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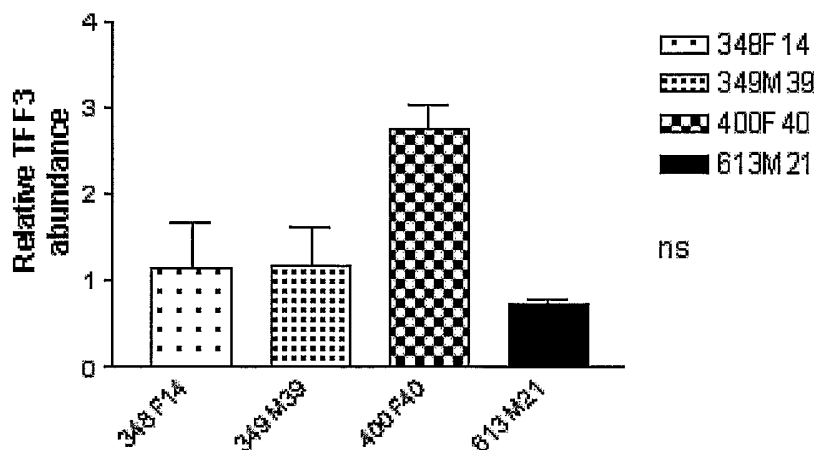
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(54) Title: USE OF TREFOIL POLYPEPTIDES IN THE TREATMENT OF DIABETES



(57) Abstract: The present invention
relates, inter alia, to the use of TFF
peptides in the treatment of diabetes
in a subject.

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USE OF TREFOIL POLYPEPTIDES IN THE TREATMENT OF DIABETES

FIELD OF THE INVENTION

5 The present invention relates to a novel use of trefoil peptides in the treatment of diabetes and other inflammation related conditions.

BACKGROUND OF THE INVENTION

10 Mammalian trefoil factors (TFFs) comprise a group of three peptides (TFF1, TFF2 and TFF3) widely distributed in the gastrointestinal tract. These peptides are characterised by containing one (TFF1 and TFF3) or two (TFF2) trefoil domains. 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 trefoil peptides are expressed in the gastrointestinal tract in a tissue-specific manner. In humans, TFF1 and TFF2 are expressed in mucus-producing cells in the stomach and duodenum, whereas TFF3 is primarily expressed in
15 goblet cells in the small and large intestine. In the case of gastric ulceration or inflammatory bowel disease the expression of trefoil peptides is highly upregulated. This suggests that trefoil peptides may function to repair damage in the gastrointestinal tract, thus acting as naturally occurring healing factors. The importance of TFFs for normal mucosal function has also been investigated in two gene knock-out studies involving deletion of the genes
20 encoding TFF1 and TFF3, respectively, by gene-targeting techniques. The TFF3 knock-out mice employed had impaired mucosal healing and died from extensive colitis after oral administration of dextran sulphate; however, it was possible to circumvent this by luminal administration of recombinant TFF3. Although several studies have documented a protective or healing effect of trefoil peptides in gastric ulceration and colitis models, the detailed
25 mechanism of action is still largely unknown. One theory is that trefoil peptides together with mucins form stable gel complexes resistant to mechanical stress and gastrointestinal proteases. Although no direct evidence for such gel formation has so far been provided, some studies have indicated an interaction/binding between trefoil peptides and mucins.

30 Diabetes is a disease in which the body does not produce or properly use insulin, a hormone that is needed to convert sugar, starches and other food components to provide energy needed for daily life. The cause of diabetes is a mystery, although genetic, environmental and life-style factors, such as obesity and lack of exercise, appear to play roles. There are two major types of diabetes, viz. Type 1, which is an autoimmune disease in which the body does not produce insulin, most often occurring in children and young adults, and Type

2, which is a metabolic disorder resulting from the body's inability to produce sufficient insulin and/or to properly use insulin. People with Type 1 diabetes must take daily insulin injections to stay alive. Type 1 diabetes accounts for 5-10 percent of diabetes. Type 2 diabetes is the most common form of the disease, accounting for 90-95 percent of diabetes. In some parts of the world, Type 2 diabetes is nearing epidemic proportions, due to an increasing prevalence of obesity and sedentary lifestyle.

International patent application WO 92/14837 describes single-domain TFF3 (TFF3) and the use of this peptide in the treatment of gastrointestinal injury.

International patent application WO 94/17102 and US patent 5,783,416 relate to human TFF2 peptides in glycosylated form, variants thereof and a method of producing TFF2 peptides in glycosylated form. International patent application WO 02/46226 relates to glycosylated Lys99-TFF2 peptides. International patent applications WO 02/102399 and WO 02/102403 relate to the use of TFF2 and dimers of TFF peptides, e.g. TFF1 and TFF3 peptides, for increasing the viscosity of mucus layers. International patent application PCT/DK03/00083 relates to the use of TFF1 or TFF3 monomer peptides for decreasing the viscosity of mucus layers.

SUMMARY OF THE INVENTION

The present invention relates, in a broad aspect, to the prevention and treatment of conditions related to inflammation, including diabetes.

The diabetes and diabetes-related conditions which may be treated in the manner disclosed herein include, but are not limited to, diabetes characterized by the presence of elevated blood glucose levels, such as hyperglycemic disorders, for example, diabetes mellitus, including both Type 1 and Type 2 diabetes, as well as other diabetic-related disorders such as obesity, elevated cholesterol, kidney-related disorders and the like. The invention described herein may be employed to lower insulin levels, improve glucose tolerance, increase hepatic glucose utilization, normalize blood glucose levels, increase apo A-I and HDL levels, decrease fibrinogen levels, stimulate hepatic fatty acid oxidation, reduce hepatic triglyceride accumulation and normalize glucose tolerance.

In one aspect, the present invention relates to the use of a TFF peptide for the preparation of a medicament for the treatment of diabetes in a subject.

In a second aspect, the invention relates to a method for the treatment of diabetes in a subject, the method comprising administering to the subject a composition comprising a therapeutically or prophylactically effective amount of a TFF peptide, whereby at least one symptom of diabetes is alleviated.

DETAILED DESCRIPTION OF THE INVENTION

Type 1 diabetes is characterized by an initial inflammatory lymphocytic infiltration around the pancreatic islets that may be followed by lymphocytic invasion of the islets, insulinitis, resulting in beta cell destruction involving beta cell cytotoxic cytokines and T-cells.

5 These observations have a striking parallel in inflammatory diseases in the intestine, such as coeliac disease that shows association with Type 1 diabetes. The fact that TFF peptides, e.g. TFF3, play an important role in susceptibility to and recovery from inflammatory diseases in the intestine, taken together with data presented herein (*vide infra*) showing the expression of TFF peptides, e.g. TFF3, in islets, indicates that TFF peptide expression in the beta cell may
10 play a similar role in Type 1 diabetes, i.e. protection against lymphocytic infiltration and destructive invasion, and promotion of recovery from damage by proliferation and neogenesis of beta cells. Although predisposing genetic factors, in particular MHC class II genes, are involved in beta cell damage, the involvement of environmental factors (e.g. virus, bacteria or toxic chemicals) that may directly damage beta cells to release autoantigens or
15 indirectly activate the immune system to react against beta cell antigens, e.g. by molecular mimicry, is also evident. *In vivo*, the initial event is accumulation of lymphocytes in the periphery of the islets that may persist without beta cell destruction, or the lymphocytes may invade the islets leading to disruption of the architecture of, and specific destruction of, the beta cells. Mediators of beta cell destruction include proinflammatory cytokines like
20 interleukin-1 (IL-1), tumor necrosis factor- α (TNF α) and interferon- γ (IFN γ), as well as cytotoxic T-lymphocytes. IL-1, which may be the most important factor, acts via activation of NF κ B that stimulates transcription of several genes, including the gene encoding inducible nitric oxide synthase (iNOS), thereby resulting in NO formation shown to be toxic to beta cells. TNF α has little toxic effect in normal beta cells, but potentiates the toxic effect of IL-1 by
25 activation of the apoptotic pathway. IFN γ activates STAT1 that induces transcription of the interferon regulatory factor-1 (IRF-1), that in turn stimulates the transcription of iNOS and thereby potentiates the effect of IL-1.

It has been shown by the inventors of the present invention that TFF peptides are highly expressed in the human pancreatic islets. This expression is co-localised with insulin.
30 Also, expression of TFF peptides during embryonic development in the rat has been measured. Regulation of TFF peptide expression by growth hormone in neonatal islets and in INS-1 cells has been studied, as has the effects of TFF peptides on islet cell attachment and migration. It is anticipated on the basis of the resulting observations that TFF peptides exhibit both protective and regenerative effects on beta cells in Type 1 diabetes, and that
35 they accordingly may be used therapeutically for the prevention and treatment of Type 1 diabetes.

The term "TFF peptide" as employed herein refers to any protein comprising one or more trefoil domain structures, each trefoil domain structure comprising 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.

5 The terms "TFF2 peptide" and "TFF2 peptides" as employed herein refer to proteins that are substantially homologous to human TFF2, the amino acid sequence of which is shown in Fig. 8 (*vide infra*). Examples within this definition are the Asn99-TFF2 and Lys99-TFF2 variants. Both Lys99-TFF2 and Asn99-TFF2 may be glycosylated at the Asn15. The terms in question also include analogs of naturally occurring TFF2 peptides. Analogs can differ from
10 naturally occurring TFF2 by amino acid sequence differences or by modifications that do not affect sequence, or by both. Analogs of the invention will generally exhibit at least 50%, more preferably 60%, more preferably 70%, more preferably 80%, more preferably 90%, and most preferably 95% or even 99%, sequence identity with a naturally occurring TFF2 sequence.

15 The term "glycosylated Lys99-TFF2", as employed herein refers to a TFF2 peptide in which the Asn residue at position 99 (see Fig. 8) is replaced with a Lys residue, and which is glycosylated at Asn15.

20 The terms "TFF dimer peptide" and "TFF dimer peptides" refer to proteins that are substantially homologous to human TFF1 or human TFF3 in their dimer forms. Fig. 8 shows TFF1 and TFF3 in the monomer form. The TFF1 dimer consists of two TFF1 monomers linked together by a disulfide bond between the cysteine amino acid residues at position 58 in each TFF1 monomer. The TFF3 dimer consists of two TFF3 monomers linked together by a disulfide bond between the cysteine amino acid residues at position 57 of each TFF3 monomer. The terms in question also encompass analogs of naturally occurring TFF dimer peptides. Analogs can differ from naturally occurring TFF dimers by amino acid sequence differences or by
25 modifications that do not affect sequence, or by both. Analogs of the invention will generally exhibit at least 70%, more preferably 80%, more preferably 90%, and most preferably 95% or even 99%, homology with a naturally occurring TFF dimer sequence.

