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Trefoil peptides: from structure to function

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Abstract. The unique structure in which six cysteine residues in a sequence of 38 or 39 amino acid residues form three disulphide bonds in a 1-5, 2-4 and 3-6 configuration constitutes the basic elements of a trefoil domain. Today three mammalian trefoil factors (TFF1, TFF2 and TFF3) containing one or two trefoil domains are known. Trefoil factors are usually associated with the mucin layer of the gastrointestinal tract. Early studies on trefoil factors concentrated on structure elucidation and sites of expression in health and disease, whereas studies over the last 3–5 years have focused on the mechanism of action and the search for specific receptors. This review summarises our present knowledge of trefoil peptide structures, their sites of expression, and their protection and repair functions, with a focus on the mechanism by which these peptides exert their biological function.

Key words. Trefoil factors; TFF-domain; spasmolytic polypeptide; pS2; intestinal trefoil factor; epithelial restitution; ulcer; inflammatory bowel disease.

Introduction

Trefoil factors (TFFs) constitute a family of mucin-associated peptides containing one or more structurally characteristic trefoil domains [1]. A trefoil domain is defined as a sequence of 38 or 39 amino acid residues in which six cysteines are disulphide-linked in a 1-5, 2-4 and 3-6 configuration. The amino acid sequence, together with the disulphide bonds, forms a characteristic three-leaved structure which has given the peptide family its name [1]. The mammalian trefoil factors constitute the two-domain spasmolytic polypeptide (SP or TFF2) [2–4], the one-domain breast cancer-associated pS2 peptide (TFF1) [5, 6] and the one-domain intestinal trefoil factor (ITF or TFF3) [7, 8] (fig. 1). Trefoil factors are expressed in several tissues of the body but most pronouncedly in the gastrointestinal (GI) tract, where the individual trefoil factors are expressed in a tissue-specific manner. Under normal circumstances in humans pS2 and SP are expressed in the stomach [9, 10], and ITF in the small and large intestines [8]. The physiological function of trefoil factors has been discussed in several articles, e.g. [11–22], and will be further substantiated in the present review in the light of a series of new results.

History

The first of the trefoil factors to be isolated and characterised was porcine pancreatic spasmolytic polypeptide, pSP (pTFF2). This peptide was isolated back in the late seventies from pancreas at Novo Research Institute in Denmark as a component in a side fraction from the production of porcine insulin. These observations were reported for the first time in September 1979 when a UK patent application was filed [23], followed by US patent applications in March 1980 [24] and March 1981 [25], the latter containing the amino acid sequence of pSP. The detailed characterisation of pSP, including the amino acid composition, was initially published at the 17th Annual Meeting of the European Association for the Study of Diabetes in Amsterdam in September 1981 [2]. The preparation, characterisation, tissue distribution and pharmacology of pSP were published in three successive papers in *Regulatory Peptides* in March 1982 [3, 4, 26], followed by the disclosure of the complete amino acid sequence of pSP at the 4th International Symposium on Gastrointestinal Hormones in Sweden in June 1982 [27]. The experimental details leading to the amino acid sequence of pSP were published in

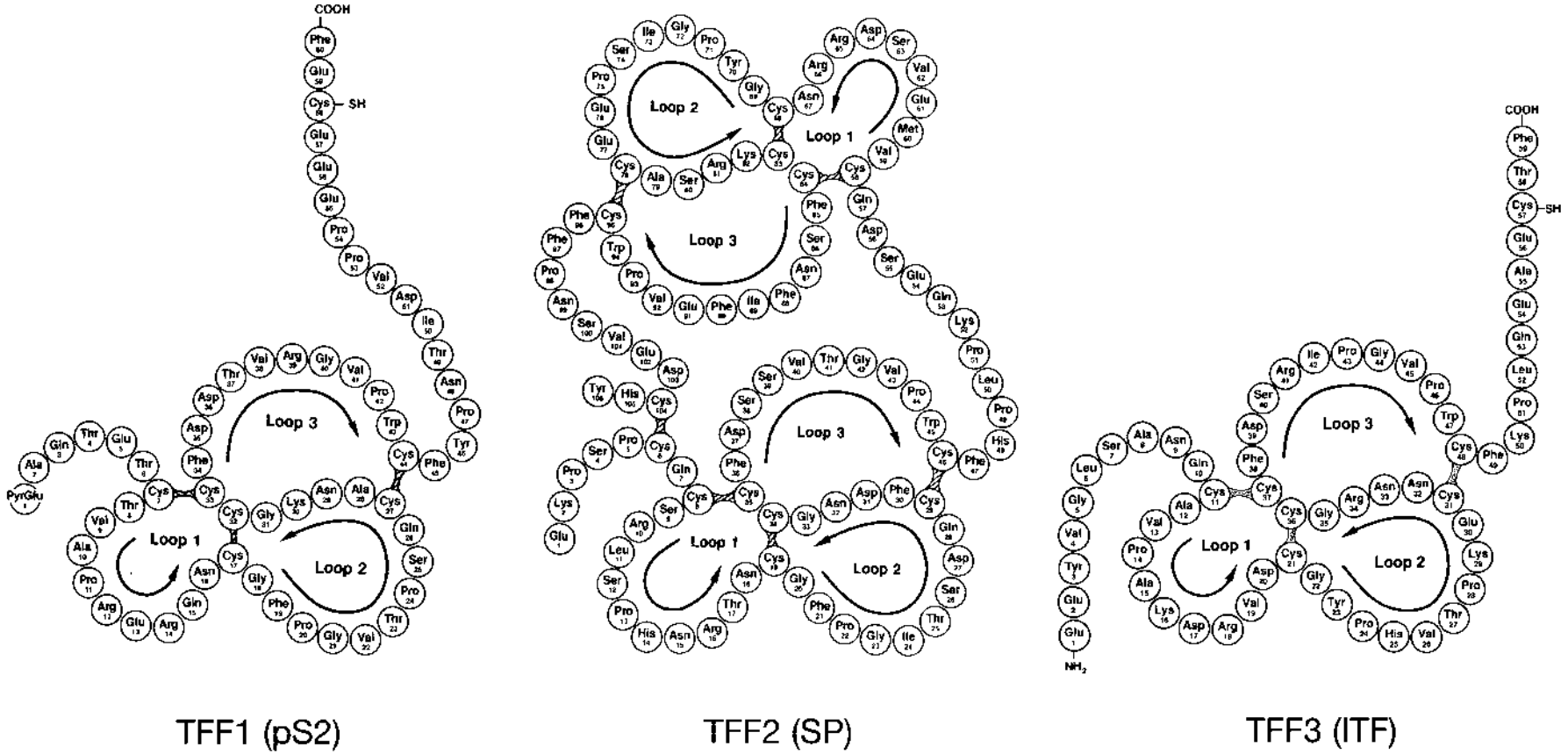

Figure 1. The three mammalian trefoil peptides: TFF1, TFF2 and TFF3. TFF1 (pS2) and TFF3 (ITF) contain one trefoil domain, and TFF2 (SP) contains two trefoil domains. The figure shows the human sequences.

