Title: ZYMOGEN-LIKE PROTEIN C POLYPEPTIDES

Precursor protein C variant

One-chain zymogen protein C variant

Activated zymogen-like protein C variant

Abstract: The invention relates to protein C variants wherein the cleavage site between the light chain and the heavy chain has been removed, in particular by substitution of K156 and/or R157, with the proviso that the substitutions are not K156R and R157K. Such variants have a lowered anticoagulant activity while retaining their amidolytic and anti-inflammatory properties.
For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.
ZYMOGEN-LIKE PROTEIN C POLYPEPTIDES

FIELD OF THE INVENTION

The present invention relates to protein C variants wherein the cleavage site between the light chain and the heavy chain has been removed. Thus, activated variants according to the invention are activated two-chain forms of protein C and include the light chain covalently linked to the activation peptide, optionally via a dipeptide which is different from the naturally occurring Lys-Arg dipeptide, and the activated heavy chain. Accordingly, in its activated form the bond between the activation peptide and the activated heavy chain is cleaved (i.e. cleavage of the bond between residues 169 and 170). Such variants are contemplated to be useful due to having a lowered anticoagulant activity while retaining their amidolytic and anti-inflammatory properties. It is thus contemplated that clinical use of such variants will be safer (compared to use of, e.g., Xigris®, commercially available from Eli Lilly) since the bleeding risks will be eliminated or at least reduced.

BACKGROUND OF THE INVENTION

Blood coagulation is a process consisting of a complex interaction of various blood components, or factors, which eventually give rise to a fibrin clot. Generally, blood components participating in the coagulation "cascade" are proenzymes or zymogens, i.e. enzymatically inactive proteins that are converted into an active form by action of an activator. Negative regulation of blood coagulation by activated protein C (APC) is accomplished enzymatically by proteolytic inactivation of the procoagulation factors Va and VIIIa (Esmon, J Biol Chem 1989; 264; 4743-4746).

Protein C is a serine protease that circulates in the plasma as a zymogen with a half-life of approximately 7 hours, and plasma levels are typically in the range of 3-5 µg/l. It is produced in vivo in the liver as a single chain precursor polypeptide of 461 amino acids. This polypeptide undergoes multiple post-translational modifications including a) cleavage of a 42 amino acid pre-pro sequence; b) cleavage of lysine and arginine residues (positions 156 and 157) to make a two-chain inactive zymogen (a 155 amino acid light chain attached via a disulfide bridge to a 262 amino acid heavy chain); c) vitamin K-dependent carboxylation of nine glutamic acid residues of the light chain resulting in nine gamma-carboxyglutamic acid residues in the N-terminal region of the light chain; and d) carbohydrate attachment at four sites (one in the light chain and three in the heavy chain). Finally, the two-chain zymogen may be
activated by removal of a dodecapeptide (the activation peptide) at the N-terminus of the heavy chain (positions 158-169) producing the activated protein C (APC).

A problem with activation of protein C via the route described above (see also the description of Figure 1 below) is incomplete removal of the Lys-Arg dipeptide in positions 156-157, resulting in incompletely processed and thus inactive protein C in a protein C product otherwise intended to be in the form of activated protein C. US 5,516,650 discloses modifications that enhance the cleavage of protein C between the light chain and the heavy chain, e.g. by introduction of the residues Arg-Arg after position 155.

Protein C is activated by limited proteolysis by thrombin in complex with thrombomodulin on the luminal surface of the endothelial cell. As explained above, activation liberates a small 12 amino acid peptide (designated the activation peptide) from the N-terminal of the heavy chain. The hAPC has an enzymatic (i.e. functional) half-life of approximately 13 minutes in plasma (FDA Clinical Review, Drotrecogin alfa (activated), BLA# 125029/0, November 21, 2001).

In the presence of its cofactor, protein S, APC proteolytically inactivates factors Va and VIIIa, thereby reducing thrombin generation (Esmon, Thromb Haemost 1993; 70; 29-35). Protein S circulates reversibly bound to another plasma protein, C4b-binding protein. Only free protein S serves as a cofactor for APC. Since C4b-binding protein is an acute phase reactant, the plasma levels of this protein varies greatly in many diseases and thus influence the anticoagulant activity of the protein C system.

The gene encoding human protein C maps to chromosome 2q13-q14 (Patracchini et al., Hum Genet 1989; 81; 191-192) spans over 11 kb, and comprises a coding region (exons II to IX) and a 5' untranslatable region encompassing exon I. The protein domains encoded by exons II to IX show considerable homology with other vitamin K-dependent coagulation proteins such as factor IX and X. Exon II codes for a signal peptide, while exon III codes for a propeptide and a 38 amino acid sequence containing 9 Glu residues. The propeptide contains a binding site for the carboxylase transforming the Glu residues into dicarboxylic acid (Gla) able to bind calcium ions, a step required for phospholipid binding (Cheung et al., Arch Biochem Biophys 1989; 274; 574-581). Exons IV, V and VI encodes a short connection sequence and two EGF-like domains, respectively. Exon VII encodes both a domain encompassing a 12 amino acid activation peptide released after activation of protein C by thrombin, and the dipeptide 156-157 which, when cleaved off, yields the mature two-chain form of the protein. Exons VIII and IX encodes the serine protease domain.
The complete amino acid sequence of human protein C has been reported by Foster et al., PNAS. USA 1986; 82; 4673-4677 and includes a signal peptide, a propeptide, a light chain, a heavy chain and an activation peptide.

APC is inhibited in the plasma by the protein C inhibitor as well as by alpha-1-antitrypsin and alpha-2-macroglobulin.

The experimental three-dimensional structure of hAPC (in a Gla-domainless form) has been determined to 2.8 Å resolution and reported by Mather et al., EMBO J 1996; 15; 6822-6831. The structure included a covalently bound inhibitor (D-Phe-Pro-Arg chloromethylketone, PPACK).

Protein C may be isolated from prothrombin concentrates produced by monoclonal antibody affinity chromatography. Furthermore, protein C may be produced recombinantly by expression from mammalian cells.

APC is used for the treatment of genetic and acquired protein C deficiency and is suggested to be used as anticoagulant in patients with some forms of Lupus, following stroke or myocardial infarction, after venous thrombosis, disseminated intravascular coagulation (DIC), septic shock, emboli such as pulmonary emboli, transplantation, such as bone marrow transplantation, burns, pregnancy, major surgery/trauma and adult respiratory stress syndrome (ARDS).

Recombinant hAPC (Xigris®) is produced and marketed by Eli Lilly. One major problem in use of hAPC (Xigris®), which has been approved by the FDA for the treatment of severe sepsis, is the severe bleeding risks associated therewith. Accordingly, Xigris® has been approved only for “adult patients with severe sepsis who have a high risk of death (at least one dysfunctional major organ, APACHE II score above 25) with low risk of bleeding complications“. Evidently, a large group of sepsis patients are not eligible for treatment with Xigris® due to the high bleeding risks, and there is thus a need for a modified product which is suitable for treatment of sepsis but which is safe to use, i.e. which has a lowered anticoagulant activity and therefore a lower bleeding risk.

It is now well established that an excessive inflammatory response accompanies the initial stages of sepsis and appears to contribute to associated organ system failure and death (Arndt et al. Intensive Care Med 27, S104-S115, 2001). Thus, the anti-inflammatory properties of an anti-sepsis drug are crucial.

The present invention provides activated zymogen-like protein C variants having a reduced anticoagulant activity as compared to hAPC yet retained anti-inflammatory proper-
ties. This opens up the possibility of using such variants as medicinal agents, e.g. in the treatment of sepsis and septic shock, since the important anti-inflammatory properties are retained and the product is safe to use due to the reduced anticoagulant activity of the activated zymogen-like protein C variants.

Thus, an object of the present invention is to provide protein C polypeptides having a reduced anticoagulant activity and which substantially retain the anti-inflammatory properties of hAPC. Such polypeptides are contemplated to be suitable for treatment of various diseases, in particular diseases where reduced inflammation is desirable, such as in sepsis.

Another problem in current sepsis treatment is the short half-life of hAPC. This means that relatively high doses and frequent administration is necessary (typically as continuous infusion, see Bernard et al. N England J M 344 (2001), pp. 699-709) to reach and sustain the desired therapeutic or prophylactic effects of hAPC. As a consequence adequate dose regulation is difficult to obtain and the need for continuous infusion of high levels of hAPC is problematic and expensive.

Thus, another object of the present invention is to provide protein C polypeptides which, in addition to the before-mentioned properties (i.e. reduced anticoagulant activity and retained anti-inflammatory activity), have an increased functional half-life as compared to hAPC. A number of suitable modifications giving rise to an increased functional half-life are described in WO 02/32461.

The above-mentioned problems are addressed by the zymogen-like protein C polypeptides disclosed herein.

**BRIEF DISCLOSURE OF THE INVENTION**

Thus, in a first aspect the present invention relates to a protein C variant wherein the cleavage site between the light chain and the heavy chain has been removed. More particularly, at least one of K156 and/or R157 have been removed, with the proviso that K156 is not removed by the substitution K156R and that R157 is not removed by the substitution R157K.

In a further aspect the present invention relates to a nucleotide sequence encoding the variant of the invention.

In still further aspects the present invention relates to an expression vector comprising the nucleotide sequence of the invention; and to a host cell comprising the nucleotide sequence of the invention or the expression vector of the invention.
In even further aspects the present invention relates to a pharmaceutical composition comprising the variant of the invention; to the variant of the invention for use as a medicament; to the use of the variant of the invention for the manufacture of a medicament for the treatment of sepsis or septic shock; and to a method for treating a patient having sepsis or septic shock (or a patient at risk of acquiring sepsis or septic shock) which comprises administering to said patient an effective amount of the variant of the invention or an effective amount of the pharmaceutical composition of the invention.

Further aspects of the present invention will be apparent from the disclosure below.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates the route by which APC is synthesized. Formation of the mature one-chain zymogen protein C molecule arises from the precursor protein C molecule by cleavage and removal of the pre-pro peptide consisting of amino acid residues -42 to -1 of SEQ ID NO:2. Next, the Lys-Arg dipeptide (residues 156 and 157) is removed to form the two-chain zymogen protein C molecule. The activation of the two-chain zymogen molecule into activated protein C involves the proteolytic cleavage of an Arg-Leu bond (residues 169 and 170). This latter cleavage releases the activation peptide (residues 158-169).

In Figure 1 the following abbreviations are used: pre-pro: pre-pro peptide; LC: light chain; KR: Lys-Arg dipeptide; AP: activation peptide; aHC: activated heavy chain; HC:

heavy chain.

Figure 2 illustrates the route by which the activated zymogen-like protein C variant of the invention is synthesized. Formation of the mature one-chain zymogen protein C variant arises from the precursor protein C variant by cleavage and removal of the pre-pro peptide consisting of amino acid residues -42 to -1. Since the naturally occurring cleavage site between the light chain and the heavy chain has been removed (illustrated by XX) the two-chain zymogen molecule is not formed. This has been experimentally confirmed by Western blotting, which for two different protein C variants each having the substitutions K156A and R157A showed that the variants were expressed as a single chain and not as two separate chains. The activation of the one-chain zymogen protein C variant into the activated zymogen-like protein C variant involves the proteolytic cleavage of an Arg-Leu bond (residues 169 and 170). This latter cleavage releases the activation peptide (residues 158-169) from the heavy chain, but not from the light chain.
In Figure 2 the following abbreviations are used: pre-pro: pre-pro peptide; LC: light
chain; XX: removed Lys-Arg dipeptide (by substitution, deletion, etc.); AP: activation pep-
tide; aHC: activated heavy chain; HC: heavy chain.

5 DETAILED DISCLOSURE OF THE INVENTION

Definitions

In the context of the present application and invention the following definitions ap-
ply:

The term “precursor protein C” or “precursor human protein C” refers to the DNA-
encoded form of protein C, i.e. it includes the signal peptide (residues –42 to –1), the light
chain (residues 1-155), the Lys-Arg dipeptide (residues 156-157) and the heavy chain (158-
419) including the activation peptide (residues 158-169), shown in SEQ ID NO:2.

The term “one-chain zymogen protein C” or “one-chain zymogen human protein C”
refers to an inactive form of protein C, which includes the light chain (residues 1-155), the
Lys-Arg dipeptide (residues 156-157) and the heavy chain (residues 158-419) including the
activation peptide (residues 158-169), shown in SEQ ID NO:1.

The term “two-chain zymogen protein C” or “two-chain zymogen human protein C”
refers to the secreted, inactive form of protein C, which includes the light chain (residues 1-
155) and the heavy chain (residues 158-419) including the activation peptide (residues 158-
169), shown in SEQ ID NO:1.