30 Modifications include *in vivo* or *in vitro* chemical derivatization of polypeptides, e.g., acetylation or carboxylation. Also included are modifications of glycosylation, e.g. arising by modifying the glycosylation patterns of a polypeptide during its synthesis and processing or in further processing steps, e.g. by exposing the polypeptide to enzymes that affect glycosylation derived from cells that normally provide such processing, e.g. mammalian glycosylation enzymes. Also encompassed are versions of the same primary amino acid sequence that have phosphorylated amino acid residues, e.g. phosphotyrosine, phosphoserine or
35 phosphothreonine.

In addition to substantially full-length polypeptides, TFF peptides in the context of the present invention include biologically active fragments of the polypeptides. As used herein, the term "fragment" refers to a TFF peptide wherein 1-16 amino acids have been deleted from the N- or C-terminal of a full length TFF peptide, while retaining all disulphide bonds of the TFF monomer or dimer peptide. Typically, fragments of TFF1 monomer are peptides lacking 1-6 amino acids in the N-terminal and/or 1-16 amino acids in the C-terminal; fragments of TFF1 dimer are peptides lacking 1-6 amino acids in the N-terminal and/or 1-2 amino acids in the C-terminal; fragments of TFF2 or glycosylated TFF2 are peptides lacking 1-5 amino acids in the N-terminal and/or 1-2 amino acids in the C-terminal; fragments of TFF3 monomer are peptides lacking 1-10 amino acids in the N-terminal and/or 1-11 amino acids in the C-terminal; fragments of TFF3 dimer are peptides lacking 1-10 amino acids in the N-terminal and/or 1-2 amino acids in the C-terminal. The ability of a candidate fragment to exhibit a biological activity of a TFF peptide can be assessed by methods known to those skilled in the art. Also encompassed by the term "fragment" are biologically active TFF peptides containing amino acids that are normally removed during protein processing, including additional amino acids that are not required for the biological activity of the polypeptide, or including additional amino acids that result from alternative mRNA splicing or alternative protein processing events.

The terms "TFF monomer peptide" or "TFF monomer peptides" refer to a protein that is substantially homologous to human TFF1 or human TFF3 in monomer forms. Fig. 8 shows TFF1 and TFF3 in the monomer form. TFF monomer peptides also encompass analogs of naturally occurring TFF monomer peptides. Analogs can differ from naturally occurring TFF monomer by amino acid sequence differences or by modifications that do not affect sequence, or by both. Analogs of the invention will generally exhibit at least 70%, more preferably 80%, more preferably 90%, and most preferably 95% or even 99%, sequence identity with a naturally occurring TFF monomer sequence.

A TFF peptide, including a fragment or analog, is biologically active if it exhibits a biological activity of a naturally occurring TFF peptide.

The term "glycosylation", as used herein, refers to post-translational modification of a peptide wherein a carbohydrate molecule is covalently attached to the peptide. The glycosylation may take place in a eucaryotic host cell, such as a yeast cell, or it may be achieved by chemical linkage *in vitro* after production of the peptide in a cell, e.g. the peptide could be produced in a bacteria and glycosylated *in vitro* afterwards.

The term "treatment", as used herein, means the administration of an effective amount of a therapeutically active compound of the invention with the purpose of preventing development of symptoms or a disease state, or with the purpose of curing or easing such symptoms or disease states which have already developed. The term "treatment" is thus meant

to include prophylactic and protective treatment. The symptoms or disease state include, but are not limited to, diseases, e.g. gastric ulcers or asthma, inherited biological disorders or conditions induced by damaging external stimuli, e.g. Inhalation of toxic or corrosive (such as acidic) chemicals.

5 Another embodiment of the present invention relates to a pharmaceutical composition for local application.

In a further embodiment the present invention relates to a pharmaceutical composition for luminal application.

10 In a further embodiment the present invention relates to a pharmaceutical composition for parenteral administration.

In a further embodiment the present invention relates to a pharmaceutical composition for oral administration.

In a further embodiment the present invention relates to a pharmaceutical composition further comprising a mucin glycoprotein preparation.

15 In a further embodiment of the invention, the TFF peptide is independently selected from the group consisting of TFF1, TFF2 and TFF3.

In a further embodiment of the invention, the TFF peptide is human.

In a further embodiment of the invention, the TFF peptide is a dimer peptide of recombinant human TFF1.

20 In a further embodiment of the invention, the TFF peptide is a dimer peptide of recombinant human TFF3.

In a further embodiment of the invention, the TFF peptide is a dimer peptide of recombinant TFF1 or recombinant TFF3.

In a further embodiment of the invention, the TFF peptide is glycosylated.

25 In a further embodiment of the invention, the TFF peptide is a monomer peptide of recombinant TFF1 or recombinant TFF3.

In a further embodiment of the invention, the TFF peptide is a monomer peptide of recombinant human TFF1.

30 In a further embodiment of the invention, the TFF peptide is a monomer peptide of human TFF3.

In a further embodiment of the invention, the TFF peptide is human TFF2.

In a further embodiment of the invention, the TFF peptide is recombinant TFF2.

In a further embodiment of the invention, the TFF peptide is recombinant human TFF2.

In a further embodiment of the invention, the TFF peptide is human Asn99-TFF2.

35 In a further embodiment of the invention, the TFF peptide is recombinant human Asn99-TFF2.

In a further embodiment of the invention, the TFF peptide is glycosylated TFF2.

In a further embodiment of the invention, the TFF peptide is non-glycosylated TFF2.

In a further embodiment of the invention, the TFF peptide is glycosylated Asn99-TFF2.

5 In a further embodiment of the invention, the TFF peptide is recombinant glycosylated Asn99-TFF2.

In a further embodiment of the invention, the TFF peptide is non-glycosylated Asn99-TFF2.

In a further embodiment of the invention, the TFF peptide is recombinant non-glycosylated Asn99-TFF2.

10 In a further embodiment of the invention, the TFF peptide is non-glycosylated Lys99-TFF2.

In a further embodiment of the invention, the TFF peptide is recombinant non-glycosylated Lys99-TFF2.

15 In a further embodiment, the present invention relates to the use of a TFF peptide for the preparation of a medicament for the treatment of diabetes in a subject. In one embodiment the diabetes is Type 1 diabetes. In another embodiment the diabetes is Type 2 diabetes.

In a further embodiment of the invention, the subject is human.

In a further embodiment of the invention, the medicament is for oral administration.

20 In a further embodiment of the invention, the medicament is for intravenous administration.

In a further embodiment of the invention, the medicament is for subcutaneous administration.

25 One aspect of the present invention provides a method for the treatment, control or prevention of diseases, disorders or conditions in a subject which comprises administering to such subject a therapeutically effective amount of a TFF peptide. TFF peptides may be useful in treating, controlling or preventing many inflammation-related diseases and conditions, including, but are not limited to, diabetes mellitus, and especially non-insulin dependent diabetes mellitus (NIDDM), hyperglycemia, low glucose tolerance, insulin resistance, obesity, lipid disorders, dyslipidemia, hyperlipidemia, hypertriglyceridemia, 30 hypercholesterolemia, low HDL levels, high LDL levels, atherosclerosis and its sequelae, vascular restenosis, irritable bowel syndrome, inflammatory bowel disease, including Crohn's disease and ulcerative colitis, other inflammatory conditions, pancreatitis, abdominal obesity, neurodegenerative disease, retinopathy, neoplastic conditions, adipose cell tumors, adipose cell carcinomas, such as liposarcoma, prostate cancer and other cancers, including gastric, 35 breast, bladder and colon cancers, angiogenesis, Alzheimer's disease, psoriasis, high blood

pressure, Syndrome X, ovarian hyperandrogenism (polycystic ovarian syndrome), and other disorders where insulin resistance is a component.

Another aspect of the invention provides a method for the treatment, control, or prevention of hypercholesterolemia, atherosclerosis, low HDL levels, high LDL levels, hyperlipidemia, hypertriglyceridemia and/or dyslipidemia, the method comprising
5 administering to a subject in need of such treatment a therapeutically effective amount of a TFF peptide.

Another aspect of the invention provides a method of treating inflammatory conditions, including inflammatory bowel disease, Crohn's disease and ulcerative colitis, by
10 administering an effective amount of a TFF peptide. Additional inflammatory diseases that may be treated by the methodology of the present invention include gout, rheumatoid arthritis, osteoarthritis, multiple sclerosis, asthma, ARDS, psoriasis, vasculitis, ischemia/reperfusion injury, frostbite and related diseases.

TFF peptides are typically produced by recombinant DNA techniques, such as
15 described in Danish patent applications no. 2000/01847 and 2000/01850. To this end, a DNA sequence encoding the TFF peptide may be isolated by preparing a genomic or cDNA library and screening for DNA sequences coding for all or part of the peptide by hybridization using synthetic oligonucleotide probes in accordance with standard techniques (cf. Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor,
20 New York, 1989). For the present purpose, the DNA sequence encoding the peptide is preferably of human origin, i.e. derived from a human genomic DNA or cDNA library.

The DNA sequences encoding the TFF peptides may also be prepared synthetically by established standard methods, e.g. the phosphoramidite method described by Beaucage and Caruthers, Tetrahedron Letters **22** (1981), 1859 - 1869, or the method described by Matthes et
25 al., EMBO Journal **3** (1984), 801 - 805. According to the phosphoramidite method, oligonucleotides are synthesized, e.g. in an automatic DNA synthesizer, purified, annealed, ligated and cloned in suitable vectors.

The DNA sequences may also be prepared by polymerase chain reaction (PCR) using specific primers, for instance as described in US 4,683,202, Saiki et al., Science **239** (1988),
30 487 - 491, or Sambrook et al., *supra*.

The DNA sequences encoding the TFF peptides are usually inserted into a recombinant vector which may be any vector that may conveniently be subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a
35 vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid. Alternatively, the vector may be one which, when

introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

The vector is suitably an expression vector in which the DNA sequence encoding the TFF peptide is operably linked to additional segments required for transcription of the DNA. In general, the expression vector is derived from plasmid or viral DNA, or may contain elements of both. The term "operably linked" indicates that the segments are arranged so that they function in concert for their intended purposes, e.g. transcription initiates in a promoter and proceeds through the DNA sequence coding for the polypeptide.

The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice, and may be derived from genes encoding proteins either homologous or heterologous to the host cell.

Examples of suitable promoters for directing the transcription of the DNA encoding the TFF peptide in mammalian cells are the SV40 promoter (Subramani et al., Mol. Cell Biol. **1** (1981), 854 -864), the MT-1 (metallothionein gene) promoter (Palmiter et al., Science **222** (1983), 809 - 814) or the adenovirus 2 major late promoter.

An example of a suitable promoter for use in insect cells is the polyhedrin promoter (US 4,745,051; Vasuvedan et al., FEBS Lett. **311**, (1992) 7 - 11), the P10 promoter (J.M. Vlak et al., J. Gen. Virology **69**, 1988, pp. 765-776), the *Autographa californica* polyhedrosis virus basic protein promoter (EP 397 485), the baculovirus immediate early gene 1 promoter (US 5,155,037; US 5,162,222), or the baculovirus 39K delayed-early gene promoter (US 5,155,037; US 5,162,222).

