Monoclonal Antibodies against Tissue Factor Pathway Inhibitor (TFPI)

Zhuozhi Wang, John Murphy, Tobias Marquardt, Dieter Moosmayer

Sequence listing submission

[0001] The Sequence Listing associated with this application is filed in electronic format *via* EFS-Web and hereby incorporated by reference into the specification in its entirety.

Field of the embodiments

[0002] Provided are isolated monoclonal antibodies and fragments thereof that bind human tissue factor pathway inhibitor (TFPI).

Background

[0003] Blood coagulation is a process by which blood forms stable clots to stop bleeding. The process involves a number of proenzymes and procofactors (or "coagulation factors") that are circulating in the blood. Those proenzymes and procofactors interact through several pathways through which they are converted, either sequentially or simultaneously, to the activated form. Ultimately, the process results in the activation of prothrombin to thrombin by activated Factor X (FXa) in the presence of Factor Va, ionic calcium, and platelets. The activated thrombin in turn induces platelet aggregation and converts fibrinogen into fibrin, which is then cross linked by activated Factor XIII (FXIIIa) to form a clot.

[0004] The process leading to the activation of Factor X can be carried out by two distinct pathways: the contact activation pathway (formerly known as the intrinsic pathway) and the tissue factor pathway (formerly known as the extrinsic pathway). It was previously thought that the coagulation cascade consisted of two pathways of equal importance joined to a common pathway. It is now known that the primary pathway for the initiation of blood coagulation is the tissue factor pathway.

[0005] Factor X can be activated by tissue factor (TF) in combination with activated Factor VII (FVIIa). The complex of FVIIa and its essential cofactor, TF, is a potent initiator of the clotting cascade.

[0006] The tissue factor pathway of coagulation is negatively controlled by tissue factor pathway inhibitor ("TFPI"). TFPI is a natural, FXa-dependent feedback inhibitor of the

FVIIa/TF complex. It is a member of the multivalent Kunitz-type serine protease inhibitors. Physiologically, TFPI binds to activated Factor X (FXa) to form a heterodimeric complex, which subsequently interacts with the FVIIa/TF complex to inhibit its activity, thus shutting down the tissue factor pathway of coagulation. In principle, blocking TFPI activity can restore FXa and FVIIa/TF activity, thus prolonging the duration of action of the tissue factor pathway and amplifying the generation of FXa, which is the common defect in hemophilia A and B.

[0007] Indeed, some preliminary experimental evidence has indicated that blocking the TFPI activity by antibodies against TFPI normalizes the prolonged coagulation time or shortens the bleeding time. For instance, Nordfang et al. showed that the prolonged dilute prothrombin time of hemophilia plasma was normalized after treating the plasma with antibodies to TFPI (Thromb. Haemost., 1991, 66(4): 464-467). Similarly, Erhardtsen et al. showed that the bleeding time in hemophilia A rabbit model was significantly shortened by anti-TFPI antibodies (Blood Coagulation and Fibrinolysis, 1995, 6: 388-394). These studies suggest that inhibition of TFPI by anti-TFPI antibodies may be useful for the treatment of hemophilia A or B. Only polyclonal anti-TFPI antibody was used in these studies.

[0008] Using hybridoma techniques, monoclonal antibodies against recombinant human TFPI (rhTFPI) were prepared and identified (*See* Yang et al., Chin. Med. J., 1998, 111(8): 718-721). The effect of the monoclonal antibody on dilute prothrombin time (PT) and activated partial thromboplastin time (APTT) was tested. Experiments showed that anti-TFPI monoclonal antibody shortened dilute thromboplastin coagulation time of Factor IX deficient plasma. It is suggested that the tissue factor pathway plays an important role not only in physiological coagulation but also in hemorrhage of hemophilia (Yang et al., Hunan Yi Ke Da Xue Xue Bao, 1997, 22(4): 297-300).

[0009] Accordingly, antibodies specific for TFPI are needed for treating hematological diseases and cancer.

[0010] Generally, therapeutic antibodies for human diseases have been generated using genetic engineering to create murine, chimeric, humanized or fully human antibodies. Murine monoclonal antibodies were shown to have limited use as therapeutic agents because of a short serum half-life, an inability to trigger human effector functions, and the production of human anti-mouse-antibodies (Brekke and Sandlie, "Therapeutic Antibodies for Human Diseases at the Dawn of the Twenty-first Century," *Nature* 2, 53, 52-62, Jan. 2003). Chimeric antibodies have been shown to give rise to human anti-chimeric antibody responses. Humanized antibodies further minimize the mouse component of antibodies. However, a

fully human antibody avoids the immunogenicity associated with murine elements completely. Thus, there is a need to develop fully human antibodies to avoid the immunogenicity associated with other forms of genetically engineered monoclonal antibodies. In particular, chronic prophylactic treatment such as hemophilia treatment would be required for humanized or preferably, fully human antibodies. An anti-TFPI monoclonal antibody has a high risk of development of an immune response to the therapy if an antibody with a murine component or murine origin is used due to numerous dosing required and the long duration of therapy. For example, antibody therapy for hemophilia A may require weekly dosing for the lifetime of a patient. This would be a continual challenge to the immune system. Thus, the need exists for a fully human antibody for antibody therapy for hemophilia and related genetic and acquired deficiencies or defects in coagulation.

[0011] Therapeutic antibodies have been made through hybridoma technology described by Koehler and Milstein in "Continuous Cultures of Fused Cells Secreting Antibody of Predefined Specificity," *Nature* 256, 495-497 (1975). Fully human antibodies may also be made recombinantly in prokaryotes and eukaryotes. Recombinant production of an antibody in a host cell rather than hybridoma production is preferred for a therapeutic antibody. Recombinant production has the advantages of greater product consistency, likely higher production level, and a controlled manufacture that minimizes or eliminates the presence of animal-derived proteins. For these reasons, it is desirable to have a recombinantly produced monoclonal anti-TFPI antibody.

[0012] In addition, because TFPI binds to activated Factor X (FXa) with high affinity, an effective anti-TFPI antibody should have a comparable affinity. Thus, it is desirable to have an anti-TFPI antibody which has binding affinity which can compete with TFPI/FXa binding.

Summary

[0013] Monoclonal antibodies having specific binding to a specific epitope of human tissue factor pathway inhibitor (TFPI) are provided. Also provided are polynucleotides which encode the anti-TFPI monoclonal antibodies. Pharmaceutical compositions comprising the anti-TFPI monoclonal antibodies and methods of treatment of genetic and acquired deficiencies or defects in coagulation such as hemophilia A and B are also provided.

Brief description of the drawings

[0014] Figure 1 depicts complex formation of Fab A and TFPI Kunitz domain 2 by size exclusion analysis.

[0015] Figure 2 depicts a cartoon representation of the interaction between human tissue factor pathway inhibitor and an antibody thereof (Fab A). Fab A with denoted variable light (V_L) and heavy (V_H) domains is represented as the lower structure. The Kunitz domain 2 (KD2) of TFPI is represented as the upper structure.

[0016] Figure 3 depicts key epitope residues Asp102 (D102), Ile105 (I105), Arg107 (R107), Cys106-Cys130 disulfide bridge, and binding of TFPI at the Fab A surface.

[0017] Figure 4 depicts a superposition of TFPI - Fab A complex and a trypsin bound Kunitz domain 2 (KD2) and shows exclusion of simultaneous binding of TFPI to factor Xa and Fab A. KD2 and Fab A are shown in cartoon representation, trypsin is shown as transparent surface. Steric hindrance of Fab A and trypsin is also indicated.

[0018] Figure 5 depicts complex formation of Fab B and TFPI Kunitz domain 1+2 by size exclusion analysis.

[0019] Figure 6 depicts two cartoon representations showing the interaction between human tissue factor pathway inhibitor and an antibody thereof (Fab B) at a first angle and at another angle rotated 90 degrees relative to the first angle. Fab B with denoted variable light (V_L) and heavy (V_H) domains is shown in the lower part of the figure (shaded in grey). TFPI Kunitz domain 1 (KD1) is shown in white and TFPI Kunitz domain 2 (KD2) is shown in black.

[0020] Figure 7 depicts key epitope residues Asp31 (D31), Asp32 (D32), Pro34 (P34), Lys36 (K36), Glu60 (E60), Cys35-Cys59 disulfide bridge, and binding of Kunitz domain 1 TFPI at the Fab B surface. Also shown, but not enumerated, is the binding of Kunitz domain 2.

[0021] Figure 8 depicts two angles of view of binding and interaction of epitope residues Glu100 (E100), Glu101 (E101), Pro103 (P103), Ile105 (I105), Arg107 (R107), Tyr109 (Y109) of Kunitz domain 2 with Fab B. Arg107 interacts with Gly33 (G33) and Cys35 (C35) of Kunitz domain 1.

[0022] Figure 9 depicts a superposition of TFPI - Fab B complex and a complex of BPTI, factor VIIa and tissue factor, and shows exclusion of simultaneous binding of TFPI to factor VIIa/tissue factor and Fab B. Steric hindrance of Fab B and factor VIIa, and Fab B and tissue factor are indicated by arrows.

[0023] Figure 10 depicts a superposition of TFPI - Fab B complex and a trypsin bound Kunitz domain 2 and shows exclusion of simultaneous binding of TFPI to factor Xa and Fab B. Steric hindrance of Fab B and trypsin, and Fab B bound Kunitz domain 1 and trypsin are indicated.

[0024] Figure 11 depicts (A) a sequence alignment of light and heavy chains of Fab A (SEQ ID NOs: 2 and 3) and Fab C (SEQ ID NOs: 6 and 7) and (B) a superposition of TFPI - Fab A X-ray structure with homology models of Fab C. (A) paratope residues are in bold text and highlighted. Paratope residue hc_Asn32 which differs in Fab A and Fab C is marked with asterisk. (B) Kunitz domain 2 (KD2) is shown as cartoon in black. The Fab structures are shown as grey ribbon. Paratope residue hc_Asn32 is shown as stick.

[0025] Figure 12 depicts (A) a sequence alignment of light and heavy chains of Fab B (SEQ ID NOs: 4 and 5) and Fab D (SEQ ID NOs: 8 and 9) and (B) a superposition of TFPI - Fab B X-ray structure with homology models of Fab D. (A) paratope residues are in bold text and highlighted. Paratope residues which differ in Fab B and Fab D are marked with asterisk. (B) Kunitz domain 1 (KD1) and Kunitz domain 2 (KD2) are shown as light grey and black cartoon, respectively. The Fab structures are shown as grey ribbon. Paratope residues which differ in Fab B and Fab D are shown as sticks.

[0026] Figure 13 depicts (A) the surface plasmon resonance (Biacore) data of Fab C and Fab D blocking FXa binding on TFPI and, (B) Surface plasmon resonance (Biacore) data of Fab C and Fab D blocking FVIIa/TF binding on TFPI.

Detailed description

Definitions

[0027] The term "tissue factor pathway inhibitor" or "TFPI" as used herein refers to any variant, isoform and species homolog of human TFPI that is naturally expressed by cells. In a preferred embodiment of the invention, the binding of an antibody of the invention to TFPI reduces the blood clotting time.

[0028] As used herein, an "antibody" refers to a whole antibody and any antigen binding fragment (i.e., "antigen-binding portion") or single chain thereof. The term includes a full-length immunoglobulin molecule (e.g., an IgG antibody) that is naturally occurring or formed by normal immunoglobulin gene fragment recombinatorial processes, or an immunologically active portion of an immunoglobulin molecule, such as an antibody fragment, that retains the specific binding activity. Regardless of structure, an antibody fragment binds with the same

antigen that is recognized by the full-length antibody. For example, an anti-TFPI monoclonal antibody fragment binds to an epitope of TFPI. The antigen-binding function of an antibody can be performed by fragments of a full-length antibody. Examples of binding fragments encompassed within the term "antigen-binding portion" of an antibody include (i) a Fab fragment, a monovalent fragment consisting of the V_L, V_H, C_L and C_{H1} domains; (ii) a $F(ab')_2$ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting of the V_H and C_{H1} domains; (iv) a Fv fragment consisting of the V_L and V_H domains of a single arm of an antibody, (v) a dAb fragment (Ward et al., (1989) Nature 341:544-546), which consists of a V_H domain; (vi) an isolated complementarity determining region (CDR); (vii) minibodies, diaboidies, triabodies, tetrabodies, and kappa bodies (see, e.g. Ill et al., Protein Eng 1997;10:949-57); (viii) camel IgG; and (ix) IgNAR . Furthermore, although the two domains of the Fv fragment, V_L and V_H, are coded for by separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the V_L and V_H regions pair to form monovalent molecules (known as single chain Fv (scFv); see e.g., Bird et al. (1988) Science 242:423-426; and Huston et al (1988) Proc. Natl. Acad. Sci. USA 85:5879-5883). Such single chain antibodies are also intended to be encompassed within the term "antigen-binding portion" of an antibody. These antibody fragments are obtained using conventional techniques known to those with skill in the art, and the fragments are analyzed for utility in the same manner as are intact antibodies.

[0029] Furthermore, it is contemplated that an antigen binding fragment may be encompassed in an antibody mimetic. The term “antibody mimetic” or “mimetic” as used herein is meant a protein that exhibits binding similar to an antibody but is a smaller alternative antibody or a non-antibody protein. Such antibody mimetic may be comprised in a scaffold. The term “scaffold” refers to a polypeptide platform for the engineering of new products with tailored functions and characteristics.

[0030] The term “epitope” refers to the area or region of an antigen to which an antibody specifically binds or interacts, which in some embodiments indicates where the antigen is in physical contact with the antibody. Conversely, the term “paratope” refers to the area or region of the antibody on which the antigen specifically binds. Epitopes characterized by competition binding are said to be overlapping if the binding of the corresponding antibodies are mutually exclusive, i.e. binding of one antibody excludes simultaneous binding of another antibody. The epitopes are said to be separate (unique) if the antigen is able to accommodate binding of both corresponding antibodies simultaneously.

[0031] The term “competing antibodies,” as used herein, refers to antibodies that bind to about, substantially or essentially the same, or even the same, epitope as an antibody against TFPI as described herein. “Competing antibodies” include antibodies with overlapping epitope specificities. Competing antibodies are thus able to effectively compete with an antibody as described herein for binding to TFPI. Preferably, the competing antibody can bind to the same epitope as the antibody described herein. Alternatively viewed, the competing antibody has the same epitope specificity as the antibody described herein.

[0032] As used herein, the terms "inhibits binding" and "blocks binding" (e.g., referring to inhibition/blocking of binding of TFPI ligand to TFPI) are used interchangeably and encompass both partial and complete inhibition or blocking. Inhibition and blocking are also intended to include any measurable decrease in the binding affinity of TFPI to a physiological substrate when in contact with an anti-TFPI antibody as compared to TFPI not in contact with an anti-TFPI antibody, e.g., the blocking of the interaction of TFPI with factor Xa or blocking the interaction of a TFPI-factor Xa complex with tissue factor, factor VIIa or the complex of tissue factor/factor VIIa by at least about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 96%, 97%, 98%, 99%, or 100%.

[0033] The terms "monoclonal antibody" or "monoclonal antibody composition" as used herein refer to a preparation of antibody molecules of single molecular composition. A monoclonal antibody composition displays a single binding specificity and affinity for a particular epitope. Accordingly, the term "human monoclonal antibody" refers to antibodies displaying a single binding specificity which have variable and constant regions derived from human germline immunoglobulin sequences. The human antibodies of the invention may include amino acid residues not encoded by human germline immunoglobulin sequences (e.g., mutations introduced by random or site-specific mutagenesis in vitro or by somatic mutation in vivo).

[0034] An "isolated antibody," as used herein, is intended to refer to an antibody which is substantially free of other antibodies having different antigenic specificities (e.g., an isolated antibody that binds to TFPI is substantially free of antibodies that bind antigens other than TFPI). An isolated antibody that binds to an epitope, isoform or variant of human TFPI may, however, have cross-reactivity to other related antigens, e.g., from other species (e.g., TFPI species homologs). Moreover, an isolated antibody may be substantially free of other cellular material and/or chemicals.

