary glands, the uterus and of the conjunctiva. They have a pivotal role in maintaining the surface integrity of these delicate epithelia as constituents of mucus gels as well as by their anti-apoptotic properties and their motogenic activity modulating cell migratory processes. The latter is important for rapid healing in particular of gastrointestinal and respiratory epithelia by a process termed "restitution". On the other hand, one of these peptides - namely TFF3 - has been detected as a new neuropeptide of the human hypothalamo-pituitary axis where it is synthesized in oxytocinergic neurons of the paraventricular and supraoptic nuclei. From there it is transported to the posterior pituitary where it is released into the blood stream. Synthesis of TFF-peptides also occurs pathologically as result to chronic inflammatory diseases, for example of the gastrointestinal tract. Aberrant synthesis of TFF-peptides is observed in many tumors.

Key words: TFF-domain, Mucins, Epithelia, Restitution, Cell migration, Ulcer, Inflammatory bowel disease, Pituitary, Neuropeptide, Oxytocin, Respiratory tract, Uterus, Salivary glands, Conjunctiva, Goblet cells

Introduction

Three trefoil factor family (TFF)-peptides (formerly P-domain peptides or trefoil factors; Hoffmann and Hauser, 1993) are known in mammals including human, namely TFF1, TFF2 and TFF3 (Table 1). The present nomenclature is based upon an agreement at a Conférence Philippe Laudat (Wright et al., 1997). These peptides consist of one (TFF1 and TFF3) or two (TFF2) TFF-domains. Remarkably, TFF1 and TFF3 form cysteine-bridged homodimers (Chinery et al., 1995; Chadwick et al., 1997; Kinoshita et al., 2000). Consequently, a pair of TFF-domains seems to represent the functional unit for TFF-peptides.

Human TFF1 (formerly pS2) via cDNA cloning of an estrogen-responsive gene from a breast cancer cell line was the first member of the family to be discovered (Masiakowski et al., 1982; Jakowlew et al., 1984). TFF2 (formerly spasmolytic polypeptide/SP) was detected almost coincidentally in porcine pancreas (Jorgensen et al., 1982; Thim et al., 1985; Rose et al., 1989); human TFF2 was characterized later (Tomasetto et al., 1990; Gött et al., 1996). The high similarity between TFF1 and TFF2 was first recognized in the course of analyzing TFF-domains from the frog integumentary mucin FIM-A.1 (formerly spasmolysin; Hoffmann, 1988). TFF3 (previously called intestinal trefoil factor/ITF or hP1.B) from rat intestine was the third mammalian TFF-peptide described (Suemori et al., 1991) and its human homolog was reported in 1993 (Hauser et al., 1993; Podolsky et al., 1993).

All three human TFF-genes are clustered on chromosome 21q22.3 where TFF1, TFF2 and TFF3 are arranged head to tail (Chinery et al., 1996; Gött et al., 1996; Hattori et al., 2000).

The minimum consensus sequence for TFF-domains reads (Hoffmann and Hauser, 1993):

$$C\text{-}X_{6\text{-}7}\text{-}R\text{-}X_2\text{-}C\text{-}G\text{-}X_8\text{-}C\text{-}X_4\text{-}C\text{-}C\text{-}X_9\text{-}W\text{-}C.$$

The six conserved cysteine residues form intramolecular disulfide bridges, which are responsible for the remarkable protease resistance of TFF-peptides, are paired in the manner C^1-C^5, C^2-C^4, C^3-C^6 (Thim, 1989; Kinoshita et al., 2000). The 3-D structures of porcine TFF2 (Carr, 1992; Gorman et al., 1992; Gajhede et al., 1992, 1993; Carr et al., 1994; De et al., 1994; Petersen et al., 1996) and the monomeric C58S mutant of human TFF1 (Polshakov et al., 1995, 1997) revealed unusual molecular surface features such as a prominent hydrophobic patch.

TFF-peptides have also been detected in amphibia (Table 1). The *Xenopus laevis* homolog of mammalian TFF1 is xP1 (Hauser and Hoffmann, 1991; Hoffmann and Hauser, 1993) and the functional equivalent of mammalian TFF2 seems to be xP4.1 consisting of four

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TFF-domains arranged in tandem (Hauser and Hoffmann, 1991; Jagla et al., 1998). The homolog of mammalian TFF3 is not known in *X. laevis* so far. The TFF-peptides xP4.2 (Botzler et al., 1999) and xP2 (Hauser et al., 1992) in *X. laevis* were discovered in the gastrointestinal tract and the skin respectively, but mammalian homologs are missing. xP4.2, a result of a gene duplication in *X. laevis*, might be a semi-homolog of mammalian TFF2 which shows a clearly different expression pattern in the esophagus and stomach when compared with xP4.1 (Botzler et al., 1999).

TFF-domains are also present in a variety of mosaic proteins. Human examples are zona pellucida proteins ZP1 and ZPB (Bork, 1993; Harris et al., 1994; Hughes and Barratt, 1999), as well as the human sugar degrading enzymes sucrase-isomaltase (Tomasetto et al., 1990), α-glucosidase (Hoefsloot et al., 1988) and maltase-glucoamylase (Nichols et al., 1998).

Normal cellular localization of TFF-peptides

Mammalian TFF-peptides were originally detected as secretory products of the gut. Recently, a number of human mucous epithelia were identified as a production site of TFF3 in particular. In these organs TFF-peptides are always co-secreted with mucins in clearly defined cell-specific distribution patterns (Table 2). TFF-peptides are also formed in minor amounts in the human brain.

TFF-peptides in mucous epithelia

TFF1/xP1

The stomach is the major source of mammalian TFF1 as well as its amphibian homolog xP1. This was shown, for example, for human (Rio et al., 1988) and *X. laevis* (Hauser and Hoffmann, 1991). Gastric TFF1/xP1 is localized within secretory granules of faveolae cells (Fig. 1), i.e. the surface epithelial cells and the pits (Rio et al., 1988; Piggott et al., 1991; Hanby et al., 1993b; Jagla et al., 1998; Newton et al., 2000) where mRNA is also detected (Tomasetto et al., 1990; Hauser and Hoffmann, 1991; Hanby et al., 1993b). Gastric faveolae in humans are known for their expression and secretion of the secretory mucin MUC5AC (Audie et al., 1993; Ho et al., 1995). Furthermore, minor amounts of TFF1 are detectable in human mucous neck cells (MNCs; Piggott et al., 1991; Hanby et al., 1999). TFF1 is also a constituent of the human gastric juice in a concentration range between 30 and 100 ng/ml (Rio et al., 1988).

Another physiologically important location of TFF1 is in the human conjunctiva (Fig. 2A), where goblet cells secrete this peptide together with the mucin MUC5AC as a constituent of the ocular mucus (Gipson and Inatomi, 1997; Langer et al., 1999).

TFF1 is also synthesized to some degree in human Brunner's glands, probably by the surface and upper duct cells but not in the acinar portion (Wright et al., 1990b; Hanby et al., 1993a; Khulusi et al., 1995). Minimal amounts of TFF1 mRNA are detectable in human salivary glands (Jagla et al., 1999; Devine et al., 2000) and the human respiratory tract (Wiede et al., 1999; dos Santos Silva et al., 2000).

TFF2/xP4.1

The stomach typically secretes the highest proportion of human TFF2 (consisting of two TFF-

Table 1. Compilation of TFF-peptides detected in various mucous epithelia of mammals and *X. laevis*, respectively.

