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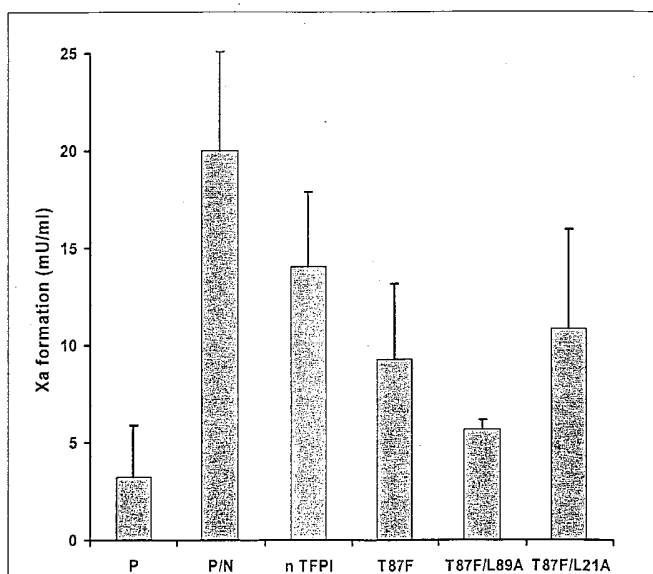
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(54) Title: VARIANT TFPI WITH INCREASED ANTI-THROMBOTIC ACTIVITY



(57) Abstract: The present invention relates to variant Tissue Factor Pathway Inhibitor - 1 alpha (TFPI-1 alpha) characterized by increased resistance to inactivation, in particular increased resistance to protease cleavage, and enhanced anti-coagulant activity as compared to a native TFPI-1 alpha protein. Such variant TFPI-1 alpha provides an anticoagulant suitable for clinical application in diseases involving TFPI and the tissue factor/factor VIIa/factor Xa-complex. In particular, diseases associated with or caused by thrombosis can be treated with variant TFPI-1 alpha.

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Variant TFPI with increased anti-thrombotic activity

Technical Field

The present invention relates to variant Tissue Factor
5 Pathway Inhibitor - 1 alpha (TFPI-1 alpha) characterized by
increased resistance to inactivation and enhanced anti-
coagulant activity as compared to a native TFPI-1 alpha
protein. More specifically, the invention relates to variant
TFPI-1 alpha characterized by increased resistance to
10 protease cleavage and increased inhibitory effect on
formation of the initiation complex of coagulation.

Background of the Invention

Arterial and venous thrombosis are directly responsible for
15 the mortality resulting from myocardial infarction, stroke,
lung emboli and a great variety of pathologies caused by
excessive thrombus formation in nearly all branches of
Clinical Medicine. In addition, arterial thrombosis
represents a major mediator of the lethal complications
20 associated with sepsis. Hence, the different types of
thrombosis are by far the most frequent cause of mortality in
Europe and North America.

Pathological alterations in fibrin formation by the blood
25 coagulation system are key factors for the genesis of
thrombosis. Blood coagulation is currently thought to be
started by a single cell membrane protein predominantly
present within the vessel wall, the so called tissue factor
(TF). TF has been shown to play a crucial role for the
30 development of arterial and venous thrombosis, as well as to
be of major relevance in the pathogenesis of sepsis and
malignant disease (Mackman et al, 2004, Arterioscler. Thromb.
Vasc. Biol.; Viles-Gonzalez 2004, Int. J. Biochem. Cell Biol.
36: 25-30; Slofstra et al, 2003, Hematol. J. 4: 295-302;
35 Mousa, 2004, Methods. Mol. Med. 93: 119-132). TF activates
factor X by forming a complex with factor VIIa, thereby
initiating the coagulation cascade.

The ternary initiation complex of coagulation thus formed is rapidly shut off by TFPI, which interrupts the coagulation cascade at its earliest stage (Mousa et al., 2004, Methods Mol. Med. 93: 133-155; Kato 2002, Arterioscler. Thromb. Vasc. Biol. 22: 539-548). This is mainly achieved by binding of the Kunitz-1 and Kunitz-2 domains of TFPI to factor Xa and factor VIIa, respectively. TFPI is expressed and stored in endothelial cells, platelets and other cells. Similarly, other elements of the coagulation cascade are countered by endogenous inhibitors, including antithrombin III, protein C, and others.

Several animal studies suggest that native TFPI might contribute to prevent pathological thrombus formation. As an example, overexpression of TFPI can prevent arterial thrombosis (Kopp et al, 2004, Blood, 103: 1653-1661). TFPI has been proposed as a therapeutic agent in humans (WO03/055442). This document discloses a TFPI analog that contains an additional N-terminal Alanine for improved expression in E. coli. Delivery of TFPI by recombinant adeno-associated virus has been proposed (WO01/09360). In US5,589,359 and WO96/04378, chimeric proteins are disclosed that comprise portions of TFPI-1 and TFPI-2. Other structural derivatives of TFPI, namely isolated Kunitz domains 1 to 3 are disclosed in WO93/14120, WO93/14121 and WO93/14122.

On the other hand, under many pathological conditions resulting in thrombosis, the anticoagulant effect of TFPI appears to be insufficient. In patients with high risk for coronary heart disease, blood levels of free TFPI are reduced (Morange et al., 2004, Circulation, 109: 1343-1348; Golino et al, 2003, Circulation 108: 2864-2869). Low blood levels of TFPI also increase the risk for venous thrombosis (Dahm et al, 2003, Blood, 101:4387-4392). A series of investigations moreover indicates that TFPI participates in the pathogenesis of sepsis, in particular in the pathological induction of

coagulation during sepsis known as disseminated intravascular coagulation (Levi et al, 2003, Cardiovasc. Res. 60:26-39; Osterud & Bjorklid 2001, Semin. Thromb. Hemost. 27: 605-617). Moreover, the participation of TFPI in a variety of further diseases has been documented, including different types of inflammatory conditions (Golino P, Cimmino G. Semin Vasc Med. 3:205-14 (2003)), tumor growth and metastasis (Mousa SA. Expert Rev Cardiovasc Ther.1, 283-91 (2003)), ischemia/reperfusion damage (Mackman N. Semin Vasc Med.3, 193-8 (2003)), and other diseases in which components of the tissue factor/factor VIIa/factor Xa-complex are implicated.

However, despite considerable efforts to block the ternary initiation complex of coagulation, i.e. any of TF, Factor X/Xa, and Factor VII/VIIa, , therapeutic intervention at this stage of the coagulation cascade or other anticoagulants, such as e.g. antithrombin, protein C have so far been of limited use for clinical application. For example, trials assessing the treatment of disseminated intravascular coagulation during sepsis by native TFPI have not been able to show improvements (Abraham et al., 2003) AMA 290:238. Also, the high amounts of TFPI necessary to inhibit fibrin formation in vivo were associated with an increased risk for bleeding hampering a clinical application of TFPI.

It was therefore the aim of the present invention to provide an inhibitor of blood coagulation suitable as a therapeutic agent, in particular an inhibitor suitable for the prevention and treatment of a disease involving TFPI and the tissue factor/factor VIIa/factor Xa-complex, such as thrombosis.

Summary of the Invention

The present invention solved this aim by providing TFPI variants having increased resistance to fragmentation by pathophysiologically relevant proteases, in particular serine proteases, and increased anti-coagulant activity. In particular, the present invention relates to:

1. A variant of the polypeptide of SEQ ID No: 1 having increased protease resistance at one or more protease cleavage sites, and an increased anti-coagulant activity as compared to the amino acid sequence according to SEQ ID No: 1.
2. The polypeptide according to item 1, wherein said one or more protease cleavage sites are one or more of cathG, HNE, MMP, thrombin and plasmin cleavage sites.
3. The polypeptide according to item 1 or 2, wherein said one or more protease cleavage sites are one or more of cathG, HNE, and MMP cleavage sites.
4. The polypeptide according to item 3, wherein said one or more protease cleavage sites are the cathG and / or HNE cleavage sites.
5. The polypeptide according to any one of item 1 to 4, comprising one or more variations affecting the cleavage site between Threonin 87 and Threonin 88 of SEQ ID No: 1.
6. The polypeptide according to any one of item 1 to 5, comprising one or more variations affecting the cleavage site between Leucine 89 and Glutamine 90 and / or Tyrosine 159 and Glycine 160 of SEQ ID No: 1.
7. The polypeptide according to any one of item 1 to 6, comprising one or more variations affecting a combination of the cleavage sites according to claims 5 and 6.
8. The polypeptide according to any one of item 1 to 7, wherein the amino acid at position 87 of SEQ ID No: 1 is substituted by Phenylalanine (T87F).

9. The polypeptide according to any one of item 1 to 8,
wherein the amino acid at position 89 of SEQ ID No: 1 is
substituted by Alanine (L89A), and / or the amino acid at
position 159 of SEQ ID No: 1 is substituted by Alanine
5 (Y159/A).

10. The polypeptide according to any one of item 1 to 9,
comprising a combination of substitutions according to claims
8 and 9.

10 11. The polypeptide according to item 10, comprising the
substitutions T87F and L89A.