Table 1. Nomenclature for mammalian trefoil factors [34].

Old locus name	New locus name	Old peptide name	New peptide name	TFF domains	Major site of expression
BCEI	*TFF1*	pS2/BCEI/pNR-2 pNR-105/Md2	TFF1	one	stomach
SML1	*TFF2*	spasmolytic polyptptide/SP	TFF2	two	stomach duodenum pancreas
-	*TFF3*	ITF/P1.B	TFF3	one	intestine

BCEI, breast cancer estrogen-inducible; SML1, spasmolysin 1; pNR-2, plasmid Newcastle regulated-2; pNR-105, plasmid Newcastle regulated-105; SP, spasmolytic polypeptide; ITF, intestinal trefoil factor; P1.B, P-domain 1.B.

January 1985 [28], and later corrected at four positions [29].

In December 1982, Masiakowski and co-workers [30] published the cloning of an estrogen-regulated gene from the MCF-7 human breast cancer cell line; however, no sequence information was, given in this work. The nucleotide sequence of the estrogen-induced element in the MCF-7 cells, called pS2, was published by two independent groups: Jakowlew and co-workers in March 1984 [5] and Prud'homme and co-workers in 1985 [6].

Three years later, with the publication of two small notes in *Biochemical Journal*, the sequence homology between the two domains of pSP and the one domain in pS2 was recognised [31, 32]. At that time the implications of the high degree of homology between pSP and pS2 could not be predicted [31]. That same year, 1988, Hoffmann published the cloning of a four-domain 'spasmolysin precursor' from the skin of the frog *Xenopus laevis* and also recognised the domain homology of this protein to pSP and pS2 [33]. Although the three peptides/proteins, i.e. porcine pancreatic SP, human breast cancer-associated pS2 and frog skin spasmolysin, were found in completely different biological sources, a theory was presented in June 1989 joining these three proteins in a new family of 'trefoil peptides' [1].

A third, and so far last, member of the mammalian trefoil peptides was identified by Suemori and co-workers [7] in 1991. This peptide was named ITF, intestinal trefoil factor.

The appellations 'trefoil peptides' [1] and the 'P-domain peptides' [9] have been widely used since 1989/90 to describe this new family of peptides and have only just recently been the subject of revision (see the 'Nomenclature' section).

Nomenclature

Since the trefoil factors were discovered independently from several different sources (for review, see, for example [11, 12]), the nomenclature has until recently been somewhat confusing. However, as a result of the work of a nomenclature discussion group at a Philippe Laudat Conference on trefoil/P-domain peptides in the autumn of 1996, the scientific organising committee of the meeting recommended the use of a TFF (TreFoil Factor) nomenclature system for the mammalian trefoil peptides [34]. According to this system, human breast cancer-associated pS2 is named TFF1, spasmolytic polypeptide TFF2 and intestinal trefoil factor TFF3. The system has been summarised in table 1, and the names have been approved by the Human Gene Mapping Workshop (HGMW). It is recommended that genes encoding the trefoil peptides be written in italics (*TFF1*, *TFF2* and *TFF3*) and that the corresponding peptides be written in non-italic uppercase letters (TFF1, TFF2 and TFF3). It is further recommended that different species be indicated by a lowercase letter prefixed to the peptide name, e.g. hTFF1 for human pS2, mTFF2 for mouse SP, rTFF3 for rat ITF etc. Since this nomenclature is rather new, dual naming will probably be necessary for the next couple of years, e.g. pSP (pTFF2), and this dual nomenclature will also be used to some degree in the present review.

Genomic structure

The human pS2 gene (h*TFF1*) was the first of the trefoil genes to be localised on chromosome 21 [35]. On the basis of expression of human pS2 (hTFF1) and human SP (hTFF2) in diffuse types of stomach carcinomas, Theisinger and co-workers [36] speculated about a 'co-ordinated activity of both genes, which may be regulated via comparable or even identical steps'. Tomasetto and co-workers [37] later mapped the genes encoding both hSP (h*TFF2*) and hpS2 (h*TFF1*) to chromosome 21 at 21q22.3 with a physical distance of less than 230 kb. The distance between the 3′ end of the h*TFF1* gene and the 5′ end of the h*TFF2* gene was later determined to 12.5 kb [38]. The genomic structure and promoter region of the third human trefoil peptide, hITF (hTFF3), were first studied by Seib and co-workers [39].

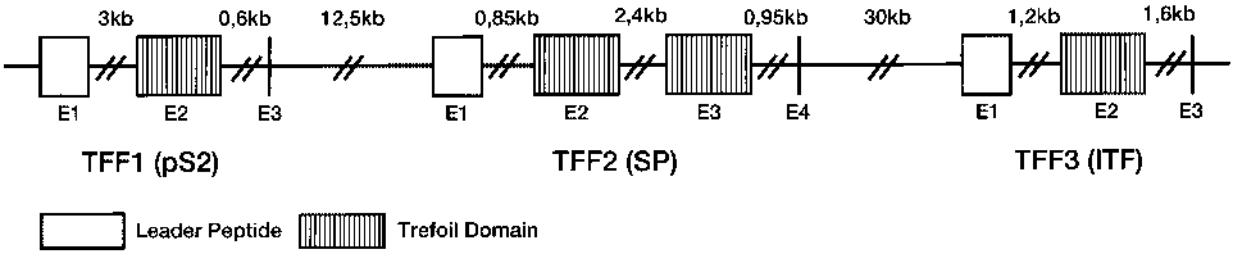

Figure 2. The region on chromosome 21q22.3 that encodes the human trefoil peptides. The regions that encode the leader peptides are gray, and the regions that encode the trefoil domains are hatched. (The figure is adapted from Seib and co-workers [44]).

