Method for producing gamma-carboxylated proteins

Technical field

The present invention relates a host cell comprising an expression vector comprising a nucleic acid molecule encoding a protein requiring gamma-carboxylation and associated expression control sequences comprising a first promoter and a nucleic acid molecule encoding a γ-glutamyl carboxylase and associated expression control sequences comprising a second promoter. The invention further relates to a method of producing a protein requiring gamma-carboxylation in high yields.

Background to the invention

Bleeding is a common clinical problem. It is a consequence of disease, trauma, surgery and medicinal treatment. It is imperative to mechanically stop the bleeding. This may be difficult or even impossible due to the location of the bleeding or because it diffuses from many (small) vessels. Patients who are bleeding may thus require treatment with agents that support haemostasis. This may be blood-derived products (haemotherapy), agents that cause the release of endogenous haemostatic agents, recombinant coagulation factors (F), or agents that delay the dissolution of blood clots.

The first line treatment among the blood derived products, often obtained from the local hospital, are whole blood for volume substitution and support of haemostasis, packed red cells for the improvement of oxygen transporting capacity, platelet concentrates to raise the number of platelets (if low or defective) and fresh frozen plasma for support of the haemostasis (blood coagulation and platelet aggregation). Second line plasma derived products that support haemostasis are plasma cryoprecipitate, prothrombin complex concentrates, activated prothrombin complex concentrates and purified coagulation factors. Several coagulation factors are today available as human recombinant proteins, inactive (coagulation factors VIII and IX) and activated (coagulation factor VIIa).

Haemophilia is an inherited or acquired bleeding disorder with either abnormal or deficient coagulation factor or antibodies directed towards a coagulation factor which inhibits the procoagulant function. The most common haemophilias are haemophilia A (lack coagulation factor VIII) and haemophilia B (factor IX). The purified or recombinant single coagulation factors are the main treatment of patients with haemophilia. Patients with inhibitory antibodies posses a treatment problem as they may also neutralise the coagulation factor that is administered to the patient. The active form of Protein C (APC) is an inhibitor of plasma coagulation by degradation of the activated coagulation factors Va and VIIIa. Recombinant APC has been shown to be an effective treatment of undue plasma coagulation in patients with sepsis.

Coagulation factors for therapeutic use can be obtained from human plasma, although the purification process is not simple and requires many steps of which several aim at eliminating contaminating viruses. But even with extensive safety measures and testing of blood-derived products, contamination with infectious viruses or prions cannot be ruled out. Because of this risk it is highly desirable to produce human therapeutic proteins from recombinant cells grown in media without animal derived components. This is not always straightforward as many proteins require a mammalian host to be produced in a fully functional form, i.e. be correctly post-translationally modified. Among the coagulation factors commercially produced in recombinant cells are FVII (NovoSeven), FVIII (Kogenate, Recombinate, Refacto) and FIX (BeneFix) (Roddie and Ludlam. Blood Rev. **11**:169 - 177, 1997) and Active Protein C (Xigris). One of the major obstacles in obtaining large amounts of fully functional recombinant human coagulation factors lies in the Gla-domain present in FII, FVII, FIX, FX and Protein C. This domain contains glutamic acid residues that are post-translationally modified by addition of carboxyl groups. The production of these factors are hampered by the fact that over-expression of them result in under-carboxylated, and hence inactive, protein. The Gla modifications are a result of the action of a vitamin K-dependent enzyme called γ-glutamyl carboxylase (GGCX). This enzyme has been extensively studied by many scientists, particularly those involved in coagulation factor research (WO-A-8803926; Wu et al. Science **254**(5038):1634-1636, 1991; Rehemtulla et al., Proc Natl Acad Sci USA **90**:4611-4615, 1993; Stanley J. Biol. Chem. **274**(24):16940-16944, 1999; Vo et al., FEBS letters **445**:256-260, 1999; Begley et

al., The Journal of Biological Chemistry **275**(46):36245-36249, 2000; Walker et al., The Journal of Biological Chemistry **276**(11):7769-7774, 2001; Bandyopadhyay, et al. Proc Natl Acad Sci USA **99**(3):1264-1269, 2002; Czerwiec et al., Eur J Biochem **269**:6162-6172, 2002; Hallgren et al., Biochemistry **41**(50):15045-15055, 2002; Harvey et al., The Journal of Biological Chemistry **278**(10):8363-8369, 2003). Attempts to increase yields by co-expressing GGCX with coagulation factor FIX has been tried by at least two scientific groups but were not successful (Rehemtulla, et al. 1993, *ibid*; Hallgren et al. 2002, *ibid*). Considering the large interest in γ-carboxylated proteins, it may be assumed that many more co-expression trials have failed and thus have not been reported.

For human FII (prothrombin) at least 8 out of 10 Glu residues have to be correctly modified in order to obtain fully functional prothrombin (Malhotra, et al., J. Biol. Chem. **260**:279-287, 1985; Seegers and Walz 'Prothrombin and other vitamin K proteins', CRC Press, 1986). Extensive efforts to obtain high production levels of rhFII have been made using several different systems such as CHO cells, BHK cells, 293 cells and vaccinia virus expression systems, but have all failed or resulted in an under-carboxylated product and thus functionally inactive prothrombin (Jørgensen et al., J. Biol. Chem. **262**:6729-6734, 1987; Russo et al., Biotechnol Appl Biochem **14**(2):222-233, 1991; Fischer et al., J Biotechnol **38**(2):129-136, 1995; Herlitschka et al. Protein Expr. Purif. **8**(3):358-364, 1996; Russo et al., Protein Expr. Purif. **10**:214-225, 1997; Vo et al. 1999, *ibid*; Wu and Suttie Thromb Res **96**(2):91-98, 1999). Earlier reported productivities for carboxylated recombinant human prothrombin are low; 20 mg/L for mutant prothrombin (Côte et al., J. Biol. Chem **269**:11374-11380, 1994), 0.55 mg/L for human prothrombin expressed in CHO cells (fully carboxylated, Jørgensen et al. 1987, *ibid*), 25 mg/L in CHO cells (degree of carboxylation not shown, Russo et al. 1997, *ibid*).