12. A polynucleotide encoding the polypeptide according to
15 any one of item 1 to 11.

13. A vector comprising the polynucleotide of item 12.

14. A host cell comprising the vector of item 13.

20 15. A polypeptide expressed by the host cell of item 14,
wherein said polypeptide is encoded by the vector of item 13.

16. A pharmaceutical composition comprising the polypeptide
25 of any one of item 1 to 11 or 15.

17. A pharmaceutical composition comprising the
polynucleotide of item 12.

30 18. A pharmaceutical composition comprising the vector of
item 13.

19. Use of the polypeptide according to any one of item 1 to
11 or 15 for the manufacture of a medicament for the
35 treatment and/or prevention of a disease involving TFPI and
the tissue factor/factor VIIa/factor Xa-complex

20. Use of the polynucleotide according to item 12 for the manufacture of a medicament for the treatment and/or prevention of a disease involving TFPI and the tissue factor/factor VIIa/factor Xa-complex.

5

21. Use of the vector according to item 13 for the manufacture of a medicament for the treatment and/or prevention of a disease involving TFPI and the tissue factor/factor VIIa/factor Xa-complex.

10

Detailed description of the invention

The polypeptide of the invention

In one embodiment the present invention relates to variants of TFPI-1 alpha protein. TFPI-1 is a glycosylated molecule comprising 276 amino acid residues (SEQ ID No: 1). In this application, the terms TFPI-1 alpha and TFPI-1 are used interchangeably. "Native" TFPI-1 alpha protein relates to a TFPI-1 polypeptide with an amino acid sequence that can be isolated from a human being. Thus, native TFPI-1 comprises the natural diversity with respect to the amino acid sequence as can be found in humans. Preferably, the native TFPI polypeptide has an amino acid sequence as set forth in SEQ ID No: 1.

25

SEQ ID No: 1 comprises, starting from the N-terminus in sequence: An N-terminal amino acid sequence followed by the Kunitz-1 domain, followed by an amino acid sequence between the Kunitz-1 and Kunitz-2 domains, the Kunitz-2 domain, an amino acid sequence between the Kunitz-2 and Kunitz-3 domain, the Kunitz-3 domain and a C-terminal amino acid sequence. Kunitz domains are distinct domains of TFPI-1 characterised by three disulfide bridges each. Kunitz domains I, II and III are commonly defined as spanning amino acid residues 26 to 76, 97 to 127 and 189 to 239 respectively, of SEQ ID No: 1.

35

The term "variant" polypeptide as used herein relates to the polypeptide according to SEQ ID No: 1, which contains a variation of the amino acid sequence, provided said variant polypeptide has increased protease resistance at one or more protease cleavage sites, and an increased anti-coagulant activity as compared to the protein with the amino acid sequence according to SEQ ID No: 1. Preferable variations comprise additions, substitutions or deletions of one or more amino acid residues. Preferably, the variant of the present invention has at least 75% homology to SEQ ID No: 1, more preferably at least 85% homology, more preferably at least 95% homology. In the context of the present invention, homology means % identity of amino acid residues when comparing the variant amino acid sequence with the native sequence. Thus, if a variant consists of 100 amino acid residues of which 90 are identical to corresponding parts of the native sequence, that variant has 90% homology. Corresponding parts of the native sequence can be determined e.g. by sequence alignment by methods commonly known in the art.

The variants are obtained by introducing amino acid variations by methods well known to the person skilled in the art. Preferably, molecular biology methods are used to introduce changes to a polynucleotide sequence encoding SEQ ID No: 1, which changes result in the desired variation in the amino acid sequence. Such molecular biology techniques are exemplified by site directed mutagenesis.

As for the amino acid deletion, the variant polypeptide may preferably be a fragment which lacks an amino acid sequence at the N-terminal end and/or the C-terminal end, or comprise deletions within the amino acid sequence. As an example, the fragment may lack part of the C-terminal sequence following the Kunitz-3 domain, and optionally in addition lack the Kunitz-3 domain, and optionally in addition lack all or part of the amino acid sequence spanning Kunitz-2 and Kunitz-3

domains in SEQ ID No: 1. Alternatively, or in addition, a fragment may also lack all or part of the N-terminal amino acid sequence preceding the Kunitz-1 domain. The fragment lacking the amino acid sequence at the N-terminal end and/or the C-terminal end can be obtained by a standard method known to the person skilled in the art.

In one embodiment, the variants carrying a deletion can in addition carry one or more variations affecting one or more protease cleavage sites.

In the context of this invention, "affecting a cleavage site" means that the resistance to cleavage by a given protease at this particular cleavage site is increased in comparison with the native sequence, in particular in comparison to SEQ ID No: 1. Increased protease resistance means that upon exposure to the protease, the variant polypeptide is cleaved to a lesser degree as compared to the native polypeptide.

The skilled person readily knows methods to determine the protease resistance for a given protease at a given cleavage site. As an example, the variant TFPI can be incubated with a given protease, and fragments analysed by SDS-PAGE. Increased protease resistance will result in a decrease in fragment formation as compared to TFPI not carrying the variation. Fragment formation can be quantified by various means, e.g. by quantification of the bands on the SDS gel by densitometry, or other methods. In case a fragment has to be identified this method can optionally be combined with N-terminal sequencing of fragments e.g. isolated from the gel. Preferred methods are specified in the examples.

Resistance to protease cleavage is considered to be increased when it is preferably at least 20% higher as compared to the native amino acid sequence, more preferably at least 40% higher, more preferably at least 60% higher, more preferably at least 80% higher, and most preferably at least 100%

higher, wherein preferably the native amino acid sequence is SEQ ID No: 1.

5 In addition to increased protease resistance, the variant of the present invention is characterized by increased anti-coagulant activity. The skilled person readily knows methods to determine anticoagulant activity of the variants of the invention. Any known method to determine blood clotting can be employed, preferably a method wherein clotting is induced
10 by recombinant tissue factor (TF). Preferred methods are provided in the examples. Preferably, anti-coagulant activity is measured in whole blood, or, alternatively, in an assay utilizing platelets and neutrophils.

15 Anti-coagulant activity is considered to be increased, when the readout of the coagulation test is preferably at least 20% higher as compared to the native amino acid sequence, more preferably at least 40% higher, more preferably at least 60% higher, and most preferably at least 80% higher, wherein
20 preferably the native amino acid sequence is SEQ ID No: 1.

Preferably, the anti-coagulant activity is enhanced 1-2 fold, more preferably 2-4 fold, most preferably 2.4-3.6-fold.

25 Preferred readouts of coagulation tests comprise the quantification of generation of the initiation complex of coagulation, more preferably the quantification of generation of factor Xa. Another preferred readout of a coagulation test is the determination of coagulation time by
30 thromboelastography, preferably in whole blood.

Preferably, the variants of the present invention comprise variations at one or more amino acid residues directly adjacent to one or more protease cleavage sites. The skilled
35 person can readily determine the cleavage sites of TFPI-1 for any protease of interest. A standard method comprises exposing the substrate, i.e. TFPI to the protease, separating

fragments, e.g. by SDS-polyacrylamide gel electrophoresis (PAGE), and N-terminal sequencing of the fragments. The N-terminal sequence can be aligned with the known amino acid sequence of the substrate, and thus the position of the cleavage site identified. Other methods than SDS-PAGE can be employed to separate cleavage fragments.

The protease cleavage sites for human neutrophil elastase (HNE), cathepsin G (cath G), and several matrix metalloproteases (MMP) have previously been identified (Higuchi et al, 1992, Blood, 79:1712-1719; Petersen et al, 1992, Thromb. Haemost. 4, 67: 537-541; Belaaouaj et al, 2000, J. Biol. Chem. 275:27123-27128; Cunningham et al, 2002, Biochem. J. 367:451-458). Table 1 provides an overview of protease cleavage sites that are of interest for the present invention within SEQ ID No: 1.

Table 1

Site*	Enzyme
K20/L21	MMP-1, MMP-7, MMP-8, MMP-9, MMP-12
A83/I84	MMP-12
K86/T87	Plasmin, Thrombin, Cath G
T87/T88	HNE
L89/Q90	Cath G
R107/G108	Plasmin
Y159/G160	Cath G
S174/T175	MMP-8, MMP-12
R199/A200	Plasmin
K249/G250	Plasmin
K254/T255	Thrombin
K256/R257	Plasmin

*The letters refer to the single letter code for amino acid residues, the number refers to the position within SEQ ID No:

1. The residue upstream and downstream of the cleavage site are specified.

It is notable that the protease cleavage sites identified in TFPI are mostly non-canonical. As an example, no canonical cleavage sites for CatG, HNE and MMP-9 are known in TFPI. It is therefore not surprising that even when exposed to proteases in vitro, no rapid cleavage (as would be expected for natural protease substrates) is observed. Examples of such natural substrates are the members of the coagulation cascade, where several serine proteases cleave their substrates within seconds or less. In contrast, in vitro cleavage of TFPI has been reported to follow a substantially slower time-course, e.g. in the range of minutes to hours (Cunningham et al, Biochem. J. 2002, 367, 451-458).