Subsequent mapping of the gene encoding hITF also placed this gene on chromosome 21q22.3, clustered with the two other members of the family [40, 41]. In 1996 Beck and co-workers [42] cloned a contiguous genomic fragment of less than 100 kb containing all three human trefoil peptide genes which formed the basis of the elucidation of the complete genomic structure of the trefoil peptide coding region in 21q22.3 [43, 44]. Figure 2 shows the 55-kb region on chromosome 21q22.3 encoding TFF1, TFF2 and TFF3 [44]. The close physical linkage between the genes encoding the three mammalian trefoil peptides seems to strengthen an old hypothesis [28] that peptides with multiple trefoil domains may have been derived from a common ancestor by gene duplication. The three trefoil genes on chromosome 21 are organised head to tail, suggesting a co-ordinated transcriptional regulation [38, 43]. As pointed out by Beck and co-workers [42], the information on the genomic structure of the trefoil region on chromosome 21 will be a valuable tool for studying gene regulation and breeding transgenic animals [45]. The close physical link between the genes encoding the trefoil peptides has to be considered, too, when evaluating results from specific trefoil peptide gene knockout experiments, so far carried out for pS2 (TFF1) [46] and ITF (TFF3) [47] (see 'Transgenic and knockout' section).

The trefoil domain

Six cysteine residues in a linear sequence can, in principle, form disulphide bridges in $5 \times 3 \times 1 = 15$ different arrangements. All of these 15 possible arrangements in proteins are known [48].

The trefoil domain was originally defined as a sequence of 38 or 39 amino acid residues containing six cysteine residues which were disulphide-linked in the configuration 1-5, 2-4 and 3-6 [1]. This definition was deduced from the preliminary assignment of disulphide bridges in porcine SP (pTFF2) and the linear alignment of the sequence of human pS2 (hTFF1) [5, 6, 49, 50], porcine SP (pTFF2) [28, 29] and two frog skin 'spasmolysins' [33]. The suggested disulphide arrangement was later confirmed by several 3-dimensional (3D) structure determinations of trefoil domains [51–57]. The 1-5, 2-4 and 3-6 disulphide bond configuration found in the trefoil domains is also found in the family of Kazal serine protease inhibitors. However, the total number of amino acid residues in the sequence containing the six cysteine residues in the Kazal inhibitors are 48–58 and thus different from the 38 or 39 residues found in the trefoil domains. The trefoil domain thus represents a new and very compact structural domain different from other well-known domains such as the 'kringle' domain in blood coagulation factors [58], the EGF domain [59] and the insulin-like growth factor domain [60]. Since the original definition was introduced, trefoil domains have been sequenced from a series of additional mammalian species (fig. 3), and new trefoil-domain containing peptides and proteins from *X. laevis* have been discovered [33, 61–64]. If, however, the original definition is maintained, the minimum requirement for a trefoil domain would be the presence of the consensus sequence of:

$$\mathbf{C}\text{-}X_{9-10}\text{-}\mathbf{C}\text{-}X_{9}\text{-}\mathbf{C}\text{-}X_{4}\text{-}\mathbf{C}\text{-}\mathbf{C}\text{-}X_{10}\text{-}\mathbf{C}$$

where **C** are cysteine residues and X other amino acid residues.

This definition will include all known mammalian trefoil domains as well as all known trefoil domains in peptides and proteins from the frog (fig. 3 and table 2). In contrast, this definition will *not* include more distant related sequences as, for example, the trefoil-like domains found in zona pellucida protein (Zpx) [65], sucrase-isomaltase [66], α-glucosidase [67] or the Kazal inhibitors.

What, then, are the actual characteristics of a trefoil domain? A linear alignment of trefoil domains (fig. 3 and table 2) would yield the following 'consensus' sequence, depending somewhat on the criteria used in defining consensus (in this case only complete conservation is included):

$$\mathbf{C}\text{-}X_{6-7}\text{-R-}X_{2}\text{-}\mathbf{C}\text{-G-X-P-}X_{6}\text{-}\mathbf{C}\text{-}X_{4}\text{-}\mathbf{C}\text{-}\mathbf{C}\text{-F-}X_{7}\text{-P-W-}\mathbf{C}\text{-F}$$

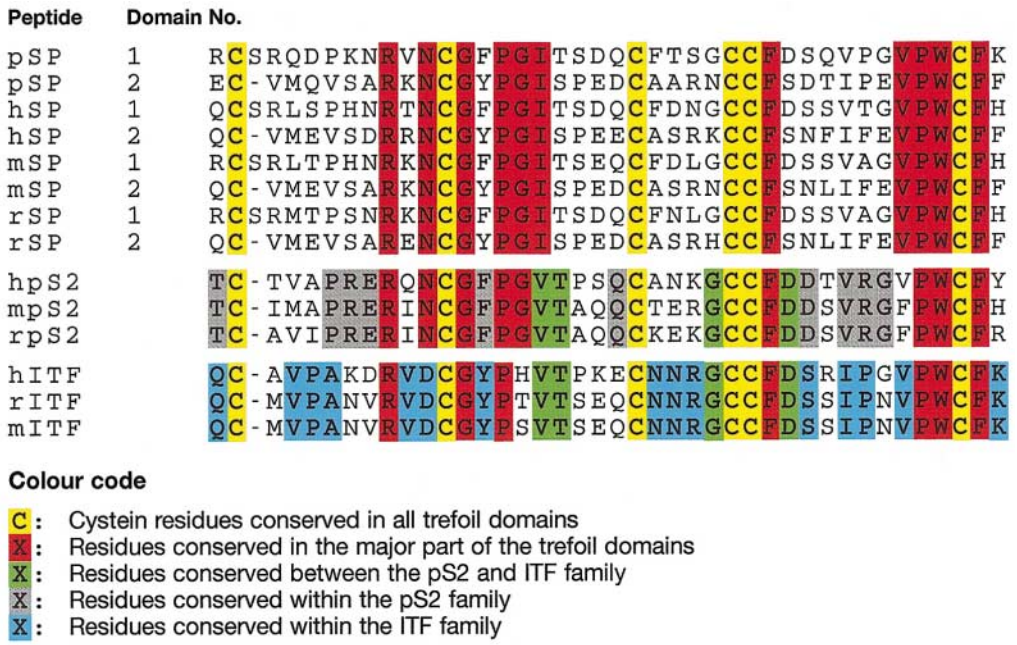

Figure 3. Sequence alignment of known mammalian trefoil domains. pSP, porcine spasmolytic polypeptide [28, 29]; hSP, human SP [9]; mSP, mouse SP [9]; rSP, rat SP [73]; hpS2, human pS2 [5, 6]; mpS2, mouse pS2 [74]; rpS2, rat pS2 [169]; hITF, human ITF [8, 111]; rITF, rat ITF [7, 70, 170] and mITF, mouse ITF [171–172].