WO 92/19636 discloses the cloning and sequence identification of a human and bovine vitamin K dependent carboxylase. The application suggests co-expressing the vitamin K dependent carboxylase and a vitamin K dependent protein in a suitable host cell in order to prepare the vitamin K dependent protein. No co-expression of the carboxylase and vitamin K dependent protein is exemplified.

There is a need for improved methods to produce activated blood clotting factors in high yields. The present invention sets out to address this need.

Summary of the invention

According to a first aspect of the invention there is provided a host cell comprising an expression vector comprising a nucleic acid molecule encoding a protein requiring gamma-carboxylation and associated expression control sequences comprising a first promoter and a nucleic acid molecule encoding a γ-glutamyl carboxylase and associated expression control sequences comprising a second promoter, wherein the first promoter is sufficiently stronger than the second promoter so that the protein requiring gamma-carboxylation and the γ-glutamyl carboxylase are expressed in a ratio of at least 10:1.

According to another aspect of the invention there is provided a cell which is engineered to express (i) a protein which requires gamma-carboxylation, and (ii) a γ-glutamyl carboxylase, wherein the proteins (i) and (ii) are expressed in a ratio between 10:1 and 500:1.

According to another aspect of the invention there is provided genetically modified eukaryotic host cell comprising:

(i) a polynucleotide sequence encoding γ-glutamyl carboxylase protein wherein said γ-glutamyl carboxylase protein encoding sequence is operably linked to expression control sequences permitting expression of γ-glutamyl carboxylase protein by said cell; and

(ii) a polynucleotide encoding a protein requiring carboxylation by the γ-glutamyl carboxylase protein operably linked to expression control sequences permitting expression of said protein requiring carboxylation by said cell;

wherein the cell is capable of expressing the γ-glutamyl carboxylase protein and the protein requiring carboxylation in the ratio of at least 1:10.

According to a further aspect of the invention there is provided a vector comprising a nucleic acid molecule encoding a protein requiring gamma-carboxylation and associated expression control sequences comprising a first promoter and a nucleic acid molecule

encoding a γ-glutamyl carboxylase and associated expression control sequences comprising a second promoter, wherein the first promoter is sufficiently stronger than the second promoter so that the protein requiring gamma-carboxylation and the γ-glutamyl carboxylase are expressed in a ratio of at least 10:1

According to yet another aspect of the invention there is provided a method for producing gamma-carboxylated protein comprising: (i) culturing a cell adapted to express a protein which requires gamma-carboxylation and γ-glutamyl carboxylase in a ratio of at least 10:1, under conditions suitable for expression of both proteins, and (ii) isolating gamma-carboxylated protein. In one embodiment the method is used for producing gamma-carboxylated human Factor IX and in another embodiment the method is used for producing gamma-carboxylated human prothrombin. In another embodiment, the gamma-carboxylated protein produced is human gamma-carboxylated Factor X.

According to another aspect of the invention there is provided a method for production of a gamma-carboxylated protein in a mammalian cell line, comprising the step of co-expressing with said protein requiring gamma-carboxylation in the mammalian cell line a γ-glutamyl carboxylase, wherein the amount of expressed protein requiring gamma-carboxylation is at least 10-fold greater than the amount of expressed γ-glutamyl carboxylase, and (ii) isolating gamma-carboxylated protein. In one embodiment the method is used for producing gamma-carboxylated human Factor IX and in another embodiment the method is used for producing gamma-carboxylated human prothrombin. In another embodiment, the gamma-carboxylated protein produced is human gamma-carboxylated Factor X.

According to a further aspect of the invention there is provided isolated gamma-carboxylated protein produced according to the above methods, and the use of isolated gamma-carboxylated protein produced according to the above methods in coagulation therapy or the use of isolated gamma-carboxylated protein produced according to the above methods for the manufacture of a medicament for use in coagulation therapy.

According to yet a further aspect of the invention there is provided a method of producing a pharmaceutical composition suitable for inducing blood clotting or promoting increased or decreased coagulation, comprising purifying active carboxylated protein expressed from a host cell adapted to express a protein requiring gamma-carboxylation and γ-glutamyl carboxylase in a ratio of between 10:1 and 500:1 and admixing the purified carboxylated protein with one or more pharmaceutically acceptable carriers or excipients and a pharmaceutical composition obtainable from the method. In one embodiment the active carboxylated protein is gamma-carboxylated human Factor IX and in another embodiment the active carboxylated protein is gamma-carboxylated human prothrombin. In another embodiment, the active carboxylated protein is gamma-carboxylated Factor X.

Brief description of Figures

Figure 1a illustrates a plasmid map of PN32 (prothrombin + GGCX) co-expression vector.

Figure 1b represents a plasmid map of Ptext5 (prothrombin) expression vector.

Figure 2 represents a plasmid map of PP6 (prothrombin + GGCX) co-expression vector.

Figure 3a represents a plasmid map of F9NopA (factor IX + GGCX) co-expression vector

Figure 3b represents a plasmid map of F9hglx (factor IX + GGCX) co-expression vector.

Detailed description of the invention

We have devised a different approach for expression of appropriately carboxylated recombinant vitamin K dependent coagulation factors at high levels, which involves co-expression of the vitamin K dependent coagulation factor and a γ-glutamyl carboxylase (GGCX) in a differential ratio. As one example we have expressed human prothrombin (rhFII) and human GGCX. Instead of using strong promoters for both rhFII and GGCX as others have tried (Rehemtulla et al., 1993, *ibid*; Hallgren et al., 2002, *ibid*), we used a strategy aiming at strong expression of FII in combination with weak or very weak expression of the GGCX, such that the amount of expressed GGCX was less than $1/10^{th}$ of the expressed rhFII. To our surprise this strategy led to high levels of secreted correctly modified rhFII and good viability of the host cells, even when the cells were grown in animal component free chemically defined medium.