Whenever the term “zymogen protein C” or “zymogen human protein C” is used,
this term refers to both the one-chain form and the two-chain form of the zymogen protein C.

The terms “activated protein C”, “activated human protein C”, “APC” and “hAPC”
are used about the activated zymogen and include the light chain (residues 1-155) and the
activated heavy chain (residues 170-419) of SEQ ID NO:1, i.e. the heavy chain without the
activation peptide.

The term “protein C” or “human protein C” encompasses all of the above-mentioned
forms of protein C, i.e. the “precursor protein C” form, the “zymogen protein C” form (the
one-chain form as well as the two-chain form) and the “activated protein C form”.

The term “activated zymogen-like protein C” is used about an activated two-chain
form of protein C, and includes the light chain (residues 1-155) covalently linked to the activa-
tion peptide (residues 158-169), optionally via a dipeptide (residues 156-157) which is dif-
f erent from the naturally occurring Lys-Arg dipeptide, and the activated heavy chain (residues
170-419) of SEQ ID NO:1. It will be understood that the “activated zymogen-like protein C” molecule is in a two-chain form wherein the bond between the activation peptide and the activated heavy chain is cleaved (i.e. cleavage of the bond between residues 169 and 170). The resulting two chains are kept together by S-S disulphide bonds.

The “pre-pro peptide”, as used herein, is constituted by amino acid residues -42 to -1 of SEQ ID NO:2.

The “light chain”, as used herein, is constituted by amino acid residues 1-155 of SEQ ID NO:1.

The “heavy chain”, as used herein, is constituted by amino acid residues 158-419 of SEQ ID NO:1. The heavy chain may be divided into two parts; the “activation peptide” which is constituted by amino acid residues 158-169 of SEQ ID NO:1 and the “activated heavy chain” which is constituted by amino acid residues 170-419 of SEQ ID NO:1.

The “cleavage site between the light chain and the heavy chain” is constituted by the Lys-Arg residues at positions 156 and 157 of SEQ ID NO:1.

When used herein, the term “Lys-Arg dipeptide” refers to residues 156 and 157 of SEQ ID NO:1.

The “Gla domain”, as used herein, is constituted by amino acid residues 1-45 of SEQ ID NO:1.

The “EGF domains”, as used herein, is constituted by amino acid residues 55-134 of SEQ ID NO:1.


The “autolysis loop” is constituted by amino acid residues 306-314 of SEQ ID NO:1.
Amino acid names and atom names (e.g. CA, CB, CD, CG, SG, NZ, N, O, C, etc.) are used as defined by the Protein Data Bank (PDB) (www.pdb.org), which is based on the IUPAC nomenclature (IUPAC Nomenclature and Symbolism for Amino Acids and Peptides (residue names, atom names, etc.), Eur. J. Biochem., 138, 9-37 (1984) together with their corrections in Eur. J. Biochem., 152, 1 (1985)).

The term “amino acid residue” is intended to include any natural or synthetic amino acid residue, and is primarily intended to indicate an amino acid residue contained in the group consisting of the 20 naturally occurring amino acids, i.e. selected from the group consisting of alanine (Ala or A), cysteine (Cys or C), aspartic acid (Asp or D), glutamic acid (Glu or E), phenylalanine (Phe or F), glycine (Gly or G), histidine (His or H), isoleucine (Ile or I), lysine (Lys or K), leucine (Leu or L), methionine (Met or M), asparagine (Asn or N), proline (Pro or P), glutamine (Gln or Q), arginine (Arg or R), serine (Ser or S), threonine (Thr or T), valine (Val or V), tryptophan (Trp or W) and tyrosine (Tyr or Y) residues.

The terminology used for identifying amino acid positions/substitutions is illustrated as follows: A39 in a given amino acid sequence indicates that position number 39 is occupied by an alanine residue. A39S indicates that the alanine residue of position 39 is substituted with a serine residue. Alternative substitutions are indicated with a “/”, e.g., A39S/T means that the alanine residue of position 39 is substituted with either a serine residue or a threonine residue. Multiple substitutions are indicated with a “+”, e.g., A39S+K251N means that the alanine residue of position 39 is substituted with a serine residue and that the lysine residue in position 251 is substituted with an asparagine residue. The insertion of an additional amino acid residue is indicated in the following way: Insertion of a serine residue after A39 is indicated by A39AS. A deletion of an amino acid residue is indicated by an asterix. For example, deletion of the alanine residue of position 39 is indicated by A39*. Unless otherwise indicated, the numbering of amino acid residues made herein is made relative to the amino acid sequence of SEQ ID NO:1.

The term “differs” or “differs from” when used in connection with specific mutations is intended to allow for additional differences being present apart from the specified amino acid difference. For instance, in addition to polypeptides wherein the cleavage site between the light chain and the heavy chain has been removed as disclosed herein, the protein C polypeptide can comprise other substitutions, insertions or deletions which are not related to this modification. Thus, the protein C variant of the invention may, if desired, contain other alterations that need not be related to the light chain-heavy chain cleavage site. Such alterations,
e.g. with the aim of introducing at least one site for conjugation to a non-polypeptide moiety, may e.g. be performed with the aim of increasing the anti-inflammatory effect, increasing the half-life and/or lowering the anticoagulant activity of the variant. Additional alterations may further include, for example, truncation of the N- and/or C-terminus by one or more amino acid residues, or addition of one or more extra residues at the N- and/or C-terminus, e.g. addition of a methionine residue at the N-terminus as well as “conservative amino acid substitutions”, i.e. substitutions performed within groups of amino acids with similar characteristics, e.g. small amino acids, acidic amino acids, polar amino acids, basic amino acids, hydrophobic amino acids and aromatic amino acids.

Examples of conservative substitutions include amino acids within the respective groups listed in the table below.

<table>
<thead>
<tr>
<th></th>
<th>Alanine (A)</th>
<th>Glycine (G)</th>
<th>Serine (S)</th>
<th>Threonine (T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Aspartic acid (D)</td>
<td>Glutamic acid (E)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Asparagine (N)</td>
<td>Glutamine (Q)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Arginine (R)</td>
<td>Histidine (H)</td>
<td>Lysine (K)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Isoleucine (I)</td>
<td>Leucine (L)</td>
<td>Methionine (M)</td>
<td>Valine (V)</td>
</tr>
<tr>
<td>6</td>
<td>Phenylalanine (F)</td>
<td>Tyrosine (Y)</td>
<td>Tryptophan (W)</td>
<td></td>
</tr>
</tbody>
</table>

The term “variant” (of a parent polypeptide) is intended to cover a polypeptide which, in addition to the light chain-heavy chain cleavage site mutation described herein, differs in one or more amino acid residues from its parent polypeptide, normally in 1-15 amino acid residues (such as in 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 amino acid residues), e.g. in 1-10, 1-8, 1-6, 1-5, 1-4 or 1-3 amino acid residues, e.g. one or two amino acid residues. The parent polypeptide in the present context is generally human protein C (SEQ ID NO:2) or a human protein C precursor comprising the propeptide sequence (SEQ ID NO:1).

The terms “mutation” and “substitution” are used interchangeably herein.

The term “modified” or “modification” includes a substitution, an insertion or a deletion.

The term “introduce” is primarily intended to mean substitution of an existing amino acid residue, but may also mean insertion of an additional amino acid residue.
The term "remove" is primarily intended to mean substitution of the amino acid residue to be removed by another amino acid residue, but may also mean deletion (without substitution) of the amino acid residue to be removed.

The term "nucleotide sequence" is intended to indicate a consecutive stretch of two or more nucleotide molecules. The nucleotide sequence may be of genomic, cDNA, RNA, semi-synthetic or synthetic origin, or any combination thereof.

" Amidolytic activity " is used in its normal meaning. A suitable in vitro amidolytic assay (entitled " Amidolytic Assay " ) is described in Example 6 herein. More particularly, a variant of the present invention is classified as having " amidolytic activity " if the variant has an amidolytic activity of at least 50% of the hAPC amidolytic activity when tested in the " Amidolytic Assay " described in Example 6 herein. Preferably, the variant has an amidolytic activity of at least 60% of the hAPC amidolytic activity, such as amidolytic activity of at least 70% of the hAPC amidolytic activity, e.g. amidolytic activity of at least 75% of the hAPC amidolytic activity, more preferably the variant has an amidolytic activity of at least 80% of the hAPC amidolytic activity, such as amidolytic activity of at least 85% of the hAPC activity, e.g. amidolytic activity of at least 90% of the hAPC amidolytic activity, even more preferably the variant has an amidolytic activity of at least 95% of the hAPC amidolytic activity, when tested in the " Amidolytic Assay " described in Example 6 herein. In one interesting embodiment, the variant has an amidolytic activity which, when tested in the " Amidolytic Assay " described in Example 6 herein, is essentially the same as the amidolytic activity of hAPC, such as in the range of 75-125%, e.g. in the range of 80-120% or in the range of 90-110% of the amidolytic activity of hAPC. In another interesting embodiment of the invention, the variant has an amidolytic activity which, when tested in the " Amidolytic Assay " described in Example 6 herein, is higher than the amidolytic activity of hAPC, such as at least 110%, e.g. at least 120% or at least 125% of the amidolytic activity of hAPC.

When used herein, the term " activity " is intended to mean " amidolytic activity ".

The terms " increased anti-inflammatory activity " , " higher anti-inflammatory activity " or " improved anti-inflammatory activity " are intended to mean that variant of the present invention prevents induction of various pro-inflammatory cytokines and adhesion molecules, such as tumor necrosis factor alpha, interleukin-1, -8 and E-selectin, either in vitro or in vivo, more efficiently than hAPC. Whether a variant has a changed anti-inflammatory activity as compared to hAPC may easily be assessed by the skilled person using the " Anti-inflammatory
Assay I” or “Anti-inflammatory Assay II” disclosed in Examples 11 and 12 herein or other similar assays.

The terms "increased resistance towards inactivation by alpha-1-antitrypsin" and "increased resistance towards inactivation by human plasma", respectively, are intended to mean a variant of the invention which is inhibited by alpha-1-antitrypsin or human plasma, respectively, to a lesser degree than hAPC. The present invention provides suitable assays which can easily be carried out by the skilled person in order to initially assess the performance of the variant in question. Thus, the “Alpha-1-Antitrypsin Inactivation Assay” (described in Example 8 herein), the “Human Plasma Inactivation Assay I” (described in Example 9 herein) and the “Human Plasma Inactivation Assay II” (described in Example 10 herein) may be used to initially assess the inactivation of the variant by alpha-1-antitrypsin and/or human plasma and hence assess the resulting change in half-life.

The term “functional in vitro half-life” is used in its normal meaning, i.e. the time at which the activity of the polypeptide is 50% of its initial value. The functional in vitro half-life may suitably be determined by the “Human Plasma Inactivation Assay II” described in Example 10 herein.

The term “increased” as used about the functional in vitro half-life is used to indicate that the functional half-life of the polypeptide is significantly increased relative to that of a reference molecule, e.g. hAPC, such as Xigris®, determined under comparable conditions.

The terms “decreased anticoagulant activity”, “reduced anticoagulant activity” and “lowered anticoagulant activity” are intended to mean a variant of the invention (i.e. a modified hAPC polypeptide or a variant thereof) with the capability to function as an anticoagulant has been reduced compared to a reference molecule, such as hAPC, e.g. Xigris®. Whether a variant of the invention has a decreased anticoagulant activity may be assessed by using the “Clotting Assay” described in Example 7 herein.

The term “reduced immunogenicity” is intended to indicate that the variant gives rise to a measurably lower immune response than a reference molecule, such as hAPC, e.g. Xigris®, as determined under comparable conditions. The immune response may be a cell or antibody mediated response (see, e.g., Roitt: Essential Immunology (8th Edition, Blackwell) for further definition of immunogenicity). Normally, reduced antibody reactivity is an indication of reduced immunogenicity. Reduced immunogenicity may be determined by use of any suitable method known in the art, e.g. in vivo or in vitro.
Variants of the invention

Modification of the cleavage site between the light chain and the heavy chain

In its broadest aspect the present invention relates to a protein C variant wherein the cleavage site between the light chain and the heavy chain has been removed. A schematic illustration of the resulting activated variant is given in Figure 2.

It has been found that by removing the naturally occurring cleavage site between the light chain and the heavy chain, i.e. the Lys-Arg dipeptide at residues 156 and 157, the resulting (activated) zymogen-like protein C molecule has a lowered anticoagulant activity compared to hAPC while still retaining the amidolytic and anti-inflammatory properties.

As will be understood by the skilled person the cleavage site between the light chain and the heavy chain may be removed in a number of ways. How the cleavage site is removed (e.g. by substitution or by deletion) is not crucial as long as it is ensured that no cleavage between the light chain and the activation peptide occurs.