[0035] As used herein, "specific binding" refers to antibody binding to a predetermined antigen. Typically, the antibody binds with an affinity of at least about 10^5 M^{-1} and binds to

the predetermined antigen with an affinity that is higher, for example at least two-fold greater, than its affinity for binding to an irrelevant antigen (e.g., BSA, casein) other than the predetermined antigen or a closely-related antigen. The phrases "an antibody recognizing an antigen" and "an antibody specific for an antigen" are used interchangeably herein with the term "an antibody which binds specifically to an antigen."

[0036] As used herein, the term "high affinity" for an IgG antibody refers to a binding affinity of at least about 10^7M^{-1}, in some embodiments at least about 10^8M^{-1}, in some embodiments at least about 10^9M^{-1}, $10^{10}M^{-1}$, $10^{11}M^{-1}$ or greater, e.g., up to $10^{13}M^{-1}$ or greater. However, "high affinity" binding can vary for other antibody isotypes. For example, "high affinity" binding for an IgM isotype refers to a binding affinity of at least about $1.0 \times 10^7M^{-1}$. As used herein, "isotype" refers to the antibody class (e.g., IgM or IgG1) that is encoded by heavy chain constant region genes.

[0037] "Complementarity-determining region" or "CDR" refers to one of three hypervariable regions within the variable region of the heavy chain or the variable region of the light chain of an antibody molecule that form the N-terminal antigen-binding surface that is complementary to the three-dimensional structure of the bound antigen. Proceeding from the N-terminus of a heavy or light chain, these complementarity-determining regions are denoted as "CDR1," "CDR2," and "CDR3," respectively. CDRs are involved in antigen-antibody binding, and the CDR3 comprises a unique region specific for antigen-antibody binding. An antigen-binding site, therefore, may include six CDRs, comprising the CDR regions from each of a heavy and a light chain V region.

[0038] As used herein, "conservative substitutions" refers to modifications of a polypeptide that involve the substitution of one or more amino acids for amino acids having similar biochemical properties that do not result in loss of a biological or biochemical function of the polypeptide. A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (e.g., threonine, valine, isoleucine), and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine). It is envisioned that the antibodies of the present invention may have conservative amino acid substitutions and still retain activity.

[0039] For nucleic acids and polypeptides, the term "substantial homology" indicates that two nucleic acids or two polypeptides, or designated sequences thereof, when optimally aligned and compared, are identical, with appropriate nucleotide or amino acid insertions or deletions, in at least about 80% of the nucleotides or amino acids, usually at least about 85%, preferably about 90%, 91%, 92%, 93%, 94%, or 95%, more preferably at least about 96%, 97%, 98%, 99%, 99.1%, 99.2%, 99.3%, 99.4%, or 99.5% of the nucleotides or amino acids. Alternatively, substantial homology for nucleic acids exists when the segments will hybridize under selective hybridization conditions to the complement of the strand. The invention includes nucleic acid sequences and polypeptide sequences having substantial homology to the specific nucleic acid sequences and amino acid sequences recited herein.

[0040] The percent identity between two sequences is a function of the number of identical positions shared by the sequences (i.e., % homology = # of identical positions / total # of positions x 100), taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences. The comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm, such as without limitation the AlignX™ module of VectorNTI™ (Invitrogen Corp., Carlsbad, CA). For AlignX™, the default parameters of multiple alignment are: gap opening penalty: 10; gap extension penalty: 0.05; gap separation penalty range: 8; % identity for alignment delay: 40. (further details found at http://www.invitrogen.com/site/us/en/home/LINNEA-Online-Guides/LINNEA-Communities/Vector-NTI-Community/Sequence-analysis-and-data-management-software-for-PCs/AlignX-Module-for-Vector-NTI-Advance.reg.us.html).

[0041] Another method for determining the best overall match between a query sequence (a sequence of the present invention) and a subject sequence, also referred to as a global sequence alignment, can be determined using the CLUSTALW computer program (Thompson et al., Nucleic Acids Research, 1994, 2(22): 4673-4680), which is based on the algorithm of Higgins et al., (Computer Applications in the Biosciences (CABIOS), 1992, 8(2): 189-191). In a sequence alignment the query and subject sequences are both DNA sequences. The result of said global sequence alignment is in percent identity. Preferred parameters used in a CLUSTALW alignment of DNA sequences to calculate percent identity via pairwise alignments are: Matrix = IUB, k-tuple = 1, Number of Top Diagonals = 5, Gap Penalty = 3, Gap Open Penalty = 10, Gap Extension Penalty = 0.1. For multiple alignments, the following CLUSTALW parameters are preferred: Gap Opening Penalty = 10, Gap Extension Parameter = 0.05; Gap Separation Penalty Range = 8; % Identity for Alignment Delay = 40.

[0042] The nucleic acids may be present in whole cells, in a cell lysate, or in a partially purified or substantially pure form. A nucleic acid is "isolated" or "rendered substantially pure" when purified away from other cellular components with which it is normally associated in the natural environment. To isolate a nucleic acid, standard techniques such as the following may be used: alkaline/SDS treatment, CsCl banding, column chromatography, agarose gel electrophoresis and others well known in the art.

Monoclonal Antibodies that bind to specific epitopes of TFPI

[0043] Provided herein are monoclonal antibodies with specific binding to human TFPI as shown in SEQ ID NO:1. In some embodiments, the anti-TFPI monoclonal antibodies inhibit binding of a TFPI ligand to TFPI. Thus, in some embodiments, the anti-TFPI monoclonal antibodies may inhibit activity of TFPI.

[0044] Provided is an isolated monoclonal antibody that binds to an epitope of human tissue factor pathway inhibitor (SEQ ID NO:1), wherein said epitope comprises one or more residues of Kunitz domain 2. In some embodiments, the isolated monoclonal antibody comprises the light chain as shown in SEQ ID NO:2 or in SEQ ID NO:4. In some embodiments, the isolated monoclonal antibody comprises the heavy chain as shown in SEQ ID NO:3 or in SEQ ID NO:5. In some embodiments, the isolated monoclonal antibody comprises the light chain as shown in SEQ ID NO:2 and the heavy chain as shown in SEQ ID NO:3. In some embodiments, the isolated monoclonal antibody comprises the light chain as shown in SEQ ID NO:4 and the heavy chain as shown in SEQ ID NO:5. In some embodiments, it is also contemplated that the isolated monoclonal antibody may comprise a light chain or heavy chain with substantial homology to those provided. For example, the isolated monoclonal antibody comprising substantial homology may comprise one or more conservative substitutions.

[0045] In some embodiments, provided is an isolated monoclonal antibody that binds to an epitope of human tissue factor pathway inhibitor (SEQ ID NO:1), wherein said epitope comprises one or more residues selected from Glu100, Glu101, Asp102, Pro103, Gly104, Ile105, Cys106, Arg107, Gly108, Tyr109, Lys126, Gly128, Gly129, Cys130, Leu131, Gly132, and Asn133 of SEQ ID NO:1 and combinations thereof.

[0046] In some embodiments, the epitope comprises residue Glu100 of SEQ ID NO:1. In some embodiments, the epitope comprises residue Glu101 of SEQ ID NO:1. In some embodiments, the epitope comprises residue Asp102 of SEQ ID NO:1. In some

embodiments, the epitope comprises residue Pro103 of SEQ ID NO:1. In some embodiments, the epitope comprises residue Gly104 of SEQ ID NO:1. In some embodiments, the epitope comprises residue Ile105 of SEQ ID NO:1. In some embodiments, the epitope comprises residue Cys106 of SEQ ID NO:1. In some embodiments, the epitope comprises residue Arg107 of SEQ ID NO:1. In some embodiments, the epitope comprises residue Gly108 of SEQ ID NO:1. In some embodiments, the epitope comprises residue Tyr109 of SEQ ID NO:1. In some embodiments, the epitope comprises residue Lys126 of SEQ ID NO:1. In some embodiments, the epitope comprises residue Gly128 of SEQ ID NO:1. In some embodiments, the epitope comprises residue Gly129 of SEQ ID NO:1. In some embodiments, the epitope comprises residue Cys130 of SEQ ID NO:1. In some embodiments, the epitope comprises residue Leu131 of SEQ ID NO:1. In some embodiments, the epitope comprises residue Gly132 of SEQ ID NO:1. In some embodiments, the epitope comprises residue Asn133 of SEQ ID NO:1.

[0047] In some embodiments, the epitope comprises residues Ile105 and Asp102 of SEQ ID NO:1. In some embodiments, the epitope comprises residues Ile105 and Leu131 of SEQ ID NO:1. In some embodiments the epitope comprises residues Ile105, Asp102 and Leu131 of SEQ ID NO:1. In some embodiments, the epitope further comprises residue Glu100, Glu101, Pro103, Gly104, Cys106, Gly108, Tyr109, Lys126, Gly128, Gly129, Cys130, Leu131, Gly132, or Asn133 of SEQ ID NO:1.

[0048] In some embodiments, provided is an isolated monoclonal antibody that binds to an epitope of human tissue factor pathway inhibitor (SEQ ID NO:1), wherein said epitope comprises two amino acid loops linked by a disulfide bridge between residues Cys106 and Cys130 of SEQ ID NO:1. In some embodiments, the epitope further comprises one or more residues selected from Glu100, Glu101, Asp102, Pro103, Gly104, Ile105, Cys106, Arg107, Gly108, Tyr109, Lys126, Gly128, Gly129, Cys130, Leu131, Gly132, and Asn133 of SEQ ID NO:1. In some embodiments, the epitope comprises residue Ile105 of SEQ ID NO:1. In other embodiments, the epitope comprises residue Asp102 of SEQ ID NO:1. In other embodiments, the epitope comprises residue Leu131 of SEQ ID NO:1. And in some embodiments, the epitope further comprises one or more residues selected from Glu100, Glu101, Asp102, Pro103, Gly104, Ile105, Cys106, Arg107, Gly108, Tyr109, Lys126, Gly128, Gly129, Cys130, Leu131, Gly132, and Asn133 of SEQ ID NO:1.

[0049] Also provided is an isolated monoclonal antibody that binds to an epitope of human tissue factor pathway inhibitor (SEQ ID NO:1), wherein said epitope comprises one or more residues of Kunitz domain 1 and one or more residues of Kunitz domain 2. In some

embodiments, the isolated monoclonal antibody comprises the light chain as shown in SEQ ID NO:6 or in SEQ ID NO:8. In some embodiments, the isolated monoclonal antibody comprises the heavy chain as shown in SEQ ID NO:7 or in SEQ ID NO:9. In some embodiments, the isolated monoclonal antibody comprises the light chain as shown in SEQ ID NO:6 and the heavy chain as shown in SEQ ID NO:7. In some embodiments, the isolated monoclonal antibody comprises the light chain as shown in SEQ ID NO:8 and the heavy chain as shown in SEQ ID NO:9. In some embodiments, it is also contemplated that the isolated monoclonal antibody may comprise a light chain or heavy chain with substantial homology to those provided. For example, the isolated monoclonal antibody comprising substantial homology may comprise one or more conservative substitutions.

[0050] In some embodiments, the residues of Kunitz domain 1 comprise one or more residues selected from Asp31, Asp32, Gly33, Pro34, Cys35, Lys36, Cys59, Glu60 and Asn62 of SEQ ID NO:1 and combinations thereof. In some embodiments, the residue of Kunitz domain 1 comprises residue Asp31 of SEQ ID NO:1. In some embodiments, the residue of Kunitz domain 1 comprises residue Asp32 of SEQ ID NO:1. In some embodiments, the residue of Kunitz domain 1 comprises residue Gly33 of SEQ ID NO:1. In some embodiments, the residue of Kunitz domain 1 comprises residue Pro34 of SEQ ID NO:1. In some embodiments, the residue of Kunitz domain 1 comprises residue Cys35 of SEQ ID NO:1. In some embodiments, the residue of Kunitz domain 1 comprises residue Lys36 of SEQ ID NO:1. In some embodiments, the residue of Kunitz domain 1 comprises residue Cys59 of SEQ ID NO:1. In some embodiments, the residue of Kunitz domain 1 comprises residue Glu60 of SEQ ID NO:1. In some embodiments, the residue of Kunitz domain 1 comprises residue Asn62 of SEQ ID NO:1.

[0051] In some embodiments, the residues of Kunitz domain 1 comprise residues Pro34 and Glu60 of SEQ ID NO:1. In some embodiments, the residues of Kunitz domain 1 comprise residues Pro34 and Lys36 of SEQ ID NO:1. In some embodiments, the residues of Kunitz domain 1 comprise residues Pro34, Lys36 and Glu60 of SEQ ID NO:1.

[0052] In some embodiments, the residues of Kunitz domain 2 comprise one or more residues selected from Glu100, Glu101, Pro103, Gly104, Ile105, Cys106, Arg107, Gly108, Tyr109, Phe114, Asn116, Glu123, Arg124, Lys126, Tyr127 and Gly128 of SEQ ID NO:1 and combinations thereof. In some embodiments, the residue of Kunitz domain 2 comprises residue Glu100 of SEQ ID NO:1. In some embodiments, the residue of Kunitz domain 2 comprises residue Glu101 of SEQ ID NO:1. In some embodiments, the residue of Kunitz domain 2 comprises residue Pro103 of SEQ ID NO:1. In some embodiments, the residue of

Kunitz domain 2 comprises residue Gly104 of SEQ ID NO:1. In some embodiments, the residue of Kunitz domain 2 comprises residue Ile105 of SEQ ID NO:1. In some embodiments, the residue of Kunitz domain 2 comprises residue Cys106 of SEQ ID NO:1. In some embodiments, the residue of Kunitz domain 2 comprises residue Arg107 of SEQ ID NO:1. In some embodiments, the residue of Kunitz domain 2 comprises residue Gly108 of SEQ ID NO:1. In some embodiments, the residue of Kunitz domain 2 comprises residue Tyr109 of SEQ ID NO:1. In some embodiments, the residue of Kunitz domain 2 comprises residue Phe114 of SEQ ID NO:1. In some embodiments, the residue of Kunitz domain 2 comprises residue Asn116 of SEQ ID NO:1. In some embodiments, the residue of Kunitz domain 2 comprises residue Glu123 of SEQ ID NO:1. In some embodiments, the residue of Kunitz domain 2 comprises residue Arg124 of SEQ ID NO:1. In some embodiments, the residue of Kunitz domain 2 comprises residue Lys126 of SEQ ID NO:1. In some embodiments, the residue of Kunitz domain 2 comprises residue Tyr127 of SEQ ID NO:1.

[0053] In some embodiments, the residue of Kunitz domain 2 comprises residues Arg107 and Glu101 of SEQ ID NO:1. In some embodiments, the residue of Kunitz domain 2 comprises residues Arg107 and Tyr109 of SEQ ID NO:1. In some embodiments, the residue of Kunitz domain 2 comprises residues Arg107, Glu101 and Tyr109 of SEQ ID NO:1. In some embodiments, the residue of Kunitz domain 2 comprises residue Gly128 of SEQ ID NO:1.

[0054] In some embodiments, the residue of Kunitz domain 2 may additionally comprise one or more residues selected from Asp102, Gly129, Cys130, Leu131, Gly132, and Asn133 of SEQ ID NO:1 and combinations thereof.

[0055] In some embodiments, the isolated monoclonal antibody comprises a residue of Kunitz domain 1 which comprises one or more residues selected from Asp31, Asp32, Gly33, Pro34, Cys35, Lys36, Cys59, Glu60 and Asn62; and a residue of Kunitz domain 2 which comprises one or more residues selected from Glu100, Glu101, Pro103, Gly104, Ile105, Cys106, Arg107, Gly108, Tyr109, Phe114, Asn116, Glu123, Arg124, Lys126, Tyr127 and Gly128.

