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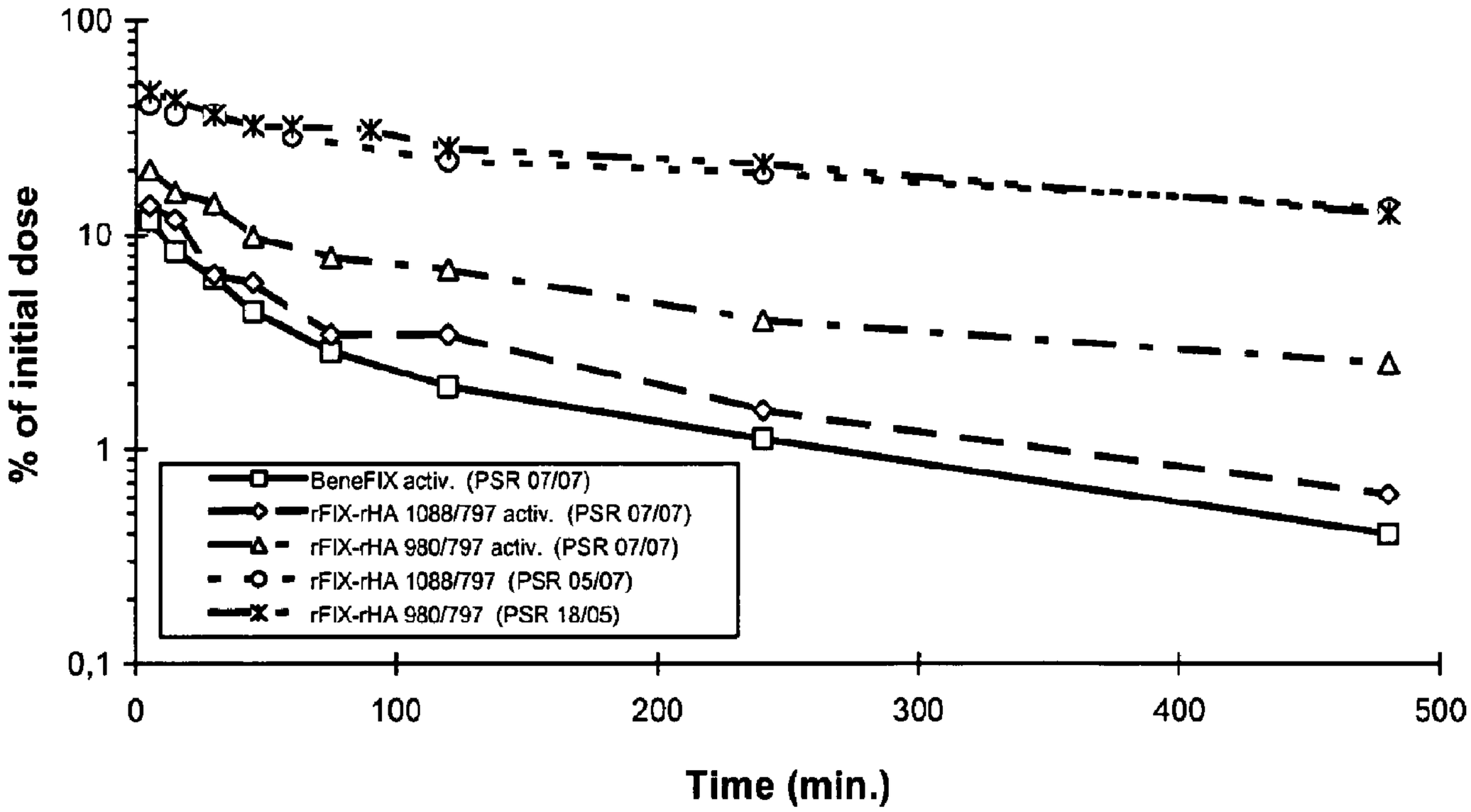
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(54) **Titre : PROTEINE DE FUSION POUVANT SUBIR UN CLIVAGE PROTEOLYTIQUE ET CONTENANT UN FACTEUR DE COAGULATION SANGUINE**
(54) **Title: PROTEOLYTICALLY CLEAVABLE FUSION PROTEIN COMPRISING A BLOOD COAGULATION FACTOR**



(57) **Abrégé/Abstract:**
The invention relates to therapeutic fusion proteins in which a coagulation factor is fused to a half-life enhancing polypeptide, and both are connected by a linker peptide that is proteolytically cleavable. The cleavage of such linkers liberates the coagulation factor from any activity-compromising steric hindrance caused by the half-life enhancing polypeptide and, thereby, allows the generation of fusion proteins with high molar specific activity when tested in coagulation-related assays. Furthermore, the fact that the linker is cleavable can enhance the rates of inactivation and/or elimination after proteolytic cleavage of the peptide linker compared to the corresponding therapeutic fusion protein linked by the non- cleavable linker having the amino acid sequence GGGGGGV.

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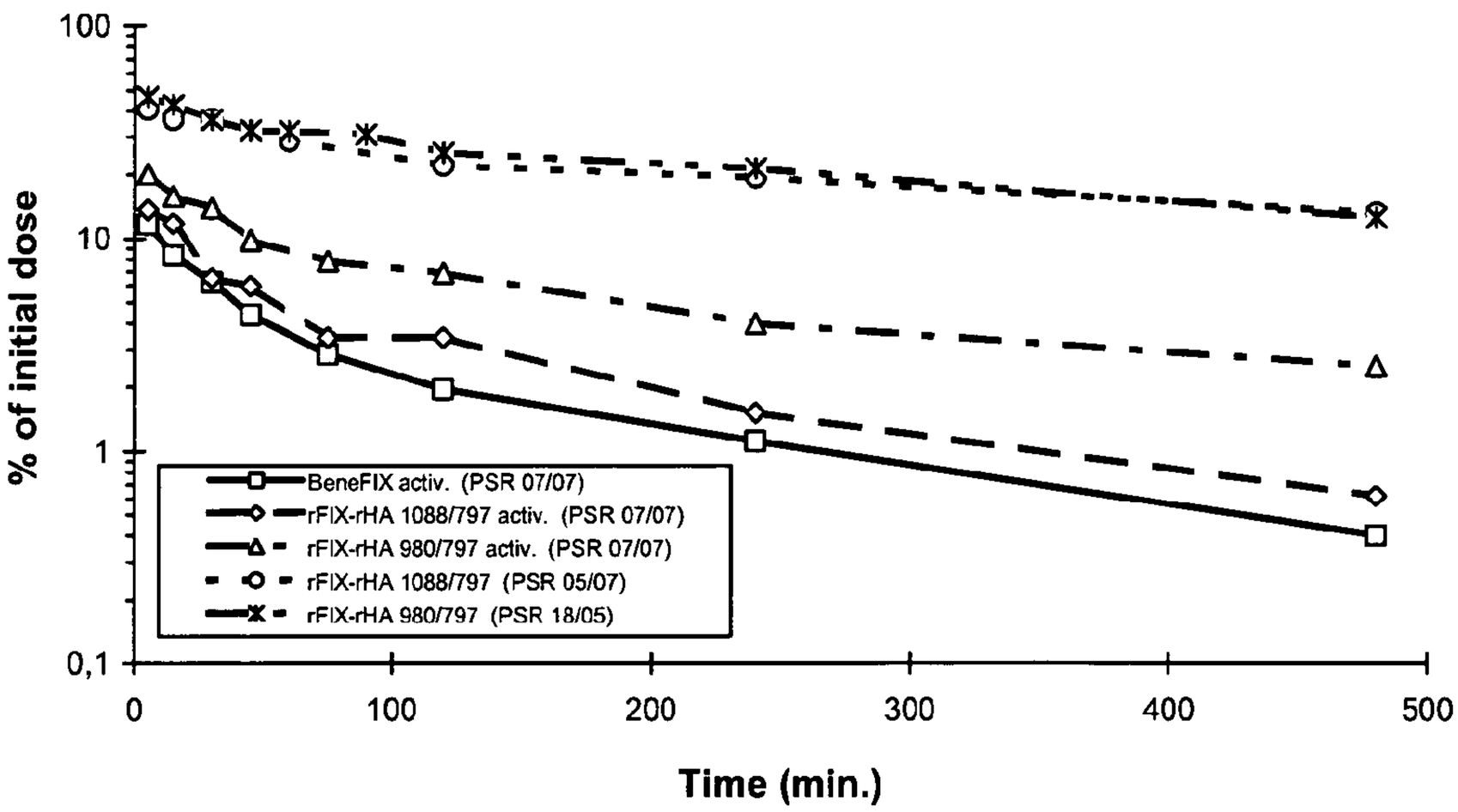
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PROTEOLYTICALLY CLEAVABLE FUSION PROTEIN COMPRISING A BLOOD COAGULATION FACTOR

Introduction

The present invention relates to the field of modified therapeutic fusion proteins with increased half-life compared to their non-modified parent therapeutic polypeptides. The invention specifically relates to coagulation factors fused to half-life enhancing polypeptides (HLEPs), which are connected by linker peptides that are proteolytically cleavable. The cleavage of such linkers liberates the therapeutic polypeptide from any activity-compromising steric hindrance caused by the HLEP and thereby allows the generation of fusion proteins, which retain a high molar specific activity of the coagulation factor. In case the therapeutic fusion proteins are zymogens, those linkers are especially preferred that liberate the therapeutic polypeptide essentially simultaneous with its activation in vivo upon exposure to the corresponding protease(s). Another aspect of the present invention is a faster inactivation rate of a given coagulation factor once the coagulation factor is activated and the peptide linker is proteolytically cleaved in a coagulation-related mode and/or a faster elimination rate of a given coagulation factor once the coagulation factor is activated and the peptide linker is proteolytically cleaved in a coagulation-related mode compared to the corresponding fusion protein without cleavable linker.

The idea of the invention is demonstrated in particular by human vitamin K-dependent polypeptides Factor IX, Factor VII, and Factor VIIa but the concept also may be applied to other coagulation factors. Any half-life enhancing polypeptide (HLEP) may be connected to the therapeutic polypeptide by a cleavable linker peptide, but albumin or immunoglobulins or fragments derived thereof like the Fc

fragment without an antigen binding domain are preferred HLEPs. The invention also relates to cDNA sequences coding for the therapeutic polypeptides and derivatives thereof genetically fused to a cDNA coding for HLEPs, such as human serum albumin linked by oligonucleotides that code for cleavable, intervening peptide linkers. Such encoded derivatives exhibit improved half-life and molar specific activities that are increased in comparison to their non-cleavable counterparts. The invention also relates to recombinant expression vectors containing such cDNA sequences, host cells transformed with such recombinant expression vectors, recombinant polypeptides and derivatives which do have biological activities comparable to the unmodified wild type therapeutic polypeptide but having improved half-lifes. The invention also relates to processes for the manufacture of such recombinant proteins and their derivatives. The invention also covers a transfer vector for use in human gene therapy, which comprises such modified DNA sequences useful to increase half-life in vivo.

Background of the invention

Several recombinant, therapeutic polypeptides are commercially available for therapeutic and prophylactic use in humans. The patients in general benefit from the specific mode of action of the recombinant active ingredients but a disadvantage often is their limited availability due to their expensive and complex manufacturing processes. A reduction of the necessary dose or the frequency of administration of such products could improve this situation. A reduced frequency of administration could improve the convenience for the patient and, therefore, also the acceptance of the therapy. Several solutions have been described to achieve the goal of an increased in vivo half-life after administration. Solutions proposed recently include the formation of fusion proteins, especially in the case of polypeptides with a short in vivo half-life that can be increased significantly by fusion to a HLEP.

Ballance et al. (WO 01/79271) described fusion polypeptides of a multitude of different therapeutic polypeptides which, when fused to human serum albumin, are

predicted to have an increased functional half-life in vivo and extended shelf-life. Long lists of potential fusion partners are described without showing by experimental data for almost any of these polypeptides that the respective albumin fusion proteins actually retain biological activity and have improved properties. Among the list of therapeutic polypeptides mentioned as Examples are Factor IX and FVII/FVIIa.. Also described are fusions of FIX and FVII/FVIIa in which there is a peptide linker between albumin and FIX or FVII/FVIIa. However, the use of cleavable linker peptides is not suggested.