This consensus sequence shows clearly that not only are the disulphide arrangement and the peptide length of the three loops conserved but also some individual amino acid residues close to the disulphide bridges, e.g. the P-W-C-F sequence in the C-terminal part of the domain. To get a more realistic picture of the characteristics of the trefoil domain, we previously tried to build a 3D model of the conserved part of the domain instead of a linear sequence alignment [51]. An example of such a 3D 'alignment' is given in figure 4, which uses the spatial co-ordinates for the first trefoil domain in human SP (N. Aghajari, unpublished observations). All atoms in residues which are conserved in mammalian trefoil domains are coloured purple. As can be seen from this model, the majority of conserved residues are located at one side of the domain. Here the residues form an 8–10 Å wide cleft [51]. We have previously suggested that this cleft could be a binding site for mucin sugar side-chains [51]. Since the primary targets of trefoil peptides are unknown, the conserved part of the trefoil domains could also be a binding site for a specific receptor or a transporter protein.

Trefoil peptide expression in mammals

When SP (pTFF2) was first isolated from porcine pancreas, this organ was thought to be a major site for trefoil peptide expression [4]. Later studies have shown that, with respect to pSP expression, the pig is unique. Among the mammalian species studied so far, the pig is the only species with a significant trefoil peptide expression in the pancreas. In the other mammalian species studied all three trefoil factors are expressed in the GI tract (for review see Poulsom [68]). Under normal circumstances pS2 (TFF1) and SP (TFF2) are expressed in the stomach [9, 10], and ITF (TFF3) in the small and large intestines and in the colon [7, 8].

In the human stomach pS2 and hSP are produced in the mucosa cells of the superficial epithelium and crypts of both the antrum and the body [9, 10, 69]. Co-expression of pS2 and hSP in the human stomach has been found in parietal, chief and mucosal neck cells [9]. In contrast, human ITF is not expressed in the stomach, and normally the expression of ITF is almost exclusively found in goblet cells of the small and large intestines and in colon [8, 70]. The expression of all three human trefoil peptides is thus restricted to cells in the GI tract which also produce mucin.

In pigs, the expression of pSP has been shown in the acinar cells of the pancreas, the mucous cells of the stomach and duodenum, and the epithelial cells in jejunum and ileum [71]. SP expression in rats has been found mainly in the antrum [72, 73], whereas the major part of immunoreactive ITF was found in duodenum, jejunum and ileum with a minor part in the colon and

Table 2. Trefoil domains in frog peptides and proteins. FIM-A.1, frog integumentary mucin A.1 [33]; FIM-C.1, frog integumentary mucin C.1 [106]; p75k, heat-stable 75-kDa protein from frog skin [64]; ×P1 and ×P4 [62]; ×P2 [63].

Peptide/ protein	Domain No.	Domain sequence
FIM-A.1	1	..D C S V A P N M - R V N C G Y P T V T E A D C R A V G C C F D S S I L N T K W C F Y..
	2	..E C S G D P T K - R I D C G F P R I T E K Q C I L R G C C F D S S I S G V K W C Y A..
	3	..E C A A D - - - - R V D C G Y S G I T Q A D C E G K G C I F D S T I P E T K W C F Y..
	4	..E C T V D P S V - R T D C G Y P G I T D K E C R E K G C C Y D E C I P D V I W C F E..
FIM-C.1	1	..H C H V K P S K - R E M C G S K G I T K K Q C K K K N C C F D P K G H G G I H C F H..
	2	..E C K M E P S K - R E D C G Y S G I T E S Q C R T K G C C F D S S I P Q T K W C F Y..
	3	..D C K V E P S Q - R V D C G F R G I T A D Q C R Q K N C C F D S S I S G T K W C F Y..
	4	..E C K M E P S K - R A D C G Y P G I T E S Q C R S K G C C F D S S I P Q T K W C F Y..
	5	..D C K V A P S S - R V D C G F G G I T A D Q C R Q R N C C F D S S I S G T K W C F Y..
	6	..M C S G P P T K - R R D C G Y P G I S S S V C I N R G C C W D N S V M N V P W C F Y..
p75k	1	..D C K G D P F K - R T D C G Y P G I T E G Q C K A K G C C F D S S I V G V K W C F F..
xP1	1	..Q C S V E R L A - R V N C G Y S G I T P Q E C T K Q G C C F D S T I Q D A P W C F Y..
xP4	1	..R C G V K P K S - R D N C G P P G I S P D E C V K K G C C F D D S D P D S I W C Y T..
	2	..I C N P A E P K A R V N C G Y P G I T S Q D C D K K G C C F N D T I P N V V W C Y Q..
	3	..D C S A V E P K K R V N C G P P G V S P D E C I K N G C C F N S D V G G V P W C F K..
	4	..Q C A V L P K A - R I N C G Y P D I T M D Q C Y K K G C C Y D S S E S D S I W C F Y..
xP2	1	..D C K G D P F K - R T D C G Y P G I T E G Q C K A K G C C F D S S I V G V K W C F F..
	2	..Q C L F S P G D - R E D C G Y S S I T P M E C M K R G C C F D A S I T G V K W C F H..

oesophagus [72]. In mice, pS2 as well as SP expression has been found in the stomach, and in the case of SP, also in the duodenum [74]. However, the cellular localisation of the two peptides in the mouse stomach seems to indicate a complementary pattern of expression [74]. Although trefoil peptides in mammals are expressed in a tissue-specific manner in the normal GI tract, the situation is somewhat different in the case of damage in the GI tract. The main conditions of damage studied have been ulceration in the stomach and the duodenum and inflammatory bowel disease. Given that tissue reacts to damage, it may not be surprising that pS2 and hSP are overexpressed in connection with mucosal damage or cancer in the stomach [36, 75–82], or that ITF may be overexpressed in colonic carcinomas [83].

However, in the case of Crohn's disease, a rather high expression of both pS2 and hSP in several parts of the intestine has been observed [84–86] in spite of the fact that the expression of these two trefoil factors is normally restricted to the stomach and doudenum. In the case of gastric damage, ITF, which is normally only expressed in the lower part of the intestine, has been shown to promote healing [87] as well as protect against induced damage [88] when given subcutaneously [87] or orally [88].

These examples seem to indicate that although trefoil factors are normally expressed in the GI tract in a tissue-specific manner, this pattern can be altered when the tissue is exposed to different kinds of damage.

Trefoil factors have also been reported to be present in several carcinomas outside their normal site of expression. For example, pS2 has been demonstrated in pancreatic carcinomas [89, 90], ovarian cysts [91, 92] and tumours in the oesophagus [93] and biliary tract [94]. As previously mentioned, pS2 was originally discovered as being induced by oestrogen in the MCF-7 breast cancer cell line [5, 6, 30, 95], and several studies have documented the expression of pS2 in breast cancer tissue [9, 78, 96–101]. However, a recent study has also identified ITF in breast cancer [102], thus adding to the examples of trefoil factor expression outside 'normal' sites.