[0056] Also provided is an isolated monoclonal antibody that binds to an epitope of human tissue factor pathway inhibitor (SEQ ID NO:1), wherein said epitope comprises two amino acid loops linked by a disulfide bridge between residues Cys35 and Cys59 of SEQ ID NO:1. In some embodiments, the epitope further comprises one or more residues of Kunitz domain 1 and one or more residues of Kunitz domain 2. In some embodiments, the residue of Kunitz domain 1 comprises one or more residues selected from Asp31, Asp32, Gly33,

Pro34, Cys35, Lys36, Cys59, Glu60, and Asn62 of SEQ ID NO:1. In some embodiments, the residue of Kunitz domain 2 comprises one or more residues selected from Glu100, Glu101, Pro103, Gly104, Ile105, Cys106, Arg107, Gly108, Tyr109, Phe114, Asn116, Glu123, Arg124, Lys126, Tyr127 and Gly128 of SEQ ID NO:1.

[0057] Also provided are antibodies which can compete with any of the antibodies described herein for binding to TFPI. For example, an antibody that binds to the same epitope as the antibodies described herein will be able to effectively compete for binding of TFPI. In some embodiments, provided is an isolated monoclonal antibody that binds to TFPI, wherein the isolated monoclonal antibody is competitive with any of the isolated monoclonal antibodies described herein. In some embodiments, the antibody is competitive with an antibody having a light chain as shown in SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6 or SEQ ID NO:8. In some embodiments, the antibody is competitive with an antibody having a heavy chain as shown in SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7 or SEQ ID NO:9. In some embodiments, the antibody is competitive with an antibody having a light chain as shown in SEQ ID NO:2 and a heavy chain as shown in SEQ ID NO:3. In some embodiments, the antibody is competitive with an antibody having a light chain as shown in SEQ ID NO:4 and a heavy chain as shown in SEQ ID NO:5. In some embodiments, the antibody is competitive with an antibody having a light chain as shown in SEQ ID NO:6 and a heavy chain as shown in SEQ ID NO:7. In some embodiments, the antibody is competitive with an antibody having a light chain as shown in SEQ ID NO:8 and a heavy chain as shown in SEQ ID NO:9.

[0058] Also provided are bispecific antibodies which can compete with any of the antibodies described herein for binding to TFPI. For example, such bispecific antibody may bind to one or more epitopes described above.

[0059] The antibody may be species specific or may cross react with multiple species. In some embodiments, the antibody may specifically react or cross react with TFPI of human, mouse, rat, guinea pig, rabbit, monkey, pig, dog, cat or other mammalian species.

[0060] The antibody may be of any of the various classes of antibodies, such as without limitation an IgG1, an IgG2, an IgG3, an IgG4, an IgM, an IgA1, an IgA2, a secretory IgA, an IgD, and an IgE antibody.

Nucleic Acids, Vectors and Host Cells

[0061] Although provided are amino acid sequences of the monoclonal antibodies, it is contemplated that nucleic acid sequences can be designed to encode any of these monoclonal

antibodies. Such polynucleotides may encode encode a light chain or a heavy chain of the anti-TFPI antibody. In some embodiments, such polynucleotides may encode both the light chain and heavy chain of the anti-TFPI antibody separated by a degradeable linkage. Further, above mentioned antibodies can be produced using expression vectors comprising the isolated nucleic acid molecules encoding any of the monoclonal antibodies and host cells comprising such vectors.

Methods of Preparing Antibodies to TFPI

[0062] The monoclonal antibody may be produced recombinantly by expressing a nucleotide sequence encoding the variable regions of the monoclonal antibody according to the embodiments of the invention in a host cell. With the aid of an expression vector, a nucleic acid containing the nucleotide sequence may be transfected and expressed in a host cell suitable for the production. Accordingly, also provided is a method for producing a monoclonal antibody that binds with human TFPI comprising:

(a) transfecting a nucleic acid molecule encoding a monoclonal antibody of the invention into a host cell,

(b) culturing the host cell so to express the monoclonal antibody in the host cell, and optionally

(c) isolating and purifying the produced monoclonal antibody, wherein the nucleic acid molecule comprises a nucleotide sequence encoding a monoclonal antibody of the present invention.

[0063] In one example, to express the antibodies, or antibody fragments thereof, DNAs encoding partial or full-length light and heavy chains obtained by standard molecular biology techniques are inserted into expression vectors such that the genes are operatively linked to transcriptional and translational control sequences. In this context, the term "operatively linked" is intended to mean that an antibody gene is ligated into a vector such that transcriptional and translational control sequences within the vector serve their intended function of regulating the transcription and translation of the antibody gene. The expression vector and expression control sequences are chosen to be compatible with the expression host cell used. The antibody light chain gene and the antibody heavy chain gene can be inserted into separate vectors or, more typically, both genes are inserted into the same expression vector. The antibody genes are inserted into the expression vector by standard methods (e.g., ligation of complementary restriction sites on the antibody gene fragment and vector, or blunt

end ligation if no restriction sites are present). The light and heavy chain variable regions of the antibodies described herein can be used to create full-length antibody genes of any antibody isotype by inserting them into expression vectors already encoding heavy chain constant and light chain constant regions of the desired isotype such that the V_H segment is operatively linked to the C_H segment(s) within the vector and the V_L segment is operatively linked to the C_L segment within the vector. Additionally or alternatively, the recombinant expression vector can encode a signal peptide that facilitates secretion of the antibody chain from a host cell. The antibody chain gene can be cloned into the vector such that the signal peptide is linked in-frame to the amino terminus of the antibody chain gene. The signal peptide can be an immunoglobulin signal peptide or a heterologous signal peptide (i.e., a signal peptide from a non-immunoglobulin protein).

[0064] In addition to the antibody chain encoding genes, the recombinant expression vectors of the invention carry regulatory sequences that control the expression of the antibody chain genes in a host cell. The term "regulatory sequence" is intended to include promoters, enhancers and other expression control elements (e.g., polyadenylation signals) that control the transcription or translation of the antibody chain genes. Such regulatory sequences are described, for example, in Goeddel; Gene Expression Technology. Methods in Enzymology 185, Academic Press, San Diego, Calif. (1990). It will be appreciated by those skilled in the art that the design of the expression vector, including the selection of regulatory sequences may depend on such factors as the choice of the host cell to be transformed, the level of expression of protein desired, etc. Examples of regulatory sequences for mammalian host cell expression include viral elements that direct high levels of protein expression in mammalian cells, such as promoters and/or enhancers derived from cytomegalovirus (CMV), Simian Virus 40 (SV40), adenovirus, (e.g., the adenovirus major late promoter (AdMLP)) and polyoma. Alternatively, nonviral regulatory sequences may be used, such as the ubiquitin promoter or ß-globin promoter.

[0065] In addition to the antibody chain genes and regulatory sequences, the recombinant expression vectors may carry additional sequences, such as sequences that regulate replication of the vector in host cells (e.g., origins of replication) and selectable marker genes. The selectable marker gene facilitates selection of host cells into which the vector has been introduced (see, e.g., U.S. Pat. Nos. 4,399,216, 4,634,665 and 5,179,017, all by Axel et al.). For example, typically the selectable marker gene confers resistance to drugs, such as G418, hygromycin or methotrexate, on a host cell into which the vector has been introduced. Examples of selectable marker genes include the dihydrofolate reductase (DHFR) gene (for

use in dhfr- host cells with methotrexate selection/amplification) and the neo gene (for G418 selection).

[0066] For expression of the light and heavy chains, the expression vector(s) encoding the heavy and light chains is transfected into a host cell by standard techniques. The various forms of the term "transfection" are intended to encompass a wide variety of techniques commonly used for the introduction of exogenous DNA into a prokaryotic or eukaryotic host cell, e.g., electroporation, calcium-phosphate precipitation, DEAE-dextran transfection and the like. Although it is theoretically possible to express the antibodies of the invention in either prokaryotic or eukaryotic host cells, expression of antibodies in eukaryotic cells, and most preferably mammalian host cells, is the most preferred because such eukaryotic cells, and in particular mammalian cells, are more likely than prokaryotic cells to assemble and secrete a properly folded and immunologically active antibody.

[0067] Examples of mammalian host cells for expressing the recombinant antibodies include Chinese Hamster Ovary (CHO cells) (including dhfr- CHO cells, described in Urlaub and Chasin, (1980) Proc. Natl. Acad. Sci. USA 77:4216-4220, used with a DHFR selectable marker, e.g., as described in R. J. Kaufman and P. A. Sharp (1982) Mol. Biol. 159:601-621), NSO myeloma cells, COS cells, HKB11 cells and SP2 cells. When recombinant expression vectors encoding antibody genes are introduced into mammalian host cells, the antibodies are produced by culturing the host cells for a period of time sufficient to allow for expression of the antibody in the host cells or secretion of the antibody into the culture medium in which the host cells are grown. Antibodies can be recovered from the culture medium using standard protein purification methods, such as ultrafiltration, size exclusion chromatography, ion exchange chromatography and centrifugation.

Use of Partial Antibody Sequences to Express Intact Antibodies

[0068] Antibodies interact with target antigens predominantly through amino acid residues that are located in the six heavy and light chain CDRs. For this reason, the amino acid sequences within CDRs are more diverse between individual antibodies than sequences outside of CDRs. Because CDR sequences are responsible for most antibody-antigen interactions, it is possible to express recombinant antibodies that mimic the properties of specific naturally occurring antibodies by constructing expression vectors that include CDR sequences from the specific naturally occurring antibody grafted onto framework sequences from a different antibody with different properties (see, e.g., Riechmann, L. et al., 1998, Nature 332:323-327; Jones, P. et al., 1986, Nature 321:522-525; and Queen, C. et al., 1989, Proc. Natl. Acad. Sci. U.S.A. 86:10029-10033). Such framework sequences can be obtained

from public DNA databases that include germline antibody gene sequences. These germline sequences will differ from mature antibody gene sequences because they will not include completely assembled variable genes, which are formed by V(D)J joining during B cell maturation. It is not necessary to obtain the entire DNA sequence of a particular antibody in order to recreate an intact recombinant antibody having binding properties similar to those of the original antibody (see WO 99/45962). Partial heavy and light chain sequence spanning the CDR regions is typically sufficient for this purpose. The partial sequence is used to determine which germline variable and joining gene segments contributed to the recombined antibody variable genes. The germline sequence is then used to fill in missing portions of the variable regions. Heavy and light chain leader sequences are cleaved during protein maturation and do not contribute to the properties of the final antibody. For this reason, it is necessary to use the corresponding germline leader sequence for expression constructs. To add missing sequences, cloned cDNA sequences can be combined with synthetic oligonucleotides by ligation or PCR amplification. Alternatively, the entire variable region can be synthesized as a set of short, overlapping, oligonucleotides and combined by PCR amplification to create an entirely synthetic variable region clone. This process has certain advantages such as elimination or inclusion or particular restriction sites, or optimization of particular codons.

[0069] The nucleotide sequences of heavy and light chain transcripts are used to design an overlapping set of synthetic oligonucleotides to create synthetic V sequences with identical amino acid coding capacities as the natural sequences. The synthetic heavy and light chain sequences can differ from the natural sequences in three ways: strings of repeated nucleotide bases are interrupted to facilitate oligonucleotide synthesis and PCR amplification; optimal translation initiation sites are incorporated according to Kozak's rules (Kozak, 1991, J. Biol. Chem. 266:19867-19870); and restricted endonuclease sites are engineered upstream of the translation initiation sites.

[0070] For both the heavy and light chain variable regions, the optimized coding, and corresponding non-coding, strand sequences are broken down into 30-50 nucleotide sections at approximately the midpoint of the corresponding non-coding oligonucleotide. Thus, for each chain, the oligonucleotides can be assembled into overlapping double stranded sets that span segments of 150-400 nucleotides. The pools are then used as templates to produce PCR amplification products of 150-400 nucleotides. Typically, a single variable region oligonucleotide set will be broken down into two pools which are separately amplified to generate two overlapping PCR products. These overlapping products are then combined by

PCR amplification to form the complete variable region. It may also be desirable to include an overlapping fragment of the heavy or light chain constant region in the PCR amplification to generate fragments that can easily be cloned into the expression vector constructs.

[0071] The reconstructed heavy and light chain variable regions are then combined with cloned promoter, translation initiation, constant region, 3' untranslated, polyadenylation, and transcription termination sequences to form expression vector constructs. The heavy and light chain expression constructs can be combined into a single vector, co-transfected, serially transfected, or separately transfected into host cells which are then fused to form a host cell expressing both chains.

[0072] Thus, in another aspect, the structural features of a human anti-TFPI antibody are used to create structurally related human anti-TFPI antibodies that retain the function of binding to TFPI. More specifically, one or more CDRs of the specifically identified heavy and light chain regions of the monoclonal antibodies of the invention can be combined recombinantly with known human framework regions and CDRs to create additional, recombinantly-engineered, human anti-TFPI antibodies of the invention.

Pharmaceutical Compositions

[0073] Also provided are pharmaceutical compositions comprising therapeutically effective amounts of anti-TFPI monoclonal antibody and a pharmaceutically acceptable carrier. "Pharmaceutically acceptable carrier" is a substance that may be added to the active ingredient to help formulate or stabilize the preparation and causes no significant adverse toxicological effects to the patient. Examples of such carriers are well known to those skilled in the art and include water, sugars such as maltose or sucrose, albumin, salts such as sodium chloride, etc. Other carriers are described for example in Remington's Pharmaceutical Sciences by E. W. Martin. Such compositions will contain a therapeutically effective amount of at least one anti-TFPI monoclonal antibody. In some embodiments, such compositions may comprise a therapeutically effective amount of one or more anti-TFPI monoclonal antibodies. In some embodiments, the pharmaceutical compositions may comprise an antibody that specifically binds to Kunitz domain 1 as described above and an antibody that specifically binds to Kunitz domain 1 and 2 as describe above.

[0074] Pharmaceutically acceptable carriers include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. The use of such media and agents for pharmaceutically active substances is known in the art. The composition is preferably formulated for parenteral

injection. The composition can be formulated as a solution, microemulsion, liposome, or other ordered structure suitable to high drug concentration. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. In some cases, it will include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, or sodium chloride in the composition.

[0075] Sterile injectable solutions can be prepared by incorporating the active compound in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by sterilization microfiltration. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle that contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, some methods of preparation are vacuum drying and freeze-drying (lyophilization) that yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

Pharmaceutical Uses

[0076] The monoclonal antibody can be used for therapeutic purposes for treating genetic and acquired deficiencies or defects in coagulation. For example, the monoclonal antibodies in the embodiments described above may be used to block the interaction of TFPI with FXa, or to prevent TFPI-dependent inhibition of the TF/FVIIa activity. Additionally, the monoclonal antibody may also be used to restore the TF/FVIIa-driven generation of FXa to bypass the insufficiency of FVIII- or FIX-dependent amplification of FXa.

[0077] The monoclonal antibodies have therapeutic use in the treatment of disorders of hemostasis such as thrombocytopenia, platelet disorders and bleeding disorders (e.g., hemophilia A, hemophilia B and hemophilia C). Such disorders may be treated by administering a therapeutically effective amount of the anti-TFPI monoclonal antibody to a patient in need thereof. The monoclonal antibodies also have therapeutic use in the treatment of uncontrolled bleeds in indications such as trauma and hemorrhagic stroke. Thus, also provided is a method for shortening the bleeding time comprising administering a therapeutically effective amount of an anti-TFPI monoclonal antibody of the invention to a patient in need thereof.