Sheffield et al. (Sheffield W.P. et al. (2004), Br. J. Haematol. 126: 565-573) expressed a murine Factor IX albumin fusion protein composed of murine FIX, a linker of 8 amino acids (GPG_4TM), murine albumin and a peptide tag of 22 amino acids, and also a human Factor IX albumin fusion protein composed of human Factor IX, a linker of 7 amino acids (G_6V) and human albumin. Using a one-stage, FIX dependent clotting assay, the molar specific activities of the murine FIX-albumin fusion protein (MFUST) and the human FIX-albumin fusion protein (HFUS) were at least two- to three-fold lower than that of their unfused counterparts, an effect attributed at least partially to a slower proteolytic activation process by FXIa. Sheffield did not use or suggest using a cleavable linker between FIX and albumin.

Several patent applications describe the fusion of therapeutic polypeptides to immunoglobulin constant regions to extend the therapeutic polypeptide´s in vivo half-life. WO 2002/04598, WO 2003/059935, WO 2004/081053, WO 2004/101740 and WO 2005/001025 include FIX as examples for the therapeutic polypeptide moiety. The latter two patent applications also describe FVII/FVIIa fused to immunoglobulin constant regions and find that fusion protein homodimers have inferior clotting activity compared to fusion proteins consisting of a monomer/dimer. Again, the use of cleavable linker peptides is not suggested.

In WO 91/09125 fusion proteins are disclosed that are joined by linkers which are cleavable by proteases of the blood coagulation cascade, but the fusion proteins are limited to those comprising fibrinolytic or antithrombotic proteins.

In WO 03/068934 chimeric molecules are described that are composed of at least one first component molecule, at least one linker and at least one second molecule, wherein the linker comprises an enzyme cleavage site to produce a non-naturally occurring linkage and cleavage site between the first and the second component molecule and wherein, upon cleavage of the chimeric molecule at the cleavage site, at least one of the component molecules is functionally active. The cleaving proteases may be coagulation factors like thrombin. Component molecules described among many others are FIX and FVIIa. However the therapeutic fusion proteins of the present invention are not disclosed, nor are improved properties of the therapeutic fusion proteins of the present invention disclosed such as increased molar specific activity, increased inactivation and/or elimination rates as compared to the therapeutic protein without cleavable linkers.

Description of the invention

There is a great medical need for coagulation factors which have a long half-life. In the prior art fusions of coagulation factors to half-life enhancing polypeptides have been suggested to achieve this goal. However, once a coagulation factor is activated during coagulation either by proteolytic cleavage of the zymogen (like FIX) or by contact of an already proteolytically "pre"-activated factor to a second polypeptide (like FVIIa binding to Tissue Factor), it is no longer desirable to maintain the long half-life of the now activated coagulation factor as this might lead to thrombotic complications, as is already the case for a wild type coagulation factor as FVIIa (Aledort L.M., J Thromb Haemost 2(10): 1700-1708 (2004)) and should be even more relevant if the activated factor would have an increased half-life. It is therefore one objective of the present invention to provide long-lived coagulation factors, which after activation or after availability of a cofactor have a half-life comparable to that of an unmodified coagulation factor.

Fusions of the coagulation factors to half-life enhancing polypeptides as described in the prior art and as also shown in example 6 and 7 suffer in general from a reduced molar specific activity of the fused coagulation factor. Another aspect of the present invention to provide coagulation factors with enhanced half-life, that show increased molar specific activity compared to the corresponding therapeutic fusion protein without a cleavable linker.

The invention is therefore about therapeutic fusion proteins comprising

a) a coagulation factor, its variants or derivatives,

b) a half-life enhancing polypeptide selected from the group consisting of albumin including variants and derivatives thereof, polypeptides of the albumin family including variants and derivatives thereof and immunoglobulins including variants and derivatives thereof and

c) a peptide linker which joins the coagulation factor and the half-life enhancing polypeptide;

wherein the peptide linker is cleavable by proteases involved in coagulation or activated by coagulation enzymes and the therapeutic fusion protein has in comparison to the respective therapeutic fusion protein linked by a non-cleavable linker having the amino acid sequence GGGGGGV

i) an increased molar specific activity in at least one coagulation-related assay and/or

ii) an increased inactivation rate of the activated coagulation factor after the peptide linker is proteolytically cleaved in a coagulation-related mode and/or

iii) an increased elimination rate of the activated coagulation factor after the peptide linker is proteolytically cleaved in a coagulation-related mode.

As a consequence of the cleavable linker, after cleavage of the peptide linker in a coagulation-related mode, the coagulation factor more closely resembles the behaviour of the native, non-fused factor and does not show an increased half-life of the active factor with potentially prothrombotic effect.

Proteolytic cleavage in a coagulation-related mode in the sense of the invention, is any proteolytic cleavage that occurs as a consequence of the activation of at least one coagulation factor or coagulation cofactor.

The term “activated coagulation factor after the peptide linker is proteolytically cleaved in a coagulation-related mode” in the sense of the invention means that the coagulation factor is either activated almost in parallel to the proteolytic cleavage of the linker peptide, or that the coagulation factor was already activated before the proteolytic cleavage of the linker peptide. Activation may occur, for example by proteolytic cleavage of the coagulation factor or by binding to a cofactor.

A further aspect of the present invention is to provide therapeutic fusion proteins comprising

a) a coagulation factor, its variants or derivatives,

b) a half-life enhancing polypeptide selected from the group consisting of albumin including variants and derivatives thereof, polypeptides of the albumin family including variants and derivatives thereof and immunoglobulins including variants and derivatives thereof and

c) a peptide linker which joins the coagulation factor and the half-life enhancing polypeptide;

wherein the peptide linker is cleavable by proteases involved in coagulation or activated by coagulation enzymes and the therapeutic fusion protein has in comparison to the respective therapeutic fusion protein linked by a non-cleavable linker having the amino acid sequence GGGGGGV

i) an increased molar specific activity in at least one coagulation-related assay and/or

ii) an increased inactivation rate of the activated coagulation factor after the peptide linker is proteolytically cleaved in a coagulation-related mode and/or

iii) an increased elimination rate of the activated coagulation factor after the peptide linker is proteolytically cleaved in a coagulation-related mode

and which have an enhanced in vivo recovery as compared to the in vivo recovery of the unmodified coagulation factor.

Preferred are therapeutic fusion proteins which have an enhanced in vivo recovery compared to the unmodified coagulation factor by at least 10%, more preferred by at least 25% and most preferred by 40% or more.

Preferred coagulation factors are vitamin-K dependent coagulation factors and fragments and variants thereof. Even more preferred are FVIIa and FIX and fragments and variants thereof.

Preferred HLEPs are albumin and fragments or variants thereof and immunoglobulins including fragments and variants thereof.

The linker region in a preferred embodiment comprises a sequence of the therapeutic polypeptide to be administered or a variant thereof, which should result in a decreased risk of neoantigenic properties (formation of a novel potentially immunogenic epitope due to the occurrence of a peptide within the therapeutic antigen which does not exist in human proteins) of the expressed fusion protein . Also in case the therapeutic protein is a zymogen (e.g. needs to be proteolytically activated) the kinetics of the peptide linker cleavage will more closely reflect the coagulation-related activation kinetics of the zymogen. Thus, in such preferred embodiments a zymogen and a corresponding linker are activated and respectively cleaved, with comparable kinetics. For this reason, the present invention also particularly relates to fusion proteins of a zymogen and a HLEP, where the kinetics of the linker cleavage by relevant proteases is not delayed by more than a factor of 3, and most preferably not by more than a factor of 2 compared to the kinetics of the zymogen activation.

In a further embodiment, the linker peptide comprises cleavage sites for more than one protease. This can be achieved either by a linker peptide that can be cleaved at

the same position by different proteases or by a linker peptide that provides two or more different cleavage sites. This may be advantageous circumstances where the therapeutic fusion protein must be activated by proteolytic cleavage to achieve enzymatic activity and where different proteases may contribute to this activation step. This is the case, for example, upon activation of FIX, which can either be achieved by FXIa or by FVIIa/Tissue Factor (TF).

Preferred embodiments of the invention are therapeutic fusion proteins wherein the linker is cleavable by the protease, that activates the coagulation factor, thereby ensuring that the cleavage of the linker is linked to the activation of the coagulation factor at a site at which coagulation occurs.

Other preferred therapeutic fusion proteins according to the invention are those, wherein the linker is cleavable by the coagulation factor which is part of the therapeutic fusion protein once it is activated, thus also ensuring that cleavage of the fusion protein is connected with a coagulatory event.

Other preferred therapeutic fusion proteins according to the invention are those, wherein the linker is cleavable by a protease, which itself is activated directly or indirectly by the activity of the coagulation factor which is part of the therapeutic fusion protein, thus also ensuring that cleavage of the fusion protein is connected with a coagulatory event.

One class of most preferred therapeutic fusion proteins are those wherein the linker is cleavable by FXIa and/or by FVIIa/TF and the coagulation factor is FIX

The gist of the invention is demonstrated in particular by the vitamin K-dependent polypeptide Factor IX, cleavable linkers and albumin as the HLEP as well as its corresponding cDNA sequences. The invention also relates to cDNA sequences coding for any other coagulation factors which can be proteolytically activated or that are involved in the activation of other zymogens or polypeptides. These cDNAs

are genetically fused to cDNA sequences coding for human serum albumin or other HLEPs, and are linked by oligonucleotides that code for intervening, cleavable peptide linkers. The expressed therapeutic fusion proteins exhibit molar specific activities which are increased in comparison to their non-cleavable counterparts. The invention also relates to recombinant expression vectors containing such fused cDNA sequences, host cells transformed with such recombinant expression vectors, recombinant therapeutic fusion proteins and derivatives that have biological activities almost comparable to the unmodified wild type therapeutic polypeptides but having improved in vivo half-life. The invention also relates to processes for the manufacture of such recombinant polypeptides and their derivatives. The invention also covers a transfer vector for use in human gene therapy, which comprises such modified DNA sequences useful to increase product levels in vivo.

Preferred therapeutic fusion proteins according to the invention are those that have a molar specific activity, in particular a molar specific activity in at least one coagulation-related assay that is at least 25% increased compared to that of the therapeutic fusion protein without a cleavable linker. More preferred are therapeutic fusion proteins in which the molar specific activity is increased by at least 50%, even more preferred those in which the molar specific activity is increased by at least 100%, in at least one of the different coagulation-related assays available.

Additional preferred embodiments of the present invention are therapeutic fusion proteins, wherein the inactivation rate of the activated coagulation factor after cleavage of the peptide linker which links the coagulation factor to the half-life enhancing polypeptide is increased by at least 10% as compared to the inactivation rate of the activated coagulation factor in a corresponding therapeutic fusion protein without a cleavable linker. More preferred are therapeutic fusion proteins in which the inactivation rate is increased by at least 25%, even more preferred those in which the inactivation rate is increased by at least 50%.

Additional preferred embodiments of the present invention are therapeutic fusion proteins, wherein the elimination rate of the coagulation factor after cleavage of the peptide linker that links the coagulation factor to the half-life enhancing polypeptide is increased by at least 10% as compared to the elimination rate of the coagulation factor in a corresponding therapeutic fusion protein without a cleavable linker. More preferred are therapeutic fusion proteins in which the elimination rate is increased by at least 25%, even more preferred those in which the elimination rate is increased by at least 50%.

Detailed description of the invention

Vitamin K-dependent polypeptides

Vitamin K-dependent polypeptides as one group of the therapeutic polypeptides are polypeptides that are γ-carboxylated enzymatically in the liver using vitamin K as a cofactor. Such vitamin K-dependent polypeptides e.g. are Factors II, VII, IX, X, Protein C, Protein S, GAS6, and Protein Z.