NAME	ORIGIN	NUMBER OF TFF-DOMAINS	TYPICAL LOCALIZATION IN HUMAN AND X. LAEVIS EPITHELIA
TFF1	mammals	1	stomach, conjunctiva
TFF2	mammals	2	stomach, Brunner's glands
TFF3	mammals	1	intestine, salivary glands, uterus, respiratory tract, conjunctiva
xP1	*X. laevis*	1	stomach
xP2	*X. laevis*	2	skin
xP4.1	*X. laevis*	4	stomach
xP4.2	*X. laevis*	4	esophagus, stomach/corpus

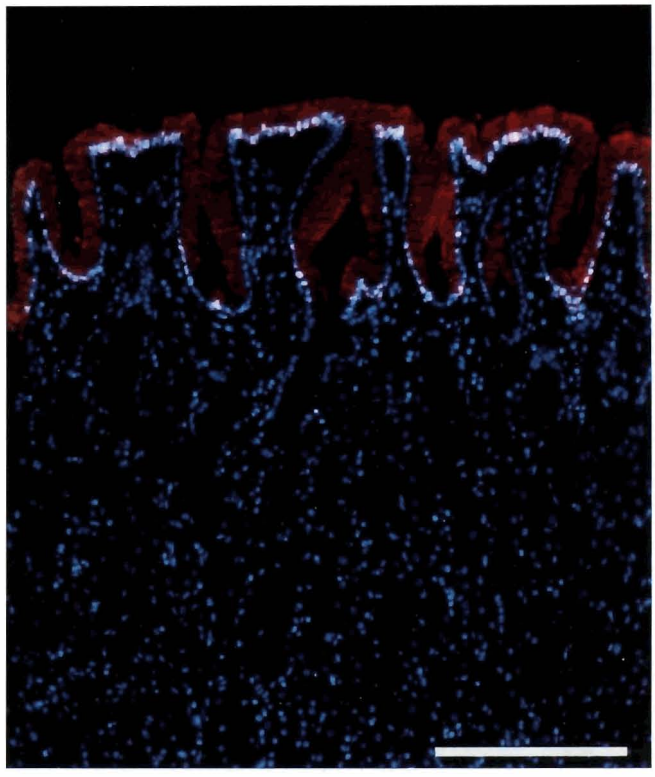

Fig. 1. Immunohistochemical localization of TFF1 in human gastric faveolae. Staining is with a polyclonal TFF1 antiserum (Novocastra, Newcastle) and immunofluorescence with Cy3-label; nuclei are counterstained with DAPI. Scale bar: 200 μm.

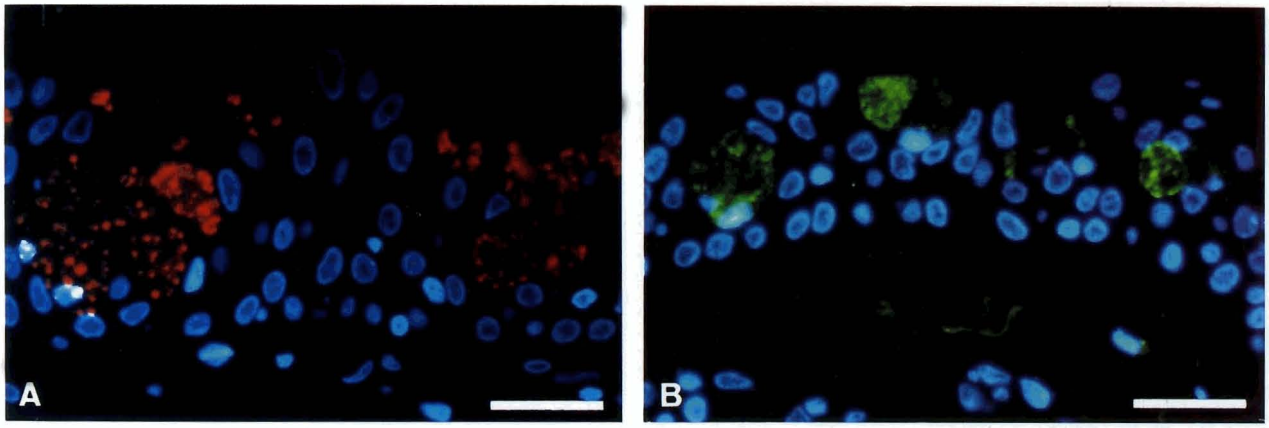

Fig. 2. Immunohistochemical localization of TFF1 or TFF3 in human conjunctival goblet cells. Staining is with a monoclonal TFF1 antiserum (Zymed Laboratories, San Francisco) and immunofluorescence with Cy3-label **(A)** or with antiserum anti-rTFF3-1 and immunofluorescence with fluorescein label **(B)**; nuclei are counterstained with DAPI. Scale bars: 50 μm. Reproduced from Langer et al. (1999) with permission of the Association for Research in Vision and Ophthalmology.

domains; Tomasetto et al., 1990) or in *X. laevis* its presumable homolog xP4.1 (consisting of four TFF-domains; Hauser and Hoffmann, 1991). Porcine stomach secretes only small amounts of TFF2 (Thim et al., 1982). Gastric TFF2/xP4.1 is predominantly released from MNCs and antral gland cells (Hanby et al., 1993b; Jagla et al., 1998). In human, these cells synthesize MUC6 as their major secretory mucin (Ho et al., 1995; Bartman et al., 1998). Only weak TFF2/xP4.1 staining was observed at the gastric surface epithelium. Thus, TFF1/xP1 and TFF2/xP4.1 show complementary cellular localization patterns within the stomach. TFF2 is present in the human gastric juice at concentrations between 1 and 20 μg/ml (May et al., 2000).

Species differences with respect to the synthesis of TFF2 in the pancreas are observed. For example, TFF2 is not detectable in normal human pancreas (Welter et al., 1992; Ohshio et al., 2000) whereas acinar cells of the porcine pancreas are the major source of TFF2 in this species (Thim et al., 1982; Rasmussen et al., 1992).

TFF2 is also expressed in human Brunner's gland duct epithelium (Wright et al., 1990b) and in Brunner's gland acini (Hanby et al., 1993a; Khulusi et al., 1995), which are known for their MUC6 synthesis (Ho et al., 1995; Bartman et al., 1998).

TFF3

This peptide has a quite different localization pattern when compared with TFF1 and TFF2 because expression is very scarce in the human stomach (Hauser et al., 1993; May and Westley). However, TFF3 is detectable in a variety of other human mucous epithelia lacking TFF1 and TFF2. A major source of TFF3 are human intestinal goblet cells (Hauser et al., 1993; Podolsky et al., 1993) which typically secrete the mucin MUC2 and MUC3 (Audie et al., 1993; Chang et al., 1994).