Two other recent studies have identified trefoil peptides in the brain. Hirota and co-workers [103] found expression of the pS2 gene in the rat brain, and Probst and co-workers have identified ITF in the human [104] and rat [105] hypothalamus. The results of further studies will indicate whether these findings identify trefoil peptides as new neuropeptides.

Trefoil peptide expression in frogs

The skin from amphibians is a rich source for isolating of biologically active peptides, and these peptides often have their counterpart in the brain and gut of mammals. In 1988 Hoffmann cloned a precursor protein from the skin of the frog *X. laevis* that contained four trefoil domains [33]. Assuming processing at two di-basic sequences of the precursor, two small peptides (spasmolysin I and II), each containing one

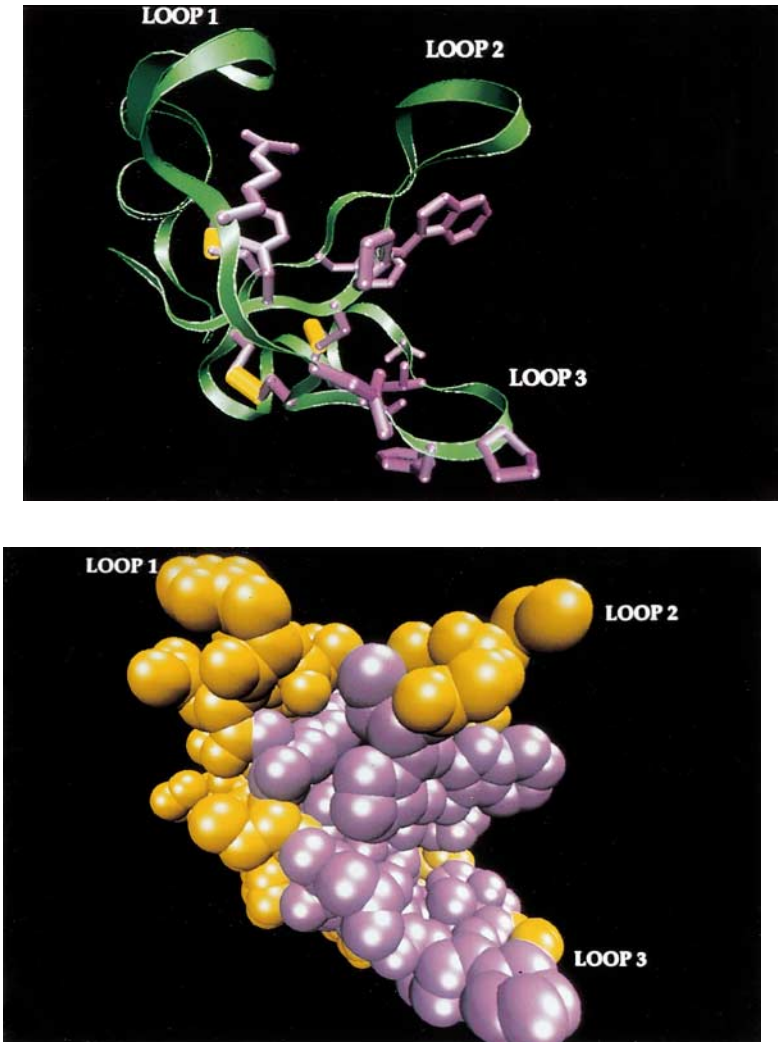

Figure 4. Three-dimensional alignment of the conserved part of mammalian trefoil domains. The figure shows (*A*: a ribbon and *B*: a space-filling) representation of the first domain in hSP (N. Aghajari, unpublished observations) including amino acid residues 1–48. The conserved residues are coloured purple. The conserved residues included in this alignment are: Arg-16, Asn-18, Gly-20, Phe-21, Pro-22, Ile-24, Thr-25, Gln-28, Phe-36, Pro-44, Trp-45 and Phe-47. (The figure is kindly provided by Rasmus Lund Jensen).

trefoil domain and one highly glycosylated spasmolysin-glycoprotein containing two trefoil domains, will be generated [33]. It has subsequently been shown that the spasmolysin precursor is probably not cleaved at the dibasic sequences and thus exists in the frog skin as a large glycosylated protein containing four trefoil domains [61]. The spasmolysin precursor was later renamed Frog Integumentary Mucin-A.1 (FIM-A.1) [61, 62]. Another member of the FIM class that contains six trefoil domains (FIM-C.1) [106] as well as a heat-stable 75-kD protein from the dermal gland of the frog [64] has recently been sequenced; the latter contains a single trefoil domain. Apart from these larger skin proteins, the existence of a small peptide with two trefoil domains, xP2, has also been described in frog skin [63]. In the stomach of *X. laevis*, genes that encode two trefoil peptides have been described [62]. One of these, xP1, contains a singe trefoil domain, whereas the other, xP4, contains four trefoil domains. Since xP1 and xP4 are secretory products of the frog stomach mucosa, they very much resemble human pS2 and SP with respect to their pattern of expression. In table 2, the sequences of all known trefoil domains in frog peptides and proteins are summarised.

The functions of trefoil domain-containing peptides and proteins in the frog are unknown; but like mammalian trefoil peptides, they are expressed in connection with mucin-producing cells, and a common function in mammals and frogs seems highly probable.

Trefoil peptide expression in ulcerated tissue

In 1990 Chambon and co-workers described increased expression of trefoil peptides in connection with gastrointestinal ulceration [84, 107]. At the same time Wright and co-workers [108] characterised an ulcer-associated cell lineage (UACL) as a specific anatomical structure appearing in close proximity to the ulcerated area. UACL is found as a bud growing from the base of the intestinal crypt, and the cells appear to arise directly from the stem cell zone [109, 110]. UACL expresses the growth factors TGFα and EGF [107] as well as all three known human trefoil peptides: SP (TFF2), pS2 (TFF1) [107] and ITF (TFF3) [111]. The sequence in which these peptides are expressed upon ulceration has been debated. The first studies of UACL seem to indicate that TGFα and EGF are produced first and stimulate neighbouring cells to express trefoil peptides [107, 108]. A recent study by Alison and co-workers [112] in which experimental ulcers were induced in the rat stomach by application of a cryoprobe seems to indicate that SP is expressed first (0.5–2 h after injury), followed by ITF (after 48 h) and later on TGFα and EGF (pS2 expression was not included in this study). Thus, trefoil peptides may be expressed as a rapid response to injury, in contrast to TGFα and EGF [113]. The development of UACL shows some resemblance to the development of Brunner's glands, and the pattern of trefoil peptide expression is very similar [109]. A cell line expressing trefoil peptides with phenotypic similarities to UACL has been described in so-called hyperplastic polyps occurring in connection with colonic lesions [114].