Variations can be limited to one side of the cleavage site, e.g. to amino acid 87 when the cleavage site lies between amino acids 87 and 88, or affect both sides of the cleavage site. However, the present invention also encompasses variations affecting amino acid residues not directly adjacent to the cleavage site, provided they show the said effect on protease resistance and anti-coagulation activity.

Preferably, the present invention relates to variants with increased resistance at the cleavage sites of one or more of cathepsin G (cathG), human neutrophil elastase (HNE), matrix metalloproteinase (MMP), thrombin and plasmin, more preferably one or more of cathG, HNE, and MMP and most preferably cathG and / or HNE.

Preferred matrix metalloproteinases comprise MMP-1, MMP-7, MMP-8, MMP-9 and MMP-12.

Preferably, single and double amino acid substitutions in the cleavage sites for HNE and cathepsin G are introduced.

In a preferred embodiment of the present invention, the variant comprises a variation affecting preferably one or more protease cleavage sites in SEQ ID No: 1 of a protease that does not cleave Kunitz domains, more preferably one or more cleavage sites of proteases cleaving within at least one of the amino acid sequences linking Kunitz domains, and most preferably the cleavage sites of one or more proteases that cleave within the amino acid sequence between the Kunitz-1 and Kunitz-2 domains. Proteases that cleave within the amino acid sequence between the Kunitz-1 and Kunitz-2 domains comprise MMP-12, Cath G, Thrombin, Plasmin and HNE.

In a further preferred embodiment of the present invention, variants comprise one or more variations affecting the cleavage sites between Threonin 87 and Threonin 88 of SEQ ID No: 1, and / or Leucine 89 and Glutamine 90, and / or Tyrosine 159 and Glycine 160 of SEQ ID No: 1. More preferably, the variants according to the present invention comprise a variation affecting the cleavage site between Threonin 87 and Threonin 88 of SEQ ID No: 1, and a variation affecting the cleavage site between Leucine 89 and Glutamine 90.

In a preferred embodiment, the amino acid at position 87 of SEQ ID No: 1 is substituted by Phenylalanine (T87F), and / or the amino acid at position 89 of SEQ ID No: 1 is substituted by Alanine (L89A), and / or the amino acid at position 159 of SEQ ID No: 1 is substituted by Alanine (Y159/A). However, any other amino acid can be substituted at these positions, which has an effect on resistance to protease cleavage, which can be experimentally determined as described above. At the non-canonical protease cleavage sites of TFPI an exchange of amino acids based on theoretical considerations only is not possible.

Most preferably, the variant comprises the substitutions T87F and L89A.

It is likely that apart from providing resistance towards proteolytic degradation, the variations introduced into the variants of the present invention elicit additional

5 functional changes in the protein, such as, e.g., providing an increased affinity for the TFPI substrates factor VIIa and Xa. Also, the variants may alternatively or in addition be characterized by an increased plasma half life. Thus, the variants generated retain the canonical anticoagulant
10 function of TFPI, but in addition increase its competence as inhibitor of the start process of fibrin formation.

The polypeptide of the invention can be produced by standard methods well known in the art. As an example, the polypeptide
15 can be produced by recombinant expression (see below). The effects of different hosts for recombinant expression on glycosylation are well known in the art. Thus, the skilled person can select the appropriate host system for the desired pattern of glycosylation.

20

The polypeptide of the present invention can be isolated and purified by the following means:

Recombinant expression of glycosylated TFPI using mammalian
25 cell hosts including mouse C127 cells, baby hamster kidney cells, Chinese hamster ovary cells and human SK hepatoma cells has been reported (Day et al, 1990, Blood 76: 1538-1545; Pedersen et al, 1990, J. Biol. Chem. 265: 16786-16793; Alternatively, the polypeptides can be chemically synthesized
30 by methods such as direct peptide synthesis using solid-phase techniques. Optionally, fragments of the polypeptide of the invention can be separately synthesized and combined using chemical methods to produce a full-length molecule.

35 According to the invention, variants of SEQ ID No: 1 also include variants in which one or more of the amino acids therein has an altered side chain. Such derivatized

polypeptides include, for example, those comprising amino acids in which free amino groups form amine hydrochlorides, p-toluene sulfonyl groups, carbobenzoxy groups; the free carboxy groups form salts, methyl and ethyl esters; free hydroxyl groups that form O-acyl or O-alkyl derivatives as well as naturally occurring amino acid derivatives, for example, 4-hydroxyproline, for proline, 5-hydroxylysine for lysine, homoserine for serine, ornithine for lysine etc. Also included are amino acid derivatives that can alter covalent bonding, for example, the disulfide linkage that forms between two cysteine residues that produces a cyclized polypeptide. The variants of the invention also comprise polypeptides cyclized by other means than a disulfide bridge.

In one embodiment, the polypeptides of the present invention may also be modified by a modifying agent. Examples of the modifying agent include polyethylene glycol (PEG), dextran, poly(N-vinyl-pyrrolidone), polypropylene glycol homopolymer, polypropylene oxide/ethylene oxide copolymer, polyoxyethylated polyol, polyvinyl alcohol, and the like. The modification of the protein with PEG can be performed by, for example, a method in which activated ester derivatives of PEG is reacted with the protein, a method in which aldehyde derivatives at the terminal portion of PEG is reacted with the protein in the presence of a reducing agent, and the like.

The polynucleotide of the invention

Another embodiment of the invention relates to a polynucleotide encoding a variant polypeptide of SEQ ID No: 1 having increased protease resistance at one or more protease cleavage sites and an increased anti-coagulant activity as compared to the amino acid sequence according to SEQ ID No: 1. The term "polynucleotide" is to be construed in the broadest sense and comprises any polymer of nucleotides. The terms "polynucleotide" and "nucleic acid" are used

interchangeably. The polynucleotide of the present invention can be in the form of DNA (deoxyribonucleic acid) which contains the bases adenine, thymine, guanine and cytosine or RNA (ribonucleic acid) which contains the bases adenine,
5 uracil, guanine and cytosine or mixtures of the two.

The polynucleotides of the present invention also include modifications of the polynucleotide wherein one or more of the bases of the nucleic acids can be replaced by modified
10 bases. These modified bases include 2-methyladenine, 1-methylguanine, 5-methylcytosine, and 5-hydroxymethylcytosine.

A polynucleotide according to the invention may be inserted into a vector. The term "vector" refers to a plasmid, virus
15 or other vehicle known in the art that can be manipulated by insertion or incorporation of a polynucleotide. Such vectors can be used for genetic manipulation (i.e., "cloning vectors") or can be used to transcribe or translate the inserted polynucleotide ("expression vectors"). A vector
20 generally contains at least an origin of replication for propagation in a cell and a promoter. Control elements, including expression control elements as set forth herein, present within an expression vector are included to facilitate proper transcription and translation (e.g.,
25 splicing signal for introns, maintenance of the correct reading frame of the gene to permit in-frame translation of mRNA and, stop codons etc.). The term "control element" is intended to include, at a minimum, one or more components whose presence can influence expression, and can also include
30 additional components, for example, leader sequences and fusion partner sequences.

As used herein, the term "expression control element" refers to one or more nucleic acid sequences that regulate the
35 expression of a nucleic acid sequence to which it is operatively linked. An expression control element operatively linked to a nucleic acid sequence controls transcription and,

as appropriate, translation of the nucleic acid sequence. Thus an expression control element can include, as appropriate, promoters, enhancers, transcription terminators, a start codon (e.g., ATG) in front of a protein-encoding
5 gene. "Operatively linked" refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner.

By "promoter" is meant a minimal sequence sufficient to
10 direct transcription. Both constitutive and inducible promoters are included in the invention (see e.g., Bitter et al., Methods in Enzymology 153:516-544, 1987). Inducible promoters are activated by external signals or agents. Also included in the invention are those promoter elements which
15 are sufficient to render promoter-dependent gene expression controllable for specific cell-types, tissues or physiological conditions; such elements may be located in the 5', 3' or intronic regions of the gene. Promoters useful in the invention also include conditional promoters. A
20 "conditional promoter" is a promoter which is active only under certain conditions. For example, the promoter may be inactive or repressed when a particular agent, such as a chemical compound, is present. When the agent is no longer present, transcription is activated or derepressed.

25 Thus, when cloning in bacterial systems, constitutive promoters such as T7 and the like, as well as inducible promoters such as pl, of bacteriophage X, plac, ptrp, ptac (ptrp-lac hybrid promoter) may be used. When cloning in
30 mammalian cell systems, constitutive promoters such as SV40, RSV, CMV including CMV-IE, and the like or inducible promoters derived from the genome of mammalian cells (e.g., metallothionein promoter) or from mammalian viruses (e.g., the mouse mammary tumor virus long terminal repeat; the
35 adenovirus late promoter) may be used. Promoters produced by recombinant DNA or synthetic techniques may also be used to

provide for transcription of the nucleic acid sequences of the invention.