Examples of suitable promoters for use in yeast host cells include promoters from yeast glycolytic genes (Hitzeman et al., J. Biol. Chem. **255** (1980), 12073 - 12080; Alber and Kawasaki, J. Mol. Appl. Gen. **1** (1982), 419 - 434) or alcohol dehydrogenase genes (Young et al., in Genetic Engineering of Microorganisms for Chemicals (Hollaender et al., eds.), Plenum Press, New York, 1982), or the TPI1 (US 4,599,311) or ADH2-4c (Russell et al., Nature **304** (1983), 652 - 654) promoters.

Examples of suitable promoters for use in filamentous fungus host cells are, for instance, the ADH3 promoter (McKnight et al., The EMBO J. **4** (1985), 2093 - 2099) or the tpiA promoter. Examples of other useful promoters are those derived from the gene encoding *A. oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, *A. niger* neutral α -amylase, *A. niger* acid stable α -amylase, *A. niger* or *A. awamori* glucoamylase (gluA), *Rhizomucor miehei* lipase, *A. oryzae* alkaline protease, *A. oryzae* triose phosphate isomerase or *A. nidulans* acetamidase. Preferred are the TAKA-amylase and gluA promoters. Suitable promoters are mentioned in, e.g., EP 238 023 and EP 383 779.

The DNA sequence encoding the TFF peptides may also, if necessary, be operably connected to a suitable terminator, such as the human growth hormone terminator (Palmiter et al., Science 222, 1983, pp. 809-814) or the TPI1 (Alber and Kawasaki, J. Mol. Appl. Gen. 1, 1982, pp. 419-434) or ADH3 (McKnight et al., The EMBO J. 4, 1985, pp. 2093-2099) terminators. The vector may further comprise elements such as polyadenylation signals (e.g. from SV40 or the adenovirus 5' Elb region), transcriptional enhancer sequences (e.g. the SV40 enhancer) and translational enhancer sequences (e.g. those encoding adenovirus VA RNAs).

The recombinant vector may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. An example of such a sequence (when the host cell is a mammalian cell) is the SV40 origin of replication.

When the host cell is a yeast cell, suitable sequences enabling the vector to replicate are the yeast plasmid 2 μ replication genes REP 1-3 and origin of replication.

The vector may also comprise a selectable marker, e.g. a gene the product of which complements a defect in the host cell, such as the gene coding for dihydrofolate reductase (DHFR) or the *Schizosaccharomyces pombe* TPI gene (described by P.R. Russell, Gene 40, 1985, pp. 125-130), or one which confers resistance to a drug, e.g. ampicillin, kanamycin, tetracyclin, chloramphenicol, neomycin, hygromycin or methotrexate. For filamentous fungi, selectable markers include amdS, pyrG, argB, niaD or sC.

To direct a TFF peptide of the present invention into the secretory pathway of the host cells, a secretory signal sequence (also known as a leader sequence, prepro sequence or pre sequence) may be provided in the recombinant vector. The secretory signal sequence is joined to the DNA sequence encoding the TFF peptide in the correct reading frame. Secretory signal sequences are commonly positioned 5' to the DNA sequence encoding the peptide. The secretory signal sequence may be that normally associated with the peptide, or may be from a gene encoding another secreted protein.

For secretion from yeast cells, the secretory signal sequence may encode any signal peptide, which ensures efficient direction of the expressed TFF peptide into the secretory pathway of the cell. The signal peptide may be naturally occurring signal peptide, or a functional part thereof, or it may be a synthetic peptide. Suitable signal peptides have been found to be the α -factor signal peptide (cf. US 4,870,008), the signal peptide of mouse salivary amylase (cf. O. Hagenbuchle et al., Nature 289, 1981, pp. 643-646), a modified carboxypeptidase signal peptide (cf. L.A. Valls et al., Cell 48, 1987, pp. 887-897), the yeast BAR1 signal peptide (cf. WO 87/02670), or the yeast aspartic protease 3 (YAP3) signal peptide (cf. M. Egel-Mitani et al., Yeast 6, 1990, pp. 127-137).

For efficient secretion in yeast, a sequence encoding a leader peptide may also be inserted downstream of the signal sequence and upstream of the DNA sequence encoding the

TFF peptide. The function of the leader peptide is to allow the expressed peptide to be directed from the endoplasmic reticulum to the Golgi apparatus and further to a secretory vesicle for secretion into the culture medium (i.e. exportation of the TFF peptide across the cell wall or at least through the cellular membrane into the periplasmic space of the yeast cell). The leader peptide may be the yeast α -factor leader (the use of which is described in e.g. US 4,546,082, US 4,870,008, EP 16 201, EP 123 294, EP 123 544 and EP 163 529). Alternatively, the leader peptide may be a synthetic leader peptide, which is to say a leader peptide not found in nature. Synthetic leader peptides may, for instance, be constructed as described in WO 89/02463 or WO 92/11378.

For use in filamentous fungi, the signal peptide may conveniently be derived from a gene encoding an *Aspergillus* sp. amylase or glucoamylase, a gene encoding a *Rhizomucor miehei* lipase or protease, or a *Humicola lanuginosa* lipase. The signal peptide is preferably derived from a gene encoding *A. oryzae* TKA amylase, *A. niger* neutral α -amylase, *A. niger* acid-stable amylase, or *A. niger* glucoamylase. Suitable signal peptides are disclosed in, e.g. EP 238 023 and EP 215 594.

For use in insect cells, the signal peptide may conveniently be derived from an insect gene (cf. WO 90/05783), such as the lepidopteran *Manduca sexta* adipokinetic hormone precursor signal peptide (cf. US 5,023,328).

The procedures used to ligate the DNA sequences coding for the TFF peptide, the promoter and optionally the terminator and/or secretory signal sequence, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for instance, Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, New York, 1989).

The host cell into which the DNA sequence encoding the TFF peptide is introduced may be any cell which is capable of producing the posttranslational modified TFF peptide, and suitable cells include yeast, fungi and higher eucaryotic cells.

Examples of suitable mammalian cell lines are the COS (ATCC CRL 1650), BHK (ATCC CRL 1632, ATCC CCL 10), CHL (ATCC CCL39) or CHO (ATCC CCL 61) cell lines. Methods of transfecting mammalian cells and expressing DNA sequences introduced in the cells are described in, e.g., Kaufman and Sharp, J. Mol. Biol. **159** (1982), 601 - 621; Southern and Berg, J. Mol. Appl. Genet. **1** (1982), 327 - 341; Loyter et al., Proc. Natl. Acad. Sci. USA **79** (1982), 422 - 426; Wigler et al., Cell **14** (1978), 725; Corsaro and Pearson, Somatic Cell Genetics **7** (1981), 603, Graham and van der Eb, Virology **52** (1973), 456; and Neumann et al., EMBO J. **1** (1982), 841 - 845.

Examples of suitable yeasts cells include cells of *Saccharomyces* spp. or *Schizosaccharomyces* spp., in particular strains of *Saccharomyces cerevisiae* or

Saccharomyces kluyveri. Methods for transforming yeast cells with heterologous DNA and producing heterologous polypeptides therefrom are described, e.g., in US 4,599,311, US 4,931,373, US 4,870,008, 5,037,743 and US 4,845,075, all of which are hereby incorporated by reference. Transformed cells are selected by a phenotype determined by a selectable marker, commonly drug resistance or the ability to grow in the absence of a particular nutrient, e.g. leucine. A preferred vector for use in yeast is the POT1 vector disclosed in US 4,931,373. The DNA sequence encoding the TFF peptide may be preceded by a signal sequence and optionally a leader sequence, e.g. as described above. Further examples of suitable yeast cells are strains of *Kluyveromyces*, such as *K. lactis*, *Hansenula*, e.g. *H. polymorpha*, or *Pichia*, e.g. *P. pastoris* (cf. Gleeson et al., *J. Gen. Microbiol.* 132, 1986, pp. 3459-3465; US 4,882,279).

Examples of other fungal cells are cells of filamentous fungi, e.g. *Aspergillus* spp., *Neurospora* spp., *Fusarium* spp. or *Trichoderma* spp., in particular strains of *A. oryzae*, *A. nidulans* or *A. niger*. The use of *Aspergillus* spp. for the expression of proteins is described in, e.g., EP 272 277, EP 238 023 and EP 184 438. The transformation of *F. oxysporum* may, for instance, be carried out as described by Malardier et al., 1989, *Gene* 78: 147-156. The transformation of *Trichoderma* spp. may be performed, for instance, as described in EP 244 234.

When a filamentous fungus is used as the host cell, it may be transformed with the DNA construct of the invention, conveniently by integrating the DNA construct in the host chromosome to obtain a recombinant host cell. This integration is generally considered to be an advantage as the DNA sequence is more likely to be stably maintained in the cell. Integration of the DNA constructs into the host chromosome may be performed according to conventional methods, e.g. by homologous or heterologous recombination.

Transformation of insect cells and production of heterologous polypeptides therein may be performed as described in US 4,745,051, US 4,879,236, US 5,155,037, 5,162,222 or EP 397,485, all of which are incorporated herein by reference. The insect cell line used as the host may suitably be a *Lepidoptera* cell line, such as *Spodoptera frugiperda* cells or *Trichoplusia ni* cells (cf. US 5,077,214). Culture conditions may suitably be as described in, for instance, WO 89/01029 or WO 89/01028, or any of the aforementioned references.

The transformed or transfected host cell described above is then cultured in a suitable nutrient medium under conditions permitting expression of the TFF peptides, after which all or part of the resulting peptide may be recovered from the culture. The medium used to culture the cells may be any conventional medium suitable for growing the host cells, such as minimal or complex media containing appropriate supplements. Suitable media are available from commercial suppliers or may be prepared according to published recipes (e.g. in catalogues of the American Type Culture Collection, ATCC). The TFF peptides produced by the cells may

then be recovered from the culture medium by conventional procedures, including separating the host cells from the medium by centrifugation or filtration, precipitating the proteinaceous components of the supernatant or filtrate by means of a salt, e.g. ammonium sulphate, purification by a variety of chromatographic procedures, e.g. ion exchange chromatography, gel-filtration chromatography, affinity chromatography or the like, dependent on the type of polypeptide in question.

In the pharmaceutical composition of the invention, the TFF peptides may be formulated by any of the established methods of formulating pharmaceutical compositions, e.g. as described in Remington's Pharmaceutical Sciences, 1985. The composition may be in a form suited for systemic injection or infusion and may, as such, be formulated with sterile water or an isotonic saline or glucose solution. The compositions may be sterilized by conventional sterilization techniques which are well known in the art. The resulting aqueous solutions may be packaged for use or filtered under aseptic conditions and lyophilized, the lyophilized preparation being combined with the sterile aqueous solution prior to administration. The composition may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as buffering agents, tonicity adjusting agents and the like, for instance sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, etc.