Human FIX

Human FIX, one member of the group of vitamin K-dependent polypeptides, is a single-chain glycoprotein with a molecular weight of 57 kDa, which is secreted by liver cells into the blood stream as an inactive zymogen of 415 amino acids. It contains 12 γ-carboxy-glutamic acid residues localized in the N-terminal Gla-domain of the polypeptide. The Gla residues require vitamin K for their biosynthesis. Following the Gla domain there are two epidermal growth factor domains, an activation peptide, and a trypsin-type serine protease domain. Further posttranslational modifications of FIX encompass hydroxylation (Asp 64), N-(Asn157 and Asn167) as well as O-type glycosylation (Ser53, Ser61, Thr159, Thr169, and Thr172), sulfation (Tyr155), and phosphorylation (Ser158).

FIX is converted to its active form, Factor IXa, by proteolysis of the activation peptide at Arg145-Ala146 and Arg180-Val181 leading to the formation of two polypeptide chains, an N-terminal light chain (18 kDa) and a C-terminal heavy chain

(28 kDa), which are held together by one disulfide bridge. Activation cleavage of Factor IX can be achieved in vitro e.g. by Factor XIa or Factor VIIa/TF. Factor IX is present in human plasma in a concentration of 5-10 µg/ml. Terminal plasma half-life of Factor IX in humans was found to be about 15 to 18 hours (White GC et al. 1997. Recombinant factor IX. Thromb Haemost. 78: 261-265; Ewenstein BM et al. 2002. Pharmacokinetic analysis of plasma-derived and recombinant F IX concentrates in previously treated patients with moderate or severe hemophilia B. Transfusion 42:190-197).

Hemophilia B is caused by non-functional or missing Factor IX and is treated with Factor IX concentrates from plasma or a recombinant form of Factor IX. As haemophilia B patients often receive at least biweekly prophylactic administrations of Factor IX to avoid spontaneous bleedings, it is desirable to increase the intervals of between administration by increasing the half-life of the Factor IX product applied. An improvement in plasma half-life would bring significant benefit to the patient. Up to now no pharmaceutical preparation of a Factor IX with improved plasma half-life is commercially available nor have any data been published showing F IX variants with prolonged in vivo half-life and almost unchanged molar specific activity in coagulation-related assays. Therefore, a great medical need still exists to develop forms of Factor IX which have a longer functional half-life in vivo.

Factor VII and Factor VIIa

FVII is a single-chain glycoprotein with a molecular weight of 50 kDa, which is secreted by liver cells into the blood stream as an inactive zymogen of 406 amino acids. FVII is converted to its active form Factor VIIa, by proteolysis of the single peptide bond at Arg152-Ile153 leading to the formation of two polypeptide chains, a N-terminal light chain (24 kDa) and a C-terminal heavy chain (28 kDa), which are held together by one disulfide bridge. In contrast to other vitamin K-dependent coagulation factors, no activation peptide is cleaved off during activation. Activation cleavage of Factor VII can be achieved in vitro, for example, by Factor Xa, Factor IXa, Factor VIIa, Factor XIIa, Factor Seven Activating Protease (FSAP), and

thrombin. Mollerup et al. (Biotechnol. Bioeng. (1995) 48: 501-505) reported that some cleavage also occurs in the heavy chain at Arg290 and/or Arg315.
Factor VII is present in plasma in a concentration of 500 ng/ml. About 1% or 5 ng/ml of Factor VII is present as activated Factor VIIa. The terminal plasma half-life of Factor VII was found to be about 4 hours and that of Factor VIIa about 2 hours.

By administering supraphysiological concentrations of Factor VIIa hemostasis can be achieved bypassing the need for Factor VIIIa and Factor IXa. The cloning of the cDNA for Factor VII (US 4,784,950) made it possible to develop activated Factor VII as a pharmaceutical. Factor VIIa was successfully administered for the first time in 1988. Ever since the number of indications of Factor VIIa has grown steadily showing a potential to become an universal hemostatic agent to stop bleeding (Erhardtsen, 2002). However, the short terminal half-life of Factor VIIa of approximately 2 hours and reduced in vivo recovery is limiting its application. Therefore, a great medical need still exists to develop forms of Factor VIIa which have an improved half-life but otherwise almost uncompromised molar specific activity, inactivation kinetics, and/or elimination kinetics after start of coagulation.

Therapeutic fusion proteins

"Therapeutic fusion proteins" in the sense of this invention are coagulation factors fused to a half-life enhancing polypeptide that upon administration to a human or animal can produce a prophylactic or therapeutic effect. These therapeutic fusion proteins may be administered to a human or an animal via intravenous, intramuscular, oral, topical, parenteral or other routes. Specific classes of therapeutic fusion proteins covered, i.e. by the examples in this invention, are coagulation factors like e.g. vitamin K-dependent polypeptides linked to half-life enhancing polypeptides like e.g. albumin and immunoglobulins and their fragments or derivatives. The expression "therapeutic fusion protein" is used interchangeable with "fusion protein".

Half-life enhancing polypeptide (HLEP)

Albumin, albumin family members and immunoglobulines and their fragments or derivatives have been described above as examples of half-life enhancing polypeptides (HLEPs). The terms “human serum albumin” (HSA) and “human albumin” (HA) are used interchangeably in this application. The terms “albumin” and “serum albumin” are broader, and encompass human serum albumin (and fragments and variants thereof) as well as albumin from other species (and fragments and variants thereof).

As used herein, “albumin” refers collectively to albumin polypeptide or amino acid sequence, or an albumin fragment or variant having one or more functional activities (e.g., biological activities) of albumin. In particular, “albumin” refers to human albumin or fragments thereof, especially the mature form of human albumin as shown in SEQ ID No:1 herein or albumin from other vertebrates or fragments thereof, or analogs or variants of these molecules or fragments thereof.

The albumin portion of the albumin fusion proteins may comprise the full length of the HA sequence as described above, or may include one or more fragments thereof that are capable of stabilizing or prolonging the therapeutic activity. Such fragments may be of 10 or more amino acids in length or may include about 15, 20, 25, 30, 50, or more contiguous amino acids from the HA sequence or may include part or all of specific domains of HA.

The albumin portion of the albumin fusion proteins of the invention may be a variant of normal HA, either natural or artificial. The therapeutic polypeptide portion of the fusion proteins of the invention may also be variants of the corresponding therapeutic polypeptides as described herein. The term “variants” includes insertions, deletions, and substitutions, either conservative or non-conservative, either natural or artificial, where such changes do not substantially alter the active site, or active domain that confers the therapeutic activities of the therapeutic polypeptides.

In particular, the albumin fusion proteins of the invention may include naturally occurring polymorphic variants of human albumin and fragments of human albumin. The albumin may be derived from any vertebrate, especially any mammal, for example human, cow, sheep, or pig. Non-mammalian albumins include, but are not limited to, hen and salmon. The albumin portion of the albumin-linked polypeptide may be from a different animal than the therapeutic polypeptide portion.

Generally speaking, an albumin fragment or variant will be at least 10, preferably at least 40, most preferably more than 70 amino acids long. The albumin variant may preferentially consist of or alternatively comprise at least one whole domain of albumin or fragments of said domains, for example domains 1 (amino acids 1-194 of SEQ ID NO:1), 2 (amino acids 195-387 of SEQ ID NO: 1), 3 (amino acids 388-585 of SEQ ID NO: 1), 1 + 2 (1-387 of SEQ ID NO: 1), 2 + 3 (195-585 of SEQ ID NO: 1) or 1 + 3 (amino acids 1-194 of SEQ ID NO: 1 + amino acids 388-585 of SEQ ID NO: 1). Each domain is itself made up of two homologous subdomains namely 1-105, 120-194, 195-291, 316-387, 388-491 and 512-585, with flexible inter-subdomain linker regions comprising residues Lys106 to Glu119, Glu292 to Val315, and Glu492 to Ala511.

The albumin portion of an albumin fusion protein of the invention may comprise at least one subdomain or domain of HA or conservative modifications thereof.

All fragments and variants of albumin are encompassed by the invention as fusion partners of a coagulation factor as long as they lead to a half-life extension of the therapeutic fusion protein in plasma of at least 25% as compared to the non-fused coagulation factor.

Besides albumin, alpha-fetoprotein, another member of the albumin family, has been claimed to enhance the half-life of an attached therapeutic polypeptide in vivo (WO 2005/024044). The albumin family of proteins, evolutionarily related serum transport proteins, consists of albumin, alpha-fetoprotein (AFP; Beattie & Dugaiczyk

1982. Gene 20:415-422), afamin (AFM; Lichenstein et al. 1994. J. Biol. Chem. 269:18149-18154) and vitamin D binding protein (DBP; Cooke & David 1985. J. Clin. Invest. 76:2420-2424). Their genes represent a multigene cluster with structural and functional similarities mapping to the same chromosomal region in humans, mice and rat. The structural similarity of the albumin family members suggest their usability as HLEPs. It is therefore another object of the invention to use such albumin family members, fragments and variants thereof as HLEPs. The term "variants" includes insertions, deletions and substitutions, either conservative or non-conservative as long as the desired function is still present.

Albumin family members may comprise the full length of the respective protein AFP, AFM and DBP, or may include one or more fragments thereof that are capable of stabilizing or prolonging the therapeutic activity. Such fragments may be of 10 or more amino acids in length or may include about 15, 20, 25, 30, 50, or more contiguous amino acids of the respective protein sequence or may include part or all of specific domains of the respective protein, as long as the HLEP fragments provide a half-life extension of at least 25% as compared to the non-fused coagulation factor. Albumin family members of the therapeutic fusion proteins of the invention may include naturally occurring polymorphic variants of AFP, AFM and DBP.

IgG and IgG-fragments may also be used as HLEPs, as long as the HLEP fragments provide a half-life extension of at least 25% as compared to the non-fused coagulation factor. The therapeutic polypeptide portion is connected to the IgG or the IgG fragments via a cleavable linker that allows high molar specific activities of the fusion protein. Examples for factor VII/VIIa and factor IX IgG fusion molecules are found, e.g., in WO 2005/001025.

It discloses i.e. a homodimer comprised of two factor VII (factor VIIa) molecules and two Fc molecules and a monomer/dimer hybrid comprised of one FVII (FVIIa) molecule and two Fc molecules, the monomer/dimer showing an about four times greater clotting activity than the homodimer. A linker

sequence of the present invention liberating the FVII (FVIIa) molecules upon cleavage by a protease of the coagulation cascade like, e.g., FXIa, FXa, or FIXa could be able to elevate the clotting activity of the constructs and especially that of the homodimer to an activity level comparable to the monomer/dimer or even higher. A FIX-Fc fusion protein with cleavable linker is exemplarily shown in SEQ ID No 93. Cleavable linkers such as those shown in table 3a and 3b may be applied in this case.

The invention specifically relates to fusion proteins comprising linking a coagulation factor or fragment or variant thereof to the N- or C-terminus of a HLEP or fragment or variant thereof such that an intervening cleavable peptide linker is introduced between the therapeutic polypeptide and the HLEP such that the fusion protein formed has an increased in vivo half-life compared to the coagulation factor which has not been linked to a HLEP and that the fusion protein has an at least 25% higher molar specific activity compared to the corresponding fusion protein with non-cleavable linker in at least one of the different coagulation-related assays available.

"Coagulation factor" as used in this application include, but is not limited to, polypeptides consisting of Factor IX, Factor VII, Factor VIII, von Willebrand Factor, Factor V, Factor X, Factor XI, Factor XII, Factor XIII, Factor I, Factor II (Prothrombin), Protein C, Protein S, GAS6, or Protein Z as well as their activated forms. Furthermore, useful therapeutic polypeptides may be wild-type polypeptides or may contain mutations. Degree and location of glycosylation or other post-translation modifications may vary depending on the chosen host cells and the nature of the host cellular environment. When referring to specific amino acid sequences, posttranslational modifications of such sequences are encompassed in this application.