Mammalian expression systems which utilize recombinant viruses or viral elements to direct expression may be engineered. For example, when using adenovirus expression vectors, nucleic acid of interest may be ligated to an adenovirus transcription/translation control complex, e.g., the late promoter and tripartite leader sequence. Alternatively, the vaccinia virus 7.5K promoter may be used.

Of particular interest are vectors based on bovine papilloma virus (BPV) which have the ability to replicate, as extrachromosomal elements. Shortly after entry of an extrachromosomal vector into mouse cells, the vector replicates to about 100 to 200 copies per cell. Because transcription of the inserted cDNA does not require integration of the plasmid into the host's chromosome, a high level of expression occurs. These vectors can be used for stable expression by including a selectable marker in the plasmid, such as the neo gene, for example. Alternatively, the retroviral genome can be modified for use as a vector capable of introducing and directing the expression of the nucleic acid of interest in host cells. High level expression may also be achieved using inducible promoters, including, but not limited to, the metallothionein RA promoter and heat shock promoters.

In yeast, a number of vectors containing constitutive or inducible promoters may be used. A constitutive yeast promoter such as ADH or LEU2 or an inducible promoter such as GAL may be used. Alternatively, vectors that facilitate integration of foreign nucleic acid sequences into a yeast chromosome, via homologous recombination for example, are known in the art and can be used.

A nucleic acid of interest according to the present invention may be inserted into an expression vector for expression in vitro (e.g., using in vitro transcription/translation assays or commercially available kits), or may be inserted into an expression vector that contains a promoter sequence which facilitates transcription and/or translation in either prokaryotes or eukaryotes (e.g., an insect cell) by transfer of an appropriate nucleic acid into a suitable cell. A cell into which a vector can be propagated and its nucleic acid transcribed, or encoded polypeptide expressed, is referred to herein as a "host cell". The term also includes any progeny of the subject host cell. Moreover, a nucleic acid of interest according to the present invention may be inserted into an expression vector for expression in vivo for somatic gene therapy for example. With these vectors, for example, retroviral vectors, lentivirus vectors, Adenovirus vectors, Adeno-associated virus vectors, plasmid expression vectors, the nucleic acids of the invention are expressed upon infection/introduction of the vector into a suitable tissue/cell of the subject to be treated.

Host cells include but are not limited to microorganisms such as bacteria, yeast, insect and mammalian organisms. For example, bacteria transformed with recombinant bacteriophage nucleic acid, plasmid nucleic acid or cosmid nucleic acid expression vectors containing a nucleic acid of interest; yeast transformed with recombinant yeast expression vectors containing a nucleic acid of interest; plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (e.g., Ti plasmid) containing a nucleic acid of interest; insect cell systems infected with recombinant virus expression vectors (e.g., baculovirus) containing a nucleic acid of interest; or animal cell systems infected with recombinant virus expression vectors (e.g., retroviruses, lentivirus, adenovirus, vaccinia virus) containing a nucleic

acid of interest, or transformed animal cell systems engineered for stable expression.

For long-term expression of polypeptides of the invention in
5 host cells, stable expression is preferred. Thus, using
expression vectors which contain viral origins of
replication, for example, cells can be transformed with a
nucleic acid of interest controlled by appropriate control
10 elements (e.g., promoter/enhancer sequences, transcription
terminators, polyadenylation sites, etc.). Optionally, the
expression vector also can contain a nucleic acid encoding a
selectable or identifiable marker conferring resistance to a
selective pressure thereby allowing cells having the vector
to be identified, grown and expanded. Alternatively, the
15 selectable marker can be on a second vector that is
cotransfected into a host cell with a first vector containing
an invention polynucleotide.

A number of selection systems may be used, including, but not
20 limited to the herpes simplex virus thymidine kinase gene,
hypoxanthine-guanine phosphoribosyltransferase gene, and the
adenine phosphoribosyltransferase genes can be employed in
tk-, hgprt or aprt cells respectively. Additionally,
antimetabolite resistance can be used as the basis of
25 selection for dhfr, which confers resistance to methotrexate;
the gpt gene, which confers resistance to mycophenolic acid;
the neomycin gene, which confers resistance to the
aminoglycoside G-418; and the hygromycin gene, which confers
resistance to hygromycin. Additional selectable genes have
30 been described, namely trpB, which allows cells to utilize
indole in place of tryptophan; hisD, which allows cells to
utilize histinol in place of histidine; and ODC (ornithine
decarboxylase) which confers resistance to the ornithine
decarboxylase inhibitor, 2-(difluoromethyl)-DL-ornithine,
35 DFMO.

As used herein, the term "transformation" means a genetic change in a cell following incorporation of DNA exogenous to the cell. Thus, a "transformed cell" is a cell into which (or a progeny of which) a DNA molecule has been introduced by means of recombinant DNA techniques.

Transformation of a host cell with DNA may be carried out by conventional techniques known to those skilled in the art. For example, when the host cell is a eukaryote, methods of DNA transformation include, for example, calcium phosphate co-precipitates, conventional mechanical procedures such as microinjection, electroporation, insertion of a plasmid encased in liposomes, and viral vectors. Eukaryotic cells also can be cotransformed with DNA sequences encoding a nucleic acid of interest, and a second foreign DNA molecule encoding a selectable phenotype, such as the those described herein. Another method is to use a eukaryotic viral vector, such as simian virus 40 (SV40) or bovine papilloma virus, to transiently infect or transform eukaryotic cells and express the protein.

When the compound of the invention is an RNA molecule, said RNA molecule can be delivered to a subject by transforming the cells of said subject with an expression vector that comprises a nucleotide sequence encoding said RNA molecule under control of sequence that allows for the transcription of the nucleotide sequence.

When the compound of the invention is polypeptide/protein, said protein molecule can be delivered to the cells of a subject by transforming said cell with an expression vector that comprises a nucleotide sequence encoding said protein under control of sequence that allows for the transcription of the nucleotide sequence.

Thus, the present invention relates to a vector, preferably an expression vector comprising a nucleic acid sequence encoding a polypeptide of the invention.

5 Furthermore, the present invention relates to a host cell comprising the vector of the invention, preferably a host cell transformed with the above mentioned expression vector.

10 The present invention also relates to polypeptides expressed by the host cell of the invention. The polypeptides can be obtained using recombinant expression methods as disclosed herein. For example, polynucleotide encoding the protein can be produced, inserted into a vector and transformed into host cells using well known techniques described herein and
15 further known in the art (Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y., 1989). Following transformation, protein may be isolated and purified in accordance with conventional methods. For example, lysate prepared from an expression host (e.g.,
20 bacteria) can be purified using HPLC, size-exclusion chromatography, gel electrophoresis, affinity chromatography, or other purification technique. Other protein purification methods known in the art additionally can be used (see e.g., Deutscher et al., Guide to Protein Purification: Methods in
25 Enzymology, Vol. 182, Academic Press, 1990).

The pharmaceutical compositions of the invention

The present invention also relates to pharmaceutical compositions comprising the polypeptide or polynucleotide of
30 the invention. In one embodiment, the pharmaceutical composition of the invention comprises the vector of the invention.

The pharmaceutical composition according to the invention can
35 also comprise further therapeutic compounds. Such further compounds comprise other substances suitable to reduce coagulant activity. Preferably, such additional compounds do

not affect factor Xa, such as salicylic acid or clopidogrel. However, substances like heparin or coumarin may also be used. The composition of the present invention may also comprise substances administered to patients suffering from cardiovascular diseases, such as β -blockers, angiotensin converting enzyme (ACE) inhibitors, or substances affecting blood lipids, such as statins.

The production of pharmaceutical compositions with an amount of one or more compounds (polypeptide / polynucleotide) according to the invention and/or their use in the application according to the invention occurs in the customary manner by means of common pharmaceutical technology methods. For this, the compounds according to the invention are processed together with suitable, pharmaceutically acceptable excipients, carriers etc. to medicinal forms suitable for the various indications and types of application. Thereby, the medicaments can be produced in such a manner that the respective desired release rate is obtained, for example a quick flooding and/or a sustained or depot effect.

A pharmaceutical composition comprising one or more polypeptides of the invention can be produced by mixing medically acceptable diluent, stabilizer, carrier, and/or other additives with the polypeptides of the present invention. At this time, in order to increase the stability of the protein in vivo, the polypeptides of the present invention may be modified by a modifying agent, such as PEG, as described above.

A pharmaceutical composition comprising the polynucleotides of the invention can comprise agents that increase cell membrane permeability and/or cellular uptake of the nucleic acids. Examples of these agents are polyamines as described for example by Antony, T. et al. (1999) Biochemistry 38: 10775-10784; branched polyamines as described for example by

Escriou, V. et al (1998) Biochem. Biophys. Acta 1368(2): 276-288; polyaminolipids as described for example by Guy-Caffey, J.K. et al. (1995) J. Biol. Chem. 270(52): 31391-31396; DOTMA as described by Felgner, P.L. et al. (1987) PNAS USA 84(21):
5 7413-7417 and cationic porphyrins as described for example by Benimetskaya, L. et al. (1998) NAR 26(23): 5310-5317.

A usual method used for transfer of a gene into animal cells is employed for the transfer of the gene for the therapy into the target tissue or cell of the subject to be treated.