The pharmaceutical composition of the present invention may also be adapted for nasal, transdermal or rectal administration. The pharmaceutically acceptable carrier or diluent employed in the composition may be any conventional solid carrier. Examples of solid carriers are lactose, terra alba, sucrose, talc, gelatin, agar, pectin, acacia, magnesium stearate and stearic acid. Similarly, the carrier or diluent may include any sustained release material known in the art, such as glyceryl monostearate or glyceryl distearate, alone or mixed with a wax. The amount of solid carrier will vary widely, but in a unit dosage form of the composition will usually be from about 25 mg to about 1 g.

The concentration of the TFF peptides in the composition may vary widely, i.e. from from about 5% to about 100% by weight. A typical concentration is in the range of 50-100% by weight. A unit dosage of the composition may contain from about 1 mg to about 200 mg, typically from about 25 mg to about 75 mg, such as about 50 mg, of the peptide.

The term "therapeutically effective amount" refers to the effective dose to be determined by a qualified practitioner, who may titrate dosages to achieve the desired response. Factors for consideration with regard to dose will include potency, bioavailability, desired pharmacokinetic/pharmacodynamic profiles, condition of treatment, patient-related factors (e.g. weight, health, age, etc.), possible co-administration of other medications, time of administration, or other factors known to a medical practitioner. The dosage of a TFF peptide

administered to a patient will vary with the type and severity of the condition to be treated, but is generally in the range of 0.1-1.0 mg/kg body weight.

The term "subject" as employed in the present context is intended to indicate any animal, in particular a mammal, such as a human, and may, where appropriate, be used interchangeably with the term "patient".

The present invention is further described by the following examples, which should not be construed as limiting the scope of the invention.

10 BRIEF DESCRIPTION OF THE DRAWINGS

The present invention is described in further detail in the examples with reference to the appended drawings, which are as follows:

15 Fig. 1. Immunocytochemical (peroxidase) staining of human pancreas for TFF1(A,B), TFF2(C,D) and TFF3 (E,F) without (A,C,E) and with (B,D,F) absorption with the corresponding peptides.

20 Fig 2. Co-localization of insulin and TFF3 in a human pancreatic islet. A section through human pancreatic tissue was stained by double immunofluorescence for insulin (green; A) and TFF3 (red; B). C shows overlay of photo A and B. Most insulin cells are also immunopositive for TFF3. A few TFF3 cells (indicated by arrows) show no immunoreactivity for insulin. Bar = 30 μ m.

25 Fig. 3. Expression of TFFs in human islets isolated from four individual donors as determined by RT-PCR using ribosomal protein L13 as housekeeping gene.

Fig. 4. Real-time PCR for TFF3 mRNA in human islets isolated from four individual donors relative to RPL13 mRNA.

30

Fig. 5. Expression of TFF3 mRNA during rat embryonic development in pancreas (A) and intestine (B), as measured by real-time PCR relative to phosphoglycerate kinase-1 (PGK) mRNA. Note the differences in scale.

Fig. 6. Effect of hGH on expression of TFF3 (A,C) and TFF1 (B,D) in neonatal rat islets (A,B) and INS-1 cells (C,D) cultured with hGH (500 ng/ml) for 4 and 24 hours, as evaluated by real-time PCR relative to PGK.

5 Fig. 7. Effect of culture of neonatal rat islets for 3 weeks in RPMI 1640 with 2% human serum (A) without any addition, (B) with addition of hGH (500 ng/ml) and (C) with addition of TFF3 (100 nM).

Fig. 8. The mammalian Trefoil Factors (TFFs), TFF1 and TFF3 in monomer form and Asn99-TFF2. The figure shows the human sequences. Dimers of TFF1 and TFF3 are formed by disulfide linkage of the cysteine amino acid residues with indicated free sulfhydryl group on two different molecules of TFF1 or TFF3, respectively. Disulphide bonds (Cys6-Cys104, Cys8-Cys35, Cys19-Cys34, Cys29-Cys46, Cys58-Cys84, Cys68-Cys83, Cys78-Cys95) in the structure of human TFF2 are represented schematically.

15

Fig. 9. Structure of human TFF3 in dimer form.

The invention described and claimed herein is not to be limited in scope by the specific embodiments herein disclosed, since these embodiments are intended as
20 illustrations of several aspects of the invention. Any equivalent embodiments are intended to be within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims.

25

EXAMPLES

Chemicals used as buffers and substrates are commercial products of at least reagent grade. Media and Solutions for the growth of filamentous fungal host cells are known
30 to the person skilled in the art and as described in, e.g., US 6,461,837 or US 5,856,163. Control sequences for the growth of expression vectors in filamentous fungal host cells are also described in US 6,461,837 or US 5,856,163, incorporated herein by reference.

Example 1

Construction of an expression system for Asn99-TFF2 and Lys99-TFF2.

An expression system expressing a mutant human TFF2 with an Asn at position 99 of the mature protein (hSP-Asn₉₉) has been described previously (Thim, L., 1993, *FEBS Letters* 5 318: 345-352).

The yeast plasmid denoted pKFN-1847 (Thim et al., *FEBS Letters*, 1993, 318: 345-352) contains an expression cassette comprising an *EcoRI* – *XbaI* DNA fragment inserted into the plasmid between the transcription-promoter (located on a *Sall* – *EcoRI* fragment) and the transcription-terminator of the *Saccharomyces cerevisiae* TPI1 gene.

10 In plasmid pKFN-1847, the *EcoRI* – *XbaI* fragment encodes a fusion protein composed of a leader sequence, a Lys-Arg cleavage site for the dibasic processing endopeptidase KEX2, and hSP-Asn₉₉. In order to construct a plasmid encoding Lys99-TFF2, the following steps are performed using standard molecular biology techniques (e.g. Sambrook, J., Fritsch, E.F. and Maniatis, T., *Molecular Cloning: A laboratory Manual*, Cold Spring Harbour Laboratory Press, New York, 1989).

15 A 688 bp DNA fragment containing the *EcoRI* – *XbaI* DNA fragment and encoding the leader-hSP fusion protein is amplified with PCR from plasmid pKFN-1847 using oligonucleotides EA-ECO: (5'-CTA TTT TCC CTT CTT ACG-3') and E147: (5'-TAA TCT TAG TTT CTA GAC TTA GTA ATG GCA GTC TCT CAC AGA CTT CGG GAA GAA GC -3'). EA-ECO corresponds to a sequence located 114 bp upstream from the *EcoRI* site of the *EcoRI* – *XbaI* DNA fragment containing the expression cassette. E147 has been designed to introduce a single nucleotide mutation in the DNA sequence encoding hSP-Asn₉₉ changing Asn₉₉ of hSP-Asn₉₉ to Lys₉₉. After digestion with *EcoRI* and *XbaI* the DNA sequence encoding hSP-Lys₉₉, hereafter referred to as Lys99-TFF2, can thus be cloned as a *EcoRI*-*XbaI* DNA fragment.

25 The *EcoRI* – *XbaI* PCR fragment containing the DNA sequence encoding the leader- Lys99-TFF2 fusion protein is ligated to the *Apal* – *EcoRI* DNA fragment of pMT742 (Egel-Mitani et al., *Gene*, 1988, 73: 113-120) containing the TPI1 promoter from *S. cerevisiae* and the *Apal* – *XbaI* vector fragment of pMT742, resulting in plasmid pEA314. The plasmid pMT742 has a similar organization as pKFN-1847. The vector construct may be constructed to contain the TAKA/NA2-tpi leader hybrid promoter, the AMG terminator, and the full-length *Aspergillus nidulans* amdS gene as a selectable marker.

30 The expression plasmid is propagated in *E. coli*, grown in the presence of ampicillin and isolated using standard techniques (Sambrook et al., 1989). The plasmid DNA is checked for

insert by appropriate restriction nucleases (e.g. *EcoRI*, *NcoI*, *Apal*, *XbaI*) and is shown by sequence analysis to contain the proper DNA sequence encoding Lys99-TFF2.

The plasmids are transformed into the host fungus and grown using standard methods known to the person skilled in the art.

- 5 Purification of LYS99-TFF2. Host cell fermentation supernatant from is concentrated from 2.5 ml to 0.25ml using a Centricon® YM-3 3000 centrifugal filter device with the method described by the manufacturer (Millipore Corporation). The concentrated sample (0.25ml) is injected onto a Vydac 214TP54 reverse-phase C4 HPLC column (0.46 x 25 cm) equilibrated at 25 °C at a flow rate of 1.0 ml/min with 0.1% (v/v) Trifluoroacetic acid in 10% (v/v)
- 10 acetonitrile. After isocratic elution in 10 min the concentration of acetonitrile in the eluting solvent is raised to 60% (v/v) over 25 minutes. Absorbance is measured at 214 nm. The peaks eluting at 27.254 min. and 28.038 min. is found by mass spectrometry analysis to represent glycosylated human Lys99-TFF2 and non-glycosylated human Lys99-TFF2, respectively.

15

Example 2

Construction of an expression system for TFF3.

Cloning of Rat TFF3 (rTFF3) and Human TFF3 (hTFF3). Rat TFF3 cDNA is cloned from a cDNA library prepared from isolated rat small intestinal cells in the AZAPII vector

- 20 (Stratagene) as previously described (Suemori, S. et al (1991) *Proc. Natl. Acad. Sci. USA.* 88, 11017-1 1021, 1991). The human TFF3 cDNA is cloned from a library prepared with normal human colon cDNA in the AgtII vector (Clontech) screened with the full-length rTFF3 cDNA as previously reported (Podolsky, D. K. et al *J. Biol. Chem.* 268, 6694-6702, 1993). Plaque-purified clones are amplified by PCR using primers prepared from the sequences
- 25 flanking the AgtI 1 insertion site (35 cycles of 95 °C for 1 min, 48 °C for 1.5 min, and 78 °C for 2 min, followed by 72 °C for 8 min). The amplified insert is subcloned into the PCR 1000 vector (Invitrogen). The vector construct may be constructed to contain the TAKA/NA2-tpi leader hybrid promoter, the AMG terminator, and the full-length *Aspergillus nidulans* amdS gene as a selectable marker.

- 30 Construction of rTFF3 and hTFF3 Secreting host strains. The host expression plasmids pHW 756 containing the rTFF3 gene and pHW 1066 containing the hTFF3 gene may be composed essentially as described for the hSP expression vector (Thim, L. et al *FEBS Lett.* 318, 345-352, 1993). The TPI promoter and terminator sequences are from *S. cerevisiae* triosephosphate isomerase gene, and the selection marker is POT from *Saccharomyces*

pombe triosephosphate isomerase gene. The 53 amino acid long signal-leader sequence is as described:

MKAVFLVI SLIGFCWAQPVTGDESSVEIPEESLIIAENTTLANVAMAERLEKR

Immediately following this sequence is the hTFF3 sequence, in accordance with Hauser et

5 al. (Hauser, F. et al. *Proc. Natl. Acad. Sci. U.S.A.* 90, 6961-6965, 1993) or the rTFF3

sequence: QEFVGLSPSQCMVPANVRVDCGYPTVTSE

QCNNRGCCFDSSIPNVPWCFLQETECTF in accordance with Chinery et al. (Chinery, R. et al *Biochem. J.* 285, 5-8, 1992). The plasmids are transformed into the host fungus and grown using standard methods known to the person skilled in the art.