We have cloned GGCX and human prothrombin into an expression vector in such a way that the prothrombin mRNA level exceeds that of GGCX mRNA by a factor of at least 10. This results in production of a large excess of prothrombin protein compared to GGCX protein.

As a further example we have expressed rhFIX using the same GGCX co-expression vectors. This resulted in cell lines producing factor IX mRNA at levels exceeding GGCX mRNA levels by a factor of at least 10 in one case. In another cell line the factor IX: GGCX mRNA ratio was approximately 4-5:1. Only the cell line giving a ratio of at least 10:1 showed substantially increased rhFIX productivity (Table 1).

Table 1. Summary of productivity and carboxylated protein:GGCX mRNA ratio

CLONE NAME/CONSTRUCT	PROTEIN PRODUCED	PRODUCTION * OF FULLY ACTIVE PROTEIN (mg/l)	CARBOXYLATED PROTEIN: GGCX, APPROX. mRNA RATIO	SOURCE OF DATA
P1E2/PN32	Human prothrombin	40	250:1	Example 3
B2F4/PP6		26	50:1	Example 5
H3B10/PP6		30	30:1	Example 5
E1A9/PText5		3.5	No GGCX	Example 3
N4D5/F9NopA	Human FIX	7.3	45:1	Example 7
P1G9/F9hglx		1.3	4:1	Example 7
IC4		1¤	No GGCX	Rehemtulla 1993, US 5,460,950

* Productivity was measured from spinner cultures under similar growth conditions.

¤ Data from Rehemtulla 1993 and U.S. Patent No. 5,460,950.

The vitamin K dependent coagulation factors (FII, FVII, FIX, FX and their activated forms FIIa or thrombin, FVIIa, FIXa, FXa) produced by the present method of co-expression with GGCX can be expected to be useful in the prevention and treatment of bleeding

following trauma, surgery or diseases of the liver, kidneys, platelets or blood coagulation factors (haemophilia). Likewise the coagulation factor Protein C and its activated form APC can be expected to be useful in the prevention and treatment of disorders of increased coagulation with or without decreased levels of Protein C. The method is also applicable to other proteins that require post-translational carboxylation.

According to a first aspect of the invention there is provided a host cell comprising an expression vector comprising a nucleic acid molecule encoding a protein requiring gamma-carboxylation and associated expression control sequences comprising a first promoter and a nucleic acid molecule encoding a γ-glutamyl carboxylase and associated expression control sequences comprising a second promoter, wherein the first promoter is sufficiently stronger than the second promoter so that the protein requiring gamma-carboxylation and the γ-glutamyl carboxylase are expressed in a ratio of at least 10:1.

In a preferred embodiment the ratio of the expressed proteins is between 10:1 and 1000:1, more preferably between 10:1 and 500:1 and still more preferably between 25:1 and 250:1. A particularly suitable ratio is around 200:1.

In separate embodiments the ratio of the two expressed proteins can be at least 10:1, 30:1, 45:1, 50:1, 100:1, 200:1, 250:1, 300:1, 400:1, 500:1 and 1000:1.

In one particular embodiment, both the nucleic acid molecule encoding the protein requiring gamma-carboxylation and associated expression control sequences, and the nucleic acid molecule encoding the γ-glutamyl carboxylase and associated expression control sequences are located on the same expression vector. In another embodiment these two nucleic acid molecules are located on separate expression vectors.

According to a further aspect of the invention there is provided a nucleic acid according to SEQ ID NO: 14 and SEQ ID NO: 15.

According to a further aspect of the invention there is provided host cells transfected or transformed with a vector comprising the sequence of SEQ. ID NO: 14 or SEQ ID NO: 15 for the expression of human Factor IX.

According to a further aspect of the invention there is provided a host cell capable of expressing human coagulation factor IX and human gamma carboxylase enzymes, wherein the nucleic acid encoding the human coagulation factor IX and the nucleic acid encoding the gamma carboxylase are operably linked to control sequences that are capable of expressing the two proteins in a ratio of at least 10:1, respectively.

According to a further aspect of the invention there is provided a non-human eukaryotic host cell adapted to express human coagulation factor IX and human gamma carboxylase enzymes in a ratio of at least 10:1. In a particular embodiment, the nucleic acid encoding the human coagulation factor IX and the nucleic acid encoding the gamma carboxylase are operably linked to control sequences that are capable of expressing the two proteins in a ratio of at least 10:1, respectively.

According to a further aspect of the invention there is provided a host cell harbouring exogenous nucleic acid comprising human coagulation factor IX encoding nucleic acid under the control of hCMV promoter and human carboxylase encoding nucleic acid under the control of SV40 promoter.

According to a further aspect of the invention there is provided a nucleic acid according to SEQ. ID NO: 1, SEQ. ID NO: 2 or SEQ ID NO: 3.

According to a further aspect of the invention there is provided host cells transfected or transformed with a vector comprising the sequence of SEQ. ID NO: 1, SEQ. ID NO: 2 or SEQ ID NO: 3 for the expression of human prothrombin.

According to a further aspect of the invention there is provided host cells capable of expressing human prothrombin and human gamma carboxylase enzymes, wherein the nucleic acid encoding the human prothrombin and the nucleic acid encoding the gamma

carboxylase are operably linked to control sequences that are capable of expressing the two proteins in a ratio of at least 10:1, respectively.

According to a further aspect of the invention there is provided a non-human eukaryotic host cell adapted to express human prothrombin and human gamma carboxylase enzymes in a ratio of at least 10:1. In a particular embodiment, the nucleic acid encoding the human prothrombin and the nucleic acid encoding the gamma carboxylase are operably linked to control sequences that are capable of expressing the two proteins in a ratio of at least 10:1, respectively.

According to a further aspect of the invention there is provided a host cell harbouring exogenous nucleic acid comprising human prothrombin encoding nucleic acid under the control of hCMV promoter and human carboxylase encoding nucleic acid under the control of SV40 promoter.