[0078] The antibodies can be used as monotherapy or in combination with other therapies to address a hemostatic disorder. For example, co-administration of one or more antibodies

of the invention with a clotting factor such as factor VIIa, factor VIII or factor IX is believed useful for treating hemophilia. In one embodiment, provided is a method for treating genetic and acquired deficiencies or defects in coagulation comprising administering (a) a first amount of a monoclonal antibody that binds to human tissue factor pathway inhibitor and (b) a second amount of factor VIII or factor IX, wherein said first and second amounts together are effective for treating said deficiencies or defects. In another embodiment, provided is a method for treating genetic and acquired deficiencies or defects in coagulation comprising administering (a) a first amount of a monoclonal antibody that binds to human tissue factor pathway inhibitor and (b) a second amount of factor VIII or factor IX, wherein said first and second amounts together are effective for treating said deficiencies or defects, and further wherein factor VII is not coadministered. The invention also includes a pharmaceutical composition comprising a therapeutically effective amount of the combination of a monoclonal antibody of the invention and factor VIII or factor IX, wherein the composition does not contain factor VII. "Factor VII" includes factor VII and factor VIIa. These combination therapies are likely to reduce the necessary infusion frequency of the clotting factor. By co-administration or combination therapy is meant administration of the two therapeutic drugs each formulated separately or formulated together in one composition, and, when formulated separately, administered either at approximately the same time or at different times, but over the same therapeutic period.

[0079] The pharmaceutical compositions may be parenterally administered to subjects suffering from hemophilia A or B at a dosage and frequency that may vary with the severity of the bleeding episode or, in the case of prophylactic therapy, may vary with the severity of the patient's clotting deficiency.

[0080] The compositions may be administered to patients in need as a bolus or by continuous infusion. For example, a bolus administration of an inventive antibody present as a Fab fragment may be in an amount of from 0.0025 to 100 mg/kg body weight, 0.025 to 0.25 mg/kg, 0.010 to 0.10 mg/kg or 0.10-0.50 mg/kg. For continuous infusion, an inventive antibody present as an Fab fragment may be administered at 0.001 to 100 mg/kg body weight/minute, 0.0125 to 1.25 mg/kg/min., 0.010 to 0.75 mg/kg/min., 0.010 to 1.0 mg/kg/min. or 0.10-0.50 mg/kg/min. for a period of 1-24 hours, 1-12 hours, 2-12 hours, 6-12 hours, 2-8 hours, or 1-2 hours. For administration of an inventive antibody present as a full-length antibody (with full constant regions), dosage amounts may be about 1-10 mg/kg body weight, 2-8 mg/kg, or 5-6 mg/kg. Such full-length antibodies would typically be administered by infusion extending for a period of thirty minutes to three hours. The frequency of the

administration would depend upon the severity of the condition. Frequency could range from three times per week to once every two or three weeks.

[0081] Additionally, the compositions may be administered to patients via subcutaneous injection. For example, a dose of 10 to 100 mg anti-TFPI antibody can be administered to patients via subcutaneous injection weekly, biweekly or monthly.

[0082] As used herein, "therapeutically effective amount" means an amount of an anti-TFPI monoclonal antibody or of a combination of such antibody and factor VIII or factor IX that is needed to effectively increase the clotting time in vivo or otherwise cause a measurable benefit in vivo to a patient in need. The precise amount will depend upon numerous factors, including, but not limited to the components and physical characteristics of the therapeutic composition, intended patient population, individual patient considerations, and the like, and can readily be determined by one skilled in the art.

Examples

Example 1. Expression and purification of recombinant TFPI (Kunitz domain 2) from E. coli.

Expression system

[0083] The destination vector (according to Gateway nomenclature), designated pD Eco5 N, was utilized. pD Eco5 N is based on the pET-16 b (Novagen), and additionally encodes a His_{10} and NusA tag as well as a Gateway cloning cassette for expression of the fusion protein consisting of His_{10}/NusA and the protein of interest.

[0084] A TFPI construct encoding a thrombin cleavage site fused to the N-terminus of Kunitz domain 2 (Lys93 to Phe154, reference Uniprot 10646) and the Gateway attachment sites (attB1-5#, attB2-3#, Invitrogen) was cloned into the pD Eco5 N vector resulting in the expression vector designated as pD Eco5 N TFPI KD2. The BL21 DE3 (Novagen) expression strain was utilized.

Amino acid sequence of expressed fusion protein using pD Eco5 N TFPI KD2, 600 AA

MGHHHHHHHH HHSSGHIEGR HMNKEILAVV EAVSNEKALP REKIFEALES ALATATKKKY
EQEIDVRVQI DRKSGDFDTF RRWLVVDEVT QPTKEITLEA ARYEDESLNL GDYVEDQIES
VTFDRITTQT AKQVIVQKVR EAERAMVVDQ FREHEGEIIT GVVKKVNRDN ISLDLGNNAE
AVILREDMLP RENFRPGDRV RGVLYSVRPE ARGAQLFVTR SKPEMLIELF RIEVPEIGEE
VIEIKAAARD PGSRAKIAVK TNDKRIDPVG ACVGMRGARV QAVSTELGGE RIDIVLWDDN
PAQFVINAMA PADVASIVVD EDKHTMDIAV EAGNLAQAIG RNGQNVRLAS QLSGWELNVM
TVDDLQAKHQ AEAHAAIDTF TKYLDIDEDF ATVLVEEGFS TLEELAYVPM KELLEIEGLD

EPTVEALRER AKNALATIAQ AQEESLGDNK PADDLLNLEG VDRDLAFKLA ARGVCTLEDL
AEQGIDDLAD IEGLTDEKAG ALIMAARNIC WFGDEATSGS GLETSLYKKA GSLVPRGSKP
DFCFLEEDPG ICRGYITRYF YNNQTKQCER FKYGGCLGNM NNFETLEECK NICEDGPNGF

Sequence components

```
His 10 tag:          MGHHHHHHHH HH
NusA tag:                         SSGHIEGR HMNKEILAVV EAVSNEKALP REKIFEALES ALATATKKKY
                     EQEIDVRVQI DRKSGDFDTF RRWLVVDEVT QPTKEITLEA ARYEDESLNL GDYVEDQIES
                     VTFDRITTQT AKQVIVQKVR EAERAMVVDQ FREHEGEIIT GVVKKVNRDN ISLDLGNNAE
                     AVILREDMLP RENFRPGDRV RGVLYSVRPE ARGAQLFVTR SKPEMLIELF RIEVPEIGEE
                     VIEIKAAARD PGSRAKIAVK TNDKRIDPVG ACVGMRGARV QAVSTELGGE RIDIVLWDDN
                     PAQFVINAMA PADVASIVVD EDKHTMDIAV EAGNLAQAIG RNGQNVRLAS QLSGWELNVM
                     TVDDLQAKHQ AEAHAAIDTF TKYLDIDEDF ATVLVEEGFS TLEELAYVPM KELLEIEGLD
                     EPTVEALRER AKNALATIAQ AQEESLGDNK PADDLLNLEG VDRDLAFKLA ARGVCTLEDL
                     AEQGIDDLAD IEGLTDEKAG ALIMAARNIC WFGDEA
Linker/translated endonulease restriction sites:
                     TSGS GLE
Translated att-site:
                     TSLYKKA GS
Thrombin site:       LVPRGS
TFPI Kunitz 2        GSLVPRGSKP DFCFLEEDPG ICRGYITRYF YNNQTKQCER FKYGGCLGNM NNFETLEECK
                     NICEDGPNGF
```

Expression

[0085] A BL21 DE3 strain transformed with pD Eco5 N #209 was grown as a pre-culture in 2x 50 mL LB medium with 200 µg/mL ampicillin for 14 h at 37 °C with an agitatation rate of 180 rpm. Next, eight shaker flasks with 400 mL Circlegrow medium (Q-Biogene), were each inoculated with 8 mL pre-culture and incubated at 37 °C, with an agitation rate of 180 rpm. At a culture density of OD600, IPTG (100 mM final concentration) was added for gene induction and further cultivated at 17 °C for 24 h with 180 rpm. The E. coli was pelleted by centrifugation (3000 g, 10 min) and stored at -80 °C.

Purification

[0086] The pelleted E. coli mass from 3.2 L of culture was resuspended in 200 mL of lysis buffer (50 mM Tris-HCl pH 8.0, 300 mM NaCl, 10% (w/w) glycerol, 40 mM imidazol, protease inhibitor cocktail Complete EDTA-free (Roche)), homogenized in a high pressure device (Microfluidics) and afterwards the lysate was centrifuged (100.000 g, 60 min, 4 °C). Several purification steps were performed using an Äkta Explorer system. The concentrated sample was applied in an initial IMAC chromatography step to two linked 5 mL units of Hi-Trap-Sepharose HP matrix (GE). Equilibration, fusion protein binding and wash of the Hi-Trap-Sepharose HP matrix was done using Buffer A (50 mM Tris HCl pH 8.0, 300 mM NaCl, 40 mM imidazol). For elution of the NusA-TFPI fusion protein, a linear gradient of imidazol

from 40 to 500 mM in Buffer B (50 mM Tris HCl pH 8.0, 150 mM NaCl) was used. The elution fractions were pooled and concentrated (by a factor of 6-7 using a Amicon ultrafiltration device) and the buffer exchanged to Tris HCl pH 8.0. The concentrated sample (6-7 mL) was further applied to size exclusion chromatoraphy using Sephacryl-100 (XK26/74) in Tris HCl pH 8.0. The fractions of the main peak containing fusion protein were pooled, concentrated by ultrafiltration (Amicon) to 5 mL volume. Thrombin (HTI) was added to the sample (ratio enzyme : fusion protein, 1:50 w/w), incubated for 5 h at 21 °C and the reaction finally stopped by PMSF (1 mM final concentration). Subsequently, a second size exclusion chromatography step (Sephacryl-100 (XK26/74) in Tris HCl pH 8.0) was performed and the peak fractions monitored by PAGE. The fractions containing the free monomeric TFPI Kunitz domain 2 were collected and concentrated (Amicon), yielding about 4 mg of product from 3.2 L E. coli culture.

Example 2. Production of a recombinant monoclonal antibody Fab A to TFPI, expression in E. coli and its purification

Expression

[0087] The Fab A was co-expressed using the expression vector pET28a and the E. coli strain BL21 Star DE3. The light and heavy chain regions encoded on the expression vector were each fused at its N-terminus to a periplasmic signal sequence. The heavy chain region also encoded at its C-terminus a His_6 tag for purification of the Fab. The transformed E. coli strain grown in the TB-Instant over-night expression medium was used for autoinduction of the recombinant protein expression (#71491, Novagen). Briefly, 10 mL of transformed E. coli culture (in a 50 mL Falcon tube) was grown as a pre-culture in LB medium with 30 µg/mL kanamycin for 14 h at 37 °C agitated with 180 rpm. Subsequently, four Erlenmeyer flasks with 500 mL TB-Instant over-night expression medium were each inoculated with 2 mL of the pre-culture and incubated for 24 h at 30 °C at 180 rpm. The cultures were centrifuged at 10,000 g at 10 °C for 30 min and the supernatant containing the Fab was immediately used for further product purification or stored at -20 or -80 °C.

[0088] Alternatively, a Fab was expressed using the expression vector pET28a and the E. coli strain BL21 Star DE3 in a 10 L bioreactor (Sartorius). A transformed E. coli culture of 500 mL was grown in LB medium with 30 µg/mL kanamycin for 17 h at 37 °C, agitated at 165 rpm and afterwards used to inoculate a stirred bioreactor with 10 L autoinduction

medium. The autoinduction medium contained the following components, per liter: 12 g tryptone, 24 g yeast extract, 9.9 g glycerol (87%), 12.54 g K2HPO4, 2.31 g KH2PO4, 0.25 g MgSO4 x 7 H2O, 1 g glucose, 2.5 g lactose, 30 mg kanamycin. The cultivation with the bioreactor was performed for 24 h (at 30 °C with 350 - max. 800 rpm) and subsequently the culture supernatant was harvested by removing the biomass by centrifugation in a centrifuge (Heraeus).

Purification

[0089] The Fab was purified using a two step chromatography procedure with an Äkta Explorer 10s device. A hollow fibre module (10 kDa cut-off threshold) was applied to concentrate 1 L of the cleared culture supernatant to a final volume of 100 mL and to equilibrate the buffer composition with Buffer A (50 mM Na-phosphate pH 8.0, 300 mM NaCl, 10 mM imidazol). In an initial immobilize metal affinity chromatography (IMAC) step with an Äkta Explorer system, the concentrated sample was applied to 5 mL Ni-NTA superflow matrix (Qiagen). Equilibration, sample binding and wash of the Ni-NTA matrix was done using Buffer A (binding was done at 21 °C, all other chromatography steps at 4 °C). For elution of the Fab, a linear gradient of imidazol from 10 to 250 mM in Buffer A was used. The fractions from the single elution peak were pooled (60 mL total volume) and concentrated to 10 mL by ultrafiltration and the buffer adjusted to PBS pH 7.4 using a Hi-Prep26/10 desalting column. Subsequently, 2 mL of an anti kappa light chain antibody matrix (Kappa Select Affinity Media, 0833.10 from BAC), equilibrated with PBS was incubated with the concentrated IMAC eluate for 1 h at room temperature under agitation. The matrix with the bound sample was transferred to a chromatography column and washed with PBS. The Fab sample was eluted with 2 mL glycine pH 2.0, neutralized with 1 M HEPES pH 7.5 and buffer adjusted to PBS with a PD10 desalting column (GE, 17-0851-01).

[0090] When the Fab was expressed in E. coli using a 10 L bioreactor (Sartorius) the following purification procedure was used. The centrifuged culture supernatant was sequentially filtered through two disposable filter modules (GE, KMP-HC-9204TT; KGF-A-0504TT) with 5 and 0.2 µm pore size. A hollow fibre module (10 kDa cut-off threshold) was applied to concentrate the cleared culture supernatant to a final volume of 1500 mL and to adjust the buffer composition to Buffer A. 25 mL of Ni-NTA superflow matrix (Qiagen, equilibrated in Buffer A) was added to the concentrated sample and incubated for 1.5 h at 21 °C. The matrix with the bound sample was transferred to an empty chromatography column (25 x 125 mm), connected to a Äkta Explorer chromatography device and washed with buffer A (approx. 250 mL). For elution of the Fab two subsequent step gradients with 5% (30 mL)

and 10 % (35 mL) Buffer B, followed by a linear elution gradient up to 100% Buffer B were applied. The pooled elution fractions (72 mL) were subsequently treated as follows: concentrated with a centrifugation ultrafiltration device (cut-off 10 kDa, Amicon) to a final volume of 20 mL, application in three portions to a desalting column (GE HiPrep, 26/10) to adjusted the buffer to PBS pH 7.4, and further concentration in a centrifugation ultrafiltration device (Amicon) to a final volume of 40 mL. The concentrated sample was incubated with 5 mL anti kappa light chain antibody matrix (Kappa Select Affinity Media, BAC, equilibrated with PBS) for 1 h at room temperature under agitation. The Sepharose matrix with the bound sample was transferred to a chromatography column and treated with the following sequence of wash steps, 4-times with 15 mL PBS; twice with 5 mL wash buffer (100 mM Na-phosphate pH 6.0, 100 mL NaCl, 500 mM arginine). The elution step consisted of 3-times 5 mL application of buffer 100 mM glycine HCl pH 3.0. The eluate was immediately neutralized with 1 M Tris HCl pH 8.0 and precipitates formed were removed by centrifugation (10 min, 3.200 g). The sample was concentrated by ultrafiltration (Amicon) and applied to a Superdex-75 prep grade 16/60 column on an Äkta Explorer chromatography system with TBS buffer. The peak fractions were analysed by PAGE and the fractions representing a heavy and light chain of Fab in a 1.1 molar ratio were pooled and again concentrated by ultrafiltration (Amicon) to a final volume of 1 mL. About 4 mg Fab A were isolated from 10 L of E. coli culture supernatant.

[0091] Analytical size exclusion chromatography (Äkta Micro system, S75 5/150 column, 100 mM Tris HCl, ph 7.5) was used to demonstrate Fab A/TFPI KD2 complex formation. Therefore, Fab A, TFPI KD2 and the mixture of Fab A plus TFPI KD2 were separately analysed (Figure 1).