10 Purification of Recombinant rTFF3. The concentration of rTFF3 in the fermentation broth and fractions obtained during the purification is measured by analytical HPLC. Aliquots (usually 50-200 μ L) are injected onto a Vydac 214TP54 reversed-phase C4 HPLC column (4.6 x 250 mm) equilibrated at 30 °C at a flow rate of 1.5 mL/min with 0.1 % (v/v) TFA in 15% (v/v) acetonitrile. After 10 min of isocratic elution, the concentration of acetonitrile in the eluting
15 solvent is raised to 55% over 40 min. Absorbance is measured at 214 nm. Three peaks eluting at 26.5, 27.3, and 28.2 min are found to represent dimer forms of rTFF3. The peptides are quantified using a calibrated TFF2 standard (Thim, L. et al *FEBS Lett.* 318, 345-352, 1993). From a 10-L fermenter, 8.7 L of fermentation broth is isolated by centrifugation. The supernatant is diluted with 14.8 L of distilled water to lower the conductivity. The sample
20 is pumped onto a Fast Flow S-Sepharose (Pharmacia) column (5 x 42 cm) with a flow rate of 600 mL/h. Prior to the application, the column is equilibrated in 50 mM formic acid buffer, pH 3.7. Rat TFF3 is eluted from the column by 50 mM formic acid, pH 3.7, containing 50 mM NaCl. Fractions of 100 mL are collected at a flow rate of 600 mL/h and analyzed for the content of rTFF3. Fractions from the previous step containing rTFF3 are pooled (2.3 L) and
25 pumped onto an Amberchrome G-71 column (5 x 10 cm). Prior to the application, the column is equilibrated in 10 mM ammonium acetate buffer, pH 4.8, at a flow rate of 0.5 L/h. After application, the column is washed with 0.5 L of equilibration buffer and eluted with 10 mM ammonium acetate buffer, pH 4.8, containing 60% (v/v) of ethanol at a flow rate of 0.1 L/h. Fractions of 10 mL are collected and pooled according to their content of rTFF3. The ethanol
30 concentration in the pool is increased from 60% (v/v) to 87% (v/v) by the addition of 2 vols of ethanol (99.9%, v/v), and rTFF3 is precipitated by cooling the resulting mixture to -25 °C for 16 h. The precipitate is collected by centrifugation for 1 h at 10000 g at -25 °C and redissolved at room temperature in 130 mL of 20 mM formic acid, pH 3.0. The sample is pumped onto a Fast Flow SP-Sepharose (Pharmacia) column (5 x 20 cm) with a flow rate of
35 50 mL/h. Prior to the application, the column is equilibrated with 20 mM formic acid, pH 3.0. Peptides are eluted from the column by a linear gradient between 1.5 L of 50 mM formic

acid, pH 3.0, and 1.5 L of formic acid, pH 3.0, containing 0.5 M NaCl. Fractions (10 mL) are collected at a flow rate of 80 ml/h, and the absorbance is measured at 280 nm. Fractions are assayed for the content of rTFF3. Fractions corresponding to rTFF3 are pooled. Rat TFF3 is further purified by preparative HPLC. Pooled fractions (900 ml) are pumped onto a Vydac 214TP1022 C4 preparative HPLC column (2.2 x 25 cm) equilibrated in 0.1% (v/v) TFA. The peptides are eluted at 25 °C and at a flow rate of 5 mL/min with a linear gradient (540 ml) formed from MeCN/H₂O/TFA (10:89.9:0.1, v/v) and MeCN/H₂O/TFA (65:34.9:0.1, v/v). UV absorption is monitored at 280 nm, and fractions corresponding to 10 mL are collected and analyzed for the content of rTFF3. Fractions containing rTFF3 are pooled, and the volume is reduced to 30% by vacuum centrifugation. From the resulting pool, rTFF3 is isolated by lyophilization. The total yield of rTFF3 from 8.7 L of fermentation medium is 236 mg, corresponding to an overall purification yield of 24%. Purification of Recombinant hTFF3. The concentration of hTFF3 in the fermentation broth and fraction, obtained during the purification, is measured in an HPLC system identical to the one described for rTFF3. In this system, two peaks eluting at 21.2 and 27.1 min are found by mass spectrometry and sequence analysis to represent a dimer and a monomer form of hTFF3 (see Results). From a 10-L fermenter, 8.0 L of fermentation broth is isolated by centrifugation. The sample is dialyzed three times (each time in 24 h) against 40 L of 10 mM formic acid, pH 2.5. The sample is pumped (0.25 L/h) onto an Fast Flow SP-Sepharose (Pharmacia) column (5 x 40 cm). The column is washed with 5 L of 20 mM formic acid, pH 2.5, and eluted with a linear gradient formed by 5 L of 20 mM formic acid, pH 2.5, and 5 L of formic acid, pH 2.5, containing 1 M of NaCl. Fractions of 100 mL are collected and analyzed for the content of hTFF3. Two forms of hTFF3 are eluted from the column: one representing a monomer form of hTFF3 (eluting at 0.5 M of NaCl) and one representing a dimer form of hTFF3 (eluting at 0.78 M of NaCl). Fractions corresponding to the two forms are pooled separately. Each fraction is divided into three equal parts (volume, approximately 700 mL) and pumped onto a Vydac 214TP1022 C4 column (2.2 x 25 cm) equilibrated in 0.1% (v/v) TFA. The peptides are eluted at a flow rate of 4 mL/min with a linear gradient (540 mL) between MeCN/H₂O/TFA (10:89.9:0.1, v/v) and MeCN/H₂O/TFA (65:34.9:0.1, v/v). UV absorption is monitored at 280 nm, and fractions corresponding to 10 mL are collected and analyzed for the content of hTFF3. Fractions from the previous step containing hTFF3 (monomer) and hTFF3 (dimer) are pooled separately, and the pH is adjusted to 3.0. The samples are applied separately onto an SP-Sepharose HiLoad 16/10 (Pharmacia) column (1.6 x 10 cm) equilibrated in 20 mM formic acid, pH 3.0, containing 40% (v/v) ethanol. The column is washed with 80 mL of equilibration buffer and eluted at a flow rate of 4 mL/min with a linear gradient between 200 mL of 20 mM formic acid, pH 3.0, 40% (v/v) ethanol, and 200 mL of 20 mM formic acid, pH

3.0, 40% (v/v) containing 1 M NaCl. Fractions (5 mL) are collected and analyzed for the content of hTFF3. Fractions containing hTFF3 (monomer) and hTFF3 (dimer), respectively, are pooled, and the peptide content is precipitated by adjusting the ethanol concentration to 90% (v/v) and cooling the mixture for 72 h at -25 °C. The precipitate is collected by centrifugation and lyophilized. The total yield from 8 L of fermentation broth is 256 mg of hTFF3 (monomer) and 133 mg of hTFF3 (dimer) corresponding to an overall purification yield of 50% and 65%, respectively, for the monomer and dimer forms.

Example 3

10 Construction of an expression system for TFF1.

Recombinant human TFF1 is prepared as previously described (Kannan, R. et al. (2001) Protein Expression and Purification 21, 92-98)

Example 4

15 Role of TFF3 in beta cell protection and neogenesis in Type 1 diabetes:

Human pancreatic islets – Human pancreatic islets samples were obtained from the multi-center European Union supported program on β -cell transplantation in diabetes directed by Professor D Pipeleers, which has been approved by central and local ethical committees.

20 The human isles preparations and isolations for the present study were from four donors (two men and two women, aged 14- 40) as previously described (Gromada, J. et al. (1998) Diabetes 47, 57-65).

Fetal rat tissues – Timed pregnant Wistar rats were obtained from Mollegaard Breeding and Research Centre, Denmark. The animal handling and experimentation were approved and carried out according to the regulations as specified under the Protection of Animals Act by the Central Authority. Fetal pancreatic and intestinal tissues were harvested at gestational days 12 (E12), 13 (E13) and 19 (E19) and isolation of total RNA from the fetal tissues was as previously described (Lee, Y.C. et al. (1999) Mol. Cell. Endo. 155, 27-35). First strand cDNA synthesis of the fetal samples (1 ug total RNA from each timed sample) was performed using Promega Access Reverse Transcription System (Promega Corp., USA) with oligo dT, and followed by quantitative polymerase chain reaction (PCR) in the LightCycler (Roche, Germany)(see below for detailed reaction conditions).

30 Pancreatic rat islet isolation - Freshly dissected pancreas from newborn Wistar rats (aged 3- 5 days)(obtained from Mollegaard Breeding and Research Centre, Denmark) was subjected to collagenase digestion, and purified on a Percoll gradient. Subsequently, islets were

handpicked under a stereomicroscope with a constriction pipette, and the pooled newborn islets (~ 8000) were cultured in RPMI 1640 medium containing L-glutamine (2 mM), penicillin (100 U/ml), and 10% newborn calf serum for 5 days as previously described (Lee, Y.C. et al. (1999) Mol. Cell. Endo. 155, 27-35)

- 5 Growth hormone (GH) treatment, RNA isolation and cDNA synthesis - Pooled pancreatic islets were then sub-divided into separate dishes containing equal number of islets cultured with RPMI 1640 containing 0.5% human serum, plus or minus human GH (500 ng/ ml)(Novo Nordisk A/S, Denmark), up to 24 hours. Total cellular RNA from the rat islets was isolated at time-points, 0, 4, and 24 hours after the addition of human GH, using RNeasy kit (Qiagen, Germany) according to manufacturer's recommended protocol. First-strand cDNA were synthesised from the timed RNA samples (1. 25 ug of total RNA each) using Promega Access Reverse Transcription System (Promega Corp., USA) with oligo dT, and followed by quantitative polymerase chain reaction (PCR).

- Effects of human GH and TFF3 on newborn rat islets attachment and spreading - Islets were pre-cultured for 7 days in 4 ml RPMI 1640 (batches of 200 islets), then were divided to four sets and cultured for further 3 weeks under the following culture conditions: (a) RPMI 1640, (b) RPMI 1640 plus human GH (500 ng/ml), (c) Trefoil factor 3 monomer (TFF3m) (10^{-7} M), (d) or Trefoil factor 3 dimer (TFF3d) (10^{-7} M). Human GH, TFF3m and TFF3d were all from Novo Nordisk A/S, Denmark.