The invention has been exemplified using prothrombin and coagulation factor IX as the proteins requiring carboxylation. However, several proteins other than prothrombin and factor IX are dependent on correct γ-carboxylation for their full biological activity. Among those known from man are the coagulation factor FVII, which at present is only commercially produced in recombinant mammalian cells at relatively low levels (approximately 10 mg/L or less). The present invention will be applicable to improve the productivity of any protein that is dependent on γ-carboxylation, such proteins include, but are not limited to: prothrombin, coagulation factor II (FII), coagulation factor VII (FVII), coagulation factor IX (FIX), coagulation factor X (FX), Protein C, Protein S, Protein Z, Bone Gla protein (also known as: BGP or osteocalcin), Matrix Gla protein (MGP), proline rich Gla polypeptide 1 (PRRG1), proline rich Gla polypeptide 2 (PRRG2), Growth arrest-specific protein 6 (Gas 6). Other suitable proteins are: FXa-like protein in venom of elapid snake (subfamily Acanthophiina) and cone snail venom (*Conus textile*).

Each of these proteins, including their nucleic acid and amino acid sequences, are well known. Table 2 identifies representative sequences of wild-type and mutant forms of the various proteins that can be used in the present invention.

Table 2.

DESCRIPTION	CDNA EMBL ACC#	SPLICE VARIANTS (PROTEIN)	MUTATIONS	GENE EMBL ACC#
Glutamate gamma carboxylase	BC013979	2; BC013979; AF253530	1 SNP (EMBL# U65896); 2 SNPs (OMIM# 137167)	U65896
Prothrombin	V00595	1; V00595	approx. 100 SNP's (EMBL# AF478696)	AF478696
Factor VII	AF466933	4; AF466933; AF272774; AR030786; AAN60063	21 SNPs (OMIM# 277500)	J02933
Factor IX	A01819	3; A01819; A34669; M19063	5 SNPs (EMBL# AF536327); 108 SNPs (OMIM# 306900)	AF536327
Factor X	BC046125	4; BC040125; M57285; AR095306; AB005892	118 SNPs (EMBL# AF503510); 14 SNPs (OMIM# 227600)	AF503510
Protein C	BC034377	7; AB083690; AB083693; I09623; S50739; S72338	57 SNPs (EMBL# AF378903); 25 SNPs (OMIM# 176860)	AF378903
Osteocalcin	AF141310	5; AF141310; AF141310; BC033656; X04143; X51699		X04143
Matrix GLA protein	BC005272	1; BC005272		
Growth arrest-specific 6; AXL stimulatory factor	BC038984	1; BC038984		
Protein Z	M55670	2; AB033749; AB033749		
Proline-rich Gla (G-carboxyglutamic acid) polypeptide 1	AF009242	2; AF009242;BC030786		
Proline-rich Gla (G-carboxyglutamic acid)	AF009243	2; AF009243; BC026032		

polypeptide 2				
Vitamin K-dependent protein S precursor	BC015801	1; BC015801	approx. 100 SNPs (EMBL# AY308744); 8 SNPs (OMIM# 176880)	AY308744

It will be appreciated that the invention is not restricted to a particular protein or protein encoding sequence of one of these proteins to be co-expressed. Moreover, and in particular with respect to blood coagulation factors, numerous mutant forms of the proteins have been disclosed in the art. The present invention is equally applicable to these mutant forms, including naturally occurring allelic variants, of the proteins as it is to wild-type sequence. In one embodiment the invention can be undertaking with any wild-type protein or one with at least 90%, preferably at least 95% sequence identity thereto.

The sequence identity between two sequences can be determined by pair-wise computer alignment analysis, using programs such as, BestFit, PILEUP, Gap or FrameAlign. The preferred alignment tool is BestFit. In practise, when searching for similar/identical sequences to the query search, from within a sequence database, it is generally necessary to perform an initial identification of similar sequences using suitable algorithms such as Blast, Blast2, NCBI Blast2, WashU Blast2, FastA, or Fasta3, and a scoring matrix such as Blosum 62. Such algorithms endeavour to closely approximate the "gold-standard" alignment algorithm of Smith-Waterman. Thus, the preferred software/search engine program for use in assessing similarity, i.e., how two primary polypeptide sequences line up is Smith-Waterman. Identity refers to direct matches, similarity allows for conservative substitutions.

The term "γ-glutamyl carboxylase" or "GGCX", as used herein, refers to a vitamin K dependent enzyme that catalyses carboxylation of glutamic acid residues.

GGCX enzymes are widely distributed, and have been cloned from many different species such as the beluga whale *Delphinapterus leucas*, the toadfish *Opsanus tau*, chicken (*Gallus gallus*), hagfish (*Myxine glutinosa*), horseshoe crab (*Limulus polyphemus*), and the cone

snail *Conus textile* (Begley et al., 2000, *ibid*; Bandyopadhyay et al. 2002, *ibid*). The carboxylase from conus snail is similar to bovine carboxylase and has been expressed in COS cells (Czerwiec et al. 2002, *ibid*). Additional proteins similar to GGCX can be found in insects and prokaryotes such as *Anopheles gambiae, Drosophila melanogaster* and *Leptospira* with NCBI accession numbers: gi 31217234, gi 21298685, gi 24216281, gi 24197548 and (Bandyopadhyay et al., 2002, *ibid*), respectively. The carboxylase enzyme displays remarkable evolutionary conservation. Several of the non-human enzymes have shown, or may be predicted to have, activity similar to that of the human GGCX we have used, and may therefore be used as an alternative to the human enzyme.

Table 3 identifies representative sequences of predicted proteins homologous to human GGXC (sorted after species origin) that can be used in the present invention.

Table 3.