As a general rule, overexpression of trefoil peptides in the GI tract is most often found in connection with ulceration as mentioned above, gastric metaplasia [76, 115, 116] or other types of mucosal damage such as Crohn's disease [84–86] or gastrointestinal cancers [77, 78, 117]. Increased trefoil peptide expression also characteristically occurs most often in the same cell types as those producing mucin, or in close association with mucin production. Trefoil peptides thus may have a 'repairing function', acting as 'naturally occurring healing factors' [11, 12, 14, 118–122].

Protection and repair function of trefoil peptides

No standard method for measuring trefoil peptide activity has been developed, and no systematic structure-activity studies have been performed so far. However, several studies have indicated that trefoil peptides can protect the epithelial layer in the GI tract against externally induced damage. Babyasky and co-workers [88] used a rat model in which gastric injury was introduced either by intragastric administration of ethanol or by subcutaneous injection of indomethacin. In both cases marked protection was obtained by oral administration of hSP (hTFF2) as well as rITF (rTFF3) when given up to 2 h before injury [88]. Nearly identical results were obtained in a study where SP (TFF2) was given before or in combination with aspirin (G. Cook, unpublished observations). Also, pS2 has been found to have a similar protective effect [123, 124]. The protective effects of trefoil peptides on intestinal epithelial barrier function have been studied by Kindon and co-workers [125] in an in vitro system where the trefoil peptides were given alone or in combination with colonic mucin glycoproteins to monolayers of colonic cells. In both cases a marked attenuation of damage was noted, and the trefoil peptides (in this study hSP and rITF) and mucin glycoproteins seemed to act in a co-operative manner [125]. A similar co-operative prophylactic effect of rITF and EGF was found by Chinery and co-workers [87] in a study where indomethacin-induced gastric damage was investigated in a rat model. Although trefoil peptides thus seem to play an important role in the defence of the GI tract [126], other protective factors such as immunoglobulins, cytokines and growth factors have to be taken into consideration, too, when evaluating the overall defence mechanism [14, 17, 127–129].

Apart from the protective effect, a series of experiments has shown that trefoil peptides participate in subsequent healing of damaged tissue – the process of epithelial restitution. In an in vitro model of epithelial restitution using wounded monolayers of confluent cells, Dignass and co-workers [130] demonstrated a 3- to 6-fold increase in the rate of epithelial migration into the wound by addition of the trefoil peptides SP and ITF, a factor which could be further increased to 15 by additional supply of intestinal mucin glycoproteins. These in vitro studies have been followed by in vivo investigations in which trefoil peptides have been given both orally and subcutaneously to rats with indomethacin- [131] or stress- [132] induced gastric lesions. Infusion (s.c.) of as little as 25 μg/kg/h of hSP has been found effective both when started 30 min before the damage [131] or at the same time as the damage [132]. In contrast to the study of Babyatsky and co-workers [88], the study by Playford and co-workers [131] failed to demonstrate any effect on protection/healing after oral administration. Although other studies have demonstrated protection/healing effects of trefoil peptides both in vivo and in vitro [133, 134] the underlying mechanism of action is far from clear [119, 122, 128] (see 'Mechanisms of action' section).

Transgenic and knockout models

To study the possible function of trefoil peptides in the mucosal repair process, a transgenic mouse model overexpressing human trefoil peptide pS2 (hTFF1) specifically in the villi of the jejunum was constructed [45]. In

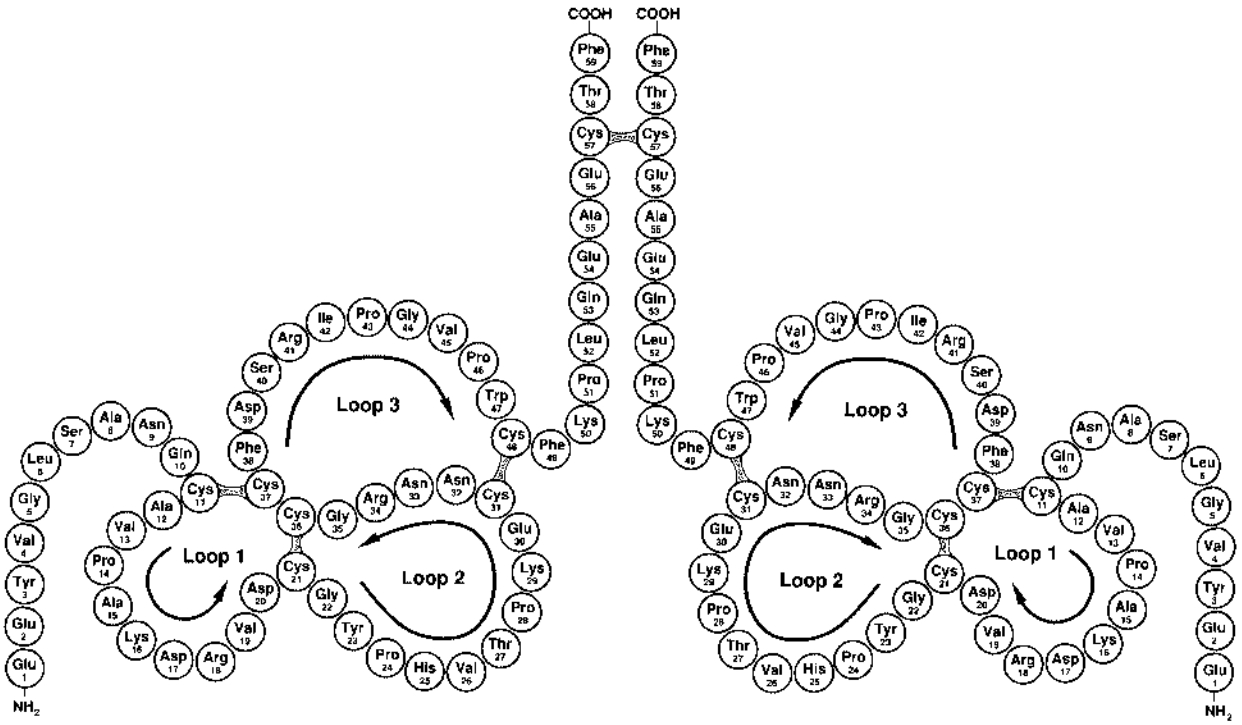

Figure 5. Structure of dimer form of human ITF. (Reprinted with permission from: Thim L., Wöldike H. F., Nielsen P. F., Christensen M., Lynch-Devaney K. and Podolsky D. K. (1995) Characterization of human and rat intestinal trefoil factor produced in yeast. Biochemistry **34:** 4757–4764), © 1997, American Chemical Society).