- 20 Cell culture of INS1 cells, GH treatment, RNA isolation and cDNA synthesis - The INS-1 cells were grown in monolayer culture in RPMI-1640 medium containing 11 mM glucose (Gibco Inc. USA) supplemented with 10 mM HEPES, 10% heat-inactivated foetal calf serum (FCS), 2 mM L-glutamine, 1 mM sodium pyruvate, 100 ug/ml streptomycin, 50 uM β -mercaptoethanol, 100 IU/ml penicillin in a humidified atmosphere (5% CO₂, 95% air) at 37 C as previously described (Asfari, M. et al. (1992) Endocrinology 130, 167-168). When the cells reached 80% confluence, they were washed once with the culture medium, and pre-incubated at 37 C in the supplemented RPMI-1640 cultured medium containing 0.1 % FCS, and seeded in 60 mm petri dishes, followed by the addition of human GH (500 ng/ml). The isolation of cellular total RNA was carried out at time 0, 4 and 24 hours after the addition of GH. Total cellular RNA isolation from the INS-1 cells and first strand cDNA synthesis of the timed RNA samples (1.25 ug total RNA per sample) were the same as for the rat islets as mentioned above.

- Standard and Real-time PCR of human, rat pancreatic islets and INS1 cells – Total RNA isolation and first strand cDNA synthesis of human islets (1 ug total RNA each sample) were performed using standard techniques.

For the standard and real-time PCR of human islets, TFF primers designed and sequences were identical to those as described by Dossinger et al (Dossinger, V. et al. (2002) Cell. Physiol. Biochem. 12, 197-206), resulting in the amplification of PCR products for TFF1 (NM_003225.1)(146 bp), TFF2 (NM_005423.1)(137 bp), and TFF3 (NM_003226.1)(124 bp),
5 respectively; and Ribosomal Protein L13 (RPL13) (NM_009438) expression in human pancreatic islets was used as reference gene for the TFFs expression in the human islet cDNA samples. The primer pairs for RPL13 were 5 - CCACCCTATGACAAGAAAAAGC - 3(forward) and 5 - ACATTCTTTTCTGCCTGTTTCC -3 (reverse) (giving rise to an amplification product of 227 bp). For the quantitation of human islet TFF3 mRNA expression,
10 standard PCR of human islet cDNA was carried out for 40 cycles using PCR master mix (Roche, Germany) annealing at 55 C [94C 2 min (94C 1 min, 55C 1.5 min, 72C, 2 min) 72 C 10 min] using a gradient PCR cycler (Eppendorf, Germany) to obtain a PCR product containing TFF3 primers-target sequence. This PCR product was serially diluted by a factor of 10 creating a series of diluted TFF3 primers-target solutions acting as the standards for
15 the quantitative study of TFF3 expression in the human islets samples.

To generate the calibration standards for the quantitation of mRNA expression in rat islets and INS1 cells, PCR of control rat islets cDNA was performed for 35 cycles with PCR master mix (Roche, Germany) in the presence of the following primers annealing at 55 C [94C 2 min (94C 1 min, 55C 1.5 min, 72C, 2 min) 72 C 10 min] using a gradient PCR cycler (Eppendorf,
20 Germany) in a total reaction volume of 50 ul. This PCR protocol was used throughout when different primer-sets were used in the individual PCR reaction to generate the corresponding primer-related DNA fragment. The primers designs were based on rat sequences for TFFs and mouse sequence for phosphoglycerate kinase 1 (PGK). The forward and reverse primer sequences for each gene product are as the following: TFF 2(SP) (M97255), 5- TCT TGG
25 TAG TGG TCC TTG TCT TG -3, 5- GAA GAT CAG GTT GGA AAA GCA G -3, (fragment size, 317 bp); TFF 1 (pS2) (D83231), 5-CAA GGT GAC CTG TGT CCT C -3, 5- CTT GCT GGT TCT CAA TGA CC -3, (size, 218 bp); TFF 3 (intestinal trefoil factor, ITF) (M80826), 5- ATG GAG ACC AGA GCC TTC T -3, 5- GGA TGC TGG AGT CAA AAC AG -3, (size, 193 bp); PGK (M15668), 5'- CTG GAA AAC CTC CGC TTT CA -3', 5'- TGG CAG ATT CAC AC
30 -3' (size, 191 bp). The PCR generated products were size-confirmed by gel-electrophoresis, cloned into pCR 2.1 -TOPO vector by TOPO cloning kit (Invitrogen A/S, Denmark), and the fidelity of the PCR products was verified and ascertained by sequence analysis (GATC Biotech AG, Constance, Germany). The PCR-fragment containing plasmids were linearised by HindIII, serial-diluted by a factor of 10, to create a series of PCR-fragment bearing diluted
35 solutions, and used subsequently as the external calibration standards for the quantification of the gene expression studies in the real-time PCR. Quantitative PCR was performed using

the LightCycler (Roche, Germany) with Quantitect SYBR Green PCR mix (Qiagen, Germany) according to the recommended protocol for the use of this reagent mix in the LightCycler (LC). The quantitative PCRs were all carried out using the same reaction conditions for denaturation, amplification, and extension [initial activation 15 min at 95 C; 3-cycling step: 5 15s denaturation at 94 C, 20s annealing at 55 C, extension 20s at 72 C; for 35 to 45 cycles]. Gel electrophoresis and/or melting curve analysis were used for the detection and confirmation of the LC PCR.

TFF3 in the endocrine pancreas:

10 Surprisingly, the inventors of the present invention found TFF3 in the endocrine human pancreas, whilst TFF1, 2 and 3 were detected in some ductal cells. Based on these observations it is suggested that TFF3 may play a role in beta cell susceptibility to lymphocytic invasion, proinflammatory cytokines and autoimmune destruction in Type 1 diabetes. Furthermore, the TFFs may be involved in beta cell proliferation and neogenesis 15 from progenitor cells present in the duct epithelium.

Expression of TFF3 in human islets:

Using antisera to human TFF1, 2 and 3 we stained sections of human pancreas (obtained from The Islet Transplantation Center in Brussels directed by D. Pipeleers). Only the 20 antiserum to TFF3 showed a specific staining of the islets that could be blocked by absorption to the peptide (Fig. 1). Double staining for insulin showed both that most beta cells expressed TFF3 in varying amounts (Fig. 2). All three TFFs were expressed in some pancreatic duct epithelial cells (not shown). Using specific primers for the three TFFs we examined the expression of the mRNA in RNA extracts of islets from four different pancreas 25 donors (obtained from The Islet Transplantation Center in Brussels directed by D. Pipeleers). In all four cases, distinct expression of TFF3 mRNA [that was quantified by real-time PCR (LightCycler) using TBP as housekeeping control] was found, whereas TFF1 was detectable in two cases and TFF2 was weakly expressed in two cases (Fig. 3 and Fig. 4).

30 Expression of TFF3 during embryonic development in the rat:

In order to determine whether TFF3 is expressed in the embryonic pancreas we compared the expression pattern in pancreas and intestine at embryonic age 12, 13 and 20 days by real-time RT-PCR (Fig. 5). As can be seen, the expression level is similar in the two organs in early development, whereas a steep increase takes place in the intestine later in 35 development, but not in the pancreas.

Regulation of TFF3 expression by growth hormone in neonatal rat islets and in INS-1 cells:

In order to determine whether the expression of TFFs is regulated by GH, we exposed neonatal rat islets and INS-1 cells (obtained from C. Wollheim, Geneva) to 500 ng/ml hGH for 4 and 24 hours, respectively. Total RNA was isolated and subjected to real-time RT-PCR (LightCycler). We found a marked upregulation of TFF3 in both islets and INS-1 cells, both after 4 and 24 hours. In contrast, there was a marked decrease in the expression of TFF1 by GH (Fig. 6).

Effects of TFF3 on islet cell attachment and migration:

In order to study the possible effect of TFF3 on islet attachment we cultured neonatal islets in tissue culture dishes in RPMI 1640 with 2% human serum, and with no addition, with addition of 500 ng/ml hGH or with addition of 10^{-7} mol/l TFF3 for 3 weeks. In the absence of any addition no attachment occurred, whereas both hGH and TFF3 resulted in attachment and migration of islet cells (Fig. 7). These data suggest that the promotion of attachment and migration by GH may at least in part be mediated by increased expression of TFF3.

CLAIMS

1. Use of a TFF peptide for the preparation of a medicament for the treatment of diabetes in a subject.

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2. The use according to claim 1, wherein the diabetes is Type 1 diabetes.

3. The use according to claim 1, wherein the diabetes is Type 2 diabetes.

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4. The use according to any one of claims 1-3, wherein the subject is a human.

5. The use according to any one of claims 1-4, wherein the medicament is for oral administration.

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6. The use according to any one of claims 1-4, wherein the medicament is for intravenous administration.

7. The use according to any one of claims 1-4, wherein the medicament is for subcutaneous administration.

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8. The use according to any one of claims 1-7, wherein said TFF peptide is independently selected from the group consisting of TFF1, TFF2 and TFF3.

9. The use according to any one of claims 1-8, wherein said TFF peptide is a monomer peptide of recombinant TFF1 or recombinant TFF3.

25

10. The use according to any one of claims 1-8, wherein said TFF peptide is a dimer peptide of recombinant TFF1 or recombinant TFF3.

11. The use according to any one of claims 1-8, wherein said TFF peptide is recombinant TFF2.

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12. The use according to any one of claims 1-11, wherein said TFF peptide is a human peptide.

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13. The use according to claim 11, wherein said TFF2 is selected from the group consisting of human Asn99-TFF2 and human Lys99-TFF2.

14. The use according to any one of claims 1-13, wherein said TFF peptide is glycosylated.

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15. The use according to any one of claims 1-13, wherein said TFF peptide is non-glycosylated.

16. A method for the treatment of diabetes in a subject, said method comprising administering to said subject a composition comprising a therapeutically or prophylactically effective amount of a TFF peptide, whereby at least one symptom of diabetes is alleviated.

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17. The method according to claim 16, wherein the diabetes is Type 1 diabetes.

15

18. The method according to claim 16, wherein the diabetes is Type 2 diabetes.

19. The method according to any one of claims 16-18, wherein said subject is a human.

20. The method according to any one of claims 16-19, wherein said TFF peptide is administered orally.

20

21. The method according to any one of claims 16-19, wherein said TFF peptide is administered intravenously.

22. The method according to any one of claims 16-19, wherein said TFF peptide is administered subcutaneously.

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23. The method according to any one of claims 16-22, wherein said TFF peptide is independently selected from the group consisting of TFF1, TFF2 and TFF3.

30

24. The method according to any one of claims 16-23, wherein said TFF peptide is a monomer peptide of recombinant TFF1 or recombinant TFF3.

25. The method according to any one of claims 16-23, wherein said TFF peptide is a dimer peptide of recombinant TFF1 or recombinant TFF3.

35

26. The method according to any one of claims 16-23, wherein said TFF peptide is recombinant TFF2.

5 27. The method according to any one of claims 16-26, wherein said TFF peptide is a human peptide.