Thus, in one embodiment of the invention, the cleavage site between the light chain and the heavy chain is removed by removing R157, with the proviso that R157 is not removed by the substitution R157K. For example, R157 may be removed by substitution, with the proviso that the substitution is not R157K. Examples of substitutions include those selected from the group consisting of R157A, R157V, R157L, R157I, R157M, R157F, R157W, R157P, R157G, R157S, R157R, R157C, R157Y, R157N, R157Q, R157H, R157D and R157E.

Preferably, R157 is substituted with an amino acid comprising a small side chain (R157A, R157G, R157S or R157T), a polar side chain (R157N or R157Q), or a negatively charged side chain (R157D or R157E). More preferably, R157 is substituted with an amino acid comprising a small side chain (R157A, R157G, R157S or R157T), in particular R157A. In an alternative embodiment R157 is deleted (R157*).

In another embodiment of the invention, the cleavage site between the light chain and the heavy chain is removed by removing K156, with the proviso that K156 is not removed by the substitution K156R. For example K156 may be removed by substitution, with the proviso that the substitution is not K156R. Examples of substitutions include those selected from the group consisting of K156A, K156V, K156L, K156I, K156M, K156F, K156W, K156P, K156G, K156S, K156R, K156C, K156Y, K156N, K156Q, K156H, K156D and K156E.

Preferably, K156 is substituted with an amino acid comprising a small side chain (K156A, K156G, K156S or K156T), a polar side chain (K156N or K156Q), or a negatively...
charged side chain (K156D or K156E). More preferably K156 is substituted with with an amino acid comprising a small side chain (K156A, K156G, K156S or K156T), in particular K156A. In an alternative embodiment K156 is deleted (K156*).

In a further interesting embodiment both of K156 and R157 are removed, with the proviso that they are not removed by the substitutions K156R and R157K. For example K156 and R157 may be removed by substitution, with the proviso that the substitutions are not K156R and R157K. Examples of substitutions include those selected from the group consisting of K156A+R157A, K156A+R157G, K156A+R157S, K156A+R157T, K156G+R157A, K156G+R157G, K156G+R157S, K156G+R157T, K156S+R157A, K156S+R157G, K156S+R157S, K156S+R157T, K156T+R157A, K156T+R157G, K156T+R157S and K156T+R157T, most preferably K156A+R157A.

Alternatively, one or both of K156 and R157 are deleted. Thus, another series of interesting modifications include K156A+R157*, K156G+R157*, K156S+R157*, K156T+R157*, K156*+R157A, K156*+R157G, K156*+R157S, K156*+R157T and K156*+R157*, most preferably K156*+R157*.

As will be understood, the variants of the invention comprise at least one amino acid modification as compared to human wild-type protein C shown in SEQ ID NO:1. Typically, the variants of the invention comprise 1-15 amino acid modification(s) relative to SEQ ID NO:1 (such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 modification(s) relative to SEQ ID NO:1), e.g. 1-10 modification(s), 1-7 modification(s) or 1-5 modification(s) relative to SEQ ID NO:1. Preferably, said modifications are substitutions.

The variants of the invention may be activated in a conventional way, e.g. by treating the one-chain zymogen protein C variant (see Figure 2) with substances such as α-thrombin, trypsin, Russell’s viper venom factor X activator, ACCC or a mixture of thrombin and thrombomodulin, which will be known to the person skilled in the art.

The activatedzymogen-like protein C polypeptides described herein retain their amidolytic activity. Thus, the activated variants described herein have at least 50% of the amidolytic activity of hAPC when tested in the “Amidolytic Assay” described in Example 6 herein. Preferably, the variants have at least 60% of the amidolytic acivity of hAPC, such as at least 70% of the amidolytic activity of hAPC, more preferably at least 80% of the amidolytic activity of hAPC, such as at least 90% of the amidolytic activity of hAPC or substantially the same amidolytic activity as hAPC, when tested in the “Amidolytic Assay” described in Example 6 herein.
In a similar way, the activated zymogen-like protein C polypeptides described herein retain their anti-inflammatory activity. Thus, the activated variants described herein have at least 50% of the anti-inflammatory activity of hAPC when tested in the “Anti-inflammatory Assay I” or “Anti-inflammatory Assay II” described in Examples 11 and 12 herein. Preferably, the variants have at least 60% of the anti-inflammatory activity of hAPC, such as at least 70% of the anti-inflammatory activity of hAPC, more preferably at least 80% of the anti-inflammatory activity of hAPC, such as at least 90% of the anti-inflammatory activity of hAPC or substantially the same anti-inflammatory activity as hAPC, when tested in the “Anti-inflammatory Assay I” or “Anti-inflammatory Assay II” described in Examples 11 and 12 herein.

As explained initially, the activated zymogen-like protein C polypeptides of the invention possess a lowered clotting activity compared to a reference APC molecule (where the reference APC molecule is hAPC or a variant of hAPC which, but for the modification of the cleavage site between the heavy chain and the light chain, is otherwise the same as the polypeptide of the invention and is produced in a comparable manner, e.g. in the same type of cell), which in turn has the consequence that the product is safer to use. Preferably, the activated variants described herein have 1-75% of the anticoagulant activity of hAPC when tested in the “Clotting Assay” described in Example 7 herein. Preferably, the variants have 1-70% (or 10-70%) of the anticoagulant activity of hAPC, such as 1-65% (or 10-65%) of the anticoagulant activity of hAPC, e.g. 1-60% (or 10-60%) of the anticoagulant activity of hAPC, more preferably 1-55% (or 10-55%) of the anticoagulant activity of hAPC, such as 1-50% (or 10-50%) of the anticoagulant activity of hAPC, e.g. 1-45% (or 10-45%) of the anticoagulant activity of hAPC, even more preferably 1-40% (or 10-40%) of the anticoagulant activity of hAPC, such as 10-35%, 10-30% or 10-25% of the anticoagulant activity of hAPC, when tested in the “Clotting Assay” described in Example 7 herein.

It will be understood that the modifications of the cleavage site between the light chain and the heavy chain discussed in this section may be combined with one or more further modifications, e.g. in the form of at least one substitution, insertion or deletion, typically at least one substitution, as described below. These additional modifications may, for example, be performed in the active site region and/or in the autolysis loop, and may e.g. be aimed at increasing the anti-inflammatory effect, increasing the half-life and/or lowering the anticoagulant activity of the resulting polypeptide. In one embodiment, such mutations may
be aimed at introducing and/or at removing at least one amino acid residue comprising an
attachment group for a non-polypeptide moiety.

The term “non-polypeptide moiety” refers to a non-polypeptide molecule that is
capable of conjugating to an attachment group of the polypeptide. Examples of such non-
polypeptide moieties include polymer molecules, sugar moieties, lipophilic compounds and
organic derivatizing agents. The non-polypeptide moiety can be directly covalently joined to
the attachment group or it can be indirectly covalently joined to the attachment group through
an intervening moiety, such as a bridge, spacer or linker moiety or moieties. Preferred exam-
iples of non-polypeptide moieties are a polymer molecule, in particular a linear or branched
polyethylene glycol or other polyalkylene glycol, and a sugar moiety, in particular an N- or O-
linked oligosaccharide generally attached by in vivo glycosylation.

In one embodiment, the protein C polypeptide may thus include at least one intro-
duced in vivo N-glycosylation site created by a substitution selected from the group con-
sisting of D172N+K174S, D172N+K174T, D189N+K191S, D189N+K191T, S190N+K192S,
S190N+K192T, K191N+K193S, K191N+K193T, K192N+L194S, K192N+L194T,
S216N+K218S, S216N+K218T, K217N+L219S, K217N+L219T, K218N+L220S,
S252N+T254S, T253N+D255S, T253N+D255T, T254N+N256S, T254N+N256T,
D255N+D257S, D255N+D257T, L296N, L296N+T298S, Y302N, Y302N+S304T, H303N,
H303N+S305T, S304N+R306S, S304N+R306T, S305N+E307S, S305N+E307T,
I348N+G350T, L349N+D351S, L349N+D351T, D351N+Q353S, D351N+Q353T,
H388N+Y390S and H388N+Y390T.

Preferred substitutions for introduction of an in vivo N-glycosylation site are se-
lected from the group consisting of D189N+K191S, D189N+K191T, S190N+K192S,

An "N-glycosylation site" has the sequence N-X-S/T/C", wherein X is any amino acid residue except proline, N is asparagine and S/T/C is either serine, threonine or cysteine, preferably serine or threonine, and most preferably threonine.

In another embodiment, the protein C polypeptide produced according to the invention includes at least one introduced amino acid residue comprising an attachment group for a non-polypeptide moiety, in particular an introduced cysteine residue. Preferably, such a cysteine residue is introduced in a position selected from the group consisting of D172, D189, S190, K191, K192, K193, D214, E215, S216, K217, K218, L220, V243, V245, S250, K251, S252, T253, T254, L296, Y302, H303, S304, S305, T315, F316, V334, S336, N337, M338, I348, L349, D351, R352, E357, G383, L386, L387 and H388; more preferably from the group consisting of D189, S190, K191, D214, K217, K251, S252, T253, Y302, S336, N337, M338, G383 and L386; such as from the group consisting of D189, K191, D214, K251, S252, T253, Y302, S336, N337, M338, G383 and L386; in particular from the group consisting of D189, D214, K251 and L386. The non-polypeptide moiety to be covalently attached to said introduced cysteine residue is preferably a polymer molecule, in particular a linear or branched polyethylene glycol or other polyalkylene oxide.

Specific examples of activated PEG polymers particularly preferred for coupling to cysteine residues include the following linear PEGs: vinylsulfone-PEG (VS-PEG), preferably vinylsulfone-mPEG (VS-mPEG); maleimide-PEG (MAL-PEG), preferably maleimide-mPEG (MAL-mPEG) and orthopyrydyl-disulfide-PEG (OPSS-PEG), preferably orthopyridyl-disulfide-mPEG (OPSS-mPEG). Such PEG or mPEG polymers will generally have a size of from about 1 kDa to about 40 kDa, such as from about 1 kDa to about 20 kDa, e.g. from about...
2 kDa to about 15 kDa, such as from about 3 kDa to about 10 kDa; for example about 5 kDa, about 6 kDa, about 10 kD, about 12 kDa or about 20 kDa.

For PEGylation to cysteine residues the protein C variant is usually treated with a reducing agent, such as dithiothreitol (DDT) prior to PEGylation. The reducing agent is subsequently removed by any conventional method, such as by desalting. Conjugation of PEG to a cysteine residue typically takes place in a suitable buffer at about pH 6-9 at temperatures of about 4°C to 25°C for periods up to about 16 hours.

In a further interesting embodiment of the invention the zymogen-like variant comprises at least one amino acid substitution of a charged amino acid residue, wherein the charged amino acid residue is located in the active site region and, at the same time, has at least 25% of its side chain exposed to the surface (as defined in Example 3 of WO 02/32461, i.e.: D172, D189, S190, K191, K192, K193, D214, E215, S216, K217, K218, L220, V243, V245, N248, S250, K251, S252, T253, T254, D255, L296, Y302, H303, S304, S305, R306, E307, K308, E309, A310, K311, R312, N313, R314, T315, F316, V334, S336, N337, M338, I348, L349, D351, R352, E357, E382, G383, L386, L387 and H388), with an amino acid residue having no charge, in particular an amino acid residue having no charge but a polar side chain (Gly, Ser, Thr, Tyr, Asn or Gln), or with an amino acid residue having an opposite charge.


Other specific examples of amino acid substitutions wherein the charged amino acid residue in question is substituted with an amino acid side chain having a polar side chain include D172G/S/T/C/Y/N/Q, D189G/S/T/C/Y/N/Q, K191G/S/T/C/Y/N/Q, K192G/S/T/C/Y/N/Q, K193G/S/T/C/Y/N/Q, D214G/S/T/C/Y/N/Q, E215G/S/T/C/Y/N/Q, K217G/S/T/C/Y/N/Q, K218G/S/T/C/Y/N/Q, K251G/S/T/C/Y/N/Q, D255G/S/T/C/Y/N/Q, R306G/S/T/C/Y/N/Q, E307G/S/T/C/Y/N/Q, K308G/S/T/C/Y/N/Q, E309G/S/T/C/Y/N/Q, R312G/S/T/C/Y/N/Q, D351G/S/T/C/Y/N/Q, R352G/S/T/C/Y/N/Q, E357G/S/T/C/Y/N/Q and E382G/S/T/C/Y/N/Q, such as D214G/S/T/C/Y/N/Q, E215G/S/T/C/Y/N/Q,
K251G/S/T/C/Y/N/Q and E357G/S/T/C/Y/N/Q, e.g. D214Q, E215Q, K251Q and E357Q, in particular K251Q. Another interesting substitution may be K251N+T253A.