10 Examples include a method using a vector for animal cells derived from virus utilized for a gene therapy such as retrovirus vectors such as Moloney mouse leukemia virus, adenovirus vectors, adeno-associated virus (AAV) vectors, herpes simplex virus vectors, and HIV vectors (with respect
15 to a vector for gene therapy, see Verma, I.M., Nature, 389: 239, 1997); calcium phosphate transfection, DEAE-dextran transfection, electroporation, the liposome method, the lipofection method, the microinjection method, and the like. Among them, the method using the retrovirus vector, the
20 adeno-associated virus vector, or the HIV vector is preferable, since permanent expression of a gene is expected due to insertion into the chromosome DNA of a target cell.

Preferably, the pharmaceutical composition of the present
25 invention is a preparation for systemic application, preferably for parenteral use, more preferably injections and infusions.

Preferably, injections are prepared either in the form of
30 vials or also as so-called ready-to-use injection preparations, for example as ready-to-use syringes or single use syringes in addition to perforation bottles for multiple withdrawals. Administration of the injection preparations can occur in the form of subcutaneous (s.c.), intramuscular
35 (i.m.), intravenous (i.v.), internodal (i.n.) or intracutaneous (i.c.) application, preferably i.v.. The respective suitable injection forms can especially be

produced as solutions, crystal suspensions, nanoparticulate or colloid-disperse systems, such as for example, hydrosols.

The injectable formulations can also be produced as concentrates which can be adjusted with aqueous isotonic dilution agents to the desired dosage of the compounds of the invention. Furthermore, they can also be produced as powders, such as for example lyophilisates, which are then preferably dissolved or dispersed immediately before application with suitable diluents. The infusions can also be formulated in the form of isotonic solutions, fat emulsions, liposome formulations, microemulsions and liquids based on mixed micells, for example, based on phospholipids. As with injection preparations, infusion formulations can also be prepared in the form of concentrates to dilute. The injectable formulations can also be applied in the form of continuous infusions as in stationary as well as in out-patient therapy, for example in the form of mini-pumps.

Albumin, plasma expanders, surface active compounds, organic solvents, pH influencing compounds, complex forming compounds or polymeric compounds can be added to the parenteral medicinal forms with the aim of decreasing the adsorption of the compounds of the present invention to materials such as injection instruments or packaging materials, for example plastic or glass.

The compounds according to the invention can be bound to nanoparticles in the preparations for parenteral use, for example on finely dispersed particles based on poly(meth)acrylates, polyacetates, polyglycolates, polyamino acids or polyether urethanes. The parenteral formulations can also be constructively modified as depot preparations, for example on the multiple unit principle, where the compounds of the present invention are incorporated in a most finely distributed and/or dispersed, suspended form or as crystal suspensions, or on the single unit principle, where the

compounds according to the invention are enclosed in a medicinal form, for example, a tablet or a seed which is subsequently implanted. Often, these implantation or depot medicaments in single unit and multiple unit medicinal forms
5 consist of so-called biodegradable polymers, such as for example, polyether urethanes of lactic and glycolic acid, polyether urethanes, polyamino acids, poly(meth)acrylates or polysaccharides.

10 Sterilized water, pH value influencing substances, such as for example organic and inorganic acids or bases as well as their salts, buffer substances for setting the pH value, agents for isotonicity, such as for example sodium chloride, monosodium carbonate, glucose and fructose, tensides and/or
15 surface active substances and emulsifiers, such as for example, partial fatty acid esters of polyoxyethylene sorbitan (Tween®) or for example fatty acid esters of polyoxethylene (Cremophor®), fatty oils such as for example peanut oil, soybean oil and castor oil, synthetic fatty acid
20 esters, such as for example ethyl oleate, isopropyl myristate and neutral oil (Miglyol®) as well as polymer adjuvants such as for example gelatin, dextran, polyvinylpyrrolidone, organic solvent additives which increase solubility, such as for example propylene glycol, ethanol, N,N-dimethylacetamide,
25 propylene glycol or complex forming compounds such as for example citrates and urea, preservatives, such as for example hydroxypropyl benzoate and hydroxymethyl benzoate, benzyl alcohol, anti-oxidants, such as for example sodium sulfite and stabilizers, such as for example EDTA, are suitable as
30 adjuvants and carriers in the production of preparations for parenteral use.

In suspensions, addition of thickening agents to prevent the settling of the compounds of the present invention from
35 tensides and peptizers, to secure the ability of the sediment to be shaken, or complex formers, such as EDTA, ensues. This can also be achieved with the various polymeric agent

complexes, for example with polyethylene glycols, polystyrol, carboxymethylcellulose, Pluronic® or polyethylene glycol sorbitan fatty acid esters. The compounds according to the invention can also be incorporated in liquid formulations in the form of inclusion compounds, for example with cyclodextrins. As further adjuvants, dispersion agents are also suitable. For production of lyophilisates, builders are also used, such as for example mannite, dextran, saccharose, human albumin, lactose, PVP or gelatin varieties.

For long-term application with a systematic release of the compounds of the present invention up to several weeks, pressed implants are suitable which are preferably formulated on the basis of so-called biodegradable polymers.

Furthermore, so-called electronically controlled release systems can also be formulated by which release of the compounds of the present invention can be selectively adjusted to individual needs.

The pharmaceutical composition of the invention can also be applied locally at a site that is particularly prone to thrombus formation, e.g. by means of an intraluminal catheter. As an example, the pharmaceutical composition can be applied at the site of vascular or heart surgery, in particular surgery performed using indwelling catheters or devices, or can be applied at a site of thrombolytic intervention.

The pharmaceutical compositions of the present invention can be applied directly, e.g. in form of an injection, or can be applied indirectly. For example, the pharmaceutical compositions can be comprised in a coating on a medical device. The coating may be a fixed layer, or may liberate the composition of the invention to the surrounding fluid or tissue. Such coating may optionally comprise further substances that result in a prolonged release of the

composition of the present invention. Such substances are well known to the skilled person.

Examples of preferred medical devices are intraluminal
5 catheters, artificial cardiac valves, hemodialysis equipment
or any other devices that extend into the lumen of blood
vessels, or are otherwise in contact with blood. A preferred
embodiment of a medical device is a stent or a vascular
10 implant with a coating comprising the polypeptide or the
polynucleotide of the invention. Stents for treatment of
vascular stenosis are particularly preferred.

The present invention also relates to the use of the
polypeptide or polynucleotide of the invention for the
15 manufacture of a medicament for the treatment and / or
prevention of a disease involving TFPI and the tissue
factor/factor VIIa/factor Xa-complex. Preferred examples of
such diseases comprise thrombosis or conditions associated
with an increased risk of thrombosis. Further preferred
20 examples comprise ischemia/reperfusion damage, cancer, and
inflammatory conditions.

Thrombosis comprises arterial and venous thrombosis that may
be the primary cause of a disease or syndrome or be
25 associated with a disease or syndrome as a secondary event.

Thrombosis is e.g. responsible for the mortality resulting
from myocardial infarction, stroke, or lung emboli. In
addition, arterial thrombosis represents a major mediator of
30 the lethal complications associated with sepsis.

In one preferred embodiment, the polypeptide and / or
polynucleotide are used for the manufacture of a medicament
for the prevention of thrombosis in patients with an
35 increased risk of thrombosis. The risk of thrombosis is
enhanced e.g. in subjects suffering from arteriosclerosis,
patients having undergone surgery, patients suffering from

inflammatory disorders, in particular disorders affecting blood vessels, bed ridden patients, patients suffering from a malignant disease as well as patients with ischemia / reperfusion damage.

5

The risk of thrombosis can be enhanced due to alterations of blood cells, plasma, blood flow and / or vessel walls. Such alterations can be determined by a range of clinical parameters, such as e.g. increased aggregation of

10 thrombocytes, erythrocytes and increased blood viscosity, elevation of pro-coagulant factors (e.g. fibrinogen) or reduction of anti-coagulant (e.g. antithrombin III, protein C) and abnormalities of fibrinolysis.

15 In addition, risk factors for arteriosclerotic diseases such as smoking, hypercholesterinaemia, hypertension as well as established vessel abnormalities such as coronary heart disease or arterial stenosis, lead to an increased risk of thrombosis.

20

Patients who are presently treated with anti-coagulants such as heparin or with substances inhibiting thrombocyte aggregation, such as salicylic acid can be regarded as patients with an increased risk of thrombosis.

25

The polypeptides and / or polynucleotides of the invention can also be used for the manufacture of a medical device, in particular a device coated or filled with said compounds.

30 Brief Description of the Drawings

Figure 1a: The isolated T87F mutant and the native TFPI (SEQ ID No: 1) were incubated for 10 min at 37°C with HNE (10 nM). While the native TFPI was nearly completely degraded, T87F

35 remained intact.

Figure 1b: After incubation of native TFPI (SEQ ID No: 1) with MMP-9 (200 nM), the native TFPI was substantially cleaved, whereas the T87F/L21A variant remained intact.