28. The method according to claim 26, wherein said TFF2 peptide is selected from the group consisting of human Asn99-TFF2 and human Lys99-TFF2.

10 29. The method according to any one of claims 16-28, wherein said TFF peptide is glycosylated.

30. The method according to any one of claims 16-28, wherein said TFF peptide is non-glycosylated.

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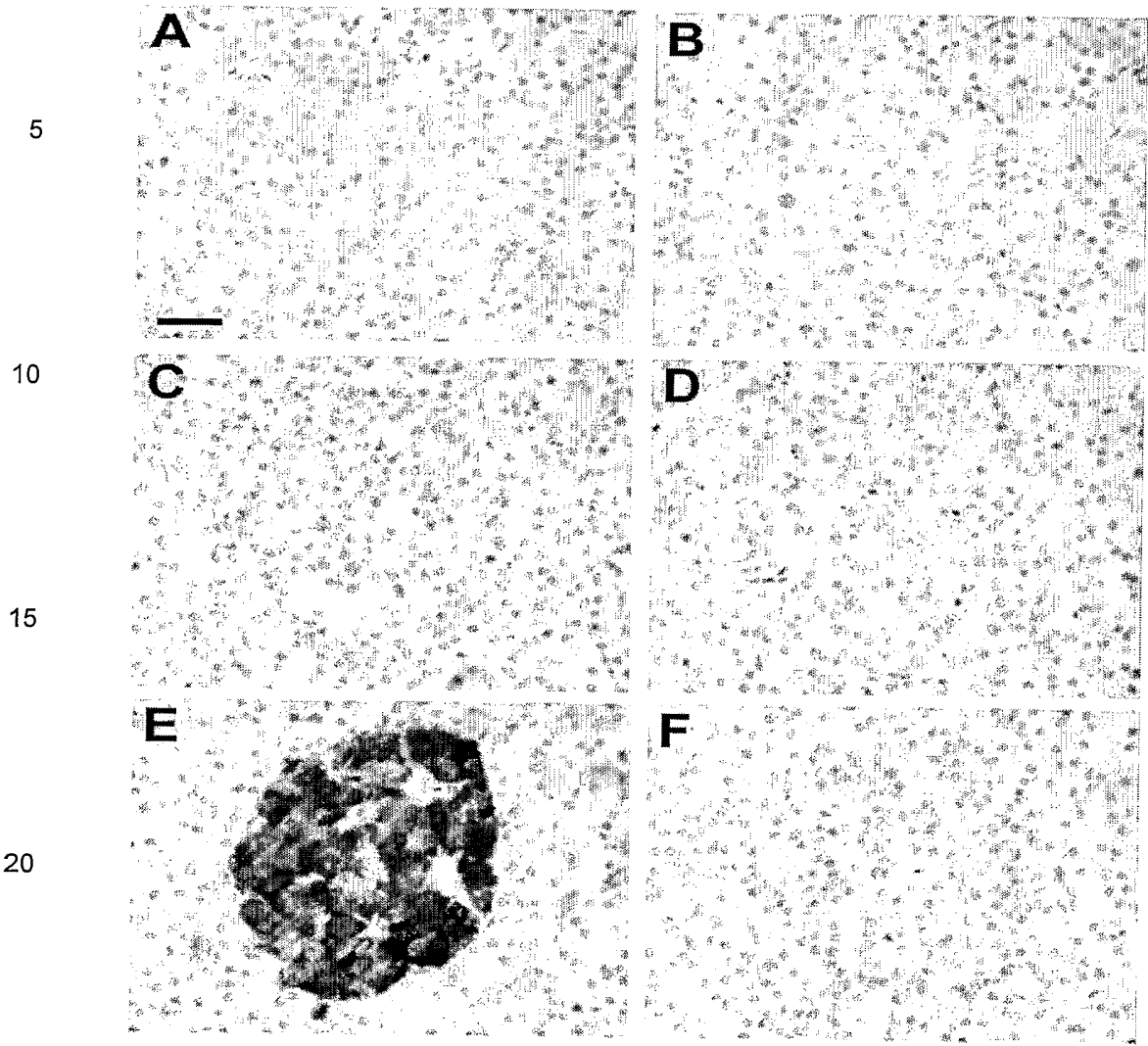
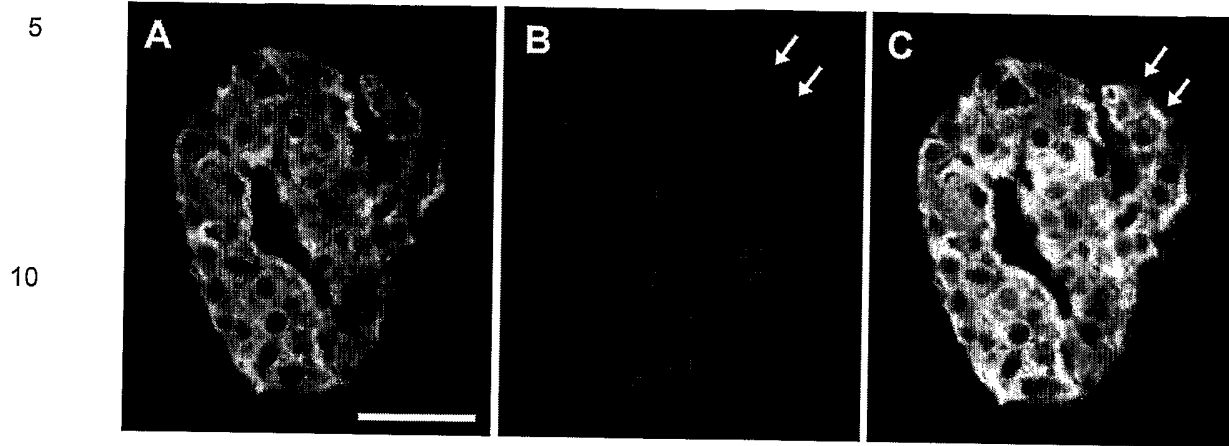


Fig. 1

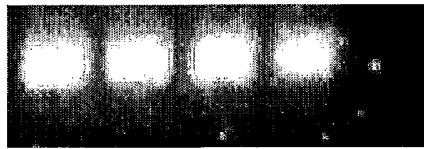
2/9



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Fig. 2

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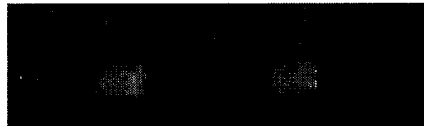
RPL13



TFF3



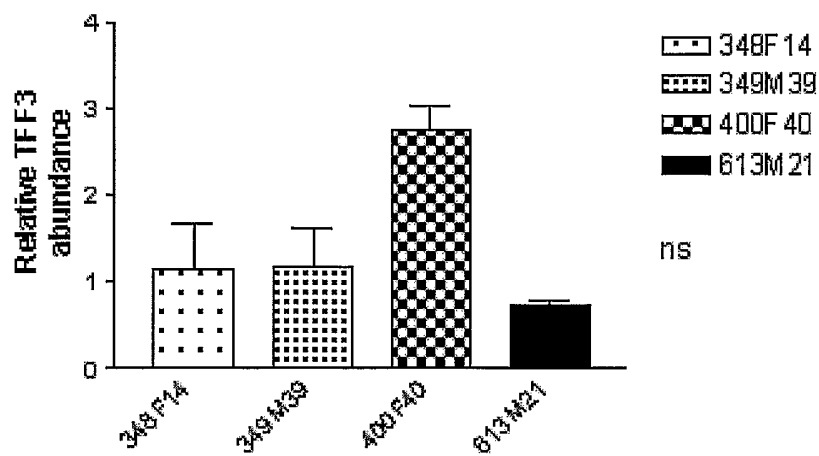
TFF2



TFF1

Fig. 3

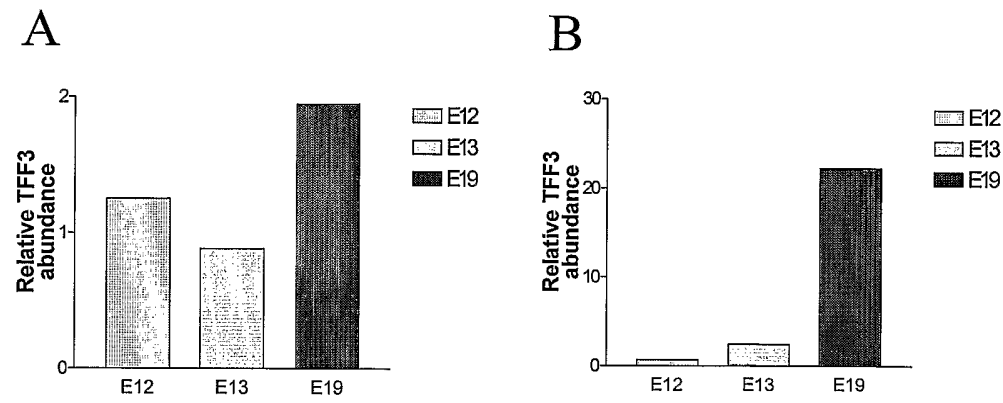
4/9



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Fig. 4

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Fig. 5

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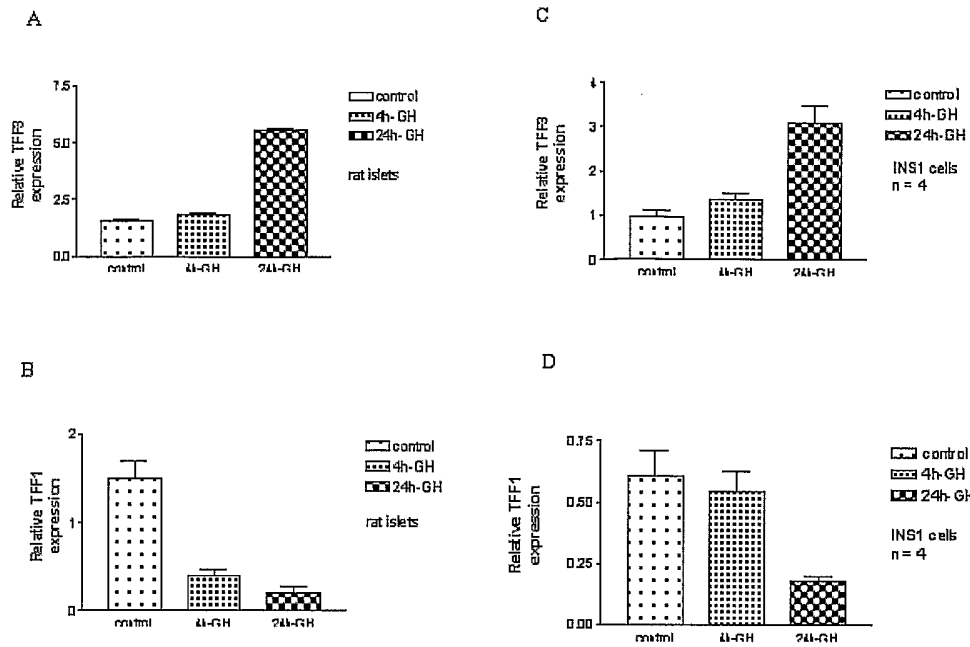
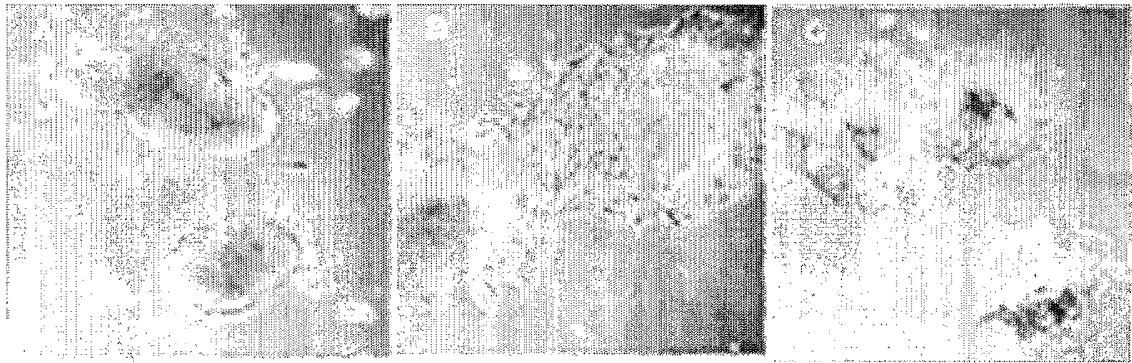