In a further embodiment, the protein C polypeptide may comprise at least one amino acid modification in the autolysis loop constituted by the amino acid residues in position 306-314 relative to SEQ ID NO:1 or 2 in order to achieve a reduced anticoagulant activity, e.g. one, two, three or four such substitutions. The autolysis loop contains a number of charged residues, namely R306, E307, K308, E309, K311, R312 and R314, and in a preferred embodiment one or more of these charged residues are substituted with an uncharged amino acid residue, e.g. A, V, L, I, F, W, P, G, S, T, Y, N or Q.

In another embodiment at least one positively charged amino acid residue in the autolysis loop is substituted with an uncharged or negatively charged amino acid residue, in particular with an uncharged amino acid residue. In a further embodiment at least one negatively charged amino acid residue in the autolysis loop is substituted with an uncharged amino acid residue.


Examples of specific substitutions which may be performed in position 308 include substitutions selected from the group consisting of K308A, K308V, K308L, K308I, K308F, K308W, K308P, K308G, K308S, K308T, K308Y, K308N and K308Q, preferably selected from the group consisting of K308A, K308V, K308L and K308I, such as K308A.

Examples of specific substitutions which may be performed in position 309 include substitutions selected from the group consisting of E309A, E309V, E309L, E309I, E309F, E309W, E309P, E309G, E309S, E309T, E309Y, E309N and E309Q, preferably selected from the group consisting of E309A, E309V, E309L and R309I, such as E309A.

Examples of specific substitutions which may be performed in position 311 include substitutions selected from the group consisting of K311A, K311V, K311L, K311I, K311F,
K311W, K311P, K311G, K311S, K311T, K311Y, K311N and K311Q, preferably selected from the group consisting of K311A, K311V, K311L and K311I, such as K311A.

Examples of specific substitutions which may be performed in position 312 include substitutions selected from the group consisting of R312A, R312V, R312L, R312I, R312F, R312W, R312P, R312G, R312S, R312T, R312Y, R312N and R312Q, preferably selected from the group consisting of R312A, R312V, R312L and R312I, such as R312A.

Examples of specific substitutions which may be performed in position 314 include substitutions selected from the group consisting of R314A, R314V, R314L, R314I, R314F, R314W, R314P, R314G, R314S, R314T, R314Y, R314N and R314Q, preferably selected from the group consisting of R314A, R314V, R314L and R314I, such as R314A.

Further information on protein C variants comprising advantageous substitutions of the type described above as well as conjugation of such variants to one or more non-polypeptide moieties is found in WO 02/32461 and PCT/DK03/00392, which are hereby incorporated herein by reference.

The protein C variants disclosed herein having at least one additional attached non-polypeptide moiety compared to hAPC have an increased functional half-life compared to the wild-type protein. Thus, preferred variants are those with an increased functional in vivo half-life as compared to hAPC, such as Xigris®. Particular preferred variants are those where the ratio between the functional in vivo half-life of said variant and the functional in vivo half-life of hAPC is at least 1.25, more preferably at least 1.50, such as at least 1.75, e.g. at least 2, even more preferably at least 3, such as at least 4, e.g. at least 5, most preferably at least 6, such as at least 7, e.g. at least 8, at least 9 or at least 10. Functional in vivo half-life may be determined in an experimental animal, such as rat, mice rabbit, dog, pig or monkey.

The increase in functional half-life may be initially assessed using a variety of the in vitro assays disclosed herein. The increased resistance towards inactivation by alpha-1-antitrypsin and/or human plasma may be determined and assessed by the “Alpha-1-Antitrypsin Inactivation Assay”, the “Human Plasma Inactivation Assay I” or the “Human Plasma Inactivation Assay II” disclosed herein. These assays enable the skilled person to assess, at an early stage of his research, whether the constructed variant can be expected to have an increased functional half-life.

Thus, a particularly interesting variant for the purposes described herein is one which has a residual activity of at least 20% when tested in the “Alpha-1-Antitrypsin Inactivation Assay” described in Example 8 herein using an inhibitor concentration of 16.6 μM. Prefera-
bly, the variant has a residual activity of at least 25% or at least 30%, such as a residual activity of at least 40%, more preferably the variant has a residual activity of at least 50%, such as a residual activity of at least 60%, even more preferably the variant has a residual activity of at least 70%, such as a residual activity at least 75%, most preferably the variant has a residual activity of at least 80%, such as at least 85%.

Alternatively, or in addition to the above-mentioned test, the suitability of a selected variant may be tested in the “Human Plasma Inactivation Assay I”. Thus, another particularly interesting variant is one which has a residual activity of at least 20% when tested in the “Human Plasma Inactivation Assay I” described in Example 9 herein. Preferably, the variant has a residual activity of at least 25% or at least 30%, such as a residual activity of at least 40%, more preferably the variant has a residual activity of at least 50%, such as a residual activity of at least 60%, even more preferably the variant has a residual activity of at least 70%, such as a residual activity at least 75%.

Alternatively, or in addition to the above-mentioned test(s), the suitability of a selected variant may be tested in the “Human Plasma Inactivation Assay II”. Thus, another particularly interesting variant is one where the ratio between the functional in vitro half-life of the said variant and the functional in vitro half-life of hAPC is at least 1.25 when tested in the “Human Plasma Inactivation Assay II” described in Example 10 herein. Preferably, the ratio is at least 1.5, such as at least 2, more preferably at least 3, such as at least 4, even more preferably at least 5, such as at least 6, most preferably at least 7, such as at least 8, in particular at least 9, such as at least 10.

For variants of the invention that are modified in the autolysis loop, it is contemplated that the autolysis loop modification may provide a further decrease in the anticoagulant activity of the zymogen-like protein C variant of the invention, i.e. in addition to a decreased anticoagulant activity as a result of other modifications in the polypeptide. This effect may conveniently be tested in the “APC Clotting Assay” described in Example 7 herein.

Methods of preparing a conjugated variant

The polypeptide variant of the present invention, optionally in glycosylated form, may be produced by any suitable method known in the art. Such methods include constructing a nucleotide sequence encoding the variant polypeptide and expressing the sequence in a suitable transformed or transfected host. Preferably, the host cell is a gamma-carboxylating host cell, in particular a mammalian cell.
In general, a conjugated variant may be produced by culturing an appropriate host cell under conditions conducive for the expression of the variant polypeptide, and recovering the variant polypeptide, wherein a) the variant polypeptide comprises at least one N- or O-glycosylation site and the host cell is an eukaryotic host cell capable of \textit{in vivo} glycosylation, and/or b) the variant polypeptide is subjected to conjugation to a non-polypeptide moiety \textit{in vitro}.

It will be understood that the conjugation should be designed so as to produce the optimal molecule with respect to the number of non-polypeptide moieties attached, the size and form of such molecules (e.g. whether they are linear or branched), and the attachment site(s) in the polypeptide. The molecular weight of the non-polypeptide moiety to be used may, e.g., be chosen on the basis of the desired effect to be achieved. For instance, if the primary purpose of the conjugation is to achieve a conjugated variant having a high molecular weight (e.g. to reduce renal clearance) it is usually desirable to conjugate as few high molecular weight non-polypeptide moieties as possible to obtain the desired molecular weight. When a high degree of shielding is desirable this may be obtained by use of a sufficiently high number of low molecular weight non-polypeptide moieties (e.g. with a molecular weight of from about 300 Da to about 5 kDa, such as a molecular weight of from 300 Da to 2 kDa).

\textit{Conjugation to a polymer molecule}

The polymer molecule to be coupled to the variant polypeptide may be any suitable polymer molecule, such as a natural or synthetic homo-polymer or hetero-polymer, typically with a molecular weight in the range of about 300-100,000 Da, such as about 500-20,000 Da, more preferably in the range of about 500-15,000 Da, even more preferably in the range of about 2-12 kDa, such as in the range of about 3-10 kDa. When the term "about" is used herein in connection with a certain molecular weight, the word "about" indicates an approximate average molecular weight and reflects the fact that there will normally be a certain molecular weight distribution in a given polymer preparation.

Examples of homo-polymers include a polyol (i.e. poly-OH), a polyamine (i.e. poly-NH$_2$) and a polycarboxylic acid (i.e. poly-COOH). A hetero-polymer is a polymer comprising different coupling groups, such as a hydroxyl group and an amine group.

Examples of suitable polymer molecules include polymer molecules selected from the group consisting of polyalkylene oxide (PAO), including polyalkylene glycol (PAG), such as polyethylene glycol (PEG) and polypropylene glycol (PPG), branched PEGs, poly-vinyl
alcohol (PVA), poly-carboxylate, poly-(vinylpyrrolidone), polyethylene-co-maleic acid anhydride, polystyrene-co-maleic acid anhydride, dextran, including carboxymethyl-dextran, or any other biopolymer suitable for reducing immunogenicity and/or increasing functional in vivo half-life. Another example of a polymer molecule is human albumin or another abundant plasma protein. Generally, polyalkylene glycol-derived polymers are biocompatible, non-toxic, non-antigenic, non-immunogenic, have various water solubility properties, and are easily excreted from living organisms.

PEG is the preferred polymer molecule, since it has only few reactive groups capable of cross-linking compared to, e.g., polysaccharides such as dextran. In particular, monofunctional PEG, e.g. methoxypolyethylene glycol (mPEG), is of interest since its coupling chemistry is relatively simple (only one reactive group is available for conjugating with attachment groups on the polypeptide). Consequently, as the risk of cross-linking is eliminated, the resulting conjugated variants are more homogeneous and the reaction of the polymer molecules with the variant polypeptide is easier to control.

To effect covalent attachment of the polymer molecule(s) to the variant polypeptide, the hydroxyl end groups of the polymer molecule must be provided in activated form, i.e. with reactive functional groups (examples of which include primary amino groups, hydrazide (HZ), thiol, succinate (SUC), succinimidyl succinate (SS), succinimidyl succinamide (SSA), succinimidyl propionate (SPA), succinimidyl butyrate (SBA), succinimidyl carboxymethylate (SCM), benzotriazole carbonate (BTC), N-hydroxysuccinimide (NHS), aldehyde, nitrophenylcarbonate (NPC), and tresylate (TRES)). Suitable activated polymer molecules are commercially available, e.g. from Nektar Therapeutics, Huntsville, AL, USA, or from Polymasc Pharmaceuticals plc, UK.

Alternatively, the polymer molecules can be activated by conventional methods known in the art, e.g. as disclosed in WO 90/13540. Specific examples of activated linear or branched polymer molecules for use in the present invention are described in the Shearwater Corporation Catalog 2001 (Polyethylene Glycol and Derivatives for Biomedical Applications, incorporated herein by reference).

Specific examples of activated PEG polymers include the following linear PEGs:

NHS-PEG (e.g. SPA-PEG, SSPA-PEG, SBA-PEG, SS-PEG, SSA-PEG, SC-PEG, SG-PEG, and SCM-PEG), and NOR-PEG, BTC-PEG, EPOX-PEG, NCO-PEG, NPC-PEG, CDI-PEG, ALD-PEG, TRES-PEG, VS-PEG, IODO-PEG, and MAL-PEG, and branched PEGs such as PEG2-NHS.
Normally, the polymer conjugation is performed under conditions aimed at reacting as many of the available polymer attachment groups with polymer molecules. This is achieved by means of a suitable molar excess of the polymer relative to the polypeptide. Typically, the molar ratios of activated polymer molecules to polypeptide are up to about 1000-1, such as up to about 200-1, or up to about 100-1. In some cases the ration may be somewhat lower, however, such as up to about 50-1, 10-1, 5-1, 2-1 or 1-1 in order to obtain optimal reaction.

Detailed information on methods for PEGylating protein C or activated protein C is found in WO 02/32461, which is hereby incorporated by reference.

**Coupling to a sugar moiety**

In order to achieve *in vivo* glycosylation of a protein C molecule comprising one or more glycosylation sites the nucleotide sequence encoding the variant polypeptide must be inserted in a glycosylating, eucaryotic expression host. The expression host cell may be selected from fungal (filamentous fungal or yeast), insect or animal cells or from transgenic plant cells. In one embodiment the host cell is a mammalian cell, such as a CHO cell, BHK or HEK, e.g. HEK 293, cell, or an insect cell, such as an SF9 cell, or a yeast cell, e.g. *S. cerevisiae* or *Pichia pastoris*, or any of the host cells mentioned hereinafter.

Covalent *in vitro* coupling of sugar moieties (such as dextran) to amino acid residues of the variant polypeptide may also be used, e.g. by means of *in vitro* coupling of sugar moieties or PEG to protein- and peptide-bound Gln-residues carried out by transglutaminases (TGases).