Species	**Data base accession #/ID**
Homo sapiens (man)	NM_000821.2 HUMGLUCARB HUMHGCA BC004422 HSU65896 AF253530.1
Papio hamadryas (red baboon)	AC116665.1
Delphinapterus leucas (white whale)	AF278713
Bos taurus (bovine)	NM_174066.2 BOVCARBOXG BOVBGCA
Ovis aries (domestic sheep)	AF312035
Rattus norvegicus (brown rat)	NM_031756.1 AF065387
Mus musculus (mouse)	NM_019802.1 AF087938

Opsanus tau (bony fishes)	AF278714.1
Conus textile (molluscs)	AY0044904.1 AF382823.2
Conus imperialis (molluscs)	AF448234.1
Conus episcopatus (molluscs)	AF448233.1
Conus omaria (molluscs)	AF448235.1
Drosophila melanogaster (fruit fly)	NM_079161.2
Anopheles gambiae (mosquito)	XM_316389.1
Secale cereale (monocots)	SCE314767
Triticum aestivum (common wheat)	AF280606.1
Triticum urartu (monocots)	AY245579.1
Hordeum vulgare (barley)	BLYHORDCA
Leptospira interrogans (spirochetes)	AE011514.1
Streptomyces coelicolor (high GC Gram+ bacteria)	SCO939109 SCO939124 AF425987.1
Streptomyces lividans (high GC Gram+ bacteria)	SLU22894
Streptomyces viginiae (high GC Gram+ bacteria)	SVSNBDE
Micrococcus luteus (high GC Gram+ bacteria)	MLSPCOPER
Chlamydomonas reinhardtii (green algae)	AF479588.1
Dictyostelium discoideum (slime mold)	AC115612.2
Coturnix coturnix (birds)	AF364329.1
Bradyrhizobium japonicum (α-protoebacteria)	AP005937.1
Rhodobacter sphaeroides (α-proteobacteria)	RSY14197
Sinorhizobium meliloti (α-proteobacteria)	RME603647 AF119834
Mesorhizobium loti (α-proteobacteria)	AP003014.2
Chromobacterium violaceum (β-proteobacteria)	AE016910.1 AE016918.1
Pseudomonas aeruginosa (γ-proteobacteria)	AE004613.1 AF165882

Xanthomonas axonopodis (γ-proteobacteria)	AE011706.1
Human herpesvirus 8	KSU52064 KSU75698 AF305694 AF360120 AF192756

Each of the above-identified GGCX proteins and GGCX proteins from other species can be used as the carboxylase enzyme in the present invention.

One way to effect differential expression of the two co-expressed proteins is to use different promoters as part of the respective expression control sequences. The art is replete with examples of different promoters and other expression control sequences that are capable of expressing heterologous proteins to differing degrees or extents. Recombinant expression technology is suitably advanced such that a person skilled in the art of protein expression is able to select promoters and other control sequences to bring about co-expression of the protein requiring carboxylation and the carboxylase in the desired ratio. The selection of which particular promoters and other expression control sequences to use is a matter of individual choice.

In one embodiment, the control sequences associated with the protein requiring gamma-carboxylation comprise a strong promoter. In one embodiment this is the human cytomegalovirus (hCMV) immediate-early promoter. A strong promoter is here defined as a promoter giving rise to more than 1000 transcripts/cell. A weak promoter is here defined as a promoter giving rise to less than 1000 transcripts/cell.

In another embodiment, the control sequences associated with the γ-glutamyl carboxylase comprise a weak promoter. In one embodiment this is SV40 early promoter. In another embodiment the protein requiring gamma-carboxylation and the γ-glutamyl carboxylase are under the control of different promoter elements with the promoter controlling expression of the γ-glutamyl carboxylase being weaker that the promoter controlling expression of the protein requiring gamma-carboxylation.

In another embodiment, the γ-glutamyl carboxylase is under the control of SV40 early promoter and the protein requiring gamma-carboxylation is under the control of the human cytomegalovirus (hCMV) immediate-early promoter. In one embodiment according to this particular aspect of the invention the protein requiring gamma-carboxylation is human Factor X. In another embodiment the protein requiring gamma-carboxylation is human prothrombin. In another embodiment the protein requiring gamma-carboxylation is human Factor IX.

The invention has been exemplified by use of the strong CMV promoter (Boshart et al. Cell **41**:521-530, 1985) to over-express Factor IX or prothrombin and the weaker SV40 promoter (Wenger et al. Anal Biochem **221**:416-418, 1994) to control the GGCX expression. Other strong promoter that could be used according to the present invention include, but are not limited to, pEF-1α [human elongation factor-1α subunit gene) (Mizushima and Nagata, Nuc Acids Res **18**:5322, 1990; Goldman et al., BioTechniques **21**:1013-1015, 1996), pRSV [Rous sarcoma virus (Gorman et al ., Proc Natl Acad Sci USA **79**:6777-6781 ,1982)] and pUbC [human ubiquitin (Schorpp et al., Nuc Acids Res **24**:1787-1788, 1996)].

It is important to ensure that the protein to be produced (protein requiring carboxylation) is in excess compared to the modification enzyme, giving a ratio of at least 10:1. Ways to achieve a low level expression of the modification enzyme (γ-glutamyl carboxylase) include:

1) Use of a weak promoter to control expression of the modification enzyme including, but not limited to, SV40 immediate early promoter, the minimized FIX promoter (Rouet et al., The Journal of Biological Chemistry **267**:20765-20773,1992) or the HSV Thymidine kinase promoter (Wenger et al., 1994, *ibid*).

2) Mutate promoter or enhancer sequences of a strong promoter to reduce promoter strength.

3) Remove or change the Kozak sequence (translation initiation signal) to reduce the translation efficiency (Kozak. Nuc Acids Res **15**:8125-8148, 1987;
Kozak. Proc Natl Acad Sci USA **87**:8301-83051987, 1990).

4) Clone nucleic acid encoding protein to be produced (protein requiring carboxylation) and nucleic acid encoding GGCX on separate vectors and transfect with a large excess of the construct containing the protein to be produced so as to generate a cell with multiple copies of the construct containing the protein to be produced

5) Clone DNA encoding protein to be produced and DNA encoding GGCX modification vectors on separate vectors, co-transfect or separately transfect, and use an amplification system to amplify the expression of the protein to be produced.

6) Isolate a stable cell line recombinantly expressing low levels of GGCX (but above endogenous levels) and use as host cell line for expression of proteins in need of γ-carboxylation.