Example 3. Crystallization and X-ray Structure Determination of TFPI-Fab A Complex

Crystallization

[0092] Co-crystals of TFPI Kunitz domain 2 and the monoclonal antibody Fab A were grown at 20 °C using the sitting-drop method. The protein complex was concentrated to 9 mg/mL and crystallized by mixing equal volumes of protein solution and well solution (15% PEG8000, Tris HCl pH 7.5) as precipitant. Crystals appeared after one day.

Data Collection and Processing

[0093] Crystals were flash-frozen in liquid nitrogen in 30% glycerol in crystallization buffer for cryo-protection. Data was collected at beamline BL14.1, BESSY synchrotron (Berlin) on a MAR CCD detector. Data was indexed and integrated with XDS (W. Kabsch (2010) Acta Cryst. D66, 125-132) or IMOSFLM (The CCP4 Suite: Programs for Protein Crystallography (1994) Acta Cryst. D50, 760-763; A.G.W. Leslie, (1992), Joint CCP4 + ESF-EAMCB Newsletter on Protein Crystallography, No. 26), prepared for scaling with POINTLESS (P.R. Evans, (2005) Acta Cryst. D62, 72-82), and scaled with SCALA (P.R. Evans, (2005) Acta Cryst. D62, 72-82). The crystal diffracted up to 2.6 Å and possessed space group $P2_12_12_1$ with cell constants a=65.7, b=114.7, c=151.9; $\alpha=\beta=\gamma=90°$, and two TFPI-Fab complexes in the asymmetric unit.

Structure Determination and Refinement

[0094] TFPI Kunitz domain 2 and the monoclonal antibody Fab co-structure was solved by molecular replacement using PHASER (A.J. McCoy et al. (2007) J. Appl. Cryst. 40, 658-674) and published X-ray structures of TFPI Kunitz domain 2 (pdb code 1tfx) and a Fab fragment (pdb code 3mxw) as search models. Prior to molecular replacement, the Fab model sequence was modified with CHAINSAW (N. Stein, (2008) J. Appl. Cryst. 41, 641 - 643). Iterative rounds of model building with COOT (P. Emsley et al. (2010) Acta Cryst. D66:486-501) and maximum likelihood refinement using REFMAC5.5 (G.N. Murshudov et al. (1997) Acta Cryst. D53, 240-255) completed the model. Regions Phe A 31 - Asn A 35, Pro B 9, Lys M 139 - Ser M 142, and Asp140 - Phe154 of KD2 showed weak electron density and were not included in the model. Data set and refinement statistics are summarized in Table 1.

[0095] **Table 1.** Data set and refinement statistics for TFPI-Fab A complex.

Wavelength	0.9184 Å
Resolution (highest shell)	46-2.6 (2.7-2.6) Å
Reflections (observed/unique)	176619 / 36076
Completeness[a]	99.9% (100.0%)
I/σ[a]	9.8 (2.5)
R_{merge}[a,b]	0.115 (0.70)
Space group	$P2_12_12_1$
Unit cell parameters	
a	65.7 Å
b	114.7 Å
c	151.9 Å
R_{cryst}[c]	0.25
R_{free}[d]	0.32
Wilson temperature factor	23.87 Å²
RMSD bond length[e]	0.009 Å
RMSD bond angles	1.4°
Protein atoms	7580
Water molecules	108

[a] The values in parentheses are for the high resolution shell.

[b] $R_{merge} = \Sigma hkl\ |I_{hkl} - \langle I_{hkl}\rangle| / \Sigma hkl\ \langle I_{hkl}\rangle$ where I_{hkl} is the intensity of reflection hkl and $\langle I_{hkl}\rangle$ is the average intensity of multiple observations.

[c] $R_{cryst} = \Sigma\ |F_{obs} - F_{calc}| / \Sigma\ F_{obs}$ where F_{obs} and F_{calc} are the observed and calculated structure factor amplitues, respectively.

[d] 5% test set

[e] RMSD, root mean square deviation from the parameter set for ideal stereochemistry

Example 4. X-ray Structure-Based Epitope Mapping of a Fab A

[0096] The complex of TFPI-Fab A (Figure 2) crystallized as two copies of the complex per asymmetric unit. The main chains of the complexes superpose with an overall root mean square deviation (RMSD) of 0.7 Å with each Fab bound to the associated TFPI epitope. Residues of TFPI in contact with Fab A (the epitope) and the respective buried surface were analysed with the CCP4 program AREAIMOL (P.J. Briggs (2000) CCP4 Newsletter No. 38). Residues with minimum 5 Å² buried surface or more than 50% buried surface have been considered contacted (Table 2). Residues of Fab A in contact with TFPI (the paratope) and the respective buried surface were analysed with AREAIMOL. Residues with minimum 5 Å² buried surface or more than 50% buried surface have been considered contacted (Table 3).

[0097] **Table 2:** Residues of TFPI in contact with Fab A. Chains C and N correspond to the TFPI of respective complex in the asymmetric unit.

Residue Nr	buried surface in Å²	buried surface in %
Glu C 100	5.6	4.3
Glu C 101	41.0	41.6
Asp C 102	50.1	85.6
Pro C 103	43.9	71.6
Gly C 104	19.1	98.9
Ile C 105	125.9	100.0
Cys C 106	59.1	93.0
Arg C 107	138.6	53.4
Gly C 108	1.5	4.4
Gly C 128	7.7	57.8
Gly C129	23.2	44.1
Cys C 130	46.2	99.5
Leu C 131	111.5	92.8
Gly C 132	24.5	48.8
Asn C 133	5.5	17.4

Residue Nr	buried surface in Å	buried surface in %
Glu N 100	31.3	20.3
Glu N 101	24.5	23.7
Asp N 102	46.7	77.0
Pro N 103	62.9	90.3
Gly N 104	21.5	89.2
Ile N 105	111.7	97.5
Cys N 106	70.2	96.4
Arg N 107	134.3	53.4
Gly N 108	6.0	12.7
Tyr N 109	7.5	4.3
Lys N 126	11.3	7.8
Tyr N 127	0.9	8.7
Gly N 128	11.0	81.4
Gly N 129	28.3	56.8
Cys N 130	42.5	100.0
Leu N 131	125.6	84.9
Gly N 132	27.2	71.7
Asn N 133	34.1	8.2

[0098] **Table 3:** residues of Fab A in contact with TFPI. Chains A, B and chains L, M represent the Fab A light and heavy chains of the respective complex in the asymmetric unit.

Residue Nr	buried surface in Å²	buried surface in %
Tyr A 37	41.3	47.5
Tyr A 96	25.8	94.8
Asp A 97	9.5	16.2
Ser A 98	5.6	11.2
Tyr A 99	42.2	57.5
Leu A 101	6.7	51.9
Asn B 32	43.4	41.4
Ser B 33	11.7	27.8
Ala B 35	3.8	100.0
Ile B 52	4.9	100.0
Tyr B 54	44.5	98.0
Arg B 56	40.2	49.7
Ser B 57	2.9	3.3
Lys B 58	16.2	14.0
Tyr B 60	64.0	79.8
Asn B 61	0.8	0.9
Arg B 62	51.3	50.4
Trp B 102	42.6	98.3
Ser B 104	24.5	100.0
Asp B 105	25.9	36.7
Trp B 108	40.2	49.5

Residue Nr	buried surface in Å²	buried surface in %
Tyr L 37	42.1	59.1
Tyr L 96	25.3	96.1
Asp L 97	21.4	29.1
Ser L 98	2.7	6.7
Tyr L 99	48.4	68.0
Leu L 101	12.4	81.0
Asn M 32	34.5	39.0
Ser M 33	6.1	17.8
Ala M 35	7.2	90.0
Ile M 52	5.4	100.0
Tyr M 54	57.0	88.3
Arg M 56	115.2	72.4
Ser M 57	4.6	4.3
Lys M 58	27.3	20.0
Tyr M 60	67.0	72.9
Asn M 61	0.8	0.9
Arg M 62	59.2	53.0
Trp M 102	33.5	100.0
Ser M 104	28.0	80.9
Asp M 105	42.5	50.0
Lys M 106	3.3	2.5
Trp M 108	75.8	53.1

[0099] The non-linear epitope recognized by the Fab A is defined by regions Glu100 - Arg109 and Lys126, Gly128 - Asn133. The paratope in the Fab A includes light chain (lc) residues lc_Tyr37, lc_Tyr96, lc_Asp97, lc_Ser98, lc_Tyr99, and lc_Leu101 and heavy chain (hc) residues hc_Asn32, hc_Ser33, hc_Ala35, hc_Ile52, hc_Tyr54, hc_Arg56, hc_Lys58,

hc_Tyr60, hc_Arg62, hc_Trp102, hc_Ser104, hc_Asp105, and hc_Trp108. CDR-L3, CDR-H2, and CDR-H3 appear to be the major interaction sites, based on the number of contacts.

[00100] The epitope consists of two loops linked by a disulfide bridge between Cys106 and Cys130 (Figure 3). The disulfide bridge stacks against hc_Trp108 of CDR-H3, while the adjacent Ile105 and Leu131 are buried in a hydrophobic cleft created by hc_Ala35, hc_Ile52, hc_Tyr54 (CDR-H2), hc_Trp102 (CDR-H3), and lc_Tyr96, lc_Ser98, lc_Tyr99, lc_Leu101 (CDR-L3). Based on the number of contacts, Ile105 and Leu131 are key epitope residues in hydrophobic contact with CDR-L3, CDR-H2, and CDR-H3.

[00101] TFPI region Glu101 - Ile105 interacts with CDR-H2. The interface is strongly characterized by hc_Tyr54, hc_Tyr60, and hc_Arg62. Hc_Tyr54 shows polar interactions with the side chain of Asp102. Hc_Tyr60 shows polar interactions with the main chain carbonyl oxygen of Glu101 and hc_Arg62 with the side chain of Asp102 and the main chain carbonyl oxygen of Gly132.

[00102] Asp102 is a key epitope residue in polar interaction with CDR-H2 hc_Tyr54 and hc_Arg62. Replacement of *wild type* hc_Asp62 to arginine in Fab A results in an affinity increase of 120 fold. Based on the X-ray structure, this can be explained by the switch from repulsion between hc_Asp62 and Asp102 to polar interaction of hc_Arg62 and Asp102, and main chain carbonyl oxygens.

[00103] The guanidinium group of Arg107 interacts directly with the side chains of hc_Asn32 and hc_Asp105 of CDR-H1 and CDR-H3, respectively. Arg107 has been shown to be essential for inhibition of factor Xa (M.S. Bajaj et al. (2001) Thromb Haemost 86(4):959-72.). Fab A occupies this critical residue and competes with Arg107 function in inhibiting factor Xa.

Example 5. Paratope comparison of Fab A and its optimized variant Fab C

[00104] To assess consistency of TFPI epitope binding by the optimized variant of Fab A, Fab C, sequence alignments of the light and heavy chains (Figure 11A) and homology models of Fab C (Figure 11B) were analysed for conservation of Fab A paratope residues in Fab C. Homology models were calculated with DS MODELER (ACCELRYS, Inc; Fiser, A. and Sali A. (2003) Methods in Enzymology, 374:463-493) using our TFPI - Fab A X-ray structure as input template structure. The homology models show nearly identical backbone conformations in comparison to Fab A with RMSD < 0.5 Å. Of 20 paratope residues observed in TFPI-Fab A complex, hc_Asn32 is the only paratope residue that differs in Fab C

where an aspartate residue is at the respective position (Figure 11). Hc_Asn32 interacts with TFPI Arg107. Asp32 of FabC should interact more tightly with TFPI given its carboxylate group and prospective interaction with the guanidinium group of Arg107. Based on high sequence conservation between Fab A and Fab C paratope residues and the expected identical backbone conformation, Fab C likely recognizes the same TFPI epitope as Fab A.

Example 6. X-ray Structure-based Rationale for Inhibition of TFPI-Factor Xa Interaction

[00105] Fab A anticipates TFPI-factor Xa interaction and inhibition. Superposition of the TFPI-Fab A complex with the structure of TFPI-trypsin (M.J. Burgering et al (1997) J Mol Biol. 269(3):395-407) shows that the TFPI region containing the Fab A epitope is crucial for the interaction with trypsin, which is a surrogate for factor Xa. Based on the X-ray structure, binding of the Fab A to the observed epitope on Kunitz domain 2 should exclude binding of factor Xa by steric hindrance (Figure 4).

Example 7. Production of recombinant TFPI (Kunitz domain 1 + 2), expression in E. coli and its purification

Expression system

[00106] The destination vector (according to Gateway nomenclature), designated pD Eco5 N is based on ET-16 b (Novagen). The vector also encodes a His_{10} and NusA tag, as well as the Gateway cloning cassette for expression of the fusion protein consisting of His_{10}/NusA and the protein of interest.

[00107] A DNA construct encoding a TEV protease cleavage site fused to the N-terminus of the Kunitz domains 1 + 2 (Asp1 to Phe154, reference Uniprot 10646, mature TFPI alpha) and the Gateway attachment sites (attB1-5#, attB2-3#, Invitrogen) was cloned into the pD Eco5 N vector resulting in the expression vector designated as pD Eco5 N TFPI KD1+2. The expression strain used was BL21 DE3 (Novagen).

Amino acid sequence of expressed fusion protein using pD Eco5 N TFPI KD1+2, 600 AA

SEQUENCE 699 AA; 78579 MW; 4D2932FF7C1E3F7E CRC64;
MGHHHHHHHH HHSSGHIEGR HMNKEILAVV EAVSNEKALP REKIFEALES ALATATKKKY
EQEIDVRVQI DRKSGDFDTF RRWLVVDEVT QPTKEITLEA ARYEDESLNL GDYVEDQIES
VTFDRITTQT AKQVIVQKVR EAERAMVVDQ FREHEGEIIT GVVKKVNRDN ISLDLGNNAE
AVILREDMLP RENFRPGDRV RGVLYSVRPE ARGAQLFVTR SKPEMLIELF RIEVPEIGEE

VIEIKAAARD PGSRAKIAVK TNDKRIDPVG ACVGMRGARV QAVSTELGGE RIDIVLWDDN
PAQFVINAMA PADVASIVVD EDKHTMDIAV EAGNLAQAIG RNGQNVRLAS QLSGWELNVM
TVDDLQAKHQ AEAHAAIDTF TKYLDIDEDF ATVLVEEGFS TLEELAYVPM KELLEIEGLD
EPTVEALRER AKNALATIAQ AQEESLGDNK PADDLLNLEG VDRDLAFKLA ARGVCTLEDL
AEQGIDDLAD IEGLTDEKAG ALIMAARNIC WFGDEATSGS GLETSLYKKA GSDYDIPTTE
NLYFQDSEED EEHTIITDTE LPPLKLMHSF CAFKADDGPC KAIMKRFFFN IFTRQCEEFI
YGGCEGNQNR FESLEECKKM CTRDNANRII KTTLQQEKPD FCFLEEDPGI CRGYITRYFY
NQQTKQCERF KYGGCLGNMN NFETLEECKN ICEDGPNGF

Sequence components

His 10 tag: MGHHHHHHHH HH

NusA tag: SSGHIEGR HMNKEILAVV EAVSNEKALP REKIFEALES ALATATKKKY
EQEIDVRVQI DRKSGDFDTF RRWLVVDEVT QPTKEITLEA ARYEDESLNL GDYVEDQIES
VTFDRITTQT AKQVIVQKVR EAERAMVVDQ FREHEGEIIT GVVKKVNRDN ISLDLGNNAE
AVILREDMLP RENFRPGDRV RGVLYSVRPE ARGAQLFVTR SKPEMLIELF RIEVPEIGEE
VIEIKAAARD PGSRAKIAVK TNDKRIDPVG ACVGMRGARV QAVSTELGGE RIDIVLWDDN
PAQFVINAMA PADVASIVVD EDKHTMDIAV EAGNLAQAIG RNGQNVRLAS QLSGWELNVM
TVDDLQAKHQ AEAHAAIDTF TKYLDIDEDF ATVLVEEGFS TLEELAYVPM KELLEIEGLD
EPTVEALRER AKNALATIAQ AQEESLGDNK PADDLLNLEG VDRDLAFKLA ARGVCTLEDL
AEQGIDDLAD IEGLTDEKAG ALIMAARNIC WFGDE

Linker/translated endonulease restriction sites: TSGS GLE
Translated att-site: TSLYKKA GS

TEV site: DYDIPTTENLYFQ

TFPI Kunitz 1+2 DSEED EEHTIITDTE LPPLKLMHSF CAFKADDGPC KAIMKRFFFN IFTRQCEEFI
YGGCEGNQNR FESLEECKKM CTRDNANRII KTTLQQEKPD FCFLEEDPGI CRGYITRYFY
NQQTKQCERF KYGGCLGNMN NFETLEECKN ICEDGPNGF

Expression

[00108] A BL21 DE3 strain transformed with pD Eco5 N TFPI KD1+2 was grown as a pre-culture in 2x 100 mL of LB medium with 200 µg/mL ampicillin for 14 h at 37 °C with agitation of 180 rpm. A Bioreactor (Sartorius Stedim Biotech) with 10 L culture volume (LB medium, 200 µg/mL ampicillin) was inoculated with 200 mL pre-culture and incubated at 37 °C, with agitation of 150 rpm. At a culture density of OD600, IPTG (isopropyl *β*-D-thiogalactoside) was added to a final concentration of 100 mM for gene induction and further cultivated at 17 °C for 24 h with a pO2 minimum level of 50% and an agitation rate of 180 – 800 rpm. The E. coli was pelleted by centrifugation (3000 g, 10 min) and stored at -80 °C.