Further information on *in vivo* and *in vitro* glycosylation of protein C and activated protein C is found in WO 02/32461 (incorporated by reference).

**Methods of preparing a protein C variant**

The polypeptide variant, optionally in glycosylated form, may be produced by any suitable method known in the art. Such methods include constructing a nucleotide sequence encoding the variant polypeptide and expressing the sequence in a suitable transformed or transfected host. Preferably, the host cell is a gamma-carboxylating host cell such as a mammalian cell. However, variant polypeptides may be produced, albeit less efficiently, by chemical synthesis or a combination of chemical synthesis or a combination of chemical synthesis and recombinant DNA technology.
A nucleotide sequence encoding a polypeptide variant of the invention, or a precursor thereof, may be constructed by isolating or synthesizing a nucleotide sequence encoding the parent protein C, such as protein C with the amino acid sequence shown in SEQ ID NO:1 and then changing the nucleotide sequence so as to effect introduction (i.e. insertion or substitution) or removal (i.e. deletion or substitution) of the relevant amino acid residue(s).

The nucleotide sequence is conveniently modified by site-directed mutagenesis in accordance with conventional methods. Alternatively, the nucleotide sequence is prepared by chemical synthesis, e.g. by using an oligonucleotide synthesizer, wherein oligonucleotides are designed based on the amino acid sequence of the desired polypeptide, and preferably selecting those codons that are favored in the host cell in which the recombinant polypeptide will be produced. For example, several small oligonucleotides coding for portions of the desired polypeptide may be synthesized and assembled by PCR, ligation or ligation chain reaction (LCR) (Barany, PNAS 88:189-193, 1991). The individual oligonucleotides typically contain 5’ or 3’ overhangs for complementary assembly.

Persons skilled in the art will be capable of selecting suitable vectors, expression control sequences and hosts for expressing the polypeptide. For example, in selecting a vector, the host must be considered because the vector must be able to replicate in it or be able to integrate into the chromosome. The vector’s copy number, the ability to control that copy number, and the expression of any other proteins encoded by the vector, such as antibiotic markers, should also be considered. In selecting an expression control sequence, a variety of factors should also be considered. These include, for example, the relative strength of the sequence, its controllability, and its compatibility with the nucleotide sequence encoding the polypeptide, particularly as regards potential secondary structures. Hosts should be selected by consideration of their compatibility with the chosen vector, the toxicity of the product coded for by the nucleotide sequence, their secretion characteristics, their ability to fold the polypeptide correctly, their fermentation or culture requirements, and the ease of purification of the products coded for by the nucleotide sequence.

The recombinant vector may be an autonomously replicating vector, i.e. a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.
The vector is preferably an expression vector in which the nucleotide sequence encoding the polypeptide of the invention is operably linked to additional segments required for transcription of the nucleotide sequence. The vector is typically derived from plasmid or viral DNA. A number of suitable expression vectors for expression in the host cells mentioned herein are commercially available or described in the literature. Useful expression vectors for eukaryotic hosts include, for example, vectors comprising expression control sequences from SV40, bovine papilloma virus, adenovirus and cytomegalovirus. Specific vectors are, e.g., pCDNA3.1(+):Hyg (Invitrogen, Carlsbad, CA, USA) and pCI-neo (Stratagene, La Jolla, CA, USA). Useful expression vectors for yeast cells include the 2μ plasmid and derivatives thereof, the POT1 vector (US 4,931,373), the pJSO37 vector described in Okkels, Ann. New York Acad. Sci. 782, 202-207, 1996, and pPICZ A, B or C (Invitrogen). Useful vectors for insect cells include pVL941, pBG311 (Cate et al., Cell 45, pp. 685-98 (1986), pBluebac 4.5 and pMelbac (both available from Invitrogen). Useful expression vectors for bacterial hosts include known bacterial plasmids, such as plasmids from E. coli, including pBR322, pET3a and pET12a (both from Novagen Inc., WI, USA), wider host range plasmids, such as RP4, phage DNAs, e.g., the numerous derivatives of phage lambda, e.g., NM989, and other DNA phages, such as M13 and filamentous single stranded DNA phages.

Other vectors for use in this invention include those that allow the nucleotide sequence encoding the variant polypeptide to be amplified in copy number. Such amplifiable vectors are well known in the art. They include, for example, vectors able to be amplified by DHFR amplification (see, e.g., US 4,470,461; Kaufman et al., Mol. Cell. Biol., 2, pp. 1304-19 (1982)), and by glutamine synthetase ("GS") amplification (see, e.g., US 5,122,464 and EP 0338841).

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

The vector may also comprise a selectable marker, e.g. a gene the product of which complements a defect in the host cell, such as the gene coding for dihydrofolate reductase (DHFR) or the Schizosaccharomyces pombe TPI gene (described by P.R. Russell, Gene 40, 1985, pp. 125-130), or one which confers resistance to a drug, e.g. ampicillin, kanamycin, tetracyclin, chloramphenicol, neomycin, hygromycin or methotrexate. For Saccharomyces cerevisiae, selectable markers include ura3 and leu2.
The term "control sequences" is defined herein to include all components which are necessary or advantageous for the expression of the variant polypeptide of the invention. Each control sequence may be native or foreign to the nucleic acid sequence encoding the polypeptide. Such control sequences include, but are not limited to, a leader sequence, polyadenylation sequence, propeptide sequence, promoter, enhancer or upstream activating sequence, signal peptide sequence, and transcription terminator. At a minimum, the control sequences include a promoter.

A wide variety of expression control sequences may be used in the present invention. Such useful expression control sequences include the expression control sequences associated with structural genes of the foregoing expression vectors as well as any sequence known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof.

Examples of suitable control sequences for directing transcription in mammalian cells include the early and late promoters of SV40 and adenovirus, e.g. the adenovirus 2 major late promoter, the MT-1 (metallothionein gene) promoter, the human cytomegalovirus immediate-early gene promoter (CMV), the human elongation factor 1α (EF-1α) promoter, the Drosophila minimal heat shock protein 70 promoter, the Rous Sarcoma Virus (RSV) promoter, the human ubiquitin C (UbC) promoter, the human growth hormone terminator, SV40 or adenovirus E1b region polyadenylation signals and the Kozak consensus sequence (Kozak, J Mol Biol 1987;196(4):947-50).

In order to improve expression in mammalian cells a synthetic intron may be inserted in the 5' untranslated region of the nucleotide sequence encoding the polypeptide. An example of a synthetic intron is the synthetic intron from the plasmid pCI-Neo (available from Promega Corporation, WI, USA).

The nucleotide sequence of the invention encoding a protein C polypeptide precursor, whether prepared by site-directed mutagenesis, synthesis, PCR or other methods, will generally include a nucleotide sequence that encodes a signal peptide. The signal peptide is present when the polypeptide is to be secreted from the cells in which it is expressed. Such signal peptide, when present, should be one recognized by the cell chosen for expression of the polypeptide. The signal peptide may be homologous (e.g. be that normally associated with human protein C) or heterologous (i.e. originating from another source than human protein C) to the polypeptide or may be homologous or heterologous to the host cell, i.e. be a signal pep-
tide normally expressed from the host cell or one which is not normally expressed from the host cell.

Suitable host cells that may be used to produce the polypeptide precursor of the invention include, in particular, mammalian cells. Examples of suitable mammalian host cells include Chinese hamster ovary (CHO) cell lines, (e.g. CHO-K1; ATCC CCL-61), Green Monkey cell lines (COS) (e.g. COS 1 (ATCC CRL-1650), COS 7 (ATCC CRL-1651)); mouse cells (e.g. NS/O), Baby Hamster Kidney (BHK) cell lines (e.g. ATCC CRL-1632 or ATCC CCL-10), and human cells (e.g. HEK 293 (ATCC CRL-1573)). Mammalian cells, such as CHO cells, may be modified to express a sialyltransferase, e.g. 1,6-sialyltransferase, e.g. as described in US 5,047,335, in order to provide improved glycosylation of the protein C polypeptide. It will be understood that in order to achieve in vivo glycosylation of a protein C molecule comprising one or more glycosylation sites, the nucleotide sequence encoding the variant polypeptide must be inserted in a glycosylating, eukaryotic expression host.

In order to increase secretion it may be of interest to produce the variant polypeptide of the invention together with an endoprotease, for example a PACE (paired basic amino acid converting enzyme) (e.g. as described in US 5,986,079), such as a Kex2 endoprotease (e.g. as described in WO 00/28065).

Methods for introducing exogenous DNA into mammalian host cells include calcium phosphate-mediated transfection, electroporation, DEAE-dextran mediated transfection, liposome-mediated transfection, viral vectors and the transfection method described by Life Technologies Ltd, Paisley, UK using Lipofectamin 2000. These methods are well known in the art and e.g. described by Ausbel et al. (eds.), 1996, Current Protocols in Molecular Biology, John Wiley & Sons, New York, USA. The cultivation of mammalian cells is conducted according to established methods, e.g. as disclosed in: Animal Cell Biotechnology, Methods and Protocols, Edited by Nigel Jenkins, 1999, Human Press Inc, Totowa, New Jersey, USA; and Harrison MA and Rae IF, General Techniques of Cell Culture, Cambridge University Press 1997.

In the production methods of the present invention, the cells are cultivated in a nutrient medium suitable for production of the variant polypeptide using methods known in the art. For example, the cells may be cultivated by shake flask cultivation, small-scale or large-scale fermentation (including continuous, batch, fed-batch, or solid state fermentations) in laboratory or industrial fermenters performed in a suitable medium and under conditions allowing the polypeptide to be expressed and/or isolated. The cultivation takes place in a suitable nu-
trient medium comprising carbon and nitrogen sources and inorganic salts, using procedures
known in the art. Suitable media are available from commercial suppliers or may be prepared
according to published compositions (e.g., in catalogues of the American Type Culture Col-
lection). When the polypeptide is secreted into the nutrient medium, the polypeptide can be
recovered directly from the medium.

The resulting variant polypeptide may be recovered by methods known in the art.
For example, the polypeptide may be recovered from the nutrient medium by conventional
procedures including, but not limited to, centrifugation, filtration, ultra-filtration, extraction or
precipitation.

The variant polypeptides may be purified by a variety of procedures known in the art
including, but not limited to, chromatography (e.g., ion exchange, affinity, hydrophobic,
chromatofocusing, and size exclusion), electrophoretic procedures (e.g., preparative isoelec-
tric focusing), differential solubility (e.g., ammonium sulfate precipitation) or extraction (see,
e.g., Protein Purification (2nd Edition), Janson and Ryden, editors, Wiley, New York, 1998).

**Pharmaceutical compositions and use**

In a further aspect the present relates to a variant of the invention for use as a medica-
ment. More particularly, the present invention relates to the use of a variant of the invention
for the manufacture of a medicament for the treatment of sepsis or septick shock. Analog-
gously, the present invention also relates to a method for treating a sepsis patient (or a patient
at risk of acquiring sepsis), or to a method for treating a patient having septick shock (or at risk
of acquiring septick shock), which comprises administering to said patient an effective
amount of the variant of the invention.

The present invention also relates to the use of a variant of the invention for the manu-
facture of a medicament for the treatment of a hypercoagulable state or acquired protein C
deficiency, e.g. when said hypercoagulable state or protein C deficiency is associated with
sepsis; transplantations, such as bone marrow transplantation; burns; pregnancy; major sur-
gery; trauma; and adult respiratory distress syndrome (ARDS).

The present invention further relates to the use of a variant of the invention for the
manufacture of a medicament for the treatment of inflammation.

The present invention also relates to a method of treating a patient with a hypercoa-
gulable state or acquired protein C deficiency, e.g. where said hypercoagulable state or pro-
tein C deficiency is associated with sepsis; transplantations, such as bone marrow transplanta-
tion; burns; pregnancy; major surgery; trauma; and adult respiratory distress syndrome (ARDS), which comprises administering to said patient an effective amount of a variant of the invention.

Further, the present invention relates to a method of reducing inflammation in a patient, which comprises administering to said patient an effective amount of a variant of the invention.

In the present context the term “hypercoagulable state(s)” refers to excessive coagulability associated with disseminated intravascular coagulation, prethrombotic conditions, activation of coagulation, or congenital or acquired deficiency of clotting factors, such as aPC.

Sepsis is defined as a systemic inflammatory response to infection, associated with and mediated by the activation of a number of host defense mechanisms including the cytokine network, leukocytes, and the complement and coagulation/fibrinolysis systems (Mesters et al., Blood 88:881-886, 1996). Disseminated intravascular coagulation (DIC), with widespread deposition of fibrin in the microvasculature of various organs, is an early manifestation of sepsis/septic shock. DIC is an important mediator in the development of the multiple organ failure syndrome and contributes to the poor prognosis of patients with septic shock (Fourrier et al., Chest 101:816-823, 1991).