7) Introduce mutation(s) into the GGCX in order to decrease GGCX substrate affinity.

In addition to these, the person skilled in the art of recombinant protein expression will be aware of other methods that could be used to generate a host cell that expresses the protein requiring carboxylation and the carboxylase protein in a ratio of at least 10:1.

According to a further aspect of the invention there is provided a cell which is engineered or adapted to express (i) a protein which requires gamma-carboxylation, and (ii) a γ-glutamyl carboxylase, wherein the proteins (i) and (ii) are expressed in a ratio between 10:1 and 500:1. In a particular embodiment the γ-glutamyl carboxylase is expressed between 2 and 5-fold above endogenous levels (i.e. that in a non-engineered or adapted cell).

According to a further aspect of the invention there is provided a recombinant cell adapted to express (i) γ-glutamyl carboxylase protein above constitutive levels found in an equivalent unadapted cell and (ii) a protein requiring carboxylation, wherein the amount of expressed γ-glutamyl carboxylase protein and protein requiring carboxylation is in the ratio of at least 1:10.

According to a further aspect of the invention there is provided a genetically modified eukaryotic host cell comprising:

(i) a polynucleotide sequence encoding γ-glutamyl carboxylase protein wherein said γ-glutamyl carboxylase protein encoding sequence is operably linked to expression control sequences permitting expression of γ-glutamyl carboxylase protein by said cell; and

(ii) a polynucleotide encoding a protein requiring carboxylation by the γ-glutamyl carboxylase protein operably linked to expression control sequences permitting expression of said protein requiring carboxylation by said cell;

wherein the cell is capable of expressing the γ-glutamyl carboxylase protein and the protein requiring carboxylation in the ratio of at least 1:10.

According to a further aspect of the invention there is provided a cell adapted to express a protein which requires gamma-carboxylation and γ-glutamyl carboxylase, wherein the nucleic acid encoding the protein which requires gamma-carboxylation and the nucleic acid encoding the γ-glutamyl carboxylase are under the control of regulatory sequences suitable for ensuring that the amount of expressed protein which requires gamma-carboxylation is at least 10-fold the amount of the γ-glutamyl carboxylase protein.

In one embodiment, at least one of the protein which requires gamma-carboxylation and the γ-glutamyl carboxylase is expressed from nucleic acid that has been introduced into the cell by recombinant technology. An alternate way of working the invention is to express endogenous protein (protein requiring carboxylation or carboxylase), but with substitution of the endogenous control sequences (promoter etc.) with heterologous sequences to effect the desired level of expression.

The host cell is preferably a eukaryotic cell. Typical host cells include, but are not limited to insect cells, yeast cells, and mammalian cells. Mammalian cells are particularly preferred. Suitable mammalian cells lines include, but are not limited to, CHO, HEK, NS0, 293, Per C.6, BHK and COS cells, and derivatives thereof. In one embodiment the host cell is the mammalian cell line CHO-S.

Overexpression of carboxylation dependent proteins has earlier generally resulted in undercarboxylated products. This is due to the endogenous host cell carboxylation capacity

is being limited. On the other hand vast (16 to 70-fold) over expression of GGCX activity does not improve product yield (Rehemtulla et al., Proc Natl Acad Sci USA **90**: 4611-4615, 1993), (Berkner and Pudota, Proc Natl Acad Sci USA. **95**: 446-471, 1998), (Hallgren et al., Biochemistry **41**(50): 15045-15055, 2002). The reason for this is not fully understood. Our invention requires a moderate over expression of GGCX. This ensures that greater than endogenous levels of GGCX are expressed from the cell, for example, where the GGCX activity level is elevated only 1.5 to 5-fold. At this moderately elevated level, surprisingly high levels of fully carboxylated rhFII were obtained as shown in Example 1.

It will therefore be appreciated that the expression ratio of the protein requiring carboxylation and the carboxylase that distinguishes this invention from previous co-expression teachings, excludes levels of GGCX that are endogenously produced. To meet the high productivity required it is necessary to express the carboxylase and the protein requiring carboxylation at levels above those found in normal cells.

In a preferred embodiment a cell or cell line is used which has little or no constitutively expressed carboxylase and/or protein requiring carboxylation.

In one embodiment the γ-glutamyl carboxylase is expressed at less than or equal to 10% of the amount of the protein which requires gamma-carboxylation. In alternate, further embodiments the γ-glutamyl carboxylase is expressed at less than or equal to 5%, 2%, 1%, 0.5%, 0.25% 0.1%, 0.05% or 0.01% of the amount of the protein which requires gamma-carboxylation.

The degree expression of the two proteins can be measured using techniques familiar to a person skilled in the art. These include direct measurements, for example measuring biological activity of the protein, or amount of protein (e.g. using antibodies), or indirect measurements, for example via measurement of mRNA transcript levels (e.g. Taqman analysis as in Example 3). The following references disclose ways of measuring GGCX enzyme activity (Lingenfelter et al., Biochemistry **35**: 8234-8243, 1996; Berkner et al.,

Proc Natl Acad Sci USA **95**: 446-471, 1998; Hallgren et al., Biochemistry **41**(50): 15045-15055, 2002; and, Berkner et al., Proc Natl Acad Sci USA **89**: 6242-6246, 1992).

For the purposes of this invention, the ratio of expression of the two proteins is determined indirectly via mRNA transcript level (e.g., by Taqman analysis).

In one embodiment the protein which requires gamma carboxylation is a vitamin K dependent coagulation factor. In a further embodiment, the protein which requires gamma-carboxylation is preferably selected from the group consisting of: prothrombin, coagulation factor II, coagulation FII, coagulation factor VII, coagulation FVII, coagulation factor IX, coagulation FIX, coagulation factor X, coagulation FX, Protein C, Protein S, Protein Z, Bone Gla protein, Matrix Gla protein, Growth arrest-specific protein 6 and Acanthophiinae FXa-like protein.