"Coagulation factor" within the above definition includes polypeptides that have the natural amino acid sequence including any natural polymorphisms. It also includes

polypeptides with a slightly modified amino acid sequence, for instance, a modified N-terminal or C-terminal end including terminal amino acid deletions or additions, as long as those polypeptides substantially retain the activity of the respective therapeutic polypeptide. Variants included differ in one or more amino acid residues from the wild type sequence. Examples of such differences may include truncation of the N- and/or C-terminus by one or more amino acid residues (e.g. preferably 1 to 30 amino acid residues), or addition of one or more extra residues at the N- and/or C-terminus, as well as conservative amino acid substitutions, i.e. substitutions performed within groups of amino acids with similar characteristics, e.g. (1) small amino acids, (2) acidic amino acids, (3) polar amino acids, (4) basic amino acids, (5) hydrophobic amino acids, and (6) aromatic amino acids. Examples of such conservative substitutions are shown in the following table.

Table 1

(1)	Alanine	Glycine		
(2)	Aspartic acid	Glutamic acid		
(3a)	Asparagine	Glutamine		
(3b)	Serine	Threonine		
(4)	Arginine	Histidine	Lysine	
(5)	Isoleucine	Leucine	Methionine	Valine
(6)	Phenylalanine	Tyrosine	Tryptophane	

The in vivo half-life of the fusion proteins of the invention, in general determined as terminal half-life or β-half-life, is usually at least about 25%, preferable at least about 50%, and more preferably more than 100% higher than the in vivo half-life of the non-fused polypeptide.

The fusion proteins of the present invention have at least a 25%, preferably at least a 50%, more preferably an at least 100% increased molar specific activity compared to the corresponding fusion proteins without cleavable linkers.

The molar specific activity (or molar specific coagulation-related activity as considered here in particular) in this regard is defined as the activity expressed per mole (or e.g. nmole) of the therapeutic polypeptide or therapeutic fusion protein of interest. Calculation of the molar specific activity allows a direct comparison of the activity of the different constructs which is not affected by the different molecular weights or optical densities of the polypeptides studied. The molar specific activity may be calculated as exemplified in table 2 below for FIX and a FIX-HSA fusion protein.

Table 2: Calculation of molar specific activity as shown for a purified FIX-HSA fusion protein

Product	$OD_{(280nm,\ 1\%)}$	MW	Activity/Vol/OD_{280} (IU/L/OD_{280})	Molar optical density ($OD_{(280)}$ at 1 mol/L)	Calculation of molar specific activity (IU/mol)
FIX	13.3 [1)]	57 000	determined for product	75810 (= MW x $OD_{(280,\ 1\%)}$/10)	=(Activity/Vol/OD_{280}) x (OD_{280} at 1 mol/L)
HSA	5.7 [2)]	66 300		37791 (= MW x $OD_{(280,\ 1\%)}$/10)	
FIX-HSA			determined for product	113601 (= sum of molar optical density of FIX and HSA)	= (Activity/Vol/OD_{280}) x (OD_{280} at 1 mol/L)

[1)] R.G. Di Scipio et al., Biochem. 16: 698-706 (1977)

[2)] C. Chaudhury et al, J. Exp. Med. 197(3): 315-322 (2003)

In order to determine a molar specific coagulation-related activity, any assay may be used that determines enzymatic or cofactor activities that are relevant to the coagulation process.

Therefore “coagulation-related assays” in the sense of the invention is any assay which determines enzymatic or cofactor activities that are of relevance in the coagulation process or that is able to determine that either the intrinsic or the extrinsic coagulation cascade has been activated. The “coagulation-related” assay thus may be direct coagulation assays like aPTT, PT, or the thrombin generation assays. However, other assays like, e.g., chromogenic assays applied for specific coagulation factors are also included. Examples for such assays or corresponding reagents are Pathromtin® SL (aPTT assay, Dade Behring) or Thromborel® S (Prothrombin time assay, Dade Behring) with corresponding coagulation factor deficient plasma (Dade Behring), Thrombin generation assay kits (Technoclone, Thrombinoscope) using e.g. coagulation factor deficient plasma, chromogenic assays like Biophen Factor IX (Hyphen BioMed), Staclot® FVIIa-rTF (Roche Diagnostics GmbH), Coatest® Factor VIII:C/4 (Chromogenix), or others.

For purposes of this invention, an increase in any one of the above assays or an equivalent coagulation-related assay is considered to show an increase in molar specific activity. For example, a 25% increase refers to a 25% increase in any of the above or an equivalent assay.

To determine whether therapeutic fusion proteins fall within the scope of the present invention, the standard against which the molar specific activity of these proteins is compared is a construct in which the respective coagulation factor and the respective HLEP are linked by a non-cleavable linker having the amino acid sequence GGGGGGV.

In the case of FIX, aPTT assays are often used for determination of coagulation activity. Such a coagulation assay (aPTT assay) is described in example 5 in more detail. However, other coagulation-related assays or assay principles may be applied to determine molar specific activity for FIX.

Recombinant therapeutic polypeptide drugs are usually expensive and not all countries can afford costly therapies based on such drugs. Increasing the in vivo recovery of such drugs could make the use of these products cheaper and subsequently more patients would benefit from them. In the case of the fusion proteins of the present invention an increased in vivo recovery would also be a desirable advantage. "In vivo recovery" in the sense of the invention means the amount of product found in blood or plasma shortly after administration of the product. Therefore, for detection of the in vivo recovery in general the plasma content is determined a few minutes (e.g. 5 or 15 min) after administration of the product.

Although it is desirable to have a high in vivo recovery and a long half-life for a non-activated coagulation factor, it is advantageous to limit the half-life of a coagulation factor after its activation or the activation of its co-factor in order to avoid a prothrombotic risk. Therefore, after the coagulation process has been initiated, the half-life of the active coagulation factor should again be reduced. This can either be achieved by enhancing inactivation in a coagulation-related mode or by elimination of the coagulation factor.

Inactivation according to the present invention means the decrease of activity of the therapeutic polypeptide which can be caused, for example, by a complex formation of a coagulation factor and an inhibitor of the corresponding coagulation factor or by further proteolytic cleavage as known, e.g., in the case of FVIII and FV.

The inactivation rate of an activated therapeutic fusion protein is defined as the rate the activity is declining, e.g., by reaction with inhibitors or by proteolytic inactivation. The inactivation rate may be measured by following the molar specific activity of the activated coagulation factor over time in the presence of physiologic amounts of inhibitors of this coagulation factor. Alternatively, the inactivation rate may be determined after administration of the activated product to an animal followed by

testing of plasma samples at an appropriate time frame using activity and antigen assays.

When for therapeutic fusion proteins a determination is needed whether these proteins fall within the scope of the present invention, the standard against which the inactivation rate of these therapeutic proteins is compared to, is a construct in which the respective coagulation factor and the respective HLEP are joined by a non-cleavable linker having the amino acid sequence GGGGGGV.

The elimination rate of an activated therapeutic fusion protein is defined as the rate the polypeptide is eliminated from the circulation of humans or animals. The elimination rate may be determined by measuring the pharmacokinetics of the activated, therapeutic fusion protein after intravenous administration. Using an antigen assay, the elimination by direct removal from the circulation can be determined. Using an activity assay in addition, a specific removal and inactivation rate may be determined.

When for therapeutic fusion proteins a determination is needed whether these proteins fall within the scope of the present invention, the standard against which the elimination rate of these proteins is compared to, is a construct in which the respective coagulation factor and the respective HLEP are joined by the non-cleavable linker having the amino acid sequence GGGGGGV.

According to this invention, the therapeutic polypeptide moiety is coupled to the HLEP moiety by a cleavable peptide linker. The linker should be non-immunogenic and should be flexible enough to allow cleavage by proteases. The cleavage of the linker should proceed comparably fast as the activation of the therapeutic polypeptide within the fusion protein, if the fusion protein is a zymogen.

The cleavable linker preferably comprises a sequence derived from

a) the therapeutic polypeptide to be administered itself if it contains proteolytic cleavage sites that are proteolytically cleaved during activation of the therapeutic polypeptide,
b) a substrate polypeptide of this therapeutic polypeptide, or
c) a substrate polypeptide cleaved by a protease which is activated or formed by the direct or indirect involvement of the therapeutic polypeptide.

The linker region in a more preferred embodiment comprises a sequence of the therapeutic polypeptide to be applied, which should result in a decreased risk of neoantigenic properties of the expressed fusion protein. Also in case the therapeutic protein is a zymogen (e.g. needs to be proteolytically activated) the kinetics of the peptide linker cleavage will more closely reflect the coagulation-related activation kinetics of the zymogen.

In a preferred embodiment, the therapeutic polypeptide is FIX zymogen and the HLEP is albumin. In this case the linker sequence is either derived from the sequences of the activation regions of FIX, from the cleavage region of any substrate of FIX like FX or FVII or from the cleavage region of any substrate polypeptide that is cleaved by a protease in whose activation FIXa is involved.

In a highly preferred embodiment the linker peptide is derived from FIX itself. In another preferred embodiment the linker peptide is derived from FX or FVII. In another preferred embodiment the linker sequence comprises two cleavage sequences that can be cleaved by FXIa or FVIIa/TF, two physiologically relevant activators of FIX.

Exemplary combinations of therapeutic polypeptide, cleavable linker and HLEP include the constructs listed in tables 3a and 3b but are not limited to these:

Table 3a: Examples of possible constructs

Coagulation factor	Linker	HLEP	Linker derived from (with modifications, if applicable)	SEQ ID NO:
	Linker not cleavable or not sufficiently rapidly cleavable			
FIX	-	HSA		
FIX	R I	HSA		
FIX	GGGGGGV (Sheffield et al.)	HSA		94
FIX	(GGS)nGS	HSA		
FIX	$SS(GGS)_7GS$	HSA		30
FIX	$SSNGS(GGS)_3NGS(GGS)_3GGNGS$	HSA		31
	Linker with one cleavage site			
FIX (1-412)	SVSQTSKLTR AETVFPDVD	HSA	FIX	36
FIX (1-412)	SVSQTSKLTR AETVFPDVD GS	HSA	FIX	37
FIX	SVSQTSKLTR AETVFPDVD	HSA	FIX	38
FIX	SVSQTSKLTR AETVFPDVD GS GGS	HSA	FIX	95
FIX	SVSQTSKLTR AETVFPDVD GS	HSA	FIX	39
FIX	SVSQTSKLTR AETVFPDVD NGS	HSA	FIX	40
FIX	SVSQTSKLTR AETVFPDV	HSA	FIX	96
FIX	QTSKLTR AETVFPDV	HSA	FIX	97
FIX	SKLTR AETVFPDV	HSA	FIX	98
FIX	SVSQTSKLTR AETVFP	HSA	FIX	99
FIX	SVSQTSKLTR AETVF	HSA	FIX	100
FIX	QTSKLTR AETVF	HSA	FIX	101
FIX	SKLTR AETVF	HSA	FIX	102
FIX	SVSQTSKLTR AET	HSA	FIX	103
FIX	QTSKLTR AET	HSA	FIX	104
FIX	SKLTR AET	HSA	FIX	105
FIX	SVSQTSKLTR GETVFPDVD	HSA	FIX	41
FIX	SVSQTSKLTR TETVFPDVD	HSA	FIX	42
FIX	SVSQTSKLTR SETVFPDVD	HSA	FIX	43
FIX	SVSQTSKLTR LETVFPDVD	HSA	FIX	44
FIX	SVSQTSKLTR TEAVFPDVD	HSA	FIX	45
FIX	SVSQTSKLTR GEAVFPDVD	HSA	FIX	46
FIX	QTSKLTR AETVFPDVD GS	HSA	FIX	106
FIX	SKLTR AETVFPDVD GS	HSA	FIX	107
FIX	SKLTR AETVFPDVD	HSA	FIX	47
FIX	QSFNDFTR VVGGED	HSA	FIX	48
FIX	QSFNDFTR VVGGED GS	HSA	FIX	49
FIX	QSFNDFTR VVGGE	HSA	FIX	108
FIX	QSFNDFTR TVGGED	HSA	FIX	50
FIX	QSFNDFTR LVGGED	HSA	FIX	51
FIX	QSFNDFTR GVGGED	HSA	FIX	52
FIX	QSFNDFTR VVGGED NGS	HSA	FIX	53
FIX	QSFNDFTR VVGGEDN	HSA	FIX	54