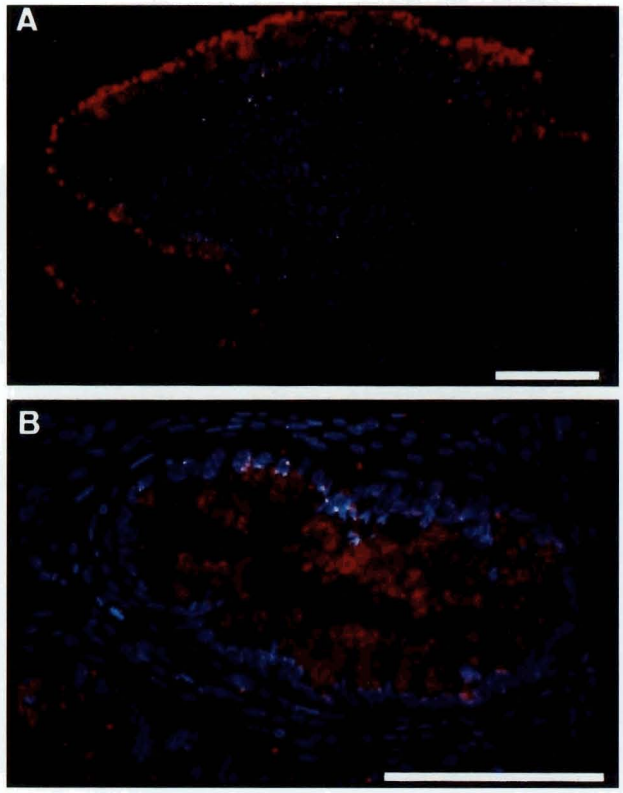

Fig. 3. Immunohistochemical localization of TFF3 in the human endocervix. Staining of epithelial cells **(A)** and gland-like structures **(B)** with antiserum anti-rTFF3-1 and immunofluorescence with Cy3-label; nuclei are counterstained with DAPI. Scale bars: 100 μm.

TFF3 is also found in the human uterus, where it is mainly released from the surface epithelium and gland-like structures of the endocervix (Fig. 3) but not from the endometrium (Hauser et al., 1993; Wiede et al., 2001).

Here, co-secretion with MUC5B probably occurs (Wickström et al., 1998).

A further physiologically important site of TFF3 production is the human respiratory tract (Wiede et al., 1999; dos Santos Silva et al., 2000), where TFF3 is secreted predominantly by mucous cells of the submucosal glands (together with MUC5B; Fig. 4) but also to some extent by goblet cells (together with MUC5AC and MUC5B; Hovenberg et al., 1996; Wickström et al., 1998; Wiede et al., 1999).

Human salivary glands represent another site of TFF3 synthesis (Fig. 5). Relatively large amounts of TFF3 are stored only within serous cells of human submandibular glands (Jagla et al., 1999). These cells are known for their MUC7 synthesis (Nielsen et al., 1996, 1997). However, TFF3 mRNA is also detectable in human sublingual (Jagla et al., 1999; Devine et al., 2000) and in parotid glands as well as in minor mucous glands (Devine et al., 2000). In contrast to the immunohistochemical localization (Fig. 5), expression of TFF3 mRNA has been reported to occur in mucous but not in serous acini of submandibular glands (Devine et al., 2000).

TFF3 is also a characteristic secretory product of human conjunctival goblet cells (Fig. 2B; Langer et al., 1999). Together with TFF1 and MUC5AC it is probably a constituent of the mucus layer of the eye's complex tear film and might contribute to its rheological properties.

TFF-peptides in the brain

TFF1 and TFF2

TFF1 is widely distributed throughout the adult rat brain with pronounced expression in the hippocampus, frontal cortex and the cerebellum (Hirota et al., 1995). Expression of TFF1 mRNA in the hippocampus reaches a maximum around birth when maturation occurs and then gradually decreases. Astrocytes, but not neurons, have been reported to represent the major site of hippocampal TFF1 synthesis in newborn rats (Hirota et al., 1995). Expression of TFF1 mRNA in cultured mouse astrocytes changed during the cell cycle (Hirota et al., 1994b) and can be markedly induced by various cytokines (IL-6, IL-7 and TNF-α; Hirota et al., 1994a). There are neither reports on localization studies of TFF1 in human brain nor are there data published concerning

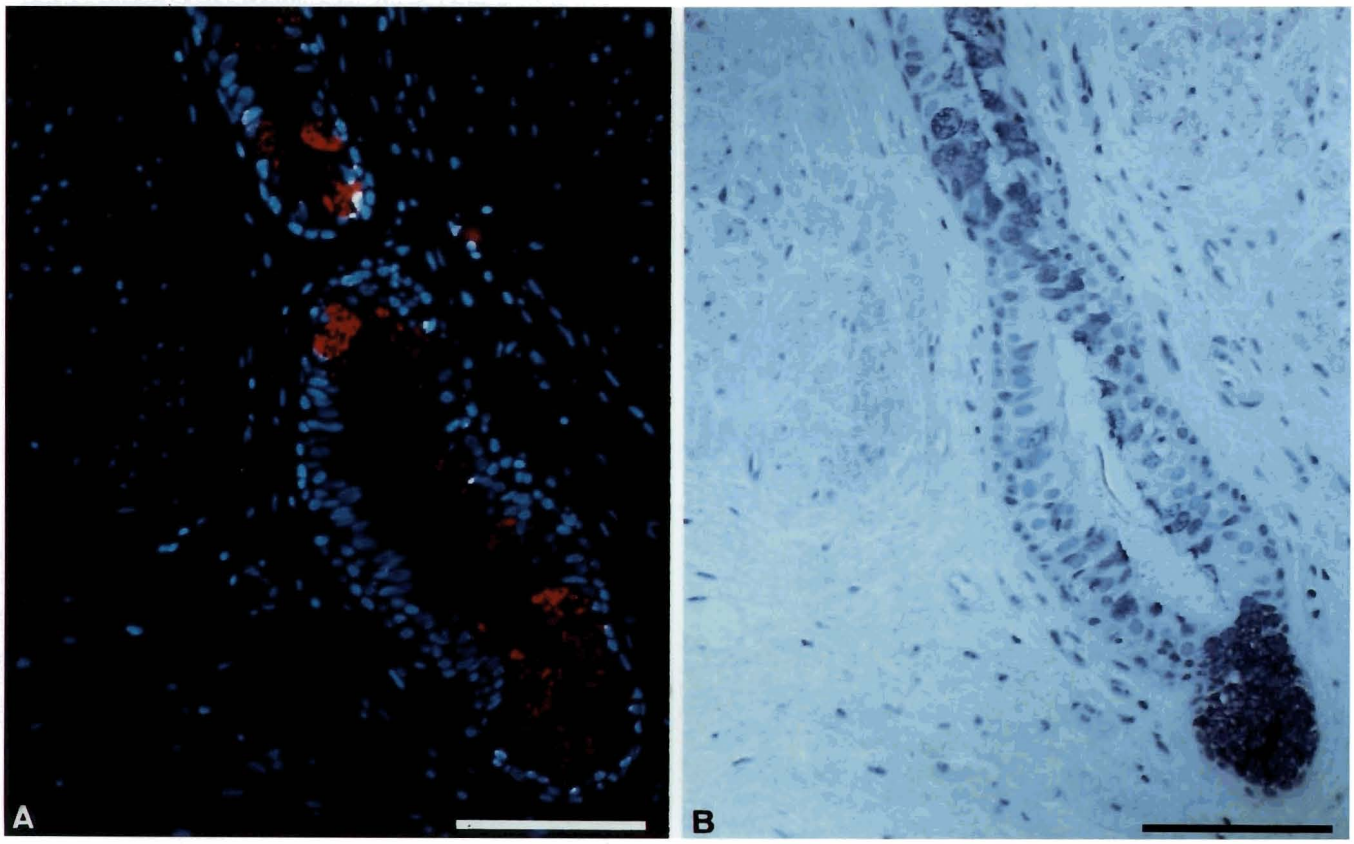

Fig. 4. Immunohistochemical localization of TFF3 in submucosal glands of human bronchi. **A.** Staining of mucous cells using antiserum anti-rTFF3-1 and immunofluorescence with Cy3-label; nuclei are counterstained with DAPI. **B.** Mucin staining of parallel section with PAS/alcian blue. Scale bars: 100 μm.

neural expression of TFF2.