Purification

[00109] The pelleted E. coli mass from 10 L culture was re-suspended in 500 mL lysis buffer [50 mM Tris HCl pH 8.0, 300 mM NaCl, 10% (w/w) glycerol, 40 mM imidazol, protease inhibitor cocktail Complete EDTA-free (Roche)], homogenized in a high pressure

device (Microfluidics) and afterwards the lysate was centrifuged (100.000 g, 60 min, 4 °C). Several purification steps were performed using an Äkta purification system. The centrifuged soluble lysate fraction was applied in an initial IMAC chromatography step to a column containing 50 mL of Ni-Sepharose HP matrix (GE). Equilibration, fusion protein binding and wash of the Hi-Trap-Sepharose HP matrix was done using Buffer A (50 mM Tris HCl pH 8.0, 300 mM NaCl, 40 mM imidazol). For elution of the NusA-TFPI fusion protein, a linear gradient of imidazol from 40 to 500 mM in Buffer B (50 mM Tris HCl pH 8.0, 150 mM NaCl) was used. The elution fractions were pooled (total volume 140 mL) and applied in fractions to a desalting column Hi Prep 26/10 (GE) (two linked column units) for exchange to a buffer with 50 mM Tris HCl pH 8.0, 150 mM NaCl, 5 mM $CaCl_2$). For removal of the Nus A tag, a proteolytic digest with His_6-tagged TEV, at an enzyme to fusion protein ratio of 1:66 w/w, was performed for 16 h at 4 °C. The sample was again applied to column containing 50 mL of Ni-Sepharose HP matrix (GE) to separate the free TFPI from uncleaved fusion protein and His-TEV. The eluate of the IMAC step was then applied to size exclusion chromatography, size exclusion chromatography (SEC, column S100, GE) to isolate a monomeric TFPI fraction which was concentrated by ultrafiltration (Amicon, unit with 3 kDa-cut off range) to about 1.5 mg/mL. The purified final TFPI Kunitz domain 1 + 2 sample ran as a double band in PAGE with an apparent molecular weight of about 18 kDa. Further analysis (SEC, western blot) revealed that only protein corresponding to the upper band was immunoreactive with the Fab B.

Example 8. Proteolytic processing and purification of Fab B from human IgG1.

Expression

[00110] The Fab B was proteolytically processed from its human IgG1 form. Fab B_IgG1 was expressed in mammalian cells (HEK 293) as a secretion protein. For IgG1 isolation, 1.6 L culture supernatant was applied to two linked columns of HiTrap MabSelectSuRE (from GE, 5 mL bed volume, flow rate 1.5 mL/min, 4 °C, for 16 h). For column wash and equilibration, a buffer consisting of PBS and 500 mM NaCl was used. Bound IgG1 was eluted (50 mM Na-acetate, 500 mM NaCl pH 3.5 followed by the same buffer with pH 3.0), neutralized (2.5 M Tris > 11) and concentrated by ultrafiltration to about 13 mg/mL.

[00111] Immobilized papain (Pierce, 20 mL slurry) was used for digest of about 270 mg (in 12.5 mL) of IgG1 using 22 fractions in 1.5 mL Eppendorf reaction tubes (incubation 16 h, 37 °C, agitation 1400 rpm). After processing the samples were centrifuged, the supernatant

was collected, the pellet washed with PBS and both supernatants and cleared wash were pooled.

[00112] The digested sample was again applied to two linked HiTrap MabSelectSuRe columns (2 x 5 mL) enabling a separation of Fc and Fab material. The pooled isolated Fab B fractions were concentrated by ultra filtration to about 8 mg/mL (total yield 120 mg). Additionally, size exclusion chromatography with Superdex75 (column 26/60, flowrate 2.5 mL/min with PBS) was used for further purification. After further concentration and sterile filtration the final yield of the Fab B was 115 mg at a concentration of 8.5 mg/mL.

[00113] Analytical size exclusion chromatography (Äkta Micro system, S75 5/150 column, 100 mM Tris HCl, pH 7.5) was used to demonstrate Fab B/TFPI KD1+2 complex formation (Figure 5). For Fab B an unexpectedly long retention time on the SEC column was observed corresponding to an apparent molecular weight of 20 kDa, which is very similar to the molecular weight detected for TFPI KD1+2.

Example 9. Production of the complex TFPI Kunitz domain 1 + 2 with Fab B

[00114] In order to form immune complex, TFPI Kunitz domain 1 + 2 and Fab B were combined at a ratio of approximately 1:1.5 (w/w). Therefore, 3.85 mg of the concentrated, monomeric TFPI Kunitz domain 1 + 2 protein (from S100 pooled fractions) was mixed with 7.4 mg Fab B (from SEC Superdex75) and incubated for 16 h at 21 °C. Complex formation was demonstrated via analytical SEC (S200/150) and Western blot. The complex was further purified by SEC (S200 26/26) in 10 mM Tris HCl pH 7.4 with 150 mM NaCl, concentrated by ultrafiltration (Amicon, unit with 5 kDa-cut off range) to 10.3 mg/mL, which was used for crystallization.

Example 10. Crystallization and X-ray Structure Determination of TFPI-Fab B complex

Crystallization

[00115] Co-crystals of a protein construct comprising TFPI - Kunitz domain 1 (KD1) and Kunitz domain 2 (KD2) and the monoclonal TFPI antibody Fab B were grown at 4 °C using the sitting-drop method. The protein complex was concentrated to 10 mg/mL and crystallized by mixing equal volumes of protein solution and well solution (20% PEG8000) as precipitant. Crystals appeared after three days.

Data Collection and Processing

[00116] Crystals were flash-frozen in liquid nitrogen in 30% glycerol in crystallization buffer for cryo-protection. Data of one crystal was collected at beamline BL14.1, BESSY synchrotron (Berlin) on a MAR CCD detector. Data was indexed and integrated with IMOSFLM (A.G.W. Leslie, (1992), Joint CCP4 + ESF-EAMCB Newsletter on Protein Crystallography, No. 26), prepared for scaling with POINTLESS (P.R. Evans, (2005) Acta Cryst. D62, 72-82), and scaled with SCALA (P.R. Evans, (2005) Acta Cryst. D62, 72-82). The crystal diffracted up to 2.3 Å and possesses space group $P2_1$ with cell constants a=80.3, b=71.9, c=108.8; β=92.5° and two TFPI-KD1, -KD2 - Fab complexes in the asymmetric unit.

Structure Determination and Refinement

[00117] The co-structure of TFPI-KD1, -KD2 and the monoclonal antibody Fab was solved by molecular replacement using PHASER (A.J. McCoy et al (2007) J. Appl. Cryst. 40, 658-674), MOLREP (A.Vagin and A.Teplyakov (1997) J. Appl. Cryst. 30, 1022-10) and in house and published X-ray structures of TFPI-KD2 (pdb code 1tfx) and a Fab fragment (pdb code 1w72) as search models. Prior to molecular replacement Fab and KD1 models were processed with CHAINSAW (N. Stein, (2008) J. Appl. Cryst. 41, 641 - 643). Iterative rounds of model building with COOT (P. Emsley et al. (2010) Acta Cryst. D66:486-501) and maximum likelihood refinement using REFMAC5.5 (G.N. Murshudov et al. (1997) Acta Cryst. D53, 240-255) completed the model. Region hc_Ser131 - hc_Ser136 of both Fabs, TFPI residues Asp1 - Leu21, Asp149 - Phe154, and the KD1 - KD2 linker residues Arg78 - Glu92 showed weak electron density and were not included in the model. Data set and refinement statistics are summarized in Table 4.

[00118] **Table 4.** Data set and refinement statistics for TFPI-Fab B complex.

Wavelength	0.9184 Å
Resolution (highest shell)	47-2.3 (2.4-2.3) Å
Reflections (observed/unique)	165457 (56223)
Completeness[a]	97.8% (97.8%)
I/σ[a]	5.8 (2.0)
R_{merge}[a,b]	0.13 (0.52)
Space group	$P2_1$
Unit cell parameters	
a	80.3 Å
b	71.9 Å
c	108.8 Å
β	92.5°
R_{cryst}[c]	0.20
R_{free}[d]	0.27
Wilson temperature factor	16.7 Å²
RMSD bond length[e]	0.008 Å
RMSD bond angles	1.3°
Protein atoms	8205
Water molecules	599

[a] The values in parentheses are for the high resolution shell.

[b] $R_{merge} = \Sigma hkl\ |I_{hkl} - \langle I_{hkl}\rangle| / \Sigma hkl\ \langle I_{hkl}\rangle$ where I_{hkl} is the intensity of reflection hkl and $\langle I_{hkl}\rangle$ is the average intensity of multiple observations.

[c] $R_{cryst} = \Sigma\ |F_{obs} - F_{calc}| / \Sigma\ F_{obs}$ where F_{obs} and F_{calc} are the observed and calculated structure factor amplitues, respectively.

[d] 5% test set

[e] RMSD, root mean square deviation from the parameter set for ideal stereochemistry

Example 11. X-ray Structure-Based Epitope Mapping

[00119] The complex of TFPI-KD1, -KD2, and Fab B (Figure 6) crystallized as two copies of the complex per asymmetric unit. The main chains of the complexes superpose with an overall RMSD of 1.0 Å with each Fab B bound to epitope of the associated TFPI-KD1 and -KD2. Both Kunitz domains interact directly or through water-mediated interactions with Fab B. KD1 and KD2 also interact with each other. Residues of TFPI in contact with Fab B (the epitope) and respective buried surface were analysed with the CCP4 program AREAIMOL (P.J. Briggs (2000) CCP4 Newsletter No. 38). Residues with minimum 5 Å² buried surface or more than 50% buried surface have been considered contacted (Table 5). Residues of Fab B in contact with TFPI (the paratope) and respective buried surface were analysed with AREAIMOL. Residues with minimum of 5 Å² buried surface or more than 50% buried surface have been considered contacted (Table 6).

[00120] **Table 5:** Residues of TFPI in contact with Fab B. Chains C, D and chains N, O correspond to the TFPI Kunitz domains 1 and Kunitz domain 2 of the respective complex in the asymmetric unit.

Residue Nr	buried surface in Å²	buried surface in %
Phe C 28	3.4	4.1
Asp C 31	26.2	47.3
Asp C 32	6.8	73.9
Gly C 33	7.6	100.0
Pro C 34	87.2	97.2
Cys C 35	45.7	93.4
Lys C 36	139.6	72.4
Ala C 37	0.9	2.2
Ile C 38	3.0	2.1
Cys C 59	11.3	34.7
Glu C 60	83.9	60.4
Gly C 61	0.5	1.4
Asn C 62	4.1	11.2

Residue Nr	buried surface in Å²	buried surface in %
Glu D 100	83.2	53.9
Glu D 101	80.8	93.4
Pro D 103	57.4	85.7
Gly D 104	16.6	68.3
Ile D 105	11.4	61.2
Cys D 106	12.5	32.2
Arg D 107	116.8	73.2
Gly D 108	18.6	49.4
Tyr D 109	133.5	74.6
Phe D 114	8.5	72.0
Asn D 116	8.3	24.5
Glu D 123	45.5	56.5
Arg D 124	9.9	6.0
Phe D 125	0.1	0.9
Lys D 126	29.2	39.0
Tyr D 127	1.3	7.1
Gly D 128	6.5	67.0

Residue Nr	buried surface in Å²	buried surface in %
Lys N 29	1.1	1.2
Asp N 31	28.9	49.4
Asp N 32	10.2	91.8
Gly N 33	12.5	100.0
Pro N 34	87.9	97.2
Cys N 35	42.1	86.0
Lys N 36	142.5	74.2
Ile N 38	4.3	3.2
Cys N 59	11.9	38.5
Glu N 60	71.7	53.0
Gly N 61	0.4	1.0
Asn N 62	7.0	20.2

Residue Nr	buried surface in Å²	buried surface in %
Glu O 100	65.1	44.3
Glu O 101	84.8	97.0
Pro O 103	60.2	84.0
Gly O 104	13.6	64.7
Ile O 105	11.6	67.4
Cys O 106	12.7	37.0
Arg O 107	101.3	69.1
Gly O 108	19.7	52.5
Tyr O 109	139.6	76.4
Thr O 111	0.1	0.1
Phe O 114	11.5	78.2
Asn O 116	13.4	34.6
Glu O 123	24.1	35.9
Arg O 124	11.1	6.7
Phe O 125	0.1	1.2
Lys O 126	35.0	52.6
Tyr O 127	1.2	8.1
Gly O 128	6.4	58.1

[00121] **Table 6:** Residues of Fab B in contact with TFPI. Chains A, B and chains L, M represent the Fab B light and heavy chains of the respective complex in the asymmetric unit.