- 5 Figure 1c: Cathepsin G (50 nM) elicited near complete degradation of the native TFPI (SEQ ID No: 1). Under the same conditions, the T87F/L89A mutant was not cleaved.

- 10 Figure 2: Factor Xa formation by a mixture of human platelets and human neutrophils (P/N) in response to collagen was reduced by addition of native TFPI (5 nM, nTFPI, SEQ ID No: 1) by 35%. The same concentration of the variant T87F lead to a reduction by 64%. A similar level of reduction was achieved by the variant T87F/L21A, whereas the variant T87F/L89A
15 resulted in 85% reduction.

- Figure 3: Thromboelastography tracings of whole blood (I), whole blood mixed with collagen (II), whole blood mixed with collagen in the presence of native TFPI (SEQ ID No: 1) (III)
20 or the variant T87F/L89A (IV). The variant resulted in a 160% prolongation of coagulation time as compared to II.

- Figure 4: Polynucleotide and amino acid sequence of a variant polypeptide of the invention comprising the variation L21A,
25 T87F, L89A and Y159A (SEQ ID No: 6 and 7). The sequences of fig. 4 also depict the N-terminal signal sequence (the first 9 amino acids), and a C-terminal strep-tag for purification (the last 23 amino acids), as is also apparent from comparing SEQ ID No: 1 with SEQ ID Nos. 6 and 7.

30

Figure 5: Schematic representation of TFPI (SEQ ID No: 1) excluding N-terminal signal peptide and C-terminal strep-tag indicating various protease cleavage sites

35 Examples

All amino acid positions relate to SEQ ID No: 1, unless specified differently.

Example 1: production of native and variant TFPI polypeptides**Bacterial strains and plasmids**

5 DH5alpha, BL21 Escherichia coli cells (Invitrogen, Germany) were employed for cloning, site directed mutagenesis and expression of TFPI. E. coli was grown in LB and SOC medium. The full-length human TFPI-1 gene (alpha isoform) (excluding the N-terminal signal peptide) was amplified from arterial
10 vessel wall cDNA using outer and inner primers, the forward outer primer 5' GTACATGCACTTTGGGCTTC-3' (SEQ ID No: 2) and the reverse outer primer 5'-TCACATATCTTTAACAAGAATTC -3' (SEQ ID No: 3). The inner primers (forward: 5'-
CGGGATCCGCTGATTCTGAGGAAG-3' (SEQ ID No: 4); reverse: 5'-
15 GGAATTCGCTGATTCTGAGGAAG-3' (SEQ ID No: 5)) contained restriction sites for EcoRI and BamHI. Said full length TFPI was cloned in the expression vector pASK-IBA3 containing the Strep-Tag coding sequence (IBA Germany). Site directed mutagenesis was performed with GeneTailor™ Site-Directed
20 Mutagenesis System (Invitrogen, Germany). All the constructs were sequenced in their entirety to verify for the correctness of the mutated sites (Seqlab, Goettingen, Germany).

25 Variant TFPI polypeptides

The following mutants of human TFPI were designed: For modification of the HNE cleavage site between Threonin 87 and Threonin 88, the amino acid in position 87 was substituted by Phenylalanin (T87F). In order to additionally modify the cath
30 G specific degradation sites between Leucin 89 and Glutamine 90, and between Tyrosine 159 and Glycine 160, the amino acids in positions 89 and 159 were both replaced by Alanine (T87F/L89A; T87F/Y159A). To alter the MMP cleavage site between residues 20 and 21, Leucine 21 was substituted by
35 Alanine (T87F/L21A). The modifications were introduced as given below.

Culture conditions and expression of TFPI protein

Luria-Bertani (LB) broth medium was used to produce TFPI native and the mutant forms of the protein. 500 ml of LB amp (100 µg/ml amp.) medium was inoculated with 10 ml of BL21 E. coli cells, transfected with the expression vectors for the variant TFPI polypeptides, from an overnight culture. The mixture was shaken in an incubator at 37 °C and 190 rpm. When this culture reached an OD600 of 0.4, 50 µl anhydrotetracycline (IBA, Germany) was added to the cell culture at a concentration of 2.0 mg/ml, to induce the expression of the recombinant protein. The cell culture was incubated for an additional 2 h at 25 °C.

Purification of TFPI

After incubation, the culture suspensions were centrifuged at 5000 rpm for 20 min at 4 °C to pellet the cells. The pellets were resuspended in 10 ml of pre-cooled buffer W (100 mM Tris/HCl, pH 8.0, 150 mM NaCl, 1.0 mM EDTA), and sonicated under ice-cooling conditions until the lysis was complete. The suspension was then centrifuged at 6,000 rpm for 15 min at 4 °C to remove the cell debris, the supernatant was additionally centrifuged at 35,000 g for 30 min at 4 °C to remove the residual proteins. After the centrifugation, the supernatant containing the TFPI protein was collected in a clean 50 ml Falcon tube. A chromatography column (0,8 x 4 cm) (BioRad) containing 2ml Strep-Tactin II (IBA, Germany) was initially washed with 10 ml of washing buffer (100 mM Tris/HCl, pH 8.0, 1.0 mM EDTA, and 0.02% w/v NaN₃) at a flow rate of 0.5 ml/min. Then, 10 ml of the supernatant containing TFPI protein was loaded onto the column. Washing buffer (30 ml) was passed through the column to elute impurities. To elute the protein from the column, 7 x 500 µl of elution buffer (100 mM Tris/HCl, pH 8.0, 1.0 mM EDTA, 0.02% w/v NaN₃, and 2.5 mM desthiobiotin) was passed through the column. The eluent was collected (0.5 ml/tube). Thirty microliters of each fraction was analyzed using a 1.5 mm 12% SDS-polyacrylamide gel. The amount of protein was also quantified

using UV spectrophotometry. To regenerate the column, it was first washed with 10 ml of washing buffer followed by 30 ml of regeneration buffer (100 mM Tris/HCl, pH 8.0, 2 mM EDTA, 0.02% (w/v) NaN₃, and 5 mM of 4-hydroxyazobenzene-2-carboxylic acid (HABA)). Finally, 40 ml of washing buffer was run through the column to remove the HABA.

Western blot

Equal amounts of the recombinant proteins were dissolved in 20 µl of Tris-HCl, 50 mM, pH 6.8, containing 2-mercaptoethanol (1%), sodium dodecyl sulfate (SDS) (2%), glycerol (20%), bromphenol blue (0.04%). The samples were heated at 100 °C for 5 min. and then were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred electrophoretically onto nitrocellulose membranes (Hibond, Amersham Pharmacia Biotech). The membranes were blocked with Tris-buffered saline containing 1% (w/v) bovine serum albumin and 0.5% (v/v) Tween 20 over night at 4 °C. The primary antibody, mouse anti hTFPI polyclonal (Santa Cruz) was used at a final concentration of 3 µg/ml for 2 hours. The secondary goat anti-mouse IgG Ab coupled with horseradish peroxidase (Santa Cruz) was diluted 2000 fold and incubated for 1.5 hours. The immunocomplexes were detected by using ECL Amersham Western blot detection System according to the manufacturer's protocol.

Example 2: in vitro resistance to protease cleavage of native and variant TFPI polypeptides

Next, it was verified whether the variants were resistant towards in vitro cleavage by the serine and matrix metalloproteases. The isolated T87F mutant and the native TFPI were incubated for 10 min at 37°C with HNE (10 nM (Calbiochem 50 µg Package)) and analyzed by Western blot as described above. While the native TFPI was nearly completely degraded, T87F remained entirely intact (Fig. 1a).

After incubation of native TFPI with MMP-9 (200 nM (Calbiochem (5 µg Package), the native TFPI was substantially cleaved as analyzed by Western blot as described above. In contrast, the T87F/L21A variant was not degraded (Fig. 1b).

5

Cathepsin G (200 nM (Sigma 0.25 U Package) elicited near complete degradation of the native TFPI as analyzed by Western blot as described above. Under the same conditions, the T87F/L89A mutant was not cleaved (Fig. 1c).

10

Thus, the variant TFPI molecules showed enhanced resistance to protease cleavage in vitro as compared to native TFPI.

15 **Example 3: Effect of the TFPI variants on the activity of the initiator complex of coagulation**

In order to assess the anticoagulant efficiency of the TFPI variants, their effects on the formation of factor Xa were evaluated in mixtures of activated human platelets and human neutrophils. Platelets were isolated from venous blood of healthy volunteers. The blood was drawn into sodium citrate (0.38%final concentration) and subsequently centrifuged at 190g for 15 minutes. The upper two thirds of the supernatant were supplemented with apyrase (0.2 U/ml) and iloprost (10 ng/ml). The suspension was centrifuged at 330g for 10 min, and the platelet pellet was recovered and resuspended in a buffer composed of 145 mM NaCl, 10 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), 5 mM KCl, 1 mM MgCl₂, and 5 mM glucose (pH 7.4; resuspension buffer). Human neutrophils were prepared by incubation of freshly obtained buffy coats with microbeads coupled to anti-CD15 antibodies (Miltenyi Biotec) for 15 min at 8°C. The suspensions were thereafter applied onto the positive selection column, and neutrophils were eluted with the antibody buffer (phosphate-buffered saline [PBS] supplemented with 0.13% EDTA [ethylenediaminetetraacetic acid] and 0.15% bovine serum albumin).