TFF3

This peptide has been detected in magnocellular neurons of the rat and human hypothalamus (Fig. 6; Probst et al., 1995, 1996). The oxytocinergic neurons in the human paraventricular and supraoptic nuclei were shown to be the sole site of TFF3 synthesis in these nuclei; the distinct population of vasopressinergic neurons is completely devoid of TFF3 (Jagla et al., 2000). Thus, TFF3 is a new neuropeptide of the hypothalamo-pituitary axis transported together with oxytocin to the neurohypophysis. TFF3 - but not TFF1 or TFF2 - is stored here in relatively large amounts awaiting release into the blood stream. Noteworthy is the M_r of TFF3 from the posterior pituitary as estimated from gel electrophoresis which appeared somewhat higher than corresponding material from the duodenum, probably due to an unknown posttranslational modification (Fig. 7). This may influence the stability of TFF3 in the blood. TFF3 is also detectable in human post mortem cerebrospinal fluid obtained from the third ventricle (Jagla et al., 2000). This observation would fit with a report claiming localization of TFF3 in human tanycytes lining the third ventricle (Griepentrog et al., 1999).

Pathological expression of TFF-peptides

Wounding response and inflammatory diseases

Aberrant expression of TFF-peptides was widely observed during various chronic inflammatory diseases; for example in ileal Crohn's disease, in colon mucosa of patients with ulcerative colitis and in gallbladder during acute cholecystis (Rio et al., 1991), in the pancreas of patients with chronic pancreatitis (Wright, 1994; Ebert et al., 1999), in gastric glands during gastric ulcer disease (Hauser et al., 1993), in various types of metaplasia (Hanby et al., 1993a; Khulusi et al., 1995; Shaoul et al., 2000), in different hyperplastic polyps (Hauser et al., 1993; Hanby et al., 1993c; Nogueira et al., 1999) and in Barrett's oesophagus (Hanby et al., 1994; Labouvie et al., 1999).

Furthermore, it was shown that expression of TFF-

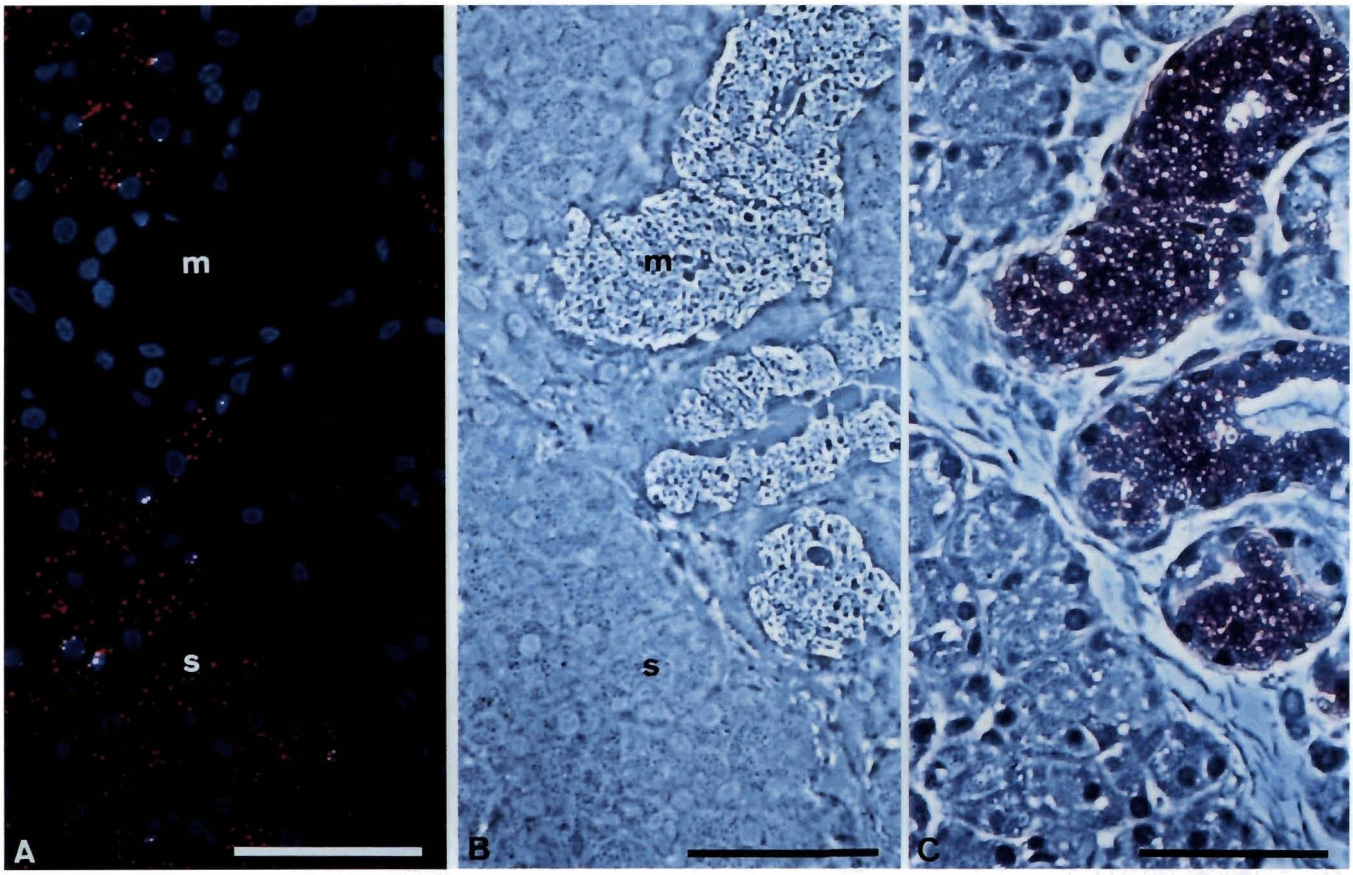

Fig. 5. Immunohistochemical localization of TFF3 in human submandibular gland. **A.** Immunofluorescence of serous cells (s) with antiserum anti-rTFF3-1 and Cy3-label; nuclei are counterstained with DAPI. **B.** Phase-contrast image of A. **C.** staining of mucous cells (m) on parallel section with PAS/alcian blue. Scale bars: 50 μm.

peptides is a typical response after gastric mucosal damage in various rat models of experimental ulceration (Alison et al., 1995; Konturek et al., 1997, 1998). Here, an ordered sequence of gene expression including epidermal growth factor receptor (EGFR), c-met, EGF, transforming growth factor alpha (TGF-α) and hepatocyte growth factor (HGF) was observed (Wong et al., 2000); in particular, TFF2 was identified as an early gastric response gene whereas TFF3 was expressed later in response. In contrast, TFF1 was expressed during the acute phase of acid-induced colitis in the rat rectum (Itoh et al., 1996). Expression of TFF-peptides was also induced in gastrointestinal tissues of embryonic mice after incisional wounds (Otto and Patel, 1999).