Residue Nr	buried surface in Å²	buried surface in %
Leu A 27	1.8	39.1
Arg A 28	20.7	13.5
Asn A 29	44.6	38.6
Tyr A 30	56.0	57.7
Tyr A 31	96.9	76.7
Tyr A 48	43.0	75.8
Tyr A 49	39.2	90.7
Asp A 50	16.5	56.7
Asn A 52	10.3	16.8
Pro A 54	10.4	34.2
Ser A 55	5.0	3.9
Asn A 65	6.3	13.4
Trp A 90	19.2	45.9
Asp A 92	13.6	10.2
Gly A 93	8.7	37.8
Gln B 1	12.6	6.8
Gly B 26	29.6	58.6
Phe B 27	21.1	57.9
Thr B 28	56.9	65.0
Arg B 30	32.0	19.2
Ser B 31	52.2	65.9
Tyr B 32	54.0	94.9
Arg B 52	8.0	6.2
Arg B 98	7.0	49.2
Tyr B 100	92.2	98.6
Arg B 101	106.6	71.6
Tyr B 102	80.2	79.4
Trp B 103	21.7	87.7
Asp B 105	15.3	42.6
Tyr B 106	13.7	15.2

Residue Nr	buried surface in Å²	buried surface in %
Leu L 27	4.2	61.7
Arg L 28	22.2	15.5
Asn L 29	43.0	33.6
Tyr L 30	58.2	67.3
Tyr L 31	103.4	80.3
Tyr L 48	48.7	83.8
Tyr L 49	37.5	88.8
Asp L 50	15.5	59.1
Asn L 52	8.6	14.8
Pro L 54	12.9	45.4
Ser L 55	9.8	8.0
Asn L 65	3.9	7.7
Gly L 67	0.1	0.3
Trp L 90	20.3	48.3
Asp L 92	1.9	1.5
Gly L 93	18.4	49.4
Val M 2	1.6	4.3
Gly M 26	34.2	62.2
Phe M 27	18.2	62.1
Thr M 28	64.4	69.7
Arg M 30	27.1	18.8
Ser M 31	51.5	63.7
Tyr M 32	55.3	95.3
Arg M 52	7.4	6.2
Arg M 98	8.5	57.0
Tyr M 100	86.9	98.3
Arg M 101	110.8	74.4
Tyr M 102	82.8	81.0
Trp M 103	18.5	91.1
Asp M 105	17.3	48.7
Tyr M 106	13.5	15.0

[00122] The Fab B recognized a non-linear epitope of KD1 and KD2 which is defined by residues Asp31 - Lys36, Cys59 (which forms a disulfide bridge with Cys35), Glu60, and Asn62 of TFPI-KD1 and Glu100, Glu101, region Pro103 - Cys106 (which forms a disulfide bridge with Cys130), residues Arg107- Tyr109, Phe114, Asn116, Glu123, Arg124, and residues Lys126 - Gly128 of TFPI-KD2. The paratope in Fab B which interacts with TFPI-KD1 includes lc_Leu27 - lc_Tyr31, lc_Asp50, lc_Asn65, lc_Trp90, lc_Asp92, lc_Gly93, and hc_Arg101 and hc_Tyr102. The paratope in Fab B which interacts with TFPI-KD2 includes lc_Tyr31, lc_Tyr48 and lc_Tyr49, and hc_Thr28, hc_Arg30 - hc_Tyr32, hc_Tyr100, hc_Arg101, hc_Trp103, and hc_Asp105. The light chain CDRs appear to be the major interaction sites for TFPI-KD1, the heavy chain CDRs appear to be the major interaction sites for TFPI-KD2, based on the number of contacts.

[00123] The non-linear epitope on TFPI-KD1 consists of two loop regions linked by a disulfide bridge between Cys35 and Cys59. The epitope is characterized by a central

hydrophobic interaction of Pro34 surrounded by a triangle of polar interactions of Asp31, Asp32, Glu60, and Lys36 with Fab B (Figure 7).

[00124] Pro34 lies in a hydrophobic cleft created by lc_Tyr30 and lc_Tyr31 of CDR-L1, lc_Trp90 of CDR-L3 and hc_Tyr102 of CDR-H3. Asp31 and Asp32 possess polar interaction with CDR-H3 and a hydrogen bond network with hc_Arg101, hc_Tyr102, and a water molecule. Hc_Tyr102 side chain is well oriented to possess hydrophobic interaction with Pro34, polar interaction with Asn31, and aromatic π-π interaction with lc_Trp90 of CDR-L3.

[00125] Interaction of Asp31 and Asp32 with CDR-H3 is a key epitope feature and orientations and interactions of hc_Tyr102 and hc_Arg101 appear crucial. Mutation of *wild type* residue hc_Lys99 to leucine resulted in 20 fold affinity increase. Hc_Leu99 is located in the hydrophobic interface between light and heavy chain and followed by the CDR-H3 loop. A polar and flexible lysine side chain is a disadvantage at this position and interferes with optimal CDR-H3 conformation and antigen interactions.

[00126] Glu60, which forms the second corner of the polar triangle, interacts with the side chains of lc_Tyr30 (CDR-L1), lc_Trp90 and main chain nitrogen of lc_Gly93 (CDR-L3).

[00127] The third corner of the triangle is formed by Lys36. Lys36 is an essential residue for inhibition of the factor VIIa/tissue factor complex by TFPI (M.S. Bajaj et al. (2001) Thromb Haemost 86(4):959-72.). In complex with Fab B, Lys36 is significantly contacted and buried by CDR-L1 lc_Leu27, lc_Arg28, lc_Asn29, lc_Tyr31, CDR-L2 lc_Asp50, and a water molecule. Interaction of Lys36 with factor VIIa/tissue factor complex while bound to Fab B and its inhibition appear excluded.

[00128] The non-linear epitope on TFPI-KD2 consists of three sections comprising residues Glu100, Glu101, Pro103 - Tyr109, Phe114; Asn116 and Glu123; Arg124, Lys126 - Gly128. The KD2-epitope forms polar and hydrophobic interactions with Fab B CDR-L1, -L2, -H1, and -H3 (Figure 8).

[00129] Glu100, Glu101, Arg107, and Tyr109 are key epitope residues providing strong polar or hydrophobic anchor points in contact with three separated surface regions of Fab B created by CDR-H1 (interaction with Glu100 and Glu101) CDR-L1, -L2, -H3 (interaction with Arg107), and CDR-L2, -H3 (interaction with Tyr109).

[00130] Arg107 is significantly contacted by lc_Tyr31, lc_Tyr49, hc_Arg101, and hc_Tyr102 of CDR-L1, -L2, -H3, respectively, and additionally interacts with Gly33 and Cys35 of KD1. Arg107 has been shown to be essential for inhibition of factor Xa (M.S. Bajaj

et al. (2001) Thromb Haemost 86(4):959-72.). Fab B occupies this critical residue and excludes Arg107 function in inhibiting factor Xa.

[00131] Glu100 and Glu101 form hydrogen bonds with CDR-H1 residues hc_Arg30, hc_Ser31, and hc_Thr28 and hc_Tyr32.

[00132] Tyr109 lies in a hydrophobic niche created by CDR-L2 lc_Tyr48, and CDR-H3 residues hc_Tyr100, and hc_Trp103, and forms a hydrogen bond with hc_Asp105.

Example 12. Paratope comparison of Fab B and its optimized variant Fab D

[00133] To assess consistency of TFPI epitope binding by the optimized variant of Fab B, Fab D, sequence alignments of the light and heavy chains (Figure 12A) and homology models of Fab D (Figure 12B) were analysed for conservation of Fab B paratope residues in Fab D. Homology models were calculated with DS MODELER (ACCELRYS, Inc; Fiser, A. and Sali A. (2003) Methods in Enzymology, 374:463-493) using our TFPI - Fab B X-ray structure as input template structure. The homology models show nearly identical backbone conformations in comparison to Fab B with RMSD < 0.5 Å. Of 29 paratope residues observed in TFPI-Fab B complex, seven residues (five light chain residues, two heavy chain residues) differ in Fab D (Figure 12). Lc_Arg28; lc_Asn29, lc_Asp92, and lc_Gly93 show main chain interactions with the TFPI epitope residues. The exchanges of these residues in Fab D are not expected to induce binding to a different TFPI epitope. The replacement of lc_Tyr48 and hc_Gln1 by a phenylalanine and glutamate in Fab D are negligible as no polar side chain interactions are observed in the X-ray structure. Hc_Arg30 shows a polar interaction with Glu100 of TFPI and is exchanged to a serine in Fab D. At this position, an arginine should be favorable over a serine to interact with TFPI. Based on expected impact of the analysed exchanges between Fab B and Fab D paratope residues, overall sequence conservation and low RMSD of Fab D homology model, Fab D is contemplated to recognize the same TFPI epitope as Fab B.

Example 13 X-ray Structure-based Rationale for Inhibition of TFPI Interaction with-Factor Xa and Factor VIIa/Tissue Factor complex

[00134] Fab B binds to both KD1 and KD2 of TFPI. KD2 binds and inhibits factor Xa. KD1 binds and inhibits factor VIIa/tissue factor complex. The X-ray structures of KD2 in complex with trypsin (M.J. Burgering et al (1997) J Mol Biol. 269(3):395-407) and BPTI in

complex with an extracellular portion of TF and factor VIIa (E. Zhang et al (1999) J Mol Biol 285(5):2089-104.) have been reported. Trypsin is a surrogate for factor Xa, BPTI is a homolog of TFPI-KD1. Superposition of the TFPI-Fab B complex with either KD2-trypsin or BPTI-factor VIIa/tissue factor reveals that antibody binding excludes binding of KD1 and KD2 to their natural ligands factor VIIa/tissue factor and factor Xa, respectively (Figure 9, Figure 10).

Example 14. Fab C and Fab D blocked TFPI binding with FXa and FVII/TF

[00135] To confirm that Fab C and Fab D can block FVIIa/TF- complex or FXa- binding on TFPI, we conducted a surface plasmon resonance (Biacore) study. A CM5 chip was immobilized with 170 RU of human TFPI using amine coupling kit (GE Healthcare). A volume of 60 µL of Fab C, Fab D or a negative control Fab was injected before 60 µL of 5 µg/mL FVIIa/TF complex or FXa was injected on the chip. After the injection of coagulation factors, 30 to 45 µL of 10 mM glycine at pH 1.5 was injected to regenerate the chip. The relative unit (RU) of coagulation factors generated after negative control Fab was designated as 100%, and the RU of coagulation factors generated after Fab C or Fab D injected was calculated. As shown in Figure 13A, at 0.3 µg/mL and 1 µg/mL concentration, Fab C binding on TFPI caused significant reduction of FXa binding to 42.6% and 5.2%, respectively. Similarly Fab D at concentration of 0.3 and 1 µg/mL reduced FXa binding to 20.8% and 7.6% respectively. The results of FVIIa/TF binding were shown in Figure 13B. At 0.3 and 1 µg/mL concentration, Fab C reduced FVIIa/TF binding to 25.1% and 10.0% respectively, whereas Fab D completely blocked FVIIa/TF binding, likely due to the direct binding of Fab D to KD1 of TFPI.

[00136] While the present invention has been described with reference to the specific embodiments and examples, it should be understood that various modifications and changes may be made and equivalents may be substituted without departing from the true spirit and scope of the invention. The specification and examples are, accordingly, to be regarded in an illustrative rather then a restrictive sense. Furthermore, all articles, books, patent applications and patents referred to herein are incorporated herein by reference in their entireties.

We claim:

1. An isolated monoclonal antibody that binds to an epitope of human tissue factor pathway inhibitor (SEQ ID NO:1), wherein said epitope comprises one or more residues selected from the group consisting of Glu100, Glu101, Asp102, Pro103, Gly104, Ile105, Cys106, Arg107, Gly108, Tyr109, Lys126, Gly128, Gly129, Cys130, Leu131, Gly132, and Asn133 of SEQ ID NO:1.

2. The isolated monoclonal antibody of claim 1, wherein said epitope comprises residue Ile105 of SEQ ID NO:1.

3. The isolated monoclonal antibody of claim 1, wherein said epitope comprises residue Ile105 and Asp102 of SEQ ID NO:1.

4. The isolated monoclonal antibody of claim 1, wherein said epitope comprises residue Ile105, Asp102 and Leu131 of SEQ ID NO:1.

5. The isolated monoclonal antibody of claims 2-4, wherein the epitope further comprises one or more residues selected from the group consisting of Glu100, Glu101, Asp102, Pro103, Gly104, Cys106, Arg107, Gly108, Tyr109, Lys126, Gly128, Gly129, Cys130, Leu131, Gly132, and Asn133 of SEQ ID NO:1.

6. An isolated monoclonal antibody that binds to an epitope of human tissue factor pathway inhibitor (SEQ ID NO:1), wherein said epitope comprises two amino acid loops linked by a disulfide bridge between residues Cys106 and Cys130 of SEQ ID NO:1.

7. The isolated monoclonal antibody of claim 6, wherein said epitope further comprises one or more residues selected from the group consisting of Glu100, Glu101, Asp102, Pro103, Gly104, Ile105, Cys106, Arg107, Gly108, Tyr109, Lys126, Gly128, Gly129, Cys130, Leu131, Gly132, and Asn133 of SEQ ID NO:1.

8. The isolated monoclonal antibody of claim 7, wherein said epitope comprises residue Ile105 of SEQ ID NO:1.

9. The isolated monoclonal antibody of claims 7, wherein the epitope comprises residue Asp102 of SEQ ID NO:1.

10. The isolated monoclonal antibody of claim 7, wherein the epitope comprises residue Leu131 of SEQ ID NO:1.

11. The isolated monoclonal antibody of claim 8-10, wherein the epitope further comprises one or more residues selected from the group consisting of Glu100, Glu101, Asp102,

Pro103, Gly104, Ile105, Cys106, Arg107, Gly108, Tyr109, Lys126, Gly128, Gly129, Cys130, Leu131, Gly132, and Asn133 of SEQ ID NO:1.

12. An isolated monoclonal antibody that binds to an epitope of human tissue factor pathway inhibitor (SEQ ID NO:1), wherein said epitope comprises one or more residues of Kunitz domain 1 and one or more residues of Kunitz domain 2.

13. The isolated monoclonal antibody of claim 12, wherein the residue of Kunitz domain 1 comprises one or more residues selected from the group consisting of Asp31, Asp32, Gly33, Pro34, Cys35, Lys36, Cys59, Glu60 and Asn62.

14. The isolated monoclonal antibody of claim 13, wherein the residue of Kunitz domain 1 comprises residue Pro34.

15. The isolated monoclonal antibody of claim 13, wherein the residues of Kunitz domain 1 comprise residues Pro34 and Glu60.

16. The isolated monoclonal antibody of claim 13, wherein the residues of Kunitz domain 1 comprise residues Pro34, Lys36 and Glu60.

17. The isolated monoclonal antibody of any of claims 14-16, wherein the residues of Kunitz domain 1 comprise one or more residues selected from the group consisting of Asp31, Asp32, Gly33, Cys35, Lys36, Cys59, Glu60 and Asn62.

18. The isolated monoclonal antibody of claim 12, wherein the residue of Kunitz domain 2 comprises one or more residues selected from the group consisting of Glu100, Glu101, Pro103, Gly104, Ile105, Cys106, Arg107, Gly108, Tyr109, Phe114, Asn116, Glu123, Arg124, Lys126, Tyr127 and Gly128.

19. The isolated monoclonal antibody of claim 18, wherein the residue of Kunitz domain 2 comprises residue Arg107.

20. The isolated monoclonal antibody of claim 18, wherein the residue of Kunitz domain 2 comprises residue Glu101.

21. The isolated monoclonal antibody of claim 18, wherein the residue of Kunitz domain 2 comprises residue Tyr109.

22. The isolated monoclonal antibody of any of clams 19-21, wherein the residues of Kunitz domain 2 further comprise one or more residues selected from the group consisting of Glu100, Glu101, Pro103, Gly104, Ile105, Cys106, Arg107, Gly108, Tyr109, Phe114, Asn116, Glu123, Arg124, Lys126, Tyr127 and Gly128

23. The isolated monoclonal antibody of claim 12, wherein the residue of Kunitz domain 1 comprises one or more residues selected from the group consisting of Asp31, Asp32, Gly33, Pro34, Cys35, Lys36, Cys59, Glu60 and Asn62 and wherein the residue of Kunitz domain 2 comprises one or more residues selected from the group consisting of Glu100, Glu101, Pro103, Gly104, Ile105, Cys106, Arg107, Gly108, Tyr109, Phe114, Asn116, Glu123, Arg124, Lys126, Tyr127 and Gly128.

24. An isolated monoclonal antibody that binds to an epitope of human tissue factor pathway inhibitor (SEQ ID NO:1), wherein said epitope comprises two amino acid loops linked by a disulfide bridge between residues Cys35 and Cys59 of SEQ ID NO:1.

25. The isolated monoclonal antibody of claim 24, wherein said epitope further comprises one or more residues of Kunitz domain 1 and one or more residues of Kunitz domain 2.

26. The isolated monoclonal antibody of claim 25, wherein the residue of Kunitz domain 1 comprises one or more residues selected from the group consisting of Asp31, Asp32, Gly33, Pro34, Cys35, Lys36, Cys59, Glu60 and Asn62.

27. The isolated monoclonal antibody of claim 25, wherein the residue of Kunitz domain 2 comprises one or more residues selected from the group consisting of Glu100, Glu101, Pro103, Gly104, Ile105, Cys106, Arg107, Gly108, Tyr109, Phe114, Asn116, Glu123, Arg124, Lys126, Tyr127 and Gly128.