FIX	PERGDNNLTRIVGGQE GS	HSA	FX	109
FIX	PERGDNNLTRIVGGQE	HSA	FX	61
FIX	PERGDNNLTRIVGGQ	HSA	FX	110
FIX	DNNLTRIVGGQ	HSA	FX	111
FIX	SVSQTSKLTRAETVFPDVD	Fc	FIX	62
FIX	QSFNDFTRVVGGEDN	Fc	FIX	63
FIX (1-412)	SVSQTSKLTRAETVFPDVD	Fc	FIX	64
FIX	ASKPQGRIVGG	HSA delDAH	FVII	112
FIX	KRNASKPQGRIVGGKV	HSA	FVII	65
FIX	PEEPQLRMKNNEEAED	HSA	FVIII	66
FIX	DNSPSFIQIRSVAKKHPKT	HSA	FVIII	67
FIX	LSKNNAIEPRSFSQNSRHPS	HSA	FVIII	68
FIX	DEDENQSPRSFQKKTRHYFIA	HSA	FVIII	69
FIX	SPHVLRNRAQSGSVPQ	HSA	FVIII	70
FVII or FVIIa	PEEPQLRMKNNEEAEDYDDDLTDS	HSA	FVIII	71
FVII or FVIIa	DDDNSPSFIQIRSVAKKHPKTWVHYAAEEED	HSA	FVIII	72
FVII or FVIIa	LSKNNAIEPRSFSQNSRHPSTRQKQFNA	HSA	FVIII	73
FVII or FVIIa	DEDENQSPRSFQKKTRHYFIAA	HSA	FVIII	74
FVII or FVIIa	DYGMSSSPHVLRNRAQSGSVPQFKKVVFQEFT	HSA	FVIII	75
FVIII	Derived from cleavage sites of FVIII, FIX, or Fibrinogen	HSA	FVIII, FIX or Fgn	
VWF	Derived from cleavage sites of VWF, FVIII, or FIX	HSA	FIX, FVIII, VWF	
VWF	DIYDEDENQSPRSFQKKTRHYFIA	HSA	FVIII	76
VWF	DNSPSFIQIRSVAKKHP	HSA	FVIII	77
VWF	LSKNNAIEPRSFSQNSRHPS	HSA	FVIII	78

In the case of linkers derived from the N-terminal region of the FIX activation peptide, according to the natural polymorphism T148-A148 the sequences may also contain A instead of T at this position.

Table 3b: Examples of possible constructs with two or more cleavage sites

Coagulation factor	Linker	HLEP	Linker derived from (partially incl. Modifications)	SEQ ID NO:
	Linker with two cleavage sites			
FIX	SVSQTSKLTRAETVFPDVTQPERGDNNLTRIVGGQE	HSA	FIX, FX	**79**
FIX	SKLTRAETVFPDNNLTRIVGGQE	HSA	FIX, FX	**80**
FIX	RAETVFPDVTQPERGDNNLTRIVGGQE	HSA	FIX, FX	**81**
FIX	RAETVFPERGDNNLTRIVGGQE	HSA	FIX, FX	**82**
FIX	SVSQTSKLTRAETVFPDVDYVNNLTRIVGGQE	HSA	FIX, FX	**83**
FIX	SVSQTSKLTRAETVFPDVDNNLTRIVGGQE	HSA	FIX, FX	**84**
FIX	SVSQTSKLTRAETVFPDVDNNLTRIVGGQE	HSA	FIX, FX	**85**
FIX	SVSQTSKLTRAETVFPDVDYVNSTEAETILDNITQSTQSFNDFTRVVGGEDA	HSA	FIX	**86**
FIX	SVSQTSKLTRAETVFPDVQSFNDFTRVVGGED	HSA	FIX	**87**
FIX	SVSQTSKLTRAETVFPDVDSFNDFTRVVGGED	HSA	FIX	**88**
FIX	SVSQTSKLTRAETVFPDVNASKPQGRIVGGKV	HSA	FIX and FVII	**89**
FIX	SVSQTSKLTRAETVFPDVNASKPQGRLVGGKV	HSA	FIX and FVII	**90**
FIX	SVSQTSKLTRAETVFPDVNASKPQGRTVGGKV	HSA	FIX and FVII	**91**
FIX	SVSQTSKLTRAETVFPDVD	Fc		**92**

Variants and fragments of the described linkers are also encompassed in the present invention as long as the linker can still be cleaved by the protease or the proteases, that cleave the linkers of tables 3a and 3b or by the type of proteases

defined above. The term “variants” includes insertions, deletions and substitutions, either conservative or non-conservative.

Other combinations of the cleavage sequences described above and their variants shall be included in the present invention.

In another embodiment, amino acid substitutions are included that change the post-translational modification pattern of the peptide linker. These can be, for example, substitutions of amino acids that are glycosylated, sulphated, or phosphorylated.

In another embodiment of the invention the peptide linker between the therapeutic polypeptide and the HLEP moiety contains consensus sites for the addition of posttranslational modifications. Preferably such modifications consist of glycosylation sites. More preferably, such modifications consist of at least one N-glycosylation site of the structure Asn - X – Ser/Thr, wherein X denotes any amino acid except proline. Even more preferably such N-glycosylation sites are inserted close to the amino and/or carboxy terminus of the peptide linker such that they are capable of shielding potential neoepitopes which might develop at the sequences where the therapeutic polypeptide moiety is transitioning into the peptide linker or where the peptide linker is transitioning into the albumin moiety sequence.

Brief Description of the Figures

Figure 1: In vitro activation of FIX-albumin fusion proteins by FXIa at 37°C at a molar ratio of FXIa to fusion protein of about 1:500. One fusion protein with non-cleavable linker (1478/797) and two fusion proteins with cleavable linker (1088/797 and 1089/797) were used. Samples were analyzed by SDS-PAGE under reducing conditions followed by Coomassie blue staining

Figure 2: Pharmakokinetics of activated rec FIX and FIX-albumin fusion proteins with and without cleavable linker in comparison to non-activated fusion proteins.

Figure 3: Inactivation of activated rec FIX or FIX-albumin fusion protein by AT. Residual FIX activity was determined after 120 min using a non-activated partial thromboplastin time assay.

Examples:

Example 1: Generation of cDNAs encoding FIX and FIX - albumin fusion proteins

Factor IX coding sequence was amplified by PCR from a human liver cDNA library (ProQuest, Invitrogen) using primers We1403 and We1404 (SEQ ID NO 5 and 6). After a second round of PCR using primers We1405 and We1406 (SEQ ID NO 7 and 8) the resulting fragment was cloned into pCR4TOPO (Invitrogen). From there the FIX cDNA was transferred as an EcoRI Fragment into the EcoRI site of pIRESpuro3 (BD Biosciences) wherein an internal XhoI site had been deleted previously. The resulting plasmid was designated pFIX-496 and was the expression vector for factor IX wild-type.

For the generation of albumin fusion constructs the FIX cDNA was reamplified by PCR under standard conditions using primers We2610 and We2611 (SEQ ID NO 9 and 10) deleting the stop codon and introducing an XhoI site instead. The resulting FIX fragment was digested with restriction endonucleases EcoRI and XhoI and ligated into an EcoRI / BamH1 digested pIRESpuro3 together with one XhoI / BamH1 digested linker fragment as described below.

Two different glycine / serine linker fragments without internal cleavage sites were generated: Oligonucleotides We2148 and We2150 (SEQ ID NO 11 and 12) were annealed in equimolar concentrations (10 pmol) under standard PCR conditions, filled up and amplified using a PCR protocol of a 2 min. initial denaturation at 94°C followed by 7 cycles of 15 sec. of denaturation at 94°C, 15 sec. of annealing at 55°C and 15 sec. of elongation at 72°C, and finalized by an extension step of 5 min at 72°C. The same procedure was performed using oligonucleotides We2156 and We2157 (SEQ ID NO 13 and 14). The resulting linker fragments were digested with restriction endonucleases XhoI and BamH1 and used separately in the above

described ligation reaction. The resulting plasmids therefore contained the coding sequence for FIX and a C-terminal extension of a glycine / serine linker.

Two different cleavable linker fragments derived from the activation sites of FIX were generated: Oligonucleotides We2335 and We2336 (SEQ ID NO 15 and 16), containing the activation cleavage site of the FIX light chain / activation peptide border region, were annealed, filled, and amplified as described above. The resulting linker fragment was digested with restriction endonucleases XhoI and BamH1 and used in the above described ligation reaction. The resulting plasmid therefore contained the coding sequence for FIX and a C-terminal extension of a cleavable FIX sequence (amino acids 136 to 154 of SEQ ID NO 2). In a subsequent site directed mutagenesis reaction with a commercially available mutagenesis kit (QuickChange XL Site Directed Mutagenesis Kit, Stratagene) using oligonucleotides We2636 and We2637 (SEQ ID NO 17 and 18) the XhoI site was deleted.

For generation of the second cleavable linker fragment derived from FIX, the same procedure was performed using oligonucleotides We2337 and We2338 (SEQ ID NO 19 and 20) for linker construction. The resulting linker fragment was digested with restriction endonucleases XhoI and BamH1 and used in the above described ligation reaction. The resulting plasmid now contained the coding sequence for FIX and a C-terminal extension of a cleavable FIX sequence derived from the activation cleavage site of the FIX activation peptide / heavy chain border region (amino acids 173 to 186 of SEQ ID NO 2). Oligonucleotides We2638 and We 2639 (SEQ ID NO 21 and 22) were used for deletion of the XhoI site as described above.

In the next cloning step the above generated plasmids were digested with BamH1 and a BamH1 fragment containing the cDNA of mature human albumin was inserted. This fragment had been generated by PCR on an albumin cDNA sequence using primers We1862 and We1902 (SEQ ID NO 23 and 24) under standard conditions.

The final plasmids with non-cleavable glycine/serine linkers were designated pFIX-980 (SEQ ID NO 30) and pFIX-986 (SEQ ID NO 31), respectively. The final plasmids with cleavable linkers derived from FIX sequences were designated pFIX-1088 (SEQ ID NO 40) and pFIX-1089 (SEQ ID NO 49), respectively. Their linker sequences and the C-terminal FIX and N-terminal albumin sequences are outlined below. Proteolytic cleavage sites within the linkers are indicated with arrows, the FIX derived linker sequences are underlined.

	...FIX		Albumin...
pFIX-980	...KEKTKLT	$SS(GGS)_7$ GS	DAHKSEV...
pFIX-986	...KEKTKLT	$SSNGS(GGS)_3$ $NGS(GGS)_3$ GGNGS	DAHKSEV...
pFIX-1088	...KEKTKLT	SVSQTSKLTR ↓ AETVFPDVD GS	DAHKSEV...
pFIX-1089	...KEKTKLT	QSFNDFTR ↓ VVGGED GS	DAHKSEV...

For expression in CHO cells the coding sequences for the FIX albumin fusion protein were transferred into vectors pIRESneo3 (BD Biosciences) or pcDNA3.1 (Invitrogen), respectively.

For efficient processing of the propeptide in cells expressing FIX in high amounts coexpression of furin is required (Wasley LC et al. 1993. PACE/Furin can process the vitamin K-dependent pro-factor IX precursor within the secretory pathway. J. Biol. Chem. 268:8458-8465). Furin was amplified from a liver cDNA library (Ambion) using primers We1791 and We1792 (SEQ ID NO 25 and 26). A second round of PCR using primers We1808 and We1809 (SEQ ID NO 27 and 28) yielded a furin fragment where the carboxyterminal transmembrane domain (TM) was deleted and a stop codon introduced; this fragment was cloned into pCR4TOPO (Invitrogen). From there the furinΔTM cDNA was transferred as an EcoRI/NotI

Fragment into the EcoRI/NotI sites of pIRESpuro3 (BD Biosciences) wherein an internal XhoI site had been deleted previously. The resulting plasmid was designated pFu-797. This plasmid was cotransfected with all FIX constructs in a 1:5 (pFu-797 : pFIX-xxx) molar ratio. The amino acid sequence of the secreted furin encoded by pFu-797 is given as SEQ-ID NO 29.