The discovery that all three TFF-peptides are typically secreted by a specific gland-like structure termed the ulcer-associated cell line (UACL) was certainly a major hallmark (Wright et al., 1990a,b; Hauser et al., 1993). This mucin-secreting glandular structure appears during a variety of chronic inflammatory conditions and contains a clearly defined proliferative zone about two-thirds of the way up to the duct. It develops from stem cells, most commonly in the small intestine, particularly in Crohn's disease and duodenal ulcer disease. The UACL is thought to represent a natural repair kit which is activated after mucosal damage (Wright, 1998). It has also been detected in the colon, in pancreatic ducts in chronic pancreatitis, in gall bladder in chronic cholecystitis, in the fallopian tube in chronic salpingitis and in inflammatory nasal polypi (Wright, 1998). Interestingly, TFF-genes show different expression patterns within the UACL, i.e. TFF1 in the upper ductular portion and surface cells, TFF2 (together with EGF) in the lower duct and acinar portion and TFF3 (together with TGF-α, lysozyme and neutral mucins) throughout the gland (Ahnen et al., 1994; Patel et al., 1994; Wright, 1998).

Hyperplastic neuroendocrine cells adjacent to the UACL express significant amounts of TFF1 (Wright et al., 1993). Noteworthy is that this leads to a release of TFF1 into the immediate local circulation and not to a luminal co-secretion with mucins.

Tumors

Haphazard pathological expression of TFF-peptides has been observed in many epithelial tumors (Henry et al., 1991; Luqmani et al., 1992) including the breast (Rio et al., 1987; Poulsom et al., 1997), oesophagus (Labouvie et al., 1999), stomach (Luqmani et al., 1989; Theisinger et al., 1991; Machado et al., 2000), biliary tract (Seitz et al., 1991; Welter et al., 1993), pancreas (Welter et al., 1992; Ohshio et al., 2000), colon (Welter et al., 1994; Taupin et al., 1996; Uchino et al., 1997; Häckel et al., 1998), lung (Higashiyama et al., 1994), ovary (Wysocki et al., 1990), endometrium (Koshiyama et al., 1997), cervix (Olatinwo et al., 1996), skin (Hanby et al., 1998) and various neuroendocrine tumors (Bonkoff et al., 1995; Wang et al., 1997, 1998).

A general prognostic value of aberrant TFF-peptide expression in tumors is under debate. For example, TFF3 is significantly up-regulated in colorectal carcinomas with a mucinous phenotype known to bear an especially poor prognosis (Taupin et al., 1996) whereas TFF1 expression in breast cancer seems to be associated with a longer survival of the patients (Ribiereas et al., 1998; Balleine and Clarke, 1999). Besides aberrant expression, also somatic mutations and loss of heterozygosity, in particular of the TFF1 gene, were detected in gastric adenoma and carcinoma (Park et al., 2000). This suggests that TFF1 is a gastric-specific tumor-suppressor gene.

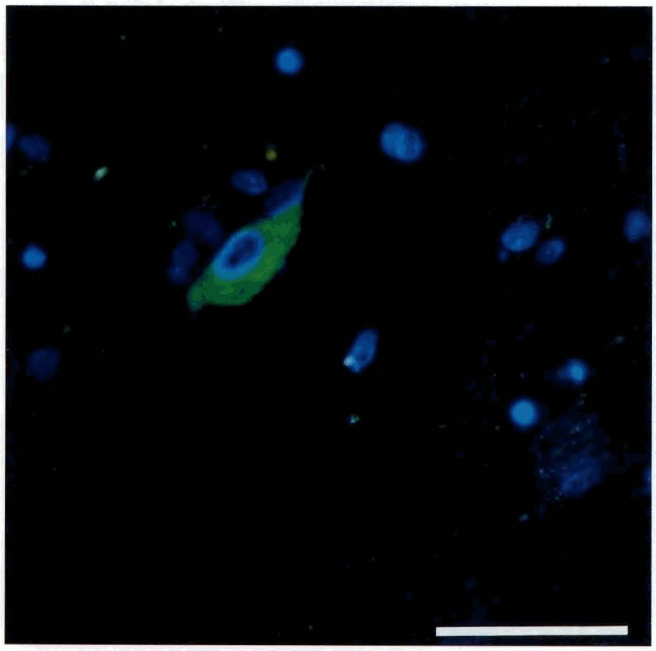

Fig. 6. Immunohistochemical localization of TFF3 in human hypothalamus. Immunofluorescence of magnocellular neuron in the paraventricular nucleus with antiserum anti-hTFF3-1 and fluorescein label; nuclei are counterstained with DAPI. Scale bar: 50 μm.

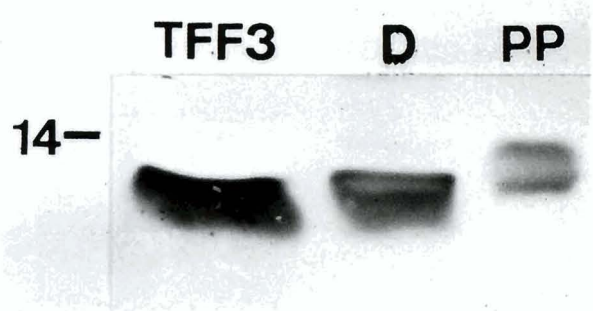

Fig. 7. Detection of TFF3 in extracts of human duodenum (D) or posterior pituitary (PP) after SDS-polyacrylamide gel electrophoresis (15%) and subsequent Western blot analysis using affinity purified antiserum anti-hTFF3-2. Recombinant human TFF3 (kindly provided by Dr. L. Thim) is used as a control. The molecular size standard is shown on the left.

Biological effects of TFF-peptides

Protective and healing effects in vivo

There are numerous in vivo studies which clearly document positive effects of all three TFF-peptides on the repair of ulcerated areas in the gastrointestinal tract. Additionally, transgenic animals lacking TFF1 or TFF3 show abnormalities of these gastrointestinal mucosae.

Subcutaneous infusion of dimeric TFF1 (50 μg.kg^{-1}.h^{-1}) prevents indomethacin-induced gastric damage in rats by about 70%, whereas monomeric C58S mutant TFF1 reduced injury by only about 30% (Marchbank et al., 1998). This protective effect of TFF1 is in line with results from transgenic mice overexpressing TFF1 in the jejunum, where the amount of jejunal damage caused by indomethacin was markedly reduced when compared to control animals (Playford et al., 1996). Transgenic mice lacking TFF1 failed to develop a functional antral and pyloric gastric mucosa. All TFF1(-/-) mice developed antropyloric adenoma, 30% of which progressed to carcinomas (Lefebvre et al., 1996). Noteworthy is that about 70% of these animals failed to express gastric TFF2 (but had normal pancreatic TFF2 synthesis) indicating coordinated expression of TFF1 and TFF2 in the stomach as already suggested from their genomic analysis (Gött et al., 1996).

Subcutaneous infusion of recombinant human TFF2 (50 μg.kg^{-1}.h^{-1}) in rats was shown to reduce indomethacin- and restraint-induced gastric damage (Playford et al., 1995) as well as stress-induced ulcerations (Konturek et al., 1997) by about 50%. A tenfold higher subcutanous dose of 500 μg.kg^{-1}.h^{-1} human recombinant TFF2 does not further reduce indomethacin-induced damage (McKenzie et al., 1997). Oral administration of up to 200 μg.kg^{-1} TFF2 had no effect (Playford et al., 1995); only significantly higher oral/intragastric doses of TFF2 showed protective effects in the rat. For example, 5 mg of recombinant human TFF2 reduced indomethacin-induced gastric damage by about 40% and ethanol-induced gastric injury by about 70% (Babyatsky et al., 1996). An oral dose of about 3 mg recombinant human TFF2 protects rats against aspirin-induced gastric injury by about 70% (Cook et al., 1998). A daily intrarectal application of 0.5 mg recombinant human TFF2 for five days reduced the inflammatory index by 50% in a rat model of dinitrobenzene sulfonic acid-induced colitis (Tran et al., 1999).