this model a marked difference in the amount of damage caused by indomethacin was observed when the transgenic mice were compared with controls, thus supporting the theory that trefoil peptides play an essential role in the gastrointestinal repair process [45]. In contrast, transgenic mice overexpressing pS2 and secreting it into the milk did not seem to have any mammary gland dysplasia, and pS2 expression in the milk did not have any effect on the physiology or development of the suckling young or the transgenic mother [135]. In a study of mice overexpressing TGFα in the gastric mucosa, a marked increase in the mucosal surface cells producing pS2 was observed [136]. When the TGFα gene was knocked out, the normal increase in trefoil peptide expression following gastric ulceration was not observed [137], indicating that induction of trefoil peptide expression after injury depends on the presence of TGFα [137].

In two recent studies the genes encoding mouse pS2 and mouse ITF, respectively, were deleted by gene-targeting techniques. Mice lacking ITF had impaired mucosal healing and died from extensive colitis after oral administration of dextran sulfate [47]. Although expression of the two other gastrointestinal trefoil peptides, SP and pS2, was normal, the ITF-deficient mice still suffered from poor epithelial regeneration after injury [47], indicating that the two other trefoil peptides, at least in this model, cannot compensate for the missing ITF. Furthermore, luminal administration of recombinant ITF to the ITF-deficient mice resulted in normal healing [47] – an elegant proof of the protective role of ITF in maintaining a normal mucosal barrier. In the other gene knockout experiment, mice lacking pS2 were studied [46] (pS2-deficient mice have so far not been studied directly with respect to the mucosal healing function). Disruption of the pS2 gene did not affect the expression of ITF, whereas in most animals studied a decreased SP mRNA level in the stomach was observed. So in this model, too, the absence of pS2 did not seem to be compensated by increased expression of the other trefoil peptides. The pS2-deficient mice showed adenomatous changes in the antropyloric part of the stomach, and after 5 months 30% of the animals developed multifocal carcinomas [46]. These results prompted Lefebvre and co-workers [46] to suggest that apart from being responsible for normal differentiation in the gastric mucosa, pS2 may also act as a tumour suppressor specific to gastric tissue. However, further studies are needed to fully evaluate the possible role of pS2 and the other trefoil peptides as tumour suppressors.

Mechanisms of action

The mechanisms by which trefoil peptides exert their main functions – gastric and intestinal protection and healing – has been the subject of several discussion papers, e.g. [16, 17, 19, 68, 118, 119, 121, 123, 126]. One of the theories is that trefoil peptides together with mucin glycoproteins form stable gel complexes resistant to gastrointestinal proteases and to mechanical stress [12, 51]. In fact, trefoil peptides are extremely resistant to gastrointestinal proteases [24, 26], and an increase in the viscosity of purified, solubilised mucin preparations has been observed following the addition of ITF (TFF3) or SP (TFF2) peptides (H. Kindon et al., personal communication). Furthermore, the 3D structure of the trefoil domain contains a binding pocket that may accumulate the sugar side-chain of a mucin glycoprotein [51]. It should be emphasised, however, that no direct proof of such a complex formation has so far been given. As noted by Polshakov and co-workers [138], the binding pocket could just as easily accommodate a hydrophobic interaction with a protein side-chain and thus form part of a protein binding site. Considering the in vitro study by Dignass and co-workers [130], in which the motogenic effect of trefoil peptides could be potentiated by mucin glycoproteins, the motogenic effect demonstrated for pS2 [139], and the fact that the mucin glycoproteins and trefoil peptides are produced in the same cells and often upregulated simultaneously upon injury, the hypothesis that these components act together to protect and heal the gastrointestinal tract seems well founded.

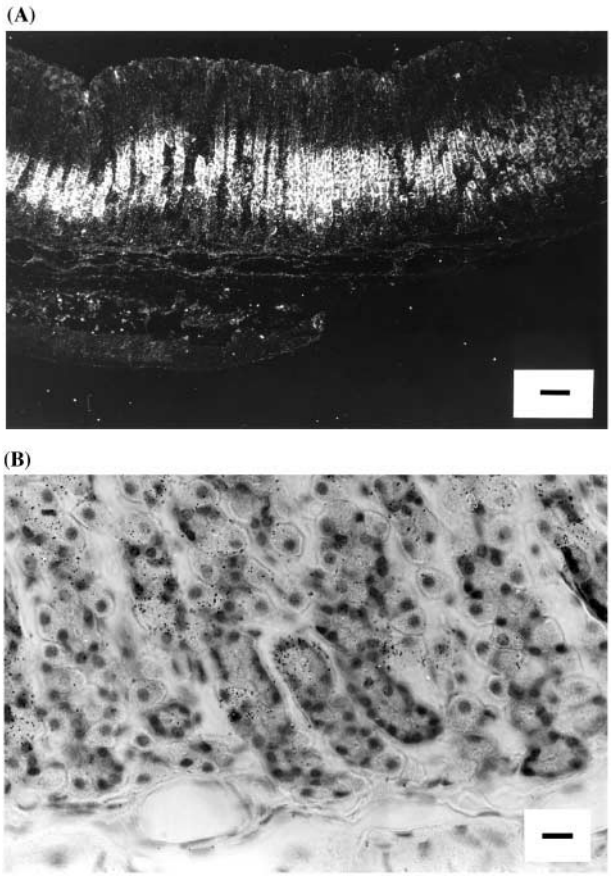

Figure 6. Autoradiograph of the corbus fundic part of the rat stomach. (*A*) The photo was taken using dark field microscopy 15 min after inferior vena cava injection of ^{125}I-pSP. The light part of the photo demonstrates binding of radioactivity in the neck part of the gland containing mucous neck cells. No part of the radioactivity was taken up by the surface epithelium. Bar inset = 80 μm. (*B*) Larger magnification showing the neck part of the corbus fundic glands. Grains are observed in the mucous neck cells and in some parietal cells, whereas the zymogenic cells are negative. Bar inset = 20 μm. (Photos are kindly provided by Steen S. Poulsen).