In one particular embodiment, the protein which requires gamma-carboxylation is Factor IX. In another particular embodiment, the protein which requires gamma-carboxylation is prothrombin. In another embodiment, the protein which requires gamma-carboxylation is Factor X.

The present invention has general application to proteins that require carboxylation from any source. However, if the expressed protein is to be used for human therapeutic purposes, human proteins are particularly preferred.

In one embodiment the γ-glutamyl carboxylase is of mouse, rat, bovine or conus snail origin. In another embodiment, the γ-glutamyl carboxylase is a human protein.

According to a further aspect of the invention there is provided a method for producing gamma-carboxylated protein comprising: (i) culturing a cell adapted to express a protein which requires gamma-carboxylation and γ-glutamyl carboxylase in a ratio of at least 10:1, under conditions suitable for expression of both proteins, and (ii) isolating gamma-carboxylated protein.

According to a further aspect of the invention there is provided a method for production of a gamma-carboxylated protein in a mammalian cell line, comprising the step of co-expressing with said protein requiring gamma-carboxylation in the mammalian cell line a γ-glutamyl carboxylase, wherein the amount of expressed protein requiring gamma-carboxylation is at least 10-fold greater than the amount of expressed γ-glutamyl carboxylase; and (ii) isolating gamma-carboxylated protein.

A method for producing a gamma-carboxylated protein comprising:
a) genetic modification of a eukaryotic cell to introduce a first polynucleotide encoding a protein that requires carboxylation and accompanying expression control sequences and a second polynucleotide encoding a γ-glutamyl carboxylase and accompanying expression control sequences to produce a eukaryotic host cell capable of co-expression of the protein that requires carboxylation and a γ-glutamyl carboxylase proteins in a ratio of at least 10:1; b) cultivating the cell in suitable culture medium under conditions which allow the first and second polynucleotide sequences to be expressed; and c) isolation of the carboxylated protein from the medium or host cells.

Expression vectors usually include an origin of replication, a promoter, a translation initiation site, optionally a signal peptide, a polyadenylation site, and a transcription termination site. These vectors also usually contain one or more antibiotic resistance marker gene(s) for selection. Suitable expression vectors may be plasmids, cosmids or viruses such as phage or retroviruses. The coding sequence of the polypeptide is placed under the control of an appropriate promoter (i.e., HSV, CMV, TK, RSV, SV40 etc), control elements and transcription terminator (these are the associated expression control sequences) so that the nucleic acid sequence encoding the polypeptide is transcribed into RNA in the host cell transformed or transfected by the expression vector construct. The coding sequence may or may not contain a signal peptide or leader sequence for secretion of the polypeptide out of the host cell. Preferred vectors will usually comprise at least one multiple cloning site. In certain embodiments there will be a cloning site or multiple cloning site situated between the promoter and gene to be expressed. Such cloning sites can be used to create N-terminal fusion proteins by cloning a second nucleic acid sequence into the cloning site so that it is contiguous and in-frame with the gene sequence. In other

embodiments there may be a cloning site or multiple cloning site situated immediately downstream of the gene to facilitate the creation of C-terminal fusions in a similar fashion to that for N-terminal fusions described above.

The host cell can be genetically modified (have extra nucleic acids introduced) by numerous methods, well known to a person skilled in the art, such as transfection, transformation and electroporation.

The invention also extends to purified gamma carboxylated protein produced by the methods of the present invention and their use in coagulant therapy.

According to yet another aspect of the invention there is provided a method of promoting increased or decreased coagulation in a subject comprising administering a pharmacologically effective amount of an isolated gamma-carboxylated protein obtained by the above-described methods to a patient in need thereof.

According to a further aspect of the invention there is provided a method of producing a pharmaceutical composition suitable for inducing blood clotting, comprising purifying active carboxylated protein expressed from a host cell adapted to express a protein requiring gamma-carboxylation and γ-glutamyl carboxylase in a ratio of at least 10:1 and admixing the purified carboxylated protein with one or more pharmaceutically acceptable carriers or excipients.

Protein-based therapeutics are usually stored frozen, refrigerated, at room temperature, and/or or in a freeze-dried state.

The compositions of the invention may be obtained by conventional procedures using conventional pharmaceutical excipients, well known in the art, but will most likely be in a form suitable for injection, either parenterally or directly into the wound site.

Aqueous suspensions generally contain the active ingredient in finely powdered form together with one or more suspending agents, such as sodium carboxymethylcellulose,

methyl-cellulose, hydroxypropylmethylcellulose, sodium alginate, polyvinyl-pyrrolidone, gum tragacanth and gum acacia; dispersing or wetting agents such as lecithin or condensation products of an alkylene oxide with fatty acids (for example polyoxethylene stearate), or condensation products of ethylene oxide with long chain aliphatic alcohols, for example heptadecaethyleneoxycetanol, or condensation products of ethylene oxide with partial esters derived from fatty acids and a hexitol such as polyoxyethylene sorbitol monooleate, or condensation products of ethylene oxide with long chain aliphatic alcohols, for example heptadecaethyleneoxycetanol, or condensation products of ethylene oxide with partial esters derived from fatty acids and a hexitol such as polyoxyethylene sorbitol monooleate, or condensation products of ethylene oxide with partial esters derived from fatty acids and hexitol anhydrides, for example polyethylene sorbitan monooleate. The aqueous suspensions may also contain one or more preservatives (such as ethyl or propyl p-hydroxybenzoate, anti-oxidants (such as ascorbic acid), colouring agents, flavouring agents, and/or sweetening agents (such as sucrose, saccharine or aspartame).

Oily suspensions may be formulated by suspending the active ingredient in a vegetable oil (such as arachis oil, olive oil, sesame oil or coconut oil) or in a mineral oil (such as liquid paraffin). The oily suspensions may also contain a thickening agent such as beeswax, hard paraffin or cetyl alcohol. Sweetening agents such as those set out above, and flavouring agents may be added to provide a palatable oral preparation. These compositions may be preserved by the addition of an anti-oxidant such as ascorbic acid.