Example 2: Transfection and expression of FIX and FIX-albumin fusion proteins

Plasmids were grown up in E.coli TOP10 (Invitrogen) and purified using standard protocols (Qiagen). HEK-293 cells were transfected using the Lipofectamine 2000 reagent (Invitrogen) and grown up in serum-free medium (Invitrogen 293 Express) in the presence of 50 ng/ml Vitamin K and 4 µg/ml Puromycin. Transfected cell populations were spread through T-flasks into roller bottles or small-scale fermenters from which supernatants were harvested for purification.

Alternatively, CHO K1 or DG44 cells (Invitrogen) were transfected using the Lipofectamine 2000 reagent (Invitrogen) and grown up in serum-free medium (Invitrogen CD-CHO) in the presence of 50 ng/ml Vitamin K and 500-750 ng/ml Geneticin. High expressing clones were selected and spread through T-flasks into roller bottles or small-scale fermenters from which supernatants were harvested for purification.

Example 3: Purification of FIX and FIX - albumin fusion proteins

Cell culture harvest containing FIX or FIX albumin fusion protein was applied on a Q-Sepharose FF column previously equilibrated with 50 mM TrisxHCl / 100 mM NaCl buffer pH 8.0. Subsequently, the column was washed with equilibration buffer containing 200 mM NaCl. Elution of the bound FIX or FIX fusion protein was achieved by a salt gradient using 50 mM TrisxHCl / 200 mM NaCl buffer pH 8.0 as a basis. The eluate was further purified by column chromatography on a hydroxylapatite resin. For this purpose, the eluate of the Q-Sepharose™ FF column was loaded on a hydroxylapatite chromatography column equilibrated with 50 mM TrisxHCl / 100 mM NaCl buffer pH 7.2. The column was washed with the same

buffer and FIX or FIX-HSA were eluted using a potassium phosphate gradient at pH 7.2. The eluate was dialyzed to reduce the salt concentration and used for biochemical analysis as well as for determination of the pharmacokinetic parameters. FIX antigen and activity were determined as described in example 5.

Example 4: Alternative purification scheme of FIX and FIX - albumin fusion proteins

As described in example 3, cell culture harvest containing FIX or FIX albumin fusion protein was purified by chromatography on Q-Sepharose FF. The Q-Sepharose eluate was further purified by chromatography on a Heparin-Fractogel™ column. For this purpose, the Heparin-Fractogel column was equilibrated using 50 mM Tris x HCl, 50 mM NaCl pH 8.0 buffer (EP), the Q-Sepharose FF eluate was applied and the column was washed with equilibration buffer containing 75 mM NaCl. FIX or FIX albumin fusion protein, respectively, was eluted using EP adjusted to 300 mM NaCl. The Heparin-Fractogel eluate was further purified by chromatography on a hydroxylapatite chromatography column as described in example 3. The purified FIX resp. FIX albumin fusion protein concentrate was subjected to FIX activity and antigen determination according to example 5 and characterized by further in vitro and in vivo investigations.

Example 5: Determination of FIX activity and antigen

FIX activity was determined as clotting or coagulation activity (FIX:C) using commercially available aPTT reagents (Pathromtin SL and FIX depleted plasma, Dade Behring). An internal substandard calibrated against the WHO International FIX concentrate Standard (96/854) was used as a reference.

FIX antigen (FIX:Ag) was determined by an ELISA acc. to standard protocols known to those skilled in the art. Briefly, microtiter plates were incubated with 100 µL per well of the capture antibody (Paired antibodies for FIX ELISA 1:200, Cedarlane, but other sources of appropriate antibodies may also be applied) overnight at ambient temperature. After washing plates three times with washing buffer B (Sigma P3563), each well was incubated with 200 µL blocking buffer C

(Sigma P3688) for one hour at ambient temperature. After another three wash steps with buffer B, serial dilutions of the test sample in buffer B as well as serial dilutions of a substandard (SHP) in buffer B (volumes per well: 100 µL) were incubated for two hours at ambient temperature. After three wash steps with buffer B, 100 µL of a 1:200 dilution of the detection antibody (Paired antibodies for FIX ELISA, peroxidase labelled, Cedarlane) in buffer B were added to each well and incubated for another two hours at ambient temperature. After three wash steps with buffer B, 100 µL of substrate solution (TMB, Dade Behring, OUVF) were added per well and incubated for 30 minutes at ambient temperature in the dark. Addition of 100 µL undiluted stop solution (Dade Behring, OSFA) prepared the samples for reading in a suitable microplate reader at 450 nm wavelength. Concentrations of test samples were then calculated using the standard curve with standard human plasma as reference.

Example 6: Comparison of FIX-activity/FIX-antigen ratio of different FIX-albumin fusion proteins in cell culture supernatant

Cell culture supernatants of HEK cells transfected with DNA constructs coding for FIX-albumin fusion proteins that contained different linker peptides were subjected to FIX activity and antigen testing as described above (see example 5). The ratio of FIX:C to FIX:Ag was calculated representing a measure directly proportional to molar specific activity of the different constructs.

The results shown in table 4 indicate that there is an increase in activity/antigen ratio upon introduction of cleavable linkers into the FIX-HSA molecule. It also shows that the cleavable linker peptide should have a length of more than two amino acids in order to provide clearly increased activity/antigen ratios.

Table 4: FIX:C/FIX:Ag ratios of FIX-albumin fusion proteins containing different linker peptides

FIX-HSA construct	Linker	FIX:C/FIX:Ag	Fold increase compared to fusion protein 980/797 with non-cleavable linker (GGGGGGV)
1182/797	None	< 0.031	
1366/797	RI	< 0.068	
1478/863	GGGGGGV (Sheffield et al.)	0.041	--
980/797	$SS(GGS)_7GS$	0.070	1.7
986/797	SSNGS(GGS)3NGS (GGS)3GGNGS	0.076	1.9
[illegible]	[illegible]	[illegible]	[illegible]
1483/863	SVSQTSKLT**R** AETVFPDVD GSGGS	0.688	16.8
1088/797	SVSQTSKLT**R** AETVFPDVD GS	0.832	20.3
1365/797	SVSQTSKLT**R** AETVFPDVD	0.630	15.4
1482/863	SVSQTSKLT**R** AETVFP	0.482	11.8
1087/797	SVSQTSKLT**R** AETVFPDVD GS (FIX deltaKLT)	0.472	11.5
1089/797	QSFNDFT**R** VVGGED GS	0.532	13.0
1091/797	PERGDNNLT**R** IVGGQE GS	0.111	2.7

5

Example 7: Comparison of FIX and FIX - albumin fusion proteins in respect to molar specific activity, terminal in vivo half-life and in vivo recovery in rats or rabbits

Purified recombinant wild type FIX (rFIX 496/797) and FIX-albumin fusion proteins (rFIX 980/797, rFIX 986/797, rFIX- 1088/797 and rFIX 1089/797) were tested for FIX activity in a clotting assay as described above. In parallel, the difference of the optical density at 280 and 320 nm was determined as a measure for protein concentration (OD280-320). The ratios of activity per OD280-320 were calculated and based on the molar optical densities the molar specific activities were calculated. In the following table 5 the results are summarized.

Table 5: Molar specific activities of wt FIX compared to FIX–albumin fusions

	Linker	Optical density (OD280-320)	FIX clotting activity (IU/mL)	Activity/Vol/OD (IU/mL/OD)	**Molar specific activity* (IU/nmol)**
rFIX, wt (496/797)	-	0,3798	21,2	55,8	**4,23**
rFIX-HSA (non-cleavable, 1478/863	GGGGGGV (Sheffield et al.)	2,9189	5,8	2,0	**0,23**
rFIX-HSA (non-cleavable, 980/797)	SS $(GGS)_7$ GS	1,1122	3,4	3,0	**0,35**
rFIX-HSA (non-cleavable, 986/797)	SS NGS (GGS)3 NGS (GGS)3 GGN GS	0,8107	3,2	4,0	**0,45**
rFIX-HSA (cleavable, 1088/797)	FXIa cleavable	0,3421	11,9	34,8	**3,95**
rFIX-HSA (cleavable, 1089/797)	FXIa cleavable	0,4512	11,3	25,0	**2,84**

* Molar specific activity based on activity, optical density and the following molar optical densities:

Molar optical density of FIX: OD(280nm, 1 mol/L) = 75 810

Molar optical density of albumin: OD(280nm, 1 mol/L) = 37 791

Molar optical density of FIX-albumin fusion protein: OD(280nm, 1 mol/L) = 113 601

Taking the results summarized in Table 5 into account, it is surprising that two constructs that were generated according to the present invention show highly increased molar specific activities compared to the fusion proteins with non-

cleavable linkers. In addition, the molar specific activity of these constructs was only moderately decreased compared to wild type rFIX.

In vitro investigations of the proteolytic cleavage reactions by Factor XIa (FXIa) confirmed that FIX-albumin fusion proteins containing a cleavable linker like e.g. construct no. 1088/797 or 1089/797 are activated and in parallel the linker is cleaved resulting in release of the albumin moiety (Figure 1). The fusion protein with non-cleavable linker did not show a corresponding release of the albumin moiety.

In the case of FVIIa as cleaving protease in the presence of tissue factor, the FIX-albumin fusion proteins 1088/797 or 1089/797 containing a cleavable linker also showed release of the albumin moiety in parallel to release of the FIX activation peptide (Data not shown).

In addition to determination of molar specific coagulation activity, the polypeptides no. 496/797, 980/797, 986/797, 1088/797 and 1089/797 described above were administered intravenously to narcotized CD / Lewis rats (6 rats per substance) and/or rabbits (4 rabbits per substance) with a dose of 50 IU/kg body weight. Blood samples were drawn prior to test substance administration and at appropriate intervals starting at 5 minutes after administration of the test substances. FIX antigen content was subsequently quantified by an ELISA assay specific for human Factor IX (see above). The mean values of the respective groups were used to calculate in vivo recovery after 5 min. Half-lives for each protein were calculated using the time points of the beta phase of elimination (terminal half-life) according to the formula $t_{1/2} = \ln 2 / k$, whereas k is the slope of the regression line obtained upon plotting FIX:Ag levels in logarithmic scale and time in linear scale.

Calculated in vivo half-lives are summarized in table 6. In rats as well as in rabbits the in vivo half-lives of the FIX-albumin fusion proteins were found to be significantly increased in comparison to non-fused wild-type recombinant FIX

prepared inhouse or in comparison to the commercially available recombinant FIX product BeneFIX®. The in vivo half-lives of the albumin fusion proteins compared to BeneFIX® were increased to about 200-400%, depending on the animal species or construct used (Table 6).

To evaluate the in vivo recovery, the FIX antigen levels measured per mL of plasma at their maximum concentrations after intravenous administration (t = 5 min) were related to the amount of product applied per kg. Alternatively, a percentage was calculated by relating the determined antigen level (IU/mL) 5 min post infusion to the theoretical product level expected at 100 % recovery (product applied per kg divided by an assumed plasma volume of 40 mL per kg). The in vivo recoveries (IVR) of the FIX-albumin fusion proteins were significantly higher than the in vitro recoveries of rFIX (496/797) or BeneFIX® (Table 7).

Table 6:

Terminal in vivo half-lives of FIX preparations derived from recombinant expression (BeneFIX®, rFIX 496/797) and FIX albumin fusion proteins (rFIX 980/797, rFIX 986/797, rFIX 1088/797, and rFIX 1089/797) after intravenous administration of 50 IU/kg into rats and/or 50 IU/kg into rabbits, respectively.