Porcine TFF2 was also able to prevent indomethacin-induced gastric damage in rats by 40% when infused subcutaneously (50 μg kg^{-1}.h^{-1}; McKenzie et al., 1997). In another study, subcutaneous injection of porcine TFF2 (50 μg.kg^{-1} three times a day) reduced indomethacin-induced gastric ulcers in rats by 38%; surprisingly, oral application of porcine TFF2 (2 mg per day in the drinking water) showed an even better protection by about a 62% decrease of ulcerated area (Poulsen et al., 1999). In contrast, strong aggravation of mercaptamine-induced duodenal ulcers was observed in rats after subcutanous as well as oral application of porcine TFF2 (Poulsen et al., 1999).

5 mg orally applied recombinant rat TFF3, produced in yeast, reduced indomethacin-induced gastric damage in rats by about 60% and ethanol-induced gastric injury by more than 90% (Babyatsky et al., 1996). In contrast, subcutaneous infusion of recombinant rat TFF3 produced in *Escheria coli* (150 μg.kg^{-1}.h^{-1}) had no protective effect in a rat model of indomethacin- and restraint-induced gastric damage; however, there was a dramatic reduction in the amount of gastric lesions by 80% when TFF3 infusion was accompanied by infusion of human EGF (1 μg.kg^{-1}.h^{-1}; Chinery and Playford, 1995). This implies a synergistic effect of TFF3 and EGF; infusion of EGF had no significant effect on its own. Further evidence for the protective effect of TFF3 was obtained from transgenic mice lacking TFF3 which showed impaired mucosal healing. The otherwise trivial colonic injury induced by 2.5% dextran sodium sulfate is lethal in these animals; rectal application of recombinant TFF3 resulted in reconstitution of normal healing of acetic acid-induced lesions (Mashimo et al., 1996). Remarkably, these TFF3(-/-) animals showed reduced expression of gastric TFF1 and TFF2, once again demonstrating co-ordinated expression of the TFF-peptides in the stomach (Taupin et al., 1999).

Taken together, there is strong evidence for protective and healing functions of TFF-peptides, at least in the gastrointestinal tract. Noteworthy is that systemic subcutaneous application of TFF-peptides to the basolateral side of the mucosa seems to be more effective than oral application in most studies.

Action as motogens in in vitro models of restitution

Rapid repair, particularly of the gastric and respiratory mucosae, is observed after superficial injury of these epithelia by a process termed "restitution" (Silen and Ito, 1985). This healing process starts within minutes after damage well before proliferation or extensive inflammatory processes occur and is defined by migration, for example, of gastric surface mucous cells to cover the denuded area (Lacy, 1988; Erjefält et al., 1995). The TFF-peptide familiy was proposed to have a role in cell migratory processes because expression of TFF-peptides is rapidly up-regulated after wounding or during ulcerations. A classical in vitro model to investigate restitution is to monitor migration of cultured rat intestinal epithelial IEC-6 cells after in vitro wounding (McCormack et al., 1992). Since then positive motogenic effects have been documented for all three TFF-peptides using various cell lines.

Recombinant dimeric human TFF1 ($5x10^{-7}$ M) significantly increased migration of cultured HT29 cells on plastic dishes whereas the monomeric C58S mutant TFF1 had no motogenic effect, even at much higher concentrations (Marchbank et al., 1998).

A similar motogenic effect on the human colonic

carcinoma cell lines HT29 and LIM1215 was observed for recombinant human TFF2 at concentrations >10^{-6} M (Playford et al., 1995; Wilson and Gibson, 1997). Furthermore, human recombinant glycosylated TFF2 ($6.8x10^{-6}$ M) was reported to dramatically stimulate migration of HT29 cells through 8-μm pore polycarbonate filters in a transwell motility assay (Efstathiou et al., 1999). Recombinant human TFF2 also enhanced repair of a wounded monolayer of IEC-6 cells ($8.4x10^{-6}$ M TFF2; Dignass et al., 1994) and primary oxyntic cultures (Kato et al., 1999).

Migration of HT29 cells on plastic dishes could also be enhanced by 10^{-8} M recombinant TFF3 and a marked synergistic effect was reported with 10^{-8} M EGF (Chinery and Playford, 1995). Recombinant human or rat TFF3 also significantly induced restitution of IEC-6 cells ($7.6x10^{-5}$ M TFF3; Dignass et al., 1994), LIM1215 cells ($3.4x10^{-5}$ M TFF3; Wilson and Gibson, 1997) and primary oxyntic cultures (Kato et al., 1999).

In line with the motogenic effect of TFF-peptides is the observation that transfection of an adenocarcinoma cell line with TFF1 enhances dispersed growth in a 3-D collagen gel and reduces extracellular matrix deposition (Williams et al., 1996). A similar tendency to form smaller and more dispersed colonies in a 3-D collagen gel was reported for a colon carcinoma cell line stably transfected with a vector expressing TFF3 (Uchino et al., 2000).

Effect on apoptosis

$5x10^{-8}$ M TFF2 reduced apoptosis of MCF-7 breast cancer cells by 30% after 19 days in culture (Lalani et al., 1999). Furthermore, a TFF3-expressing HT29 cell line was resistant to induced apoptosis, and exogenous TFF3 at a concentration of about $7.6x10^{-5}$ M protected HCT116 and IEC-6 cells from apoptosis (Taupin et al., 2000). The anti-apoptotic effect required intact TFF3 dimer and phosphorylation of the EGFR (Kinoshita et al., 2000). These results obtained in vitro are in line with the in vivo observation that TFF3(-/-) mice have increased intestinal apoptosis (Taupin et al., 2000). TFF3 prevented also IEC-18 cells from apoptosis and induced activation of transcription factor NF-κB (Chen et al., 2000).

These results of clear anti-apoptotic effects of TFF2 and TFF3 stand in direct contrast to the previous report of Efstathiou et al. (1998), who describe a detachment of HT29 cells and an increase in the number of apoptotic nuclei after treatment with recombinant TFF3 (10^{-9} and 10^{-8} M).

Effect on nitric oxide synthesis

Binding of a biotinylated TFF3 fusion protein was reported to increase the level of type II nitric oxide (NO) synthase as well as the NO production in IEC-18 cells (Tan et al., 1999).

The TFF-receptor problem and signaling cascades triggered by TFF-peptides

There are no clear molecular cloning data published in the last 15 years unambigously describing TFF-receptors in spite of circumstantial evidence for their existence. Early studies reported that porcine TFF2 bound to the small intestine of the rat inhibiting adenylate cyclase activity (Frandsen et al., 1986; Frandsen, 1988). Further binding sites were found in the gastric faveolar epithelium and colonic crypts of histological sections using a ß-galactosidase-TFF3 fusion protein (Chinery et al., 1993). TFF2 or TFF3 cross-linked to rat jejunal membrane preparations led to the identification of a protein complex showing TFF3-stimulated tyrosine phosphorylation with a M_r of about 45,000 or 28,000 after reduction; in vitro autoradiography of [^{125}I]-labeled TFF3 revealed binding to rat gastric mucous neck cells as well as jejunal and colonic crypts (Chinery and Cox, 1995a). Further in situ binding assays using a biotinylated TFF3 fusion protein also stained rat gastric mucous neck cells and small (but not large) intestinal crypt cells; ligand blotting showed that this TFF3 fusion protein bound to a glycosylated membrane protein from rat small intestine with a M_r after reduction of about 50.000 (Tan et al., 1997). This size of a putative TFF3-receptor is similar to that claimed by Podolsky (1997). Binding sites on mucous neck cells and Paneth cells of the small intestine were also observed in vivo after intravenous administration of porcine [^{125}I]-TFF2 in rats (Poulsen et al., 1998); in this study, pyloric glands and Brunner's glands were also labeled. In recent studies a ß-subunit of the fibronectin receptor and a transmembrane protein (M_r: 224,000) with similarity to CRP-ductin/muclin/Ebnerin were characterized from porcine stomach due to their binding capacity of porcine TFF2 (Thim and Mortz, 2000). Thus, the characterization of putative TFF-receptors is certainly more complex than expected and far away from being finished.