The isolated neutrophils and platelets were incubated in resuspension buffer for 5 min with collagen (type I, 12 $\mu\text{g/ml}$) at 37°C. The medium contained 0.88 U/ml factor VII of a coagulation factor concentrate (factors II, VII, IX, X (Beriplex P/N 500; Aventis-Behring)), together with 8 mM CaCl_2 and 125 $\mu\text{g/ml}$ (final concentrations) of the chromogenic substrate S2222 (Chromogenix). After incubation for 1 min, the increase in the optical density at 405 nm was registered in 5 subsequent 360 s intervals in an ELISA reader (Dynatech MR 7000). A standard curve was prepared using dilutions of tissue factor concentrates.

Addition of native TFPI (5 nM) to the suspensions reduced the factor Xa formation by 35% (Fig. 2). At the same concentration, variant TFPI T87F diminished the factor Xa generation by 64%. Additional mutation at position 21 (T87F/L21A) did not augment the inhibitory influence of T87F (55% reduction), indicating that the mutation in position 21 did not enhance the anticoagulant influence of TFPI at the particular selected in vitro conditions. The variant TFPI T87F/L89A elicited the strongest inhibition of the factor Xa formation, lowered its generation by 85% (Fig. 2).

Thus, in a particular in vitro setting in the presence of neutrophils the variant TFPI polypeptides exhibited an enhanced capability of reducing the initiator complex of coagulation.

Example 4: Effect of the TFPI variants on whole blood coagulation

The effect of the TFPI variants was also tested under ex vivo conditions in whole blood. The coagulation time (fibrin formation) was determined mechanically by thrombelastography (TEG) (roTEG, Instrumentation Laboratories). 0.3 ml of the (citrated) blood containing corn trypsin inhibitor (32 $\mu\text{g/ml}$) was pipetted into a plastic cup that had been prewarmed to

37°C. Either collagen (type I, 12 µg/ml) or vehicle was added. The coagulation process was started by addition of 60 µl of a 100 mM CaCl₂ solution (suspended in 10 mM Hepes, pH 7.4). Mineral oil was placed on top of the sample. The TEG tracings were registered under standardized conditions as established by the manufacturer. The fibrin formation time r (the distance between the start of the recalcification and the point where the amplitude of the tracing reaches 2 mm) was markedly accelerated by addition of collagen (Fig. 3; II vs. I). The time r was prolonged by 44% in the presence of the native TFPI (III). However, addition of T87F/L89A prolonged the coagulation time by 160% (IV). In conclusion, the T87F/L89A mutant enhances the anticoagulant efficiency by 2.4-3.6-fold.

Claims

1. A variant of the polypeptide of SEQ ID No: 1 having
5 increased protease resistance at one or more protease cleavage sites, and an increased anti-coagulant activity as compared to the amino acid sequence according to SEQ ID No: 1.
- 10 2. The polypeptide according to claim 1, wherein said one or more protease cleavage sites are one or more of cathepsin G, human neutrophil elastase, matrix metalloproteinase, thrombin and plasmin cleavage sites.
- 15 3. The polypeptide according to claim 1 or 2, wherein said one or more protease cleavage sites are one or more of cathepsin G, human neutrophil elastase, and matrix metalloproteinase cleavage sites.
- 20 4. The polypeptide according to claim 3, wherein said one or more protease cleavage sites are the cathepsin G and/or human neutrophil elastase cleavage sites.
- 25 5. The polypeptide according to any one of claims 1 to 4, comprising one or more variations affecting the cleavage site between Threonin 87 and Threonin 88 of SEQ ID No: 1.
- 30 6. The polypeptide according to any one of claims 1 to 5, comprising one or more variations affecting the cleavage site between Leucine 89 and Glutamine 90 and/or Tyrosine 159 and Glycine 160 of SEQ ID No: 1.

7. The polypeptide according to any one of claims 1 to 6, comprising one or more variations affecting a combination of the cleavage sites according to claims 5 and 6.
- 5 8. The polypeptide according to any one of claims 1 to 7, wherein the amino acid at position 87 of SEQ ID No: 1 is substituted by Phenylalanine (T87F).
- 10 9. The polypeptide according to any one of claims 1 to 8, wherein the amino acid at position 89 of SEQ ID No: 1 is substituted by Alanine (L89A), and/or the amino acid at position 159 of SEQ ID No: 1 is substituted by Alanine (Y159A).
- 15 10. The polypeptide according to any one of claims 1 to 9, comprising a combination of substitutions according to claims 8 and 9.
- 20 11. The polypeptide according to claim 10, comprising the substitutions T87F and L89A.
12. A polynucleotide encoding the polypeptide according to any one of claims 1 to 11.
- 25 13. A vector comprising the polynucleotide of claim 12.
14. A host cell comprising the vector of claim 13.
- 30 15. A polypeptide obtainable by expressing the polynucleotide of claim 12 or vector of claim 13 in a host cell and isolating said polypeptide from said cell.
- 35 16. A pharmaceutical composition comprising the polypeptide of any one of claims 1 to 11 or 15.

17. A pharmaceutical composition comprising the polynucleotide of claim 12.
18. A pharmaceutical composition comprising the vector of
5 claim 13.
19. Use of the polypeptide according to any one of claims 1 to 11 or 15 for the manufacture of a medicament for the treatment and/or prevention of a disease involving TFPI
10 and the tissue factor/factor VIIa/factor Xa-complex
20. Use of the polynucleotide according to claim 12 for the manufacture of a medicament for the treatment and/or prevention of a disease involving TFPI and the tissue
15 factor/factor VIIa/factor Xa-complex.
21. Use of the vector according to claim 13 for the manufacture of a medicament for the treatment and/or prevention of a disease involving TFPI and the tissue
20 factor/factor VIIa/factor Xa-complex.

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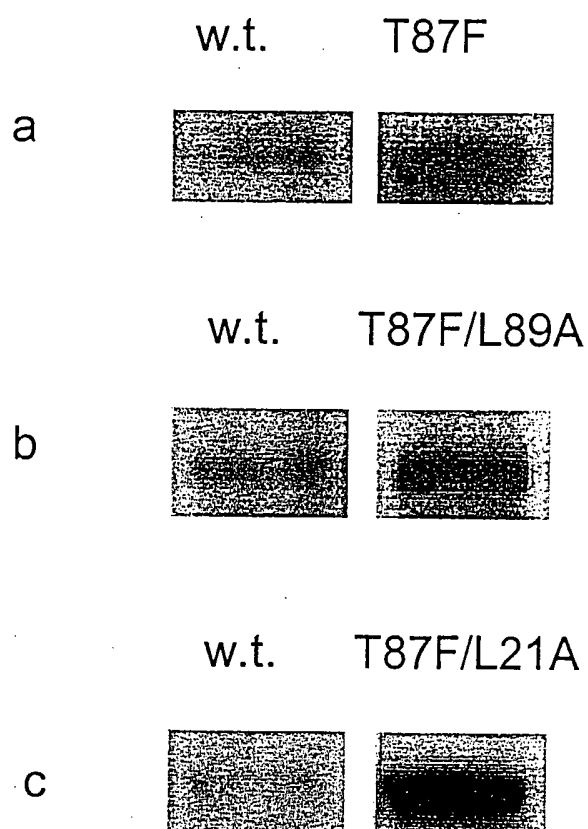