Several encouraging pre-clinical studies using protein C in various animal models of sepsis have been reported. A study in a baboon sepsis model by Taylor et al. (J. Clin. Invest. 79:918-25, 1987), used plasma-derived human activated protein C. The animals were treated prophylactically (i.e., the aPC was given at the start of the two hour infusion of the LD100 E. coli). Five out of five animals survived 7 days and were considered permanent survivors to the experimental protocol. In control animals receiving an identical infusion of E. coli, five out of five animals died in 24 to 32 hours. The efficacious dose was 7 to 8 mg/kg.

In a lipopolysaccaride (LPS; E. coli) sepsis model in rats (Murakami et al., Blood 87:642-647, 1996), the pulmonary vascular injury induced by LPS was inhibited by human plasma derived activated protein C at a dose of 100 µg/kg. Furthermore, in a ligation and puncture sepsis model in rabbits, Okamoto et al. (Gastroenterology 106:A747, 1994), demonstrated that plasma-derived human activated protein C was effective in protecting the animals from coagulopathy and organ failure at a dose of 12 µg/kg/hr for nine hours. Due to the species specificity of aPC, results obtained in these animals are not necessarily predictive to the treatment of humans. The efficacious dose level of human activated protein C is extremely
variable and unpredictable depending upon the animal model selected. For example, the serum half-life of human activated protein C in humans is 30 to 40 minutes, compared to a half-life of 8 to 10 minutes in Baboons and 90 minutes in rabbits.

There have been numerous recent attempts to treat sepsis in humans, for the most part using agents that block inflammatory mediators associated with the pathophysiology of this disease. However, clinical studies with a variety of agents that block inflammatory mediators have been unsuccessful (reviewed in Natanson et al., Ann. Intern. Med 120:771-783, 1994 and Gibaldi, Pharmacotherapy 13:302-308, 1993). Since many of the mediators involved in inflammation are compensatory responses, and therefore have salutary effects, some investigators have suggested that blocking their action may not be appropriate (see, e.g., Parrillo, N. Engl. J. Med. 328:1471-1477, 1993).

More recently, phase III trials for the treatment of sepsis (Bernard et al., N Engl J Med, 344, 699-709, 2001) has been completed. Patients suffering from severe sepsis were given doses of 24 μg/kg/h for a total duration of 96 hours as infusion. A total of 1520 persons were involved in the trial and it was found that the 28 days mortality rate was reduced from 31% to 25%.

A "patient" for the purposes of the present invention includes both humans and other mammals. Thus the methods are applicable to both human therapy and veterinary applications.

The variant is administered to patients in an effective dose. By "effective dose" herein is meant a dose that is sufficient to produce the desired effects in relation to the condition for which it is administered. The exact dose will depend on the disorder to be treated, and will be ascertainable by one skilled in the art using known techniques. As mentioned above, in the treatment of severe sepsis 24 μg/kg/h of hAPC is administered for 96 hours as continuous infusion, which corresponds to a total amount of protein of about 230 mg for a patient having a body weight of about 100 kg.

The variants disclosed herein are, due to their potentially increased anti-inflammatory effect and/or increased functional half-life, contemplated to be more efficient, in particular in the treatment of sepsis, than the corresponding hAPC. The increased efficacy means that the effective dose needed to obtain the desired effect for a particular disorder will be smaller (less protein need to be administered) than the effective dose of hAPC or, alternatively, that by using the same dose as presently being used for hAPC, the treatment of patients with the variants disclosed herein will be more efficient in that a decrease in the mortality
rate, as compared to a similar treatment with hAPC, will be observed. Moreover, the variants disclosed herein which have an increased functional half-life will permit the use of a reduced amount and/or and less frequent administration, such as bolus injections, of the variants. For example, the variants may be administered by either a bolus or infusion or as a combination thereof with doses which range from 1 µg/kg body weight as a bolus every 2nd hour for several days (e.g. for 96 hours) to 1 mg/kg body weight as a bolus once every 4th day. Preferably, as low a dose as possible is administered as less frequent as possible, e.g. 1-500 µg/kg body weight, preferably 1-250 µg/kg body weight, such as 1-100 µg/kg body weight, more preferably 1-50 µg/kg body weight is administered as a bolus every 4-96 hours, e.g. every 8-96 hours, such as every 16-96 hours, every 24-96 hours, every 40-96 hours, every 48-96 hours, every 56-96 hours, every 72-96 hours.

In a further aspect the present invention relates to a pharmaceutical composition comprising the variant of the invention and pharmaceutically acceptable carrier of excipient.

In the present context, the term “pharmaceutically acceptable” means that the carrier or excipient, at the dosages and concentrations employed, will not cause any unwanted or harmful effects in the patients to which they are administered. Such pharmaceutically acceptable carriers and excipients are well known in the art (see Remington's Pharmaceutical Sciences, 18th edition, A. R. Gennaro, Ed., Mack Publishing Company [1990]; Pharmaceutical Formulation Development of Peptides and Proteins, S. Frokjaer and L. Hovgaard, Eds., Taylor & Francis [2000]; and Handbook of Pharmaceutical Excipients, 3rd edition, A. Kibbe, Ed., Pharmaceutical Press [2000]).

The variant can be used “as is” and/or in a salt form thereof. Suitable salts include, but are not limited to, salts with alkali metals or alkaline earth metals, such as sodium, potassium, calcium and magnesium, as well as e.g. zinc salts. These salts or complexes may by present as a crystalline and/or amorphous structure.

The pharmaceutical composition of the invention may be administered alone or in conjunction with other therapeutic agents. These agents may be incorporated as part of the same pharmaceutical composition or may be administered separately from the variant, either concurrently or in accordance with another treatment schedule. In addition, the variant or pharmaceutical composition may be used as an adjuvant to other therapies.

The pharmaceutical composition of the invention may be formulated in a variety of forms, e.g. as a liquid, gel, lyophilized, or as a compressed solid. The preferred form will de-
pend upon the particular indication being treated and will be readily able to be determined by one skilled in the art.

The administration of the formulations of the present invention can be performed in a variety of ways, including, but not limited to, orally, subcutaneously, intravenously, intracerebrally, intranasally, transdermally, intraperitoneally, intramuscularly, intrapulmonary, vaginally, rectally, intraocularly, or in any other acceptable manner. The formulations can be administered continuously by infusion, although bolus injection is acceptable, using techniques well known in the art, such as pumps or implantation. In some instances the formulations may be directly applied as a solution or spray.

**Parenteral compositions**

An example of a pharmaceutical composition is a solution designed for parenteral administration. Although in many cases pharmaceutical solution formulations are provided in liquid form, appropriate for immediate use, such parenteral formulations may also be provided in frozen or in lyophilized form. In the former case, the composition must be thawed prior to use. The latter form is often used to enhance the stability of the active compound contained in the composition under a wider variety of storage conditions, as it is recognized by those skilled in the art that lyophilized preparations are generally more stable than their liquid counterparts. Such lyophilized preparations are reconstituted prior to use by the addition of one or more suitable pharmaceutically acceptable diluents such as sterile water for injection or sterile physiological saline solution.

In case of parenterals, they are prepared for storage as lyophilized formulations or aqueous solutions by mixing, as appropriate, the polypeptide having the desired degree of purity with one or more pharmaceutically acceptable carriers, excipients or stabilizers typically employed in the art (all of which are termed "excipients"), for example buffering agents, stabilizing agents, preservatives, isotonifiers, non-ionic detergents, antioxidants and/or other miscellaneous additives.

Buffering agents help to maintain the pH in the range which approximates physiological conditions. They are typically present at a concentration ranging from about 2 mM to about 50 mM Suitable buffering agents for use with the present invention include both organic and inorganic acids and salts thereof such as citrate buffers (e.g., monosodium citrate-disodium citrate mixture, citric acid-trisodium citrate mixture, citric acid-monosodium citrate mixture, etc.), succinate buffers (e.g., succinic acid-monosodium succinate mixture, succinic
acid-sodium hydroxide mixture, succinic acid-disodium succinate mixture, etc.), tartrate buffers (e.g., tartaric acid-sodium tartrate mixture, tartaric acid-potassium tartrate mixture, tartaric acid-sodium hydroxide mixture, etc.), fumarate buffers (e.g., fumaric acid-monosodium fumarate mixture, fumaric acid-disodium fumarate mixture, monosodium fumarate-disodium fumarate mixture, etc.), gluconate buffers (e.g., gluconic acid-sodium glyconate mixture, gluconic acid-sodium hydroxide mixture, gluconic acid-potassium glyconate mixture, etc.), oxalate buffer (e.g., oxalic acid-sodium oxalate mixture, oxalic acid-sodium hydroxide mixture, oxalic acid-potassium oxalate mixture, etc.), lactate buffers (e.g., lactic acid-sodium lactate mixture, lactic acid-sodium hydroxide mixture, lactic acid-potassium lactate mixture, etc.) and acetate buffers (e.g., acetic acid-sodium acetate mixture, acetic acid-sodium hydroxide mixture, etc.). Additional possibilities are phosphate buffers, histidine buffers and trimethylamine salts such as Tris.

Preservatives are added to retard microbial growth, and are typically added in amounts of e.g. about 0.1%-2% (w/v). Suitable preservatives for use with the present invention include phenol, benzyl alcohol, meta-cresol, methyl paraben, propyl paraben, octadecyldimethylbenzyl ammonium chloride, benzalkonium halides (e.g. benzalkonium chloride, bromide or iodide), hexamethonium chloride, alkyl parabens such as methyl or propyl paraben, catechol, resorcinol, cyclohexanol and 3-pentanol.

Isotonicifiers are added to ensure isotonicity of liquid compositions and include polyhydric sugar alcohols, preferably trihydric or higher sugar alcohols, such as glycerin, erythritol, arabitol, xylitol, sorbitol and mannitol. Polyhydric alcohols can be present in an amount between 0.1% and 25% by weight, typically 1% to 5%, taking into account the relative amounts of the other ingredients.

Stabilizers refer to a broad category of excipients which can range in function from a bulking agent to an additive which solubilizes the therapeutic agent or helps to prevent denaturation or adherence to the container wall. Typical stabilizers can be polyhydric sugar alcohols (enumerated above); amino acids such as arginine, lysine, glycine, glutamine, asparagine, histidine, alanine, ornithine, L-leucine, 2-phenylalanine, glutamic acid, threonine, etc., organic sugars or sugar alcohols, such as lactose, trehalose, stachyose, mannitol, sorbitol, xylitol, ribitol, myoinositol, galactitol, glycerol and the like, including cyclitols such as inositol; polyethylene glycol; amino acid polymers; sulfur-containing reducing agents, such as urea, glutathione, thiocitric acid, sodium thioglycolate, thioglycerol, α-monothioglycerol and sodium thiosulfate; low molecular weight polypeptides (i.e. <10 residues); proteins such as human
serum albumin, bovine serum albumin, gelatin or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; monosaccharides such as xylose, mannose, fructose and glucose; disaccharides such as lactose, maltose and sucrose; trisaccharides such as raffinose, and polysaccharides such as dextran. Stabilizers are typically present in the range of from 0.1 to 10,000 parts by weight based on the active protein weight.

Non-ionic surfactants or detergents (also known as "wetting agents") may be present to help solubilize the therapeutic agent as well as to protect the therapeutically polypeptide against agitation-induced aggregation, which also permits the formulation to be exposed to shear surface stress without causing denaturation of the polypeptide. Suitable non-ionic surfactants include polysorbates (20, 80, etc.), polyoxamers (184, 188 etc.), Pluronic® polyols, polyoxyethylene sorbitan monoesters (Tween®-20, Tween®-80, etc.).

Additional miscellaneous excipients include bulking agents or fillers (e.g. starch), chelating agents (e.g. EDTA), antioxidants (e.g., ascorbic acid, methionine, vitamin E) and cosolvents.

The active ingredient may also be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example hydroxymethylcellulose, gelatin or poly-(methylmethacrylate) microcapsules, in colloidal drug delivery systems (for example liposomes, albumin microspheres, microemulsions, nanoparticles and nanocapsules) or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences, supra.

Parenteral formulations to be used for in vivo administration must be sterile. This is readily accomplished, for example, by filtration through sterile filtration membranes.