However, there are numerous publications describing different signaling cascades triggered by TFF-peptides. 10^{-7} M recombinant human dimeric TFF3 was reported to decrease tyrosine phosphorylation of mitogen-activated protein (MAP) kinases ERK1/2 in IEC-6 cells within 1 minute; this ERK inhibition was claimed to be blocked by sodium orthovanadate (Kanai et al., 1998). This is in sharp contrast to a previous report which detected increased ERK phosphorylation by TFF3 monomer in IEC-6 cells for the first time (Boxberger et al., 1998). Furthermore, $7.6x10^{-7}$ M recombinant dimeric TFF3 (or alternatively $8.3x10^{-7}$ M recombinant TFF2) also stimulated phosphorylation of ERK1 in KATO-III cells; maximum activation occured 5 minutes after stimulation and could be inhibited by the protein kinase inhibitor genistein (Taupin et al., 1999). Later on, the TFF-induced phosphorylation of ERK1, which is thought to be triggered by the Ras/MEK pathway, was shown to parallel the motogenic effect and did not

require TFF3 dimerization (Kinoshita et al., 2000). Thus, the motogenic effect of TFF-peptides is perfectly in line with the well known fact that ERK phosphorylation is essential for cell migration processes leading to phosphorylation of myosin light-chain kinase (Klemke et al., 1997).

Surprisingly, the TFF-triggered ERK activation is accompanied often by phosphorylation of the EGFR. For example, TFF2 ($8.3x10^{-7}$ M) or TFF3 (10^{-2} M and $7.6x10^{-7}$ M) caused transient EGFR phosphorylation in HT29, COS-1 or AGS cells (Liu et al., 1997; Taupin et al., 1999; Kinoshita et al., 2000). All attempts, however, have failed to demonstrate direct binding of porcine TFF2 or human TFF3 to the EGFR (Otto et al., 1996; Taupin et al., 1999). The activation of the EGFR was shown recently not to be necessary for the motogenic activity of TFF3 (Kinoshita et al., 2000); but it could be responsible for the synergistic effect of EGF and TFF3 on migration of HT29 cells in vitro and for protection in vivo (Chinery and Playford, 1995); it could also be the reason for the modulatory effect of TFF3 upon EGF responses on ion transport phenomena (Chinery and Cox, 1995b).

Furthermore, TFF3 triggered phosphorylation of the EGFR is required to prevent p53-independent apoptosis in HT29 cells through a signaling pathway dependent on phosphatidylinositol-3-kinase (PI-3-K), and protein kinase B (PKB)/Akt inhibiting the cleavage of poly(ADP-ribose) polymerase (PARP; Taupin et al., 2000). This EGFR/PI-3-K-dependent anti-apoptotic pathway was also activated by dimeric TFF3 in AGS cells after induction of p53-dependent apoptosis with etoposide; a mutant TFF3 not capable of dimer formation was inactive in protecting the cells from apoptosis (Taupin et al., 2000). Recently, Chen et al. (2000) reported on an anti-apoptopic effect of dimeric TFF3 for IEC-18 cells via an alternative signaling cascade requiring PI-3-K, PKB and activation of transcription factor NF-κB.

Thus, TFF3 seems to have a complex function. On the one hand, a single TFF-domain is capable of triggering migration processes by activating the MAPK pathway independently of the EGFR. On the other hand, dimeric TFF3 is required to inhibit apoptosis in an EGFR-dependent fashion (Kinoshita et al., 2000); phosphorylation of the EGFR by TFF3 probably occurs via transactivation or with the help of adaptor molecules.

The very high concentration of 10^{-2} M TFF3 was reported to induce a sustained tyrosine phosphorylation of ß- and γ-catenins in HT29 cells which follows a transient phosphorylation of the EGFR (maximum after about 10 seconds; Liu et al., 1997). Treatment of these cells with 10^{-9} or 10^{-8} M TFF3 for 24 hours reduced the levels of E-cadherin, α-catenin, ß-catenin and the adenomatous polyposis coli (APC) gene product leading to a significant disturbance of cell aggregation, detachment of cells from the substratum and translocation of APC from the cytoplasm to the nucleus with nucleolar accentuation; the down-regulation of APC, E-cadherin, α-catenin and ß-catenin could be inhibited by tyrphostin A25, an inhibitor of protein tyrosin kinases (Liu et al., 1997; Efstathiou et al., 1998). TFF3 thus seems to be capable of triggering protein tyrosine phosphorylation with involvement of the cadherin-catenin complex. Noteworthy is that phosphorylation of ß-catenin is known to be strictly regulated during cell migration (Müller et al., 1999). This link to cell migration processes is further supported by the observation that E-cadherin is essential for the motogenic effect of TFF2 (Efstathiou et al., 1999). The nuclear translocation of APC was suggested to influence ribosomal RNA synthesis and mitosis (Neufeld and White, 1997; Deka et al., 1999).

Interaction with mucins and the rheology of mucus

TFF-peptides are co-secreted with mucins and are intimate constitutents of mucus gels. It was postulated, as a consequence of this co-localization, that TFF-peptides interact with mucins as "link-peptides" influencing the rheological properties of these complex viscous biopolymers (Hauser et al., 1993). The TFF1 dimer was shown to be strongly associated with gastric mucins even after caesium chloride density gradient centrifugation in the presence of 6M guanidine hydrochloride (Newton et al., 2000).

A direct interaction of TFF1 and cysteine-rich domains at the C-termini of mouse Muc2 and mouse Muc5AC has been reported only recently (Tomasetto et al., 2000). These results are in contrast to previous

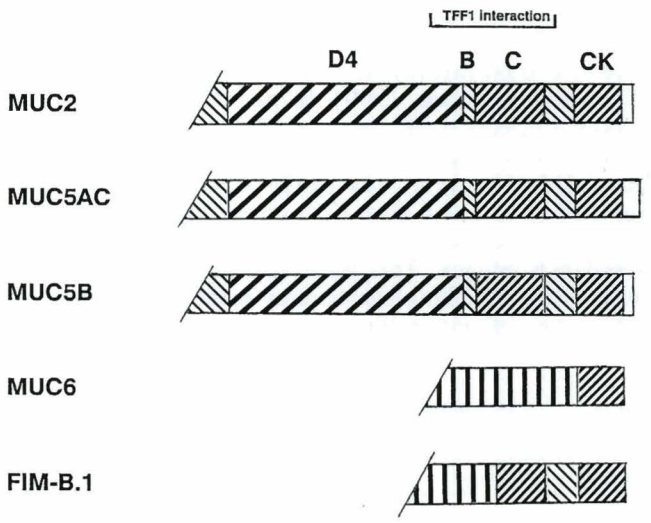

Fig. 8. Schematic representation of the C-termini of the vWF-type mucins MUC2, MUC5AC, MUC5B, MUC6 (Buisine et al., 1998) and FIM-B.1 (Probst et al., 1990) and their possible interaction with TFF1 based upon the report by Tomasetto et al. (2000). Cysteine-rich modules are hatched and domains homologous with vWF are indicated: D4-domain, B-like domain, C-domain, and cysteine knot motif (CK). Highly O-glycosylated serine/threonine-rich sequences are indicated by vertical bars.