	Rat experiments PSR18-05, PSR06-05, PSR02-05		**Rabbit experiment** PSK11-05	
	Terminal half-life (h)	relative to BeneFIX [%]	Terminal half-life (h)	relative to BeneFIX [%]
rFIX 496/797	4,5*	91	n.t.	n.t.
rFIX 980/797	11,6*	234	36,9° 29,3° (2nd exp.)	410 326
rFIX 986/797	10,5*	212	n.t.	n.t.
rFIX 1088/797	8,3*	168	30,3°	337
rFIX 1089/797	10,5*	212	n.t.	n.t.
BeneFIX	4,95* (mean of 5,3 and 4,6)	100	9,0°	100

* Determined between 120 and 1440 min

° Determined between 4 and 96 h

Table 7:

In vivo recoveries (amount of substance 5 minutes post administration) of recombinant FIX preparations (BeneFIX, rFIX 496/797) and FIX albumin fusion proteins (rFIX 1088/797, rFIX 1089/797) after intravenous administration of 50 IU /kg into rats. The percentage of in vivo recovery was calculated based on an assumed plasma volume of 40 mL/kg.

	rat experiment	
	in vivo recovery IU/dL per IU/kg / [%]*	relative to BeneFIX [%]
rFIX 496/797	0.462 / 18.5	74.6
rFIX 1088/797	1.034 / 41.4	166.5
rFIX 1089/797	1.063 / 42.5	171.2
BeneFIX	0.621 / 24.8	100

* Calculated based on a plasma volume of 40 mL/kg

Example 8: In vitro activation of FIX albumin fusion proteins with/without cleavable linker (1088/797 and 980/797) and determination of pharmacokinetics in rats

FIX-albumin fusion proteins and rec FIX were activated in vitro using commercially available Factor XIa (Kordia). Briefly, identical molar amounts of FIX or FIX-albumin fusion protein (3.0×10^{-6} mol/L) were activated at 37°C in solution in the presence of FXIa (1.9×10^{-8} mol/L) and $CaCl_2$ (1,5 mmol/L) buffered at pH 6.8. After complete activation as shown by SDS-PAGE the reaction was stopped by addition of a 5x molar excess of C1-Inhibitor (Berinert P) based on the amount of FXIa. The samples were stored frozen below –70°C until start of pharmacokinetic investigation.

A pharmacokinetic investigation of the activated FIX and the FIX-albumin fusion proteins was performed in rats as described in example 7 and the results were compared to a pharmacokinetic results covering non-activated fusion proteins.
It turned out that the activated fusion proteins demonstrated significantly reduced half-lifes as well as AUC's compared to the non-activated molecules (Figure 2). Upon activation the FIX-fusion protein with cleavable linker (1088/797) showed a pharmacokinetic behaviour very similar to activated rec FIX (BeneFIX) whereas the activated fusion protein with non-cleavable linker (980/797) resulted in a higher initial as well as terminal half-life compared to activated fusion protein 1088/797 with cleavable linker. Therefore, the results clearly demonstrate that the cleavable linker results in increased elimination of the coagulation factor after activation and, therefore, avoids accumulation of potentially thrombogenic, activated fusion proteins with extended half-lives.

Example 9: Comparison of FIX - albumin fusion proteins with/without cleavable linker in respect to inactivation rate of the activated coagulation factors by antithrombin III (AT)

FIX fusion proteins with (1088/797) and without (980/797) cleavable linker were activated by incubation with FXIa as described in example 8. The activated factors were incubated with AT for 120 min and residual FIXa activity was determined using a manual FIX clotting assay method without activation (naPTT, see below). As control samples the activated FIX-albumin fusion proteins were used in presence of the same amount of AT but without incubation.

The F IX activity was determined with the aid of a non-activated partial thromboplastin time assay (naPTT) using FIX deficient plasma from Dade Behring. The samples were prediluted in a buffer of pH 6.8 containing His, Gly, Sucrose, and Tween™ 80. The whole determination was performed using coagulometers acc. to Schnitger & Gross™. A mixture of 0.1 ml F IX deficient plasma, 0.1 ml sample, and 0.1 ml of 0,1 % Phospholipids (Rhone-Poulenc-Nattermann, 1:3 prediluted in imidazole buffer supplemented with 1 % HSA) was incubated for 2 minutes at 37 °C. The coagulation reaction was initiated by adding 0.1 ml 0.025 mol/l $CaCl_2$ solution and the clotting time was determined.

Figure 3 shows the results of a corresponding inactivation experiment. In the case of the fusion protein with cleavable linker (1088/797) an increase in clotting time from 210 to 540 sec (factor of 2.57x) demonstrated an accelerated inactivation process of FIXa activity by AT compared to a fusion protein with non-cleavable linker (980/797) that only showed an increase from 196 to 411 sec (factor of 2.10 x). Most probably, the albumin residue sterically affects the AT dependent inactivation process in the case of the fusion protein with non-cleavable linker whereas in the case of the fusion protein with cleavable linker the albumin residue is cleaved off resulting in an accelerated inactivation by AT.

Claims:

1. Therapeutic fusion protein comprising
   a) a coagulation factor,
   b) a half-life enhancing polypeptide selected from the group consisting of albumin, polypeptides of the albumin family, and immunoglobulins without an antigen binding domain, and
   c) a peptide linker which joins the coagulation factor to the N- or C-terminus of the half-life enhancing polypeptide;

wherein the peptide linker is cleavable by proteases involved in coagulation or activated by coagulation enzymes and in that the therapeutic fusion protein has in comparison to the respective therapeutic fusion protein linked by a non-cleavable linker having the amino acid sequence GGGGGGV an increased molar specific activity in at least one coagulation-related assay and wherein the molar specific activity is increased by at least 100%.

2. Therapeutic fusion protein according to claim 1 wherein the coagulation factor is a vitamin-K dependent coagulation factor.

3. Therapeutic fusion protein according to claim 1 or 2 wherein the coagulation factor is FVIIa or FIX.

4. Therapeutic fusion protein according to any one of claims 1 to 3 wherein the half life enhancing polypeptide is an immunoglobulin without an antigen binding domain.

5. Therapeutic fusion protein according to any one of claims 1 to 4 wherein the linker is cleavable by FXIa or FVIIa/TF.

6. Therapeutic fusion protein according to any one of claims 1 to 5, wherein the linker is cleavable by the protease or proteases, which activate the coagulation factor.

7. Therapeutic fusion protein according to claim 6, wherein the kinetics of the linker cleavage by the protease or proteases is not delayed by more than a factor of 3 compared to the kinetics of the activation of said coagulation factor.

8. Therapeutic fusion protein according to any one of claims 1 to 7, wherein the linker is cleavable by the protease or proteases that are activated directly or indirectly by the activity of the coagulation factor.

9. Therapeutic fusion protein according to any one of claims 1 to 8, wherein the linker is cleavable by FXIa or by FVIIa/TF and the coagulation factor is FIX.

10. Therapeutic fusion protein according to any one of claims 1 to 9, wherein the linker is cleavable by FXa or by FVIIa/TF and the coagulation factor is FVIIa.

11. Therapeutic fusion protein according to any one of claims 1 to 10, wherein the linker comprises a sequence selected from the group consisting of: SEQ ID NOS: 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111 and 112.

12. Therapeutic fusion protein according to any one of claims 1 to 11 for use as a medicament for the treatment or prevention of a blood coagulation disorder.

13. A polynucleotide encoding a therapeutic fusion protein according to any one of claims 1 to 12.

14. A plasmid or vector comprising a nucleic acid according to claim 13.

15. A plasmid or vector according to claim 14, which is an expression vector.

16. A plasmid or vector according to claim 15, wherein the vector is a transfer vector for use in human gene therapy.

17. A host cell comprising a polynucleotide according to claim 13 or a plasmid or vector according to any one of claims 14 to 16.

18. A method of producing a therapeutic fusion protein according to any one of claims 1 to 11, comprising culturing host cells according to claim 17 under conditions such that the therapeutic fusion protein is expressed and recovering the therapeutic fusion protein from the host cells or from the culture medium.

19. A pharmaceutical composition comprising a therapeutic fusion protein according to any one of claims 1 to 11, a polynucleotide according to claim 13, or a plasmid or vector according to any one of claims 14 to 16; and a pharmaceutically acceptable carrier.

20. A use of a therapeutic fusion protein according to any one of claims 1 to 11, of a polynucleotide according to claim 13, of a plasmid or vector according to any one of claims 14 to 16, or of a host cell according to claim 17 for the treatment or prevention of a blood coagulation disorder.

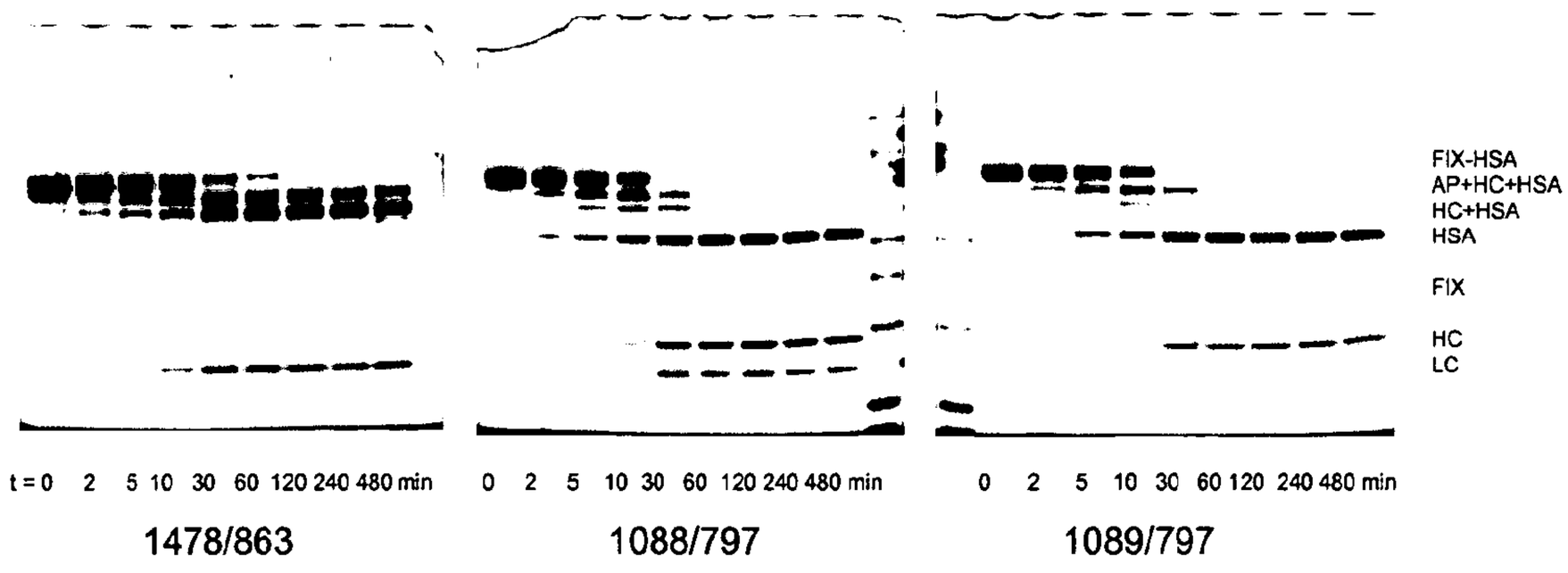
21. A use of a therapeutic fusion protein according to any one of claims 1 to 11, of a polynucleotide according to claim 13, of a plasmid or vector according to any one of claims 14 to 16, or of a host cell according to claim 17 for the manufacture of a medicament for the treatment or prevention of a blood coagulation disorder.