Sustained release preparations

Suitable examples of sustained-release preparations include semi-permeable matrices of solid hydrophobic polymers containing the variant, the matrices having a suitable form such as a film or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate) or poly(vinylalcohol)), polylactides, copolymers of L-glutamic acid and ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the ProLease® technology or Lupron Depot® (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(−)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for long periods
such as up to or over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated polypeptides remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37°C, resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S-S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

All references cited herein are hereby incorporated by reference in their entirety for all purposes. The invention is further illustrated by the following non-limiting examples.

**EXAMPLES**

**Example 1 - Construction of protein C expression vector**

A gene encoding the human protein C precursor was constructed by assembly of synthetic oligonucleotides by PCR using methods similar to the ones described in Stemmer et al. (1995) *Gene* 164, pp. 49-53. The native Protein C signal sequence was maintained in order to allow secretion of the gene product. The synthetic gene was designed with a NheI site at the 5′-end and a XbaI site at the 3′-end and subcloned behind the CMV promoter in pcDNA3.1/Hygro (Invitrogen) using these sites. The Protein C precursor sequence in the resulting plasmid, termed pCR4, is given in SEQ ID NO:2.

Furthermore, in order to test for a higher gene expression, the synthetic gene was cloned into the KpnI-XbaI sites of pcDNA3.1/Hygro containing an intron (from pCI-Neo (Promega)) in the 5′ untranslated region of the gene. The resulting plasmid was termed pRC2.

**Example 2 – Site directed mutagenesis**

All protein C variants are constructed using Quick-Change (Stratagene). Primers are purchased from TAG Technology (Copenhagen) containing the appropriate mutations. The PCR reactions are performed according to the manufacturer’s manual and the plasmids are transformed into TG1 competent cells. Plasmid preparations are made on single clones and the sequences are verified using a DNA sequencer 3100 genetic Analyser (ABI)
Example 3 – Production

Transfection of CHO K1 cells for expression of Protein C and variants thereof

Typically, CHO K1 cells are grown to near confluence in T-25 tissue culture flasks. On the day of transfection the medium on the cells is changed with 5 ml of fresh medium (e.g. MEMα (Invitrogen Gibco #32571-028), 10% FCS, 100 U/ml of penicillin, 100 µg/ml streptomycin and 5 µg/ml Vitamin K).

Using a derivative of the pcDNA3.1/Hygro (Invitrogen) expression plasmid, encoding the appropriate protein C variant, the cells are transfected using the Lipofectamin2000 (Invitrogen #116687-019) transfection agent according to the manufactures instructions. After 24 hours, 360 µg/ml Hygromycin B selection is applied to the cells. During the next few days the selective medium is changed daily until stop of cell shedding. At confluence in T-25 tissue culture flask the cells are passed to a T-175 tissue culture flask and allowed to propagate under selection until confluence. The cells in the resulting pool of stable transfectants are dilution cloned and cryo-preserved for future use.

Dilution cloning

Cells are trypsinised and counted in a hemacytometer. A dilution with approximately 10 cells/ml is made and 100 µl aliquots are dispensed into the wells of a 96 well tissue culture plate. After a week wells with single clones are identified by microscopy and marked. At confluence productive clones are screened by ELISA analysis of 24 hours culture supernatants. The expression level of productive clones are then quantified by ELISA analysis of supernatants from confluent T-25 tissue culture flasks. The best clones are then transferred to T-175 tissue culture flasks, propagated to confluence and cryo-preserved.

Production of protein C and variants thereof

Typically, the clone with the highest expression level is chosen for production purposes. After thawing, the clone is propagated to confluence in MEMα (Invitrogen Gibco #32571-028), 10% FCS, 100 U/ml of penicillin, 100 µg/ml streptomycin and 5 µg/ml Vitamin K in a T-175 tissue culture flask and used as inoculum for one tissue culture roller bottle. The cells are propagated in roller bottles until confluent in MEMα (Invitrogen Gibco #32571-028), 10% FCS, 100 U/ml of penicillin, 100 µg/ml streptomycin and 5 µg/ml Vitamin K. Then the medium is changed to UltraCHO (BioWhittaker #12-724Q), 0,1% ExCyte (Serologicals Proteins Inc. # 81-129-1), 100 U/ml of penicillin, 100 µg/ml streptomycin and 5
μg/ml Vitamin K for four days with a medium shift after 2 days. Finally, the medium is changed to the production medium, DMEM/F-12 (Invitrogen # 11039-021), 1:100 ITS-A, 0.1% ExCyte (Serologicals Proteins Inc. # 81-129-1), 100 U/ml of penicillin, 100 μg/ml streptomycin and 5 μg/ml Vitamin K and the medium is harvested daily for purification purposes.

Example 4 - Purification

Frozen supernatants (10-20 liters) are thawed at 4°C. The supernatants are concentrated and diafiltered into 20 mM tris-HCl, pH 7.4, 150 mM NaCl (equilibration buffer) using a TFF-system (Benchscale, Millipore) equipped with pellicon Biomax 10kDa mwco membranes. The diafiltered supernatant is then applied onto 20 ml of equilibrated Q-sepharose FF matrix packed in a XK26 column at 20 ml/min. The column is washed with the equilibration buffer until a stable baseline (measured by the absorbance at 280 nm) is obtained. Elution of protein C is performed by using 20 mM tris-HCl, pH 7.4, 120 mM NaCl and 15mM CaCl₂ at a flow rate of 10ml/min.

Fractions are analysed by SDS-page, and protein C-containing fractions were pooled. The conductivity is adjusted by the addition of NaCl either as a solid or as a stock solution for optimal binding onto a phenyl sepharose 6 FF (Pharmacia).

The sample is then applied onto 10 ml matrix packed in a XK16/10 column equilibrated with 20 mM tris-HCl, pH 7.4, 1 M NaCl, 10mM CaCl₂.

The column is washed until stable base line with the equilibration buffer. By applying a step gradient of 20 mM tris-HCl, pH 7.4, 1 M NaCl, 5 mM EDTA, protein C is specifically eluted from the column. Protein C-containing fractions are pooled and prepared for activation.

Example 5 – Activation

The protein C polypeptides are activated using the venom protein C activator, ACC-C (Nakagaki et al., Thrombosis Research 58:593-602, 1990). The zymogen forms are incubated at 37°C for about 60 min in 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 5 mM EDTA, using a final concentration of 1 ng/ml of ACC-C. The activation process is checked using the "Amidolytic Assay" and polyacrylamide gel electrophoresis analysis.
Example 6 – Determination of amidolytic activity

Amidolytic Assay

Amidolytic activity is determined using the peptide substrate SPECTROZYME PCa with the formula H-D-Lys(γ-Cbo)-Pro-Arg-pNA.2AcOH (American Diagnostica Inc, product # 336) at a final concentration of 0.5 mM. Assays are performed at 23°C in 50 mM Tris-HCl (pH 8.3), 100 mM NaCl, 5 mM CaCl₂ and 0.1% BSA. The rate of hydrolysis is recorded for 3 min at 405 nm as the change in absorbance units/min in a plate reader. Xigris® (hAPC) is used as a standard.

Example 7 – Determination of anticoagulant activity

APC Clotting Assay

Anticoagulant activity is assessed by monitoring the prolongation of clotting time in the activated partial thromboplastin time (APTT) assay using Nycoplastin (Nycomed, product no. 1002448) together with Normal Hemostasis Reference Plasma (American Diagnostica Inc., catalogue no. 258N). Coagulation is started by mixing the APTT reagent containing hAPC with the normal hemostasis reference plasma at 37°C and measuring the clotting time by manual mixing. In order to compare the anticoagulant activity of the variants and hAPC, equal amounts – based on amidolytic activity – of the variants and hAPC should be used in the assay.

Example 8 – Inactivation by alpha-1-antitrypsin

Alpha-1-Antitrypsin Inactivation Assay

hAPC is incubated with 16.6 or 42.3 μM human alpha-1-antitrypsin (Sigma) in 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM CaCl₂ containing 0.1% BSA at 37°C. After 20 hours incubation a 15 μl sample of the incubated mixtures is added to 110 μl 50 mM Tris-HCl (pH 8.3), 100 mM NaCl, 5 mM CaCl₂ in microplates and assayed for amidolytic activity as described in the “Amidolytic Assay”. The remaining activity is calculated by normalizing with the activity obtained in samples lacking alpha-1-antitrypsin but otherwise incubated under identical conditions.
Example 9 – Inactivation by human plasma

**Human Plasma Inactivation Assay I**

hAPC is incubated in 90% normal human plasma (Sigma Diagnostics, Accuclot™ reference plasma) containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 5 mM CaCl₂ at 37°C. Aliquots are removed after 200 min and assayed for amidolytic activity as described in the “Amidolytic Assay”. The residual amidolytic activity after 200 min is expressed in percentage of the amidolytic activity measured at the start of the experiment.

Example 10 – In vitro half-life in human plasma

**Human Plasma Inactivation Assay II**

hAPC is incubated in 90% normal human plasma (Sigma Diagnostics, Accuclot™ reference plasma) containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 5 mM CaCl₂ at 37°C. Aliquots are removed at various time-points and assayed for amidolytic activity as described in the “Amidolytic Assay”. The residual amidolytic activity at the various time-points is expressed in percentage of the amidolytic activity measured at the start of the experiment. The functional *in vitro* half-life (expressed in minutes) is calculated as the time at which 50% of the amidolytic activity is still present.

Example 11 – Determination of anti-inflammatory activity

**Anti-inflammatory Assay I**

The anti-inflammatory properties of the polypeptides are investigated using recombinant tumor necrosis factor α (TNFα) (catalogue number: 210-TA, R&D Systems, Minneapolis, USA) stimulated human umbilical vein endothelial cells (HUVEC) (catalogue number: CC-2519, Clonetics, San Diego, USA). HUVEC is stimulated using 1 ng/ml TNFα for about 7 hours. Then, various concentrations (0 – 200 nM) of hAPC are incubated for up to 20 hours. The cells are removed by trypsinization and analysed using flow cytometry measuring the surface expression of ICAM-1, VCAM-1 and/or E-selectin. For E-selectin quantification a FITC-conjugated anti-human E-selectin monoclonal antibody (CD62E) (catalog number: BBA21, R&D Systems, Minneapolis, USA) is used. The anti-inflammatory activity of hAPC is determined by calculating the hAPC concentration needed to suppress the TNFα stimulation of E-selectin to 50% compared to the effect of TNFα obtained without hAPC. This hAPC concentration is used to indicate the half maximum inhibitory concentration (IC₅₀), and these values are determined for hAPC and each individual variant.
**Example 12 - Determination of anti-inflammatory activity**

**Anti-inflammatory Assay II**

As an alternative to the assay disclosed in Example 1115 above, one may assess the anti-inflammatory property of hAPC by the inhibition of TNF alpha production from LPS stimulated monocytes. In this assay monocytic THP-1 cells (10⁵) (ATCC, Rockville MD) are pre-incubated with various concentrations (0-1600 nM) of hAPC for 3 hours. Then, cells are stimulated using 0.5ug/ml LPS (Cat# 314, List Biological Laboratories) for 4 hours. TNF alpha production in the cell culture supernatant is subsequently measured using a TNF alpha ELISA (Cat# DY210, R and D systems). The anti-inflammatory activity of hAPC is determined by calculating the hAPC concentration needed to obtain 50 % reduction in LPS induced TNF alfa production as compared to the effect of LPS alone. This hAPC concentration is used to indicate the half maximum inhibitory concentration (IC₅₀), and these values are determined for hAPC and each individual variant.
CLAIMS

1. A protein C variant wherein the cleavage site between the light chain and the heavy chain has been removed.

2. The variant of claim 1, comprising 1-15 amino acid modifications relative to the amino acid sequence of human wild-type protein C (SEQ ID NO:1).

3. The variant of claim 1 or 2, wherein R157 is removed, with the proviso that R157 is not removed by the substitution R157K.

4. The variant of claim 3, wherein R157 is removed by substitution, with the proviso that the substitution is not R157K.

5. The variant of claim 1 or 2, wherein K156 is removed, with the proviso that K156 is not removed by the substitution K156R.

6. The variant of claim 5, wherein K156 is removed by substitution, with the proviso that the substitution is not K156R.

7. The variant of claim 1 or 2, wherein both K156 and R157 are removed, with the proviso that K156 and R157 are not removed by the substitutions K156R and R157K.

8. The variant of claim 7, wherein K156 and R157 are removed by substitution, with the proviso that the substitutions are not K156R and R157K.

9. The variant of any of the preceding claims, wherein the variant is in activated form.

10. The variant of claim 9, wherein the variant has been activated by cleavage of the bond between R169 and L170.

11. The variant of claim 9 or 10, wherein said variant has at least 50% of the amidolytic activity of human wild-type protein C in its activated form (hAPC), when tested in the “Amidolytic Assay” described in Example 6 herein.