suggestions that TFF-peptides might interact with the oligosaccharide moiety of mucins (Gajhede et al., 1993). Probably, both the B-like as well as the C-domains homologous with von Willebrand factor (vWF) are involved in the direct interaction with TFF1 as illustrated in Fig. 8. The molecular function of the C-domain in vWF or mucins is not yet known. However, a deletion of the C1C2 region prevented secretion of vWF (Voorberg et al., 1991). A direct interaction of TFF-domains with the vWF-type mucins MUC6 and frog integumentary mucin FIM-B.1 cannot be expected based on the results of Tomasetto et al. (2000) because FIM-B.1 contains only a truncated C-domain and no B-like domain and both the B-like domain and the C-domain are completely missing in MUC6 (Fig. 8).

Whether TFF-peptides interact with mucins during the secretory pathway, influencing the proteolytic cleavage or oligomerization of certain mucins, is currently not known. The direct interaction of TFF-peptides and some vWF-type mucins however might explain preliminary observations that TFF-peptides change the rheological properties of mucous gels (Dignass et al., 1994). The report on specific TFF-peptide/mucin interactions by Tomasetto et al. (2000) is also a challenge to understand the molecular architecture of frog skin mucus consisting of the TFF-domain containing mucins FIM-A.1 and FIM-C.1 as well as the vWF-type mucin FIM-B.1 (Hoffmann, 1988; Hauser et al., 1990; Hauser and Hoffmann, 1992; Joba and Hoffmann, 1997).

Behavioral effects

Synthetic TFF3/monomer was tested in two different models for behavioral effects after bilateral application into the basolateral nucleus of the rat amygdala (Schwarzberg et al., 1999). In passive avoidance tests maximal effects were observed about 1 day after TFF3 application regardless of if a retrieval test or a consolidation test was performed. Surprisingly, application of a low dose (2x6 pg) decreased avoidance latency wheras a high dose (2x60 pg) increased avoidance latency. This bi-directional effect was also observed using the elevated plus-maze test, where the locomotor activity on the open arms was increased after a low dose injection and decreased after a high-dose injection. Generally, the results of both tests can be explained by anxiolytic or anxiogenic effects after low dose or high dose TFF3 injections, respectively.

Table 2. Predominant TFF-peptide/secretory mucin combinations observed in various human mucin-producing cells.

CELL	TFF-PEPTIDE/MUCIN
Gastric surface cell	TFF1/MUC5AC
Brunner's glands	TFF1+TFF2/MUC6
Gastric mucous neck cell	TFF2/MUC6
Intestinal goblet cell	TFF3/MUC2
Conjunctival goblet cell	TFF1+TFF3/MUC5AC
Respiratory goblet cell	TFF3/MUC5AC+MUC5B
Respiratory submucosal glands	TFF3/MUC5B
Endocervical epithelial cell	TFF3/MUC5B
Submandibular gland: serous cell	TFF3/MUC7

Possible physiological functions of TFF-peptides

A key role of TFF-peptides for maintenance of the surface integrity of mucous epithelia is widely accepted now. However, this major role is probably realized at least in a complex threefold manner: as typical constituents of mucuos gels; by modulating cell migratory processes; and by regulating apoptosis.

TFF-peptide interaction with mucins could influence the rheology of the delicate mucus; for example of the cervix and the respiratory tract as well as the tear film of the eye. This might be of significance to explain certain pathological conditions (e.g. of the respiratory tract) where rheological properties of mucus are changed. A first hint for the precise regulation of the rheology of these viscous biopolymers might be the fact that each of the mucous epithelia secretes its own characteristic mucin/TFF-peptide combination (Table 2) which meets optimally the physiological needs of the specific epithelium. Furthermore, TFF-peptides could be involved in the interaction of microbia; in particular with gastric or respiratory mucus as well as with saliva. For example, *Helicobacter pylori* co-localizes with MUC5AC but not with MUC6 in the human stomach (Van den Brink et al., 2000); this is remarkable because only MUC5AC is expected to interact with TFF1 (see above). Interaction of TFF-peptides with mucins appears to influence proton permeation through gastric mucus as reported for TFF2 (Tanaka et al., 1997).

Furthermore, TFF-peptides are candidates for supporting mucosal integrity by triggering anti-apototic effects. This would be of particular benefit after mucosal damage. However, this role would probably depend on specific TFF-receptors.

The concept of a protective role of TFF-peptides by promoting restitution is strongly supported by in vitro cell migration experiments. This motogenic effect probably also requires specific TFF-receptors and could be triggered by the MAPK signaling cascade; there are some indications for a synergistic effect with EGF. TFF-receptors are expected to be located at the basolateral side of the mucosae; for example of the gastrointestinal and the respiratory tracts, because subcutaneous application of TFF-peptides in vivo is far more effective than oral application. This basolateral localization would be in line with that found for the EGFR (Wong and Wright, 1999). However, the question arises about the origin of basolateral TFF-peptides. TFF-peptides from mucous epithelia under physiological conditions are secreted apically towards the lumen; they can possibly reach the basolateral side only after mucosal damage. Thus, TFF-peptides could act as luminal surveillance peptides but without function unless mucosal damage has occured. The one physiological source capable of

releasing a TFF-peptide directly into the blood stream is the posterior pituitary from which oxytocinergic neurons secrete TFF3 (Jagla et al., 2000). A pulsatile secretion during sexual activity, parturation and suckling can be expected as well as a continuous release even in males, peaking at night in analogy to the release of oxytocin (Carter, 1992; Forsling et al., 1998). This would imply that restitution of mucous epithelia might also be a neurally-regulated process. This is still speculation.

The discovery of minute amounts of TFF-peptides in the brain is compatible with a potential role as a neurotransmitter/modulator, hormone or neurotrophic factor particularly during development. All these neuropeptide functions however require the presence of TFF-receptors. TFF3 in particular produced in oxytocinergic neurons of the human hypothalamus (Jagla et al., 2000) is the most likely candidate for acting as a neurotransmitter/modulator in various brain regions innervated by these neurons, e.g. the brain stem, spinal cord and the pontine tegmentum. Specific oxytocinergic neurons of the paraventricular nucleus are also considered to be putative satiety neurons for eating behavior. Intrahypothalamic release of TFF3 might also be responsible for morphological plasticity of the hypothalamic nuclei. Extrahypothalamic pathways connecting the hypothalamus and the limbic system in the rat might explain the fear-modulating action of TFF3 (Schwarzberg et al., 1999). Furthermore, the release of TFF3 from the posterior pituitary into the blood stream opens the question of its peripheral target organs. Additional physiological functions other than enhancing surface integrity of mucous epithelia will be of major interest.

Taken together, the motogenic and anti-apoptotic actions of TFF-peptides as well as the minute neuronal secretion particularly of TFF3 favour the existence of specific TFF-receptors. Their molecular characterization revealing the signaling cascades triggered by TFF-peptides will be the major goal for the future in order to eventually understand the molecular function of this still enigmatic peptide family.

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