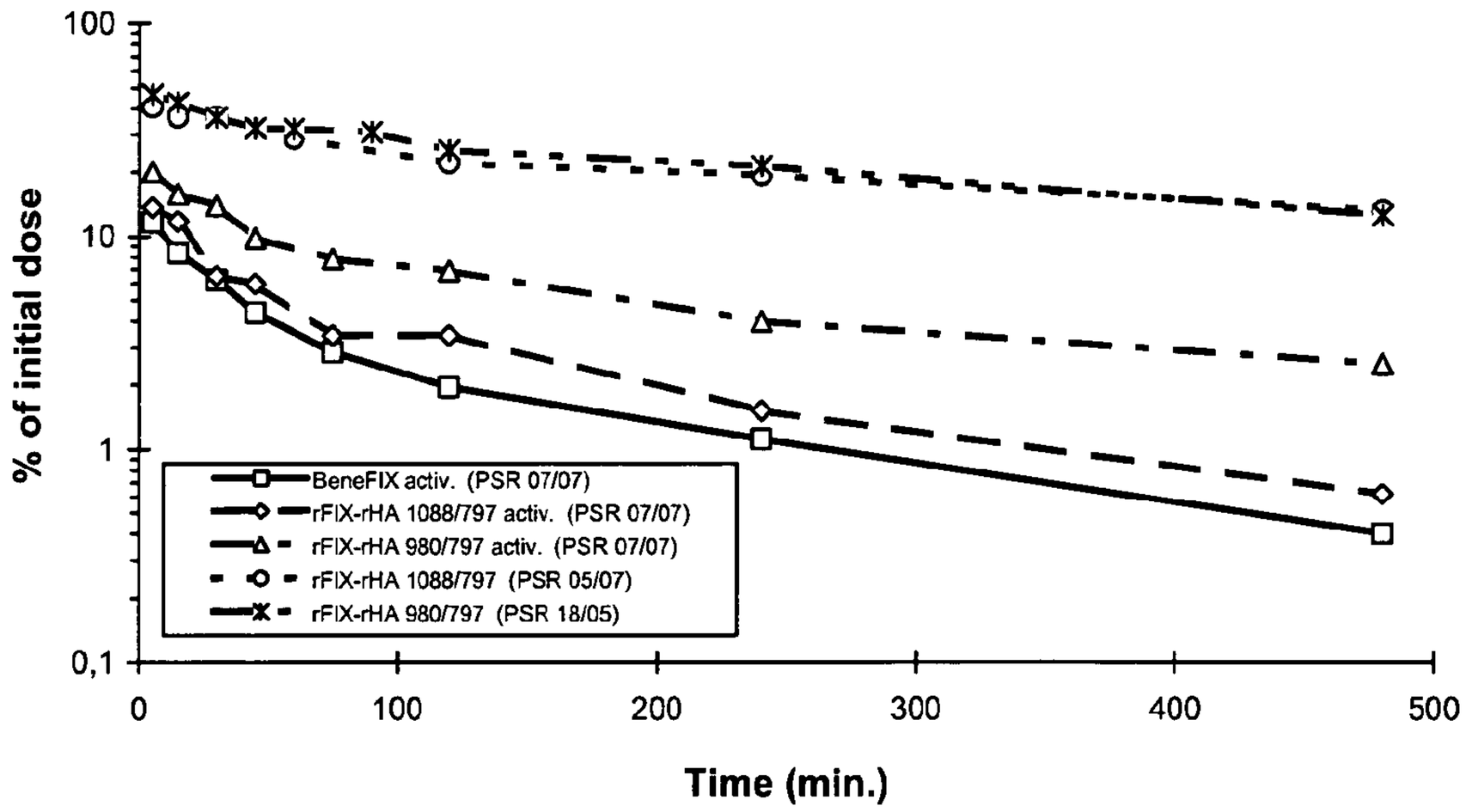
22. The use according to claim 20 or 21, wherein the blood coagulation disorder is hemophilia B.

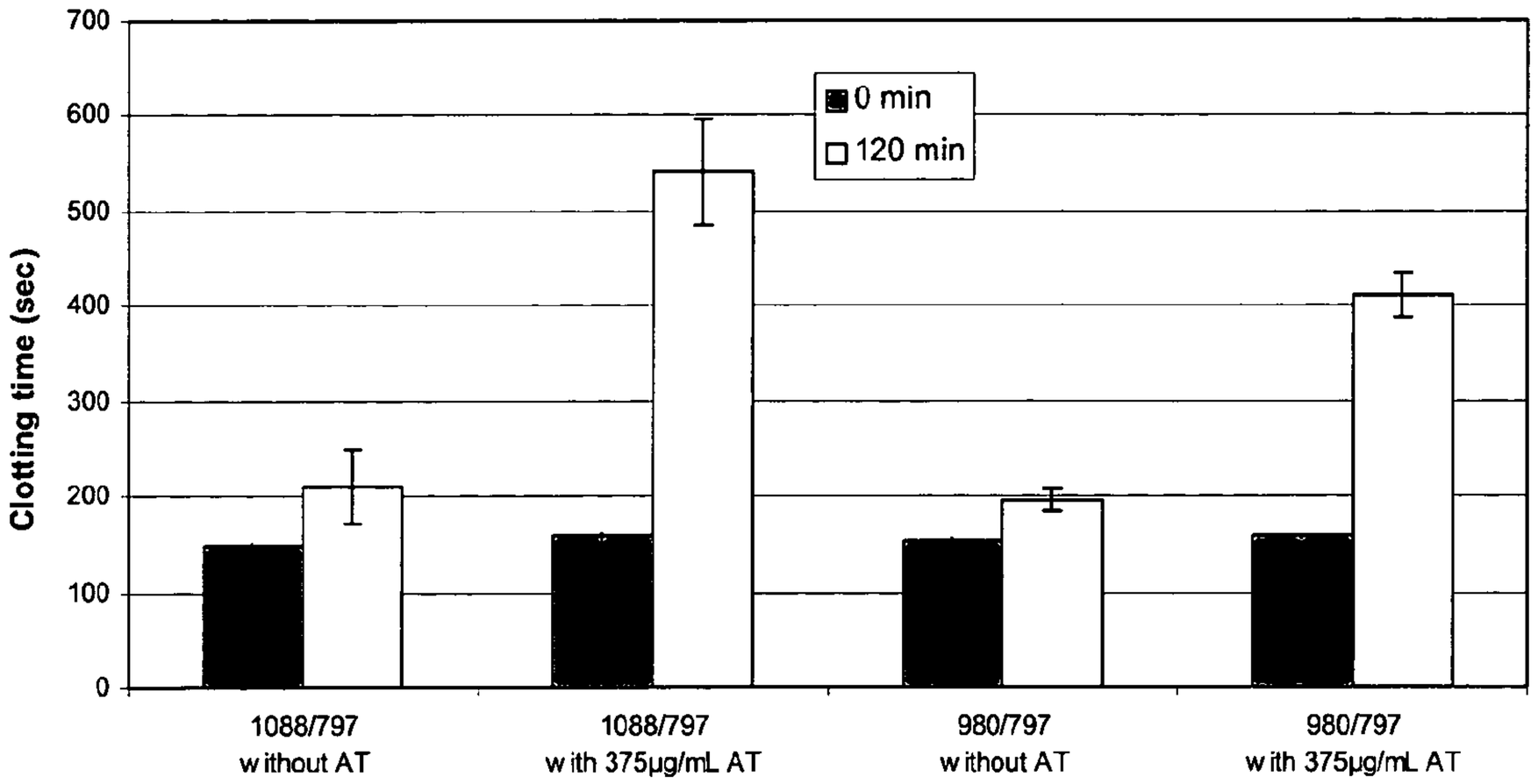
23. The use according to claim 20 or 21, wherein the blood coagulation disorder is FVII and/or FVIIa deficiency.

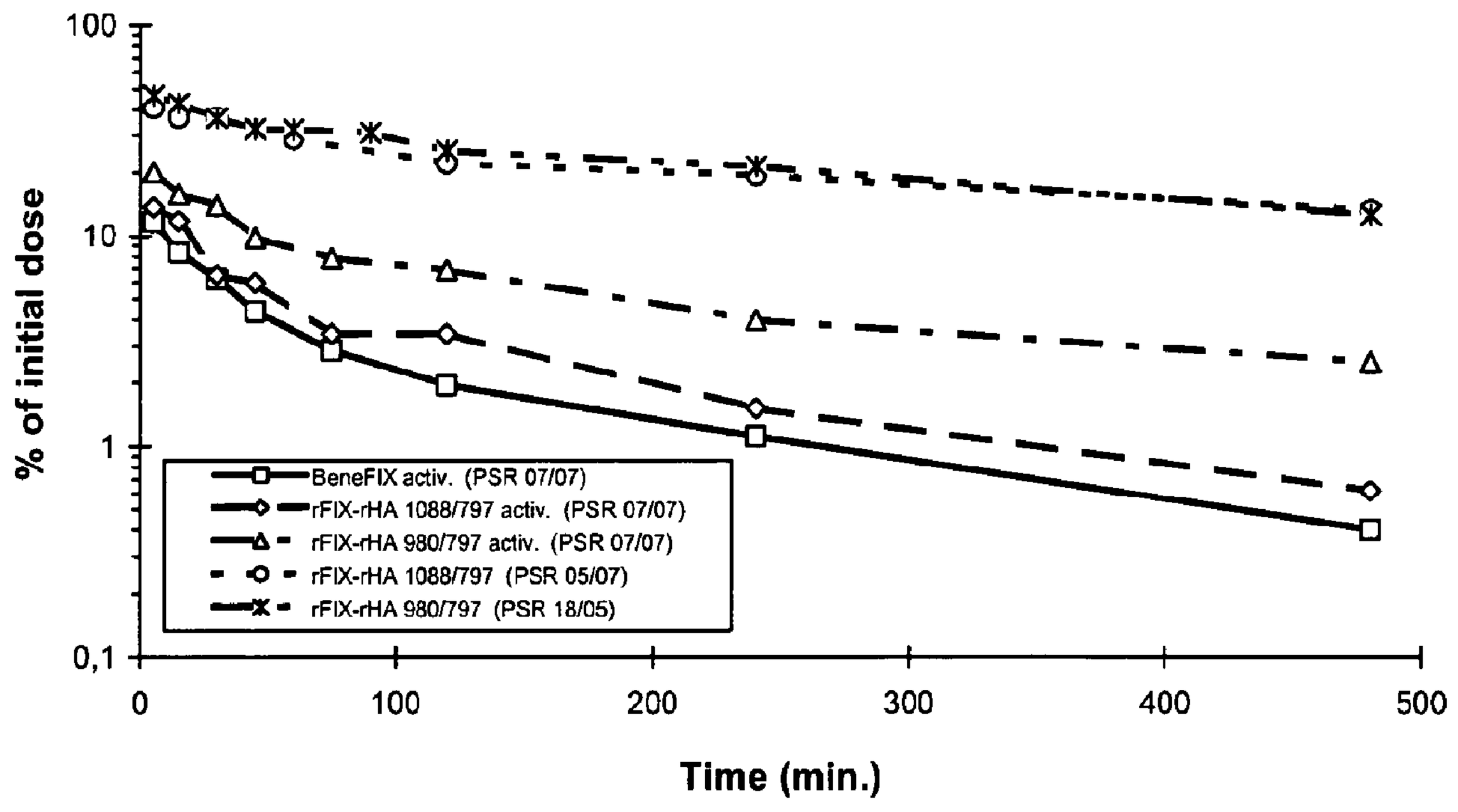
24. The use according to claim 20 or 21, wherein the blood coagulation disorder is hemophilia A.

25. The use according to any one of claims 20 to 24, wherein the treatment comprises human gene therapy.


FIX-HSA
AP+HC+HSA
HC+HSA
HSA
FIX
HC
LC
t = 0 2 5 10 30 60 120 240 480 min
0 2 5 10 30 60 120 240 480 min
0 2 5 10 30 60 120 240 480 min
1478/863
1088/797
1089/797


100
10
1
0,1
% of initial dose
0
100
200
300
400
500
Time (min.)
BeneFIX activ. (PSR 07/07)
rFIX-rHA 1088/797 activ. (PSR 07/07)
rFIX-rHA 980/797 activ. (PSR 07/07)
rFIX-rHA 1088/797 (PSR 05/07)
rFIX-rHA 980/797 (PSR 18/05)


700
600
500
400
300
200
100
0
Clotting time (sec)
0 min
120 min
1088/797
without AT
1088/797
with 375µg/mL AT
980/797
without AT
980/797
with 375µg/mL AT


100
10
1
0,1
% of initial dose
0
100
200
300
400
500
Time (min.)
BeneFIX activ. (PSR 07/07)
rFIX-rHA 1088/797 activ. (PSR 07/07)
rFIX-rHA 980/797 activ. (PSR 07/07)
rFIX-rHA 1088/797 (PSR 05/07)
rFIX-rHA 980/797 (PSR 18/05)