If the trefoil peptides act as cross-linkers for the mucin glycoproteins, one might assume that at least two trefoil domains per molecule are required to perform this action. This is only the case for one of the mammalian trefoil peptides, SP (fig. 1). Whether the other two trefoil peptides, pS2 and ITF, exist in monomer or dimer form in vivo is controversial. When human ITF was prepared in a yeast recombinant system, both the monomer form (fig. 1) and the dimer form (fig. 5) were produced [140], whereas rat ITF produced in the same system only gave rise to the dimer form [140]. The monomer form of human ITF did not exist with a free –SH group, but this group was blocked with either cysteine or glutathione (author's observation). Gel filtration studies of naturally occurring rat ITF from intestinal goblet cells [141] and neoplastic colonic tissue [142] showed that this peptide occurs in a 6.6-kDa form, i.e. as a monomer. In human tissue biopsy samples both the monomer and dimer forms of pS2 and ITF were found [143]. We can assume that pS2 and ITF exist both in monomer and dimer form depending on the redox potential of the surroundings.

Early studies indicated that trefoil peptides act as growth factors [144] or in some way modify [145] or modulate the effect of other growth factors [146]. However, the concentration needed for pSP to act as a growth factor for MCF-7 cells is in the order of 100 nM [144], which is a factor of 100 to 1000 higher than that of classical growth factors. Later studies have demonstrated that pSP may function as a growth factor for the MCF-7 cells but only in the presence of extracellular glutathione [147]. The interesting observation that trefoil peptides may act as chemotaxins for *Helicobacter pylori* [148] seems to need further studies to be fully evaluated.

In rat and cat models orally administered pSP were shown to inhibit pentagastrin-induced gastric acid secretion [26], although later studies failed to demonstrate this effect [149]. In a recent study Tanaka and co-workers [150] have shown that hSP interacts with the gastric mucin in a manner that inhibits proton permeation through the mucus layer.

One of the puzzling facts in the mechanism of action of trefoil peptides is that both orally administered peptide [88] as well as small amounts of subcutaneously infused peptide [131] seem to be active in mucosal protection/healing. It may not be surprising that some effects are seen when trefoil peptides are given orally in sufficiently high quantities, since at least some of the peptide can reach the target area by passive diffusion. However, the protective effect of subcutaneously infused doses as small as 25 μg/kg/h [131] *is* surprising and seems to require the presence of specific receptors or transporter proteins close to the target site. That such sites actually exist was recently demonstrated by autoradiographic investigation of tissue samples following intravenous infusion of radiolabelled trefoil peptides (S. Poulsen, unpublished observations). A surprisingly large portion of the radiolabelled peptide (in this study ^{125}I-pSP) was taken up by the GI tract, most pronouncedly by the stomach, where it specifically binds to the mucous neck cells and pyloric glands. Figure 6 shows autoradiographs of the corbus fundic part of the stomach where radiolabelled peptide is observed in the mucous neck cells and some parietal cells. These results suggest the presence of specific receptors or transporter proteins on these cells.

It is not clear whether these binding sites resemble the pSP receptor-like binding sites from rat intestinal mucosal cells previously characterised by Frandsen and co-workers [151, 152] or the ITF-binding protein characterised by Chinery and co-workers [153] from rat intestinal epithelial membranes. If further studies lead to the isolation and characterisation of specific trefoil peptide receptors, such receptors would be an interesting target for the treatment of gastrointestinal disorders such as ulceration and inflammatory bowel disease.

Production of trefoil peptides

Three methods are in principle available for producing trefoil peptides and fragments: peptides synthesis, preparation from natural sources and recombinant technology.

No full-length trefoil peptide has so far been produced by peptide synthesis, and establishing the three disulphide bridges in the trefoil domains will probably result in a very low yield from such a process. Peptide synthesis has, however, been successfully used to generate several small peptide fragments for antibody production [10, 96, 154, 155] and subsequent radioimmunoassays [156] and ELISAs [157–159].

Porcine pancreas has been a valuable natural source for isolating pSP (pTFF2) [2, 3, 23–25], and with a content of 50 mg/kg of tissue, the purification was relatively easy [3]. From 1979 to 1985, porcine pancreas was the only source for the production of large amounts of peptide for physiological [4, 160, 161], pharmacological [26] and structural [27–29, 51–54, 162, 163] studies of trefoil peptides. The content of pS2 in human gastric juice and in MCF-7 cell media is ~0.1 mg/l, and these sources have been the starting material for the purification of human pS2 [49, 50, 164]. Human and rat ITF have been prepared from colonic and small intestinal mucosa, respectively, but the yield from these purifications was rather low (H. Kindon et al., personal communication). A recombinant yeast expression system, optimised for insulin production [165], has been successfully used for the production of human SP [166] and human and rat ITF [140]. The expression level in this system is in the order of 100 mg/l, and purification is straightforward [140, 166]. An *Escherichia coli* expression system has been used by Prud'homme and co-workers [167] for the production of human pS2 and by Chinery and co-workers for the production of rat ITF and mutants thereof [143, 168]. The expression level in the *E. coli* system is lower than that in the yeast system (0.1–1.0 mg/l), and due to the construction as an intracellular fusion protein, the downstream processing is somewhat more complicated in the *E. coli* system compared with the yeast system. In a modified *E. coli* system Chadwick and co-workers [55] obtained expression levels of ~5 mg/l of human pS2 and mutants thereof. Also, an insect cell/baculovirus system [88] and a *Bacillus subtilis* system [157] have been used for trefoil peptide production, but no expression levels are given for these systems.

Future perspectives

Increased knowledge accumulated over the last 5–10 years of the participation of trefoil peptides in protection and healing processes in the stomach and intestines demonstrates the vital importance of these peptides for the normal function of the GI tract. Especially the two gene knockout studies [46, 47] demonstrate that without normal trefoil peptide expression the dynamic process of epithelial restitution is seriously affected. Although the detailed mechanism of action of the trefoil peptides is just beginning to be uncovered, one might start to consider possible ways of interfering in epithelial restitution processes when things go wrong in the GI tract. In the case of gastric ulceration, the present strategy using antisecretory and antibiotic (e.g. against *H. pylori*) drugs seem to be very efficient and to have no major side effects. For other pathophysiological conditions in the GI tract, in which bleeding episodes and mucosal damage are major symptoms, the present treatment strategy is not equally efficient in all cases. Treatment of conditions such as duodenal ulcers, intestinal inflammation and inflammatory bowel disease, including Crohn's disease, may benefit from a more detailed

knowledge of the processes of epithelial restitution. Trefoil peptides and the way they act will no doubt be one of the central issues in understanding the processes behind epithelial restitution.

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