Powders suitable for preparation of an aqueous preparation for injection, by the addition of a suitable diluent, generally contain the active ingredient together with suitable carriers and excipients, suspending agent and one or more stabilisers or preservatives. The diluent may contain other suitable excipients, such as preservatives, tonicity modifiers and stabilizers.

The pharmaceutical compositions of the invention may also be in the form of oil-in-water emulsions. The oily phase may be a vegetable oil, such as olive oil or arachis oil, or a mineral oil, such as for example liquid paraffin or a mixture of any of these. Suitable emulsifying agents may be, for example, naturally-occurring gums such as gum acacia or gum tragacanth, naturally-occurring phosphatides such as soya bean, lecithin, an esters or

partial esters derived from fatty acids and hexitol anhydrides (for example sorbitan monooleate) and condensation products of the said partial esters with ethylene oxide such as polyoxyethylene sorbitan monooleate.

The pharmaceutical compositions of the invention may also be in the form of a sterile solution or suspension in a non-toxic parenterally acceptable diluent or solvent, which may be formulated according to known procedures using one or more of the appropriate dispersing or wetting agents and suspending agents, which have been mentioned above. A sterile injectable preparation may also be a sterile injectable solution or suspension in a non-toxic parenterally-acceptable diluent or solvent, for example a solution in 1,3-butanediol.

For further information on Formulation the reader is referred to Chapter 25.2 in Volume 5 of Comprehensive Medicinal Chemistry (Corwin Hansch; Chairman of Editorial Board), Pergamon Press 1990; or, Volume 99 of Drugs and the pharmaceutical sciences; Protein formulation and delivery (Eugen J. McNally, executive editor), Marcel Dekker Inc 2000.

The amount of active ingredient that is combined with one or more excipients to produce a single dosage form will necessarily vary depending upon the host treated and the particular route of administration. For example, a formulation intended for injection to humans will generally contain, for example, from 0.5 mg to 2 g of active agent compounded with an appropriate and convenient amount of excipients which may vary from about 5 to about 98 percent by weight of the total composition. Dosage unit forms will generally contain about 1 mg to about 500 mg of the active ingredient. Proteinaceous therapeutics are usually stored frozen or freeze-dried. For further information on Routes of Administration and Dosage Regimes the reader is referred to Chapter 25.3 in Volume 5 of Comprehensive Medicinal Chemistry (Corwin Hansch; Chairman of Editorial Board), Pergamon Press 1990.

The size of the dose for therapeutic or prophylactic purposes of a compound will naturally vary according to the nature and severity of the conditions, the age and sex of the animal or patient and the route of administration, according to well known principles of medicine. In

using a compound for therapeutic or prophylactic purposes it will generally be administered so that a daily dose in the range, for example, 0.5 mg to 75 mg per kg body weight is received, given if required in divided doses. In general lower doses will be administered when a parenteral route is employed. Thus, for example, for intravenous administration, a dose in the range, for example, 0.5 mg to 30 mg per kg body weight will generally be used. Similarly, for administration by inhalation, a dose in the range, for example, 0.5 mg to 25 mg per kg body weight will be used.

The invention will be further described by the following non-limiting examples:

The practice of the present invention will employ, unless otherwise indicated, conventional methods of molecular biology and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, *e.g.*, Sambrook *et al.*, eds., Molecular Cloning: A Laboratory Manual (3rd ed.) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (2001); Ausubel *et al.*, eds., Current Protocols in Molecular Biology, John Wiley & Sons, New York, NY (2002); Glover & Hames, eds., DNA Cloning 3: A Practical Approach, Vols. I, II, & III, IRL Press, Oxford (1995); Colowick & Kaplan, eds., Methods in Enzymology, Academic Press; Weir *et al.*, eds., Handbook of Experimental Immunology, 5th ed., Blackwell Scientific Publications, Ltd., Edinburgh, (1997).

Example 1

Amplification of cDNA encoding human FII (hPT) and human GGCX

Human liver mRNA was purchased from Clontech and cDNA synthesis was performed using the Superscript system from Invitrogen. The obtained cDNA was used as template for amplification of human FII using:

primer PTF0 5'-ATTCCTCAGTGACCCAGGAGCTGACA-3', (SEQ ID NO: 3) and

primer PTEXT 5'-CTACTCTCCAAACTGATCAATGACCTTCTGTATCCACTTCTT-3', (SEQ ID NO: 4).

Human GGCX was amplified using

primer hglx5, 5'-TCCGCAGAGCAATGGCGGTGTCT-3', (SEQ ID NO: 5) and

hglx3, 5'-CCAACATCTGGCCCCTTCAGAACT-3', (SEQ ID NO: 6).

The FII-encoding PCR product was cloned directly into the TA-TOPO treated vector pCDNA3.1V5/His (Invitrogen). Selection of a clone with hFII cDNA inserted in the correct direction gave the Ptext5 control construct (Fig. 1 b). GGCX encoding cDNA under the control of the SV40 promoter was obtained by transfer of the GGCX encoding fragment from pCDNA3.1V5/His TA-TOPO to the pZeoSV2+ vector (Invitrogen), using restriction enzymes *Bam*H1 and *Not*I. The *Eco*RV-*Not*I restriction sites downstream of the GGCX insert were removed. A blunted *Cla*I-*Bcl*I fragment from the resulting pZeoSV2-GGCX plasmid (containing the SV40 promoter and the GGCX containing insert, but not the polyadenylation site and polyadenylation signal downstream of the GGCX encoding sequence) was then cloned into the blunted *Dra*III restriction site of pCDNA3.1+ (Invitrogen). A clone with the pSV40-GGCX fragment inserted in tandem (same transcriptional direction) relative to the CMV promoter was selected and a blunted *Kpn*I-*Not*I FII encoding fragment from Ptext5 was cloned into the *Eco*RV site to obtain the PN32 construct (Fig. 1a). The DNA sequences of PN32 and Ptext5 are as in Appendix 2. All cloning methods were according to standard methods and/or manufacturers' recommended procedures.

The