12. The variant of any of claims 9-11, wherein said variant has at least 50% of the anti-inflammatory activity of hAPC when tested in the “Anti-inflammatory Assay I” or “Anti-inflammatory Assay II” described in Examples 11 and 12 herein.
13. The variant of any of claims 9-12, wherein said variant has a reduced anticoagulant activity compared to hAPC when tested in the “Clotting Assay” described in Example 7 herein.

14. The variant of claim 13, wherein said variant has 1-75% of the anticoagulant activity of hAPC.

15. The variant of any of the preceding claims, comprising at least one amino acid modification in at least one region selected from the active site region and the autolysis loop as defined herein.

16. The variant of claim 15, comprising at least one substitution in the active site region, wherein said at least one substitution is an introduction of an amino acid residue comprising an attachment group for a non-polypeptide moiety.

17. The variant of claim 16, wherein said introduced amino acid residue comprising an attachment group for a non-polypeptide moiety creates a glycosylation site.

18. The variant of claim 17, wherein said glycosylation site is an in vivo N-glycosylation site.


20. The variant of claim 16, wherein said introduced amino acid residue comprising an attachment group for a non-polypeptide moiety is a cysteine residue.


22. The variant of claim 20 or 21, wherein at least one non-polypeptide moiety is covalently attached to said introduced cysteine residue.

23. The variant of claim 22, wherein the non-polypeptide moiety is a polymer molecule.

24. The variant of claim 23, wherein the non-polypeptide moiety is a linear or branched polyethylene glycol or a polyalkylene oxide.

25. The variant of any of claims 15-24, which comprises at least one amino acid substitution of a charged amino acid residue, wherein said charged amino acid residue is located in the active site region and has at least 25% of its side chain exposed to the surface, with an amino acid residue having no charge or an amino acid residue having an opposite charge.

26. The variant of any of claims 15-25, comprising at least one amino acid modification in the autolysis loop.

27. The variant of claim 26, wherein at least one positively charged amino acid residue in the autolysis loop has been substituted with an uncharged or a negatively charged amino acid residue.

28. The variant of claim 26, wherein at least one negatively charged amino acid residue in the autolysis loop has been substituted with an uncharged amino acid residue.

29. A nucleotide sequence encoding the variant as defined in any of claims 1-28.
30. An expression vector comprising a nucleotide sequence as defined in claim 29.

31. A host cell comprising a nucleotide sequence as defined in claim 29 or an expression vector as defined in claim 30.

32. The host cell of claim 31, wherein said host cell is a glycosylating eukaryotic cell.

33. A composition comprising the variant of any of claims 1-28 and at least one pharmaceutically acceptable carrier or excipient.

34. A variant as defined in any of claims 1-28 for use as a medicament.

35. Use of a variant as defined in any of claims 1-28 for the manufacture of a medicament for the treatment of sepsis or septic shock.

36. A method for treating a patient suffering from or at risk of acquiring sepsis or septic shock, comprising administering to said patient an effective amount of the variant as defined in any of claims 1-28 or the pharmaceutical composition as defined in claim 35.
Fig. 1
Fig. 2
SEQ ID NO: 1

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gcc aac tcc ttc ctg gag
   Ala  Asn  Ser  Phe  Leu  Glu
   1      5

gag ctc cgt cac agc agc ctg gag cgg gag tgc ata gag gag atc tgt
   Glu  Leu  Arg  His  Ser  Ser  Leu  Glu  Arg  Glu  Cys  Ile  Glu  Glu  Ile  Cys
   10     15    20

gac ttc gag gag gcc aag gaa att ttc cca aat gtt gat gac aca ctg
   Asp  Phe  Glu  Glu  Ala  Lys  Glu  Ile  Phe  Gln  Asn  Val  Asp  Asp  Thr  Leu
   25     30    35

gcc ttc ttg tcc aag cac gtc gac ggt gac cag tgc ttg gtc ttg ccc
   Ala  Phe  Trp  Ser  Lys  His  Val  Asp  Gly  Asp  Glu  Cys  Leu  Val  Leu  Pro
   40     45    50

ttg gag cac cgg tgc gcc agc ctg tgc tgc ggg cac gcc agc tgc atc
   Leu  Glu  His  Pro  Cys  Ala  Ser  Leu  Cys  Gly  His  Gly  Thr  Cys  Ile
   55     60    65    70

gac ggc atc ggc gcc agc ttc agc tgc gac tgc gcc agc gcc tgg gag gcc
   Asp  Gly  Ile  Gly  Ser  Phe  Ser  Cys  Asp  Arg  Ser  Gly  Trp  Glu  Gly
   75     80    85

cgc ttc tgc cag cgc gag tgc agc ttc ctc aat tgc tgg ctg gac aac
   Arg  Phe  Cys  Glu  Arg  Val  Ser  Phe  Leu  Asn  Cys  Ser  Leu  Asp  Asn
   90     95    100

ggc ggc tgc aag cat tac tgc cta gag gag gtt ggc tgg cgg cgc tgc
   Gly  Gly  Cys  Thr  His  Tyr  Cys  Leu  Glu  Val  Gly  Trp  Arg  Arg  Cys
   105    110   115

agc tgt ggc cct ggc tac aag ctg ggg gac gac ctc ctg cag tgt cac
   Ser  Cys  Ala  Pro  Gly  Tyr  Lys  Leu  Gly  Asp  Asp  Leu  Leu  Glu  Cys  His
   120    125   130

ccc gca gtt aag ttc cct tgc tgt ggg agg ccc tgt tgg aag cgg atg gag aag
   Pro  Ala  Val  Lys  Phe  Pro  Cys  Gly  Arg  Arg  Pro  Trp  Lys  Arg  Met  Glu  Lys
   135    140   145   150

aag cgc atg cac ctc aag cga gac aca gaa gac caa gac caa gta
   Lys  Arg  Ser  His  Leu  Arg  Leu  Arg  Leu  Gln  Glu  Asp  Glu  Val
   155    160   165

gat ccg cgg ctc att gat ggg aag atg acc aag cgg gga gac aca gtc
   Asp  Pro  Arg  Leu  Ile  Asp  Gly  Lys  Met  Thr  Arg  Arg  Gly  Ser  Pro
   170    175   180

tgg cag gtt ctc ctt gac tca aag aag aag ctt gcc tgc ggg gca
   Trp  Gln  Val  Val  Leu  Leu  Asp  Ser  Lys  Lys  Lys  Leu  Ala  Cys  Gly  Ala
   185    190   195

gtg ctc atc cac ccc ttc tgg gtt ctc aca ggc gcc cac tgc atg gat
   Val  Leu  Ile  His  Pro  Ser  Trp  Val  Leu  Thr  Ala  Ala  His  Cys  Met  Asp
   200    205   210
```
gag tcc aag aag ctc ctt gtc agg ctt gga gag tat gac ctg cgg cgc
Glu Ser Lys Lys Leu Leu Val Arg Leu Gly Glu Tyr Asp Leu Arg Arg
215 220 225 230

5 tgg gag aag tgg gag ctc gac atc aag gag gtc ttc gtc cac
Trp Glu Lys Trp Glu Leu Asp Leu Asp Ile Ala Leu Leu Val His
235 240 245

ccc aac tac agc aag acc acc acc gac aat gac atc gca ctg ctg cac
10 Pro Asn Tyr Ser Lys Ser Thr Thr Asp Asp Ile Ala Leu Leu His
250 255 260

tcg gcc cag gcc acc ctc tgg cag acc ata gtg ccc atc tgc ctc
Leu Ala Gln Pro Ala Thr Leu Ser Thr Ile Val Pro Ile Cys Leu
15 265 270 275

ccg gac agc gcc ctt gca gag cgc gag ctc aat cag gcc ggc cag gag
Pro Asp Ser Gly Ala Ala Glu Arg Leu Asn Glu Ala Gly Gln Glu
280 285 290

20 acc ctc gtc aag ggc tgg ggc tac cac agc agc cga gag aag gag gcc
Thr Leu Val Ala Gly Thr Gly Tyr His Ser Ser Arg Glu Lys Glu Ala
295 300 305 310

25 aag aga aac cgc acc ttc gtc ctc aac ttc atc aag att ccc gtc gtc
Lys Arg Asn Arg Thr Phe Val Leu Asn Phe Ile Lys Ile Pro Val Val
315 320 325

ccg cac aat gag tgc agc gag gtc atg aag aac atg gtc tct gag aac
30 Pro His Asn Glu Cys Ser Glu Val Met Ser Asn Met Val Ser Glu Asn
330 335 340

35 atg ctc tgt gcc ggc atc ctc ggg gag cgg cag gtt gcc tgc gag ggc
Met Leu Cys Ala Gly Ile Leu Gly Asp Arg Glu Asa Ala Cys Gly
345 350 355

40 gac agt ggg ggg ccc atg gtc gcc tcc ttc cac ggc acc tgg ttc ctg
Asp Ser Gly Gly Pro Met Val Ala Ser Phe His Gly Thr Trp Phe Leu
360 365 370

gtg ggc ctt gtc ggc tgt gag ggc tgt ggg ctc ctt cac aac tac
Val Gly Leu Val Ser Trp Gly Glu Gly Cys Leu Leu His Asn Tyr
375 380 385 390

45 ggc gtt tac acc aac gtc agc cgc tac ctc gag tgg atc cat ggg cac
Gly Val Tyr Thr Lys Val Ser Arg Tyr Leu Asp Trp Ile His Gly His
395 400 405

atc aga gag aag gaa gcc ccc cag aag agc tgg gca cct
Ile Arg Asp Lys Ala Pro Gin Lys Ser Trp Ala Pro
410 415
tgg cag gtg gtc ctg gtg gac tca aag aag aag ctg gcc tgc ggg gca
Trp Gln Val Val Leu Leu Asp Ser Lys Lys Leu Ala Cys Gly Ala
185 190 195
5
gtg ctc atc cac ccc tcc tgg gtc atc aag gac gcc cac tgc atg gat
Val Leu Ile His Pro Ser Trp Val Leu Thr Ala Ala His Cys Met Asp
200 205 210
10
gag tcc aag aag ctc ctt gtc agg ctt gga gag tat gac ctg cgg cgc
Glu Ser Lys Leu Val Leu Val Arg Leu Glu Tyr Asp Leu Arg Arg
215 220 225 230
15
tgg gag aag tgg gag ctg gac ctg aac aag gag gtc ttc gtc cac
Trp Glu Lys Trp Glu Ala Asp Leu Asp Ile Lys Glu Val Phe Val His
235 240 245
20
ccc aac tac aac aag acc acc gcc cac aat gac gcc ctg cgg cca
Pro Asp Tyr Ser Lys Ser Thr Thr Asp Asp Ile Ala Leu Leu His
250 255 260
25
cgg acg aac gcc ctc gca gag cgc cag ctc aat cag gcc ggc cag gag
Pro Asp Ser Gly Ala Arg Leu Asp Gln Ala Gly Gly Glu
265 270 275
30
acc ctc gtt acg ggc tgg gcc tac cac agc aag cga gag aag gag gcc
Thr Leu Val Thr Gly Trp Gly Tyr His Ser Ser Arg Glu Lys Glu Ala
280 285 290
35
aag aga aac cgc acc ttc gtc ctc aac ttc aat cgg ctc tgg gtc
Lys Arg Asn Arg Thr Phe Val Leu Asp Ile Lys Ile Pro Val Val
305 310
40
ccg cac aat gag tgc aag gcc gag gtc aag aac atg gtc tct gag aac
Pro His Asn Gly Cys Met Ala Ser Met Ser Met Ser Glu Asn
320 325
45
atg ctt ctt ggc gac gtc aac ctc ctt ggc ctt ggc gag ggc ggc
Met Leu Cys Ala Leu Ile Leu Gly Ile Leu Arg Glu Asn Ala Cys Gly
335 340
50
gac agt ggg cgc atg gtc gcc tcc ttc cac gcc acc tgg ttc ctg
Asp Ser Gly Gly Pro Met Val Ala Ser Phe Gly Thr Trp Phe Leu
345 350 355
55
gtg gcc ctt ggc gtc cgg ggg ctc ggg act cac aac tac ctc gtt ggt
Val Gly Leu Val Ser Trp Gly Glu Gly Cys Gly Leu Leu His Asn Tyr
360 365 370
60
ggc gtt tac acc aac gtc aag cgc tac ctc gac gtt atc cat ggg cag
Gly Val Tyr Thr Lys Val Ser Arg Tyr Leu Asp Trp Ile His Gly His
375 380 385 390
65
atc aag gac aag gaa gcc ccc cag aac cag tgg gca cct
Ile Arg Asp Lys Glu Ala Pro Gln Lys Ser Thr Ala Pro
400 405
70
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125
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