Fig. 1

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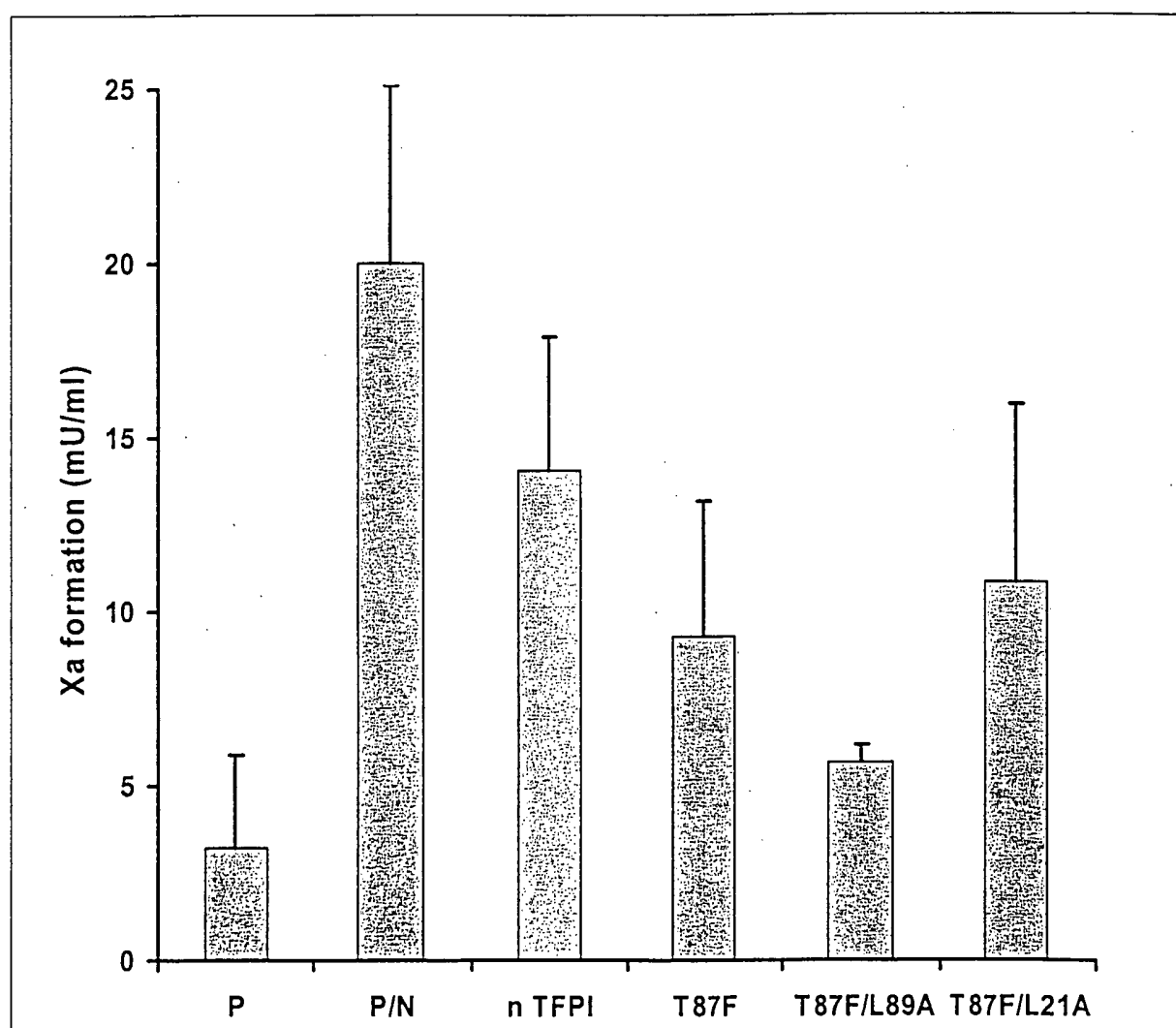


Fig. 2

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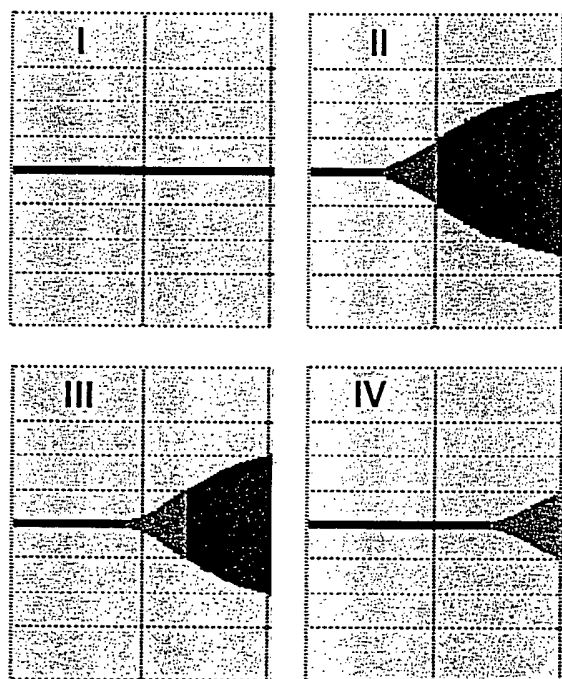


Fig. 3

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Figure 4

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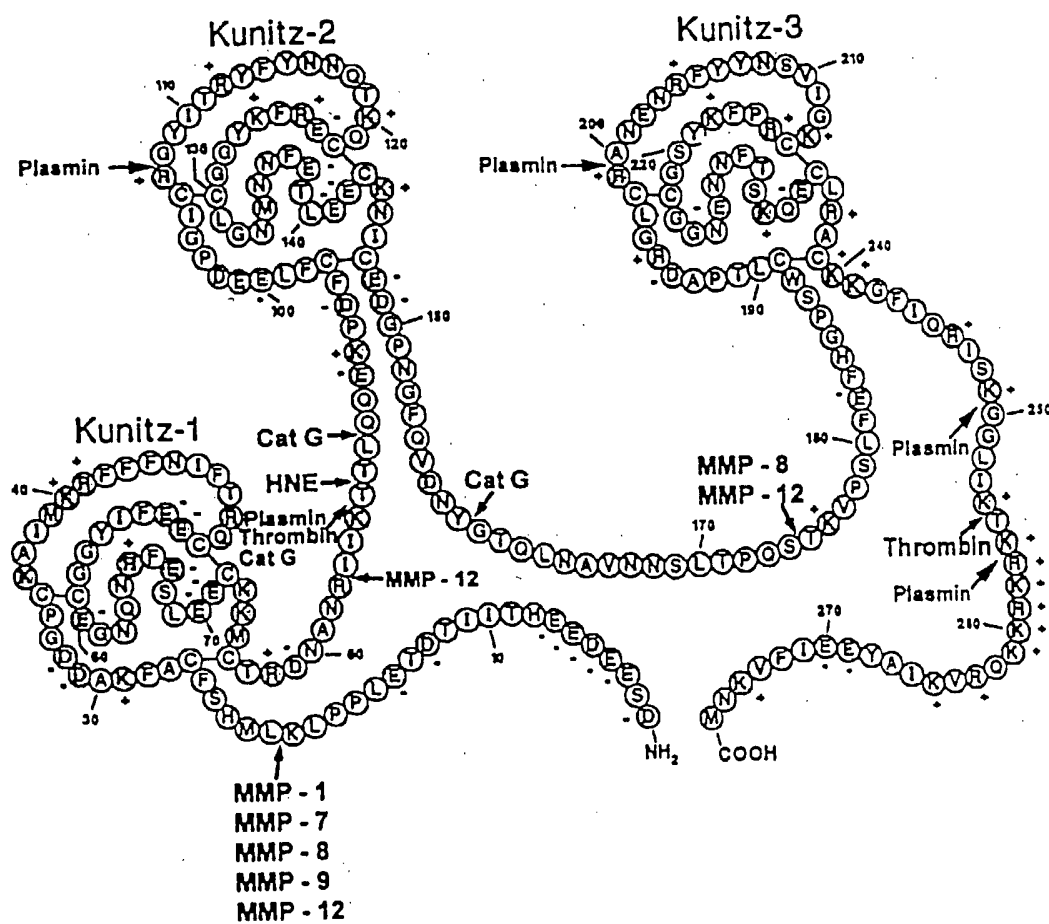


Figure 5

SEQUENCE LISTING

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<120> Variant TFPI with increased anti-thrombotic activity

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2005/013962

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/81 A61K38/57

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)

C07K A61K

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, CHEM ABS Data, Sequence Search, WPI Data, EMBASE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 96/04378 A (CHIRON CORPORATION) 15 February 1996 (1996-02-15) page 3, line 28 - line 31 page 27, line 26 - line 30; figure 4 -----	1-21
A	WO 91/02753 A (NOVO NORDISK A/S) 7 March 1991 (1991-03-07) table 2 page 3, paragraph 2 page 16, line 6 - line 27; example 1 -----	1-21
A	WO 00/11034 A (KLEESIEK, KNUT; BRINKMANN, THOMAS; PROHASKA, WOLFGANG; GOETTING, CHRIS) 2 March 2000 (2000-03-02) page 3, line 2 - line 12 figure 2 ----- -/--	1-21



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See patent family annex.

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P document published prior to the international filing date but later than the priority date claimed

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& document member of the same patent family

Date of the actual completion of the international search

6 April 2006

Date of mailing of the international search report

19/04/2006

Name and mailing address of the ISA/

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Fax: (+31-70) 340-3016

Authorized officer

Voigt-Ritzer, H

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2005/013962

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	BROZE JR G J ET AL: "REGULATION OF COAGULATION BY A MULTIVALENT KUNITZ-TYPE INHIBITOR" BIOCHEMISTRY, AMERICAN CHEMICAL SOCIETY. EASTON, PA, US, vol. 29, no. 33, 21 August 1990 (1990-08-21), pages 7539-7546, XP000268583 ISSN: 0006-2960 the whole document -----	1-21
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A	MOATTI D ET AL: "POLYMORPHISMS OF THE TISSUE FACTOR PATHWAY INHIBITOR (TFPI) GENE IN PATIENTS WITH ACUTE CORONARY SYNDROMES AND IN HEALTHY SUBJECTS. IMPACT OF THE V264M SUBSTITUTION ON PLASMA LEVELS OF TFPI" ARTERIOSCLEROSIS, THROMBOSIS, AND VASCULAR BIOLOGY, vol. 19, no. 4, April 1999 (1999-04), pages 862-869, XP000856947 ISSN: 1079-5642 the whole document -----	1-21

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2005/013962

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
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