Fig. 6

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Fig. 7

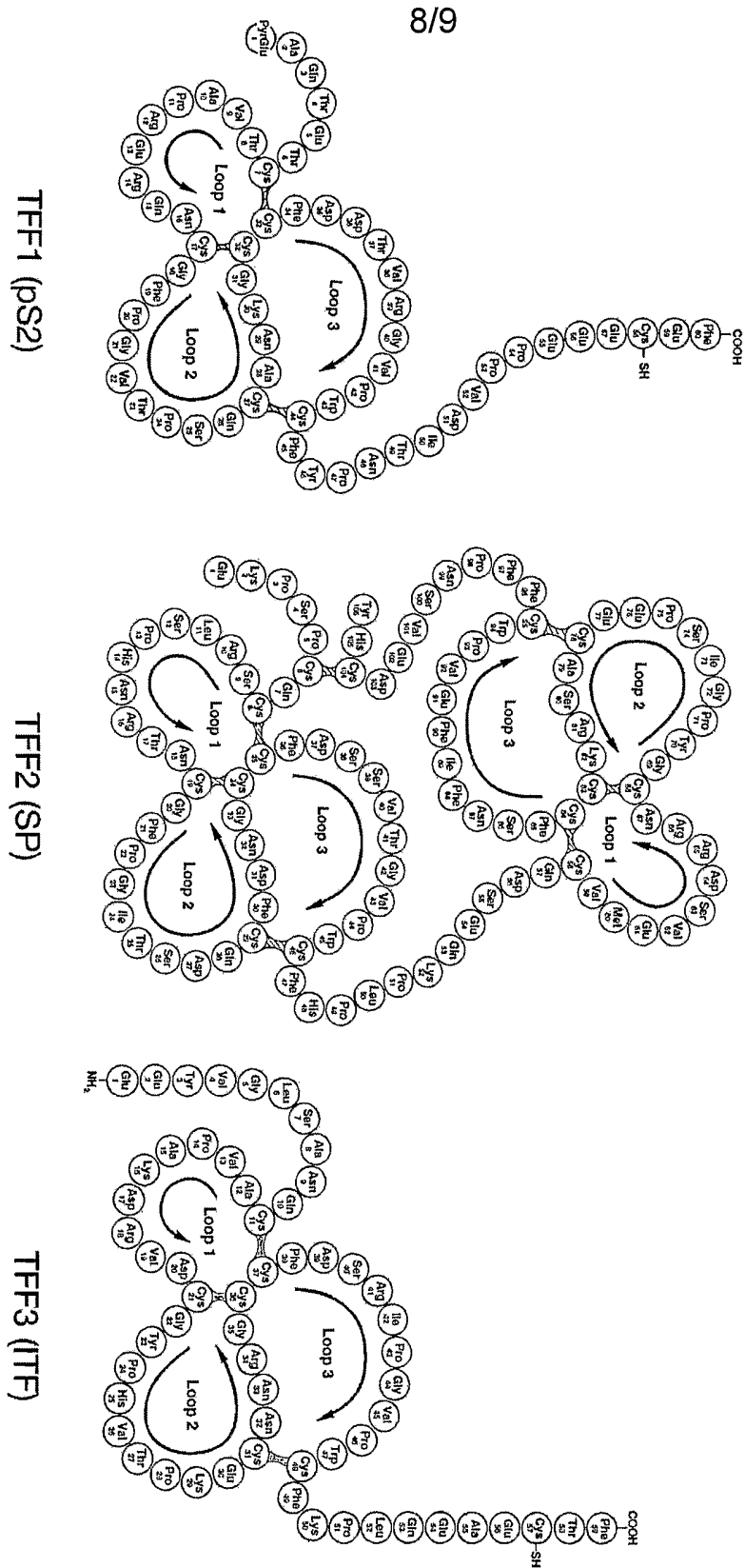


Fig. 8

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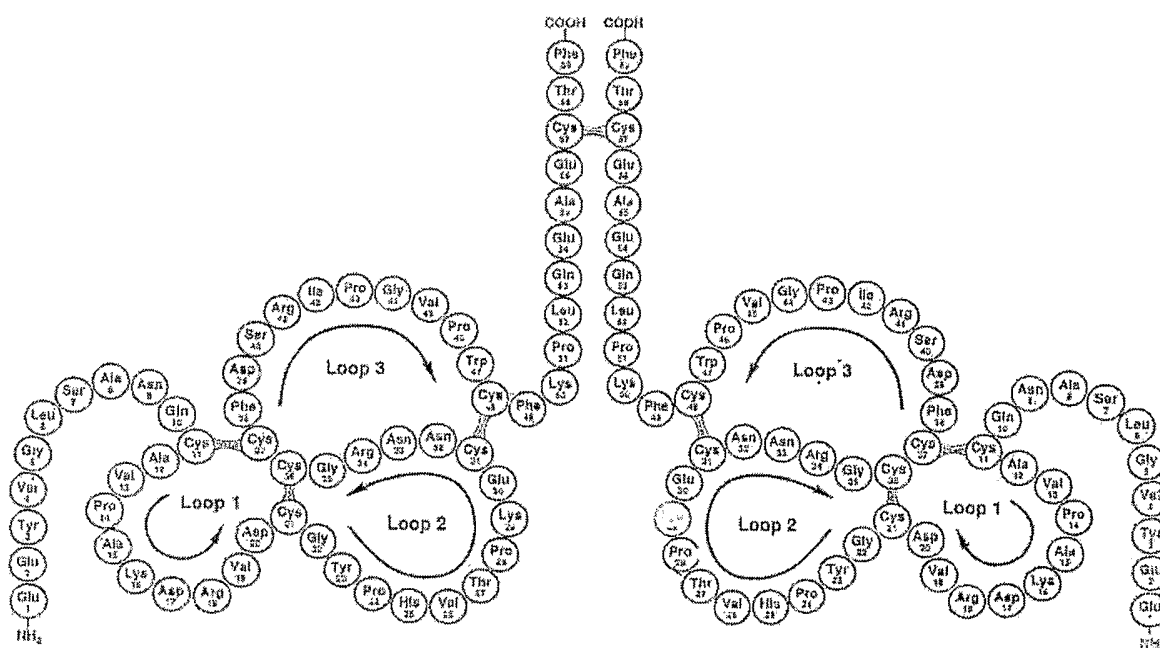


Fig. 9

INTERNATIONAL SEARCH REPORT

International Application No
PCT/DK2004/000747A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K38/22 A61P3/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, Sequence Search, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 03/094949 A (GENENTECH INC; CURAGEN CORPORATION) 20 November 2003 (2003-11-20)	1-10,12, 14-25, 27,29,30
P,A	the whole document see especially: page 19, line 19 - line 23 page 93 - page 97; examples 1,2 page 99 - page 100; example 5 page 126 - page 128; claims 10,23-27,31,32 ----- -/--	11,13, 26,28



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

° Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

& document member of the same patent family

Date of the actual completion of the international search

10 March 2005

Date of mailing of the international search report

01/04/2005

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Authorized officer

Fuchs, U

INTERNATIONAL SEARCH REPORT

International Application No

PCT/DK2004/000747

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WILLIAMS, K.L. ET AL.: "Enhanced Survival and Mucosal Repair After Dextran Sodium Sulfate-Induced Colitis in Transgenic Mice That Overexpress Growth Hormone" GASTROENTEROLOGY, vol. 120, no. 4, March 2001 (2001-03), pages 925-937, XP002320036 abstract page 932, column 2, line 40 - page 933, column 2, line 18 page 934; figure 8 page 935, column 2, line 3 - line 32	1-30
A	KINOSHITA, K. ET AL.: "Distinct Pathways of Cell Migration and Antiapoptotic Response to Epithelial Injury: Structure-Function Analysis of Human Intestinal Trefoil Factor" MOLECULAR AND CELLULAR BIOLOGY, vol. 20, no. 13, July 2000 (2000-07), pages 4680-4690, XP002320037 the whole document	1-30
A	DOSSINGER, V. ET AL.: "Down-regulation of TFF Expression in Gastrointestinal Cell Lines by Cytokines and Nuclear Factors" CELLULAR PHYSIOLOGY AND BIOCHEMISTRY, vol. 12, no. 4, 2002, pages 197-206, XP008043974 cited in the application the whole document	1-30
A	RIBIERAS, S. ET AL.: "The pS2/TFF1 trefoil factor, from basic research to clinical applications" BIOCHIMICA ET BIOPHYSICA ACTA, vol. 1378, no. 1, 19 August 1998 (1998-08-19), pages F61-F77, XP004281825 the whole document see especially: page F65, column 1, line 1 - page F68, column 2, line 26; figure 3; table 3	1-30
A	WO 03/068817 A (NOVO NORDISK A/S) 21 August 2003 (2003-08-21) cited in the application the whole document	1-30
A	WO 02/102403 A (NOVO NORDISK A/S) 27 December 2002 (2002-12-27) cited in the application the whole document	1-30

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/DK2004/000747

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 02/102399 A (NOVO NORDISK A/S) 27 December 2002 (2002-12-27) cited in the application the whole document -----	1-30

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK2004/000747

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 16-30 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/DK2004/000747

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 03094949	A	20-11-2003	US 2003208057 A1 AU 2002367927 A1 CA 2483025 A1 EP 1501529 A1 WO 03094949 A1	06-11-2003 11-11-2003 20-11-2003 02-02-2005 20-11-2003
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