28. The isolated monoclonal antibody of claim 22, wherein the residue of Kunitz domain 1 comprises one or more residues selected from the group consisting of Asp31, Asp32, Gly33, Pro34, Cys35, Lys36, Cys59, Glu60 and Asn62 and wherein the residue of Kunitz domain 2 comprises one or more residues selected from the group consisting of Glu100, Glu101, Pro103, Gly104, Ile105, Cys106, Arg107, Gly108, Tyr109, Phe114, Asn116, Glu123, Arg124, Lys126, Tyr127 and Gly128.

29. An isolated monoclonal antibody that binds to TFPI, wherein the isolated monoclonal antibody is competitive with any of the isolated monoclonal antibodies of claims 1 to 28 for binding to TFPI.

30. An isolated bispecific antibody that binds to TFPI, wherein the bispecific antibody is competitive with any of the isolated monoclonal antibodies of claims 1 to 28 for binding to TFPI.

31. A pharmaceutical composition comprising a therapeutically effective amount of the monoclonal antibody of any of claims 1-30 and a pharmaceutically acceptable carrier.

32. A pharmaceutical composition comprising a therapeutically effective amount of at least two monoclonal antibodies of any of claims 1-30 and a pharmaceutically acceptable carrier.

33. The pharmaceutical composition of claim 32, wherein a first monoclonal antibody comprises the isolated monoclonal antibody of claim 1, and a second monoclonal antibody comprises the isolated monoclonal antibody of claim 12.

34. A method for treating genetic or acquired deficiencies or defects in coagulation comprising administering a therapeutically effective amount of the pharmaceutical composition of any of claims 30-32 to a patient.

35. The method of claim 34 wherein the method treats hemophilia A, B or C.

36. A method for shortening bleeding time comprising administering a therapeutically effective amount of the pharmaceutical composition of any of claims 31-33 to a patient.

37. An isolated nucleic acid molecule encoding an antibody that binds to an epitope of human tissue factor pathway inhibitor (SEQ ID NO: 1) encoding said isolated monoclonal antibody of any of claims 1-29.

SEQUENCE LISTING

<110> Bayer HealthCare LLC
Wang, Zhuozhi
Murphy, John
Marquardt, Tobias
Moosmayer, Dieter

<120> Monoclonal Antibodies against Tissue Factor Pathway Inhibitor (TFPI)

<130> BHC 11 5 001

<160> 9

<170> PatentIn version 3.5

<210> 1
<211> 276
<212> PRT
<213> Homo Sapiens

<400> 1

Asp Ser Glu Glu Asp Glu Glu His Thr Ile Ile Thr Asp Thr Glu Leu
1 5 10 15

Pro Pro Leu Lys Leu Met His Ser Phe Cys Ala Phe Lys Ala Asp Asp
20 25 30

Gly Pro Cys Lys Ala Ile Met Lys Arg Phe Phe Phe Asn Ile Phe Thr
35 40 45

Arg Gln Cys Glu Glu Phe Ile Tyr Gly Gly Cys Glu Gly Asn Gln Asn
50 55 60

Arg Phe Glu Ser Leu Glu Glu Cys Lys Lys Met Cys Thr Arg Asp Asn
65 70 75 80

Ala Asn Arg Ile Ile Lys Thr Thr Leu Gln Gln Glu Lys Pro Asp Phe
85 90 95

Cys Phe Leu Glu Glu Asp Pro Gly Ile Cys Arg Gly Tyr Ile Thr Arg
100 105 110

Tyr Phe Tyr Asn Asn Gln Thr Lys Gln Cys Glu Arg Phe Lys Tyr Gly
115 120 125

Gly Cys Leu Gly Asn Met Asn Asn Phe Glu Thr Leu Glu Glu Cys Lys
130 135 140

Asn Ile Cys Glu Asp Gly Pro Asn Gly Phe Gln Val Asp Asn Tyr Gly
145 150 155 160

Thr Gln Leu Asn Ala Val Asn Asn Ser Leu Thr Pro Gln Ser Thr Lys
165 170 175

Val Pro Ser Leu Phe Glu Phe His Gly Pro Ser Trp Cys Leu Thr Pro

```
            180                 185                 190

Ala Asp Arg Gly Leu Cys Arg Ala Asn Glu Asn Arg Phe Tyr Tyr Asn
        195                 200                 205

Ser Val Ile Gly Lys Cys Arg Pro Phe Lys Tyr Ser Gly Cys Gly Gly
    210                 215                 220

Asn Glu Asn Asn Phe Thr Ser Lys Gln Glu Cys Leu Arg Ala Cys Lys
225                 230                 235                 240

Lys Gly Phe Ile Gln Arg Ile Ser Lys Gly Gly Leu Ile Lys Thr Lys
                245                 250                 255

Arg Lys Arg Lys Lys Gln Arg Val Lys Ile Ala Tyr Glu Glu Ile Phe
            260                 265                 270

Val Lys Asn Met
        275


<210>  2
<211>  219
<212>  PRT
<213>  Homo Sapiens

<400>  2

Asp Ile Val Met Thr Gln Ser Pro Leu Ser Leu Pro Val Thr Pro Gly
1               5                   10                  15

Glu Pro Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser Leu Val Phe Ser
            20                  25                  30

Asp Gly Asn Thr Tyr Leu Asn Trp Tyr Leu Gln Lys Pro Gly Gln Ser
        35                  40                  45

Pro Gln Leu Leu Ile Tyr Lys Gly Ser Asn Arg Ala Ser Gly Val Pro
    50                  55                  60

Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile
65                  70                  75                  80

Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Gln Gln Tyr
                85                  90                  95

Asp Ser Tyr Pro Leu Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys
            100                 105                 110

Arg Thr Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu
        115                 120                 125

Gln Leu Lys Ser Gly Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe
    130                 135                 140
```

```
Tyr Pro Arg Glu Ala Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln
145                 150                 155                 160


Ser Gly Asn Ser Gln Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser
                165                 170                 175


Thr Tyr Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu
            180                 185                 190


Lys His Lys Val Tyr Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser
        195                 200                 205


Pro Val Thr Lys Ser Phe Asn Arg Gly Glu Ala
    210                 215


<210>  3
<211>  225
<212>  PRT
<213>  Homo sapiens

<400>  3

Gln Val Gln Leu Gln Gln Ser Gly Pro Gly Leu Val Lys Pro Ser Gln
1               5                   10                  15


Thr Leu Ser Leu Thr Cys Ala Ile Ser Gly Asp Ser Val Ser Ser Asn
            20                  25                  30


Ser Ala Ala Trp Ser Trp Ile Arg Gln Ser Pro Gly Arg Gly Leu Glu
        35                  40                  45


Trp Leu Gly Ile Ile Tyr Lys Arg Ser Lys Trp Tyr Asn Arg Tyr Ala
    50                  55                  60


Val Ser Val Lys Ser Arg Ile Thr Ile Asn Pro Asp Thr Ser Lys Asn
65                  70                  75                  80


Gln Phe Ser Leu Gln Leu Asn Ser Val Thr Pro Glu Asp Thr Ala Val
                85                  90                  95


Tyr Tyr Cys Ala Arg Trp His Ser Asp Lys His Trp Gly Phe Asp Tyr
            100                 105                 110


Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly
        115                 120                 125


Pro Ser Val Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly
    130                 135                 140


Thr Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val
145                 150                 155                 160


Thr Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe
```

```
                165                 170                 175

Pro Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val
            180                 185                 190

Thr Val Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val
        195                 200                 205

Asn His Lys Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys
    210                 215                 220

Ser
225
```

<210> 4
<211> 212
<212> PRT
<213> Homo sapiens

<400> 4

```
Asp Ile Glu Leu Thr Gln Pro Pro Ser Val Ser Val Ala Pro Gly Gln
1               5                   10                  15

Thr Ala Arg Ile Ser Cys Ser Gly Asp Asn Leu Arg Asn Tyr Tyr Ala
            20                  25                  30

His Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro Val Val Val Ile Tyr
        35                  40                  45

Tyr Asp Asn Asn Arg Pro Ser Gly Ile Pro Glu Arg Phe Ser Gly Ser
    50                  55                  60

Asn Ser Gly Asn Thr Ala Thr Leu Thr Ile Ser Gly Thr Gln Ala Glu
65                  70                  75                  80

Asp Glu Ala Asp Tyr Tyr Cys Gln Ser Trp Asp Asp Gly Val Pro Val
                85                  90                  95

Phe Gly Gly Gly Thr Lys Leu Thr Val Leu Gly Gln Pro Lys Ala Ala
            100                 105                 110

Pro Ser Val Thr Leu Phe Pro Pro Ser Ser Glu Glu Leu Gln Ala Asn
        115                 120                 125

Lys Ala Thr Leu Val Cys Leu Ile Ser Asp Phe Tyr Pro Gly Ala Val
    130                 135                 140

Thr Val Ala Trp Lys Gly Asp Ser Ser Pro Val Lys Ala Gly Val Glu
145                 150                 155                 160

Thr Thr Thr Pro Ser Lys Gln Ser Asn Asn Lys Tyr Ala Ala Ser Ser
                165                 170                 175
```

Tyr Leu Ser Leu Thr Pro Glu Gln Trp Lys Ser His Arg Ser Tyr Ser
180 185 190

Cys Gln Val Thr His Glu Gly Ser Thr Val Glu Lys Thr Val Ala Pro
195 200 205

Thr Glu Cys Ser
210

<210> 5
<211> 224
<212> PRT
<213> Homo sapiens

<400> 5

Gln Val Glu Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1 5 10 15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Arg Ser Tyr
20 25 30

Gly Met Ser Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
35 40 45

Ser Ser Ile Arg Gly Ser Ser Ser Ser Thr Tyr Tyr Ala Asp Ser Val
50 55 60

Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr
65 70 75 80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
85 90 95

Ala Arg Leu Tyr Arg Tyr Trp Phe Asp Tyr Trp Gly Gln Gly Thr Leu
100 105 110

Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu
115 120 125

Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly Cys
130 135 140

Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp Asn Ser
145 150 155 160

Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val Leu Gln Ser
165 170 175

Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro Ser Ser Ser
180 185 190

Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn His Lys Pro Ser Asn

```
        195                 200                 205

Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser Cys Asp Lys Thr His
    210                 215                 220


<210>  6
<211>  219
<212>  PRT
<213>  Homo sapiens

<400>  6

Asp Ile Val Met Thr Gln Ser Pro Leu Ser Leu Pro Val Thr Pro Gly
1               5                   10                  15


Glu Pro Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser Leu Val Phe Arg
            20                  25                  30


Asp Gly Ile Thr Tyr Leu Asn Trp Tyr Leu Gln Lys Pro Gly Gln Ser
        35                  40                  45


Pro Gln Leu Leu Ile Tyr Lys Gly Ser Asn Arg Ala Ser Gly Val Pro
    50                  55                  60


Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile
65                  70                  75                  80


Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Gln Gln Tyr
                85                  90                  95


Asp Ser Tyr Pro Leu Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys
            100                 105                 110


Arg Thr Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu
        115                 120                 125


Gln Leu Lys Ser Gly Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe
    130                 135                 140


Tyr Pro Arg Glu Ala Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln
145                 150                 155                 160


Ser Gly Asn Ser Gln Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser
                165                 170                 175


Thr Tyr Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu
            180                 185                 190


Lys His Lys Val Tyr Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser
        195                 200                 205


Pro Val Thr Lys Ser Phe Asn Arg Gly Glu Cys
    210                 215
```

<210> 7
<211> 225
<212> PRT
<213> Homo sapiens

<400> 7

```
Gln Val Gln Leu Gln Gln Ser Gly Pro Gly Leu Val Lys Pro Ser Gln
1               5                  10                  15

Thr Leu Ser Leu Thr Cys Ala Ile Ser Gly Asp Ser Val Ser Ser Asp
            20                  25                  30

Ser Ala Ala Trp Ser Trp Ile Arg Gln Ser Pro Ser Arg Gly Leu Glu
        35                  40                  45

Trp Leu Gly Ile Ile Tyr Tyr Arg Ser Lys Trp Tyr Asn Arg Tyr Ala
    50                  55                  60

Val Ser Val Lys Ser Arg Ile Thr Ile Asn Pro Asp Thr Ser Lys Asn
65                  70                  75                  80

Gln Phe Ser Leu Gln Leu Asn Ser Val Thr Pro Glu Asp Thr Ala Val
                85                  90                  95

Tyr Tyr Cys Ala Arg Trp His Ser Asp Lys His Trp Gly Phe Asp Asp
            100                 105                 110

Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly
        115                 120                 125

Pro Ser Val Phe Pro Leu Ala Pro Cys Ser Arg Ser Thr Ser Glu Ser
    130                 135                 140

Thr Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val
145                 150                 155                 160

Thr Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe
                165                 170                 175

Pro Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val
            180                 185                 190

Thr Val Pro Ser Ser Asn Phe Gly Thr Gln Thr Tyr Thr Cys Asn Val
        195                 200                 205

Asp His Lys Pro Ser Asn Thr Lys Val Asp Lys Thr Val Glu Arg Lys
    210                 215                 220

Cys
225
```

<210> 8

<211> 212
<212> PRT
<213> Homo sapiens

<400> 8

Ser Tyr Glu Leu Thr Gln Pro Pro Ser Val Ser Val Ser Pro Gly Gln
1 5 10 15

Thr Ala Arg Ile Thr Cys Ser Gly Asp Asn Leu Pro Lys Tyr Tyr Ala
20 25 30

His Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro Val Val Val Ile Phe
35 40 45

Tyr Asp Val Asn Arg Pro Ser Gly Ile Pro Glu Arg Phe Ser Gly Ser
50 55 60

Asn Ser Gly Asn Thr Ala Thr Leu Thr Ile Ser Gly Thr Gln Ala Met
65 70 75 80

Asp Glu Ala Asp Tyr Tyr Cys Gln Ala Trp Trp Ser Ser Thr Pro Val
85 90 95

Phe Gly Gly Gly Thr Lys Leu Thr Val Leu Gly Gln Pro Lys Ala Ala
100 105 110

Pro Ser Val Thr Leu Phe Pro Pro Ser Ser Glu Glu Leu Gln Ala Asn
115 120 125

Lys Ala Thr Leu Val Cys Leu Ile Ser Asp Phe Tyr Pro Gly Ala Val
130 135 140

Thr Val Ala Trp Lys Ala Asp Ser Ser Pro Val Lys Ala Gly Val Glu
145 150 155 160

Thr Thr Thr Pro Ser Lys Gln Ser Asn Asn Lys Tyr Ala Ala Ser Ser
165 170 175

Tyr Leu Ser Leu Thr Pro Glu Gln Trp Lys Ser His Arg Ser Tyr Ser
180 185 190

Cys Gln Val Thr His Glu Gly Ser Thr Val Glu Lys Thr Val Ala Pro
195 200 205

Thr Glu Cys Ser
210

<210> 9
<211> 219
<212> PRT
<213> Homo sapiens

<400> 9

```
Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1               5                  10                  15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Ser Tyr
            20                  25                  30

Gly Met Asp Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
        35                  40                  45

Ser Ser Ile Arg Gly Ser Arg Gly Ser Thr Tyr Tyr Ala Asp Ser Val
    50                  55                  60

Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr
65                  70                  75                  80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

Ala Arg Leu Tyr Arg Tyr Trp Phe Asp Tyr Trp Gly Gln Gly Thr Leu
            100                 105                 110

Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu
        115                 120                 125

Ala Pro Cys Ser Arg Ser Thr Ser Glu Ser Thr Ala Ala Leu Gly Cys
    130                 135                 140

Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp Asn Ser
145                 150                 155                 160

Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val Leu Gln Ser
                165                 170                 175

Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro Ser Ser Asn
            180                 185                 190

Phe Gly Thr Gln Thr Tyr Thr Cys Asn Val Asp His Lys Pro Ser Asn
        195                 200                 205

Thr Lys Val Asp Lys Thr Val Glu Arg Lys Cys
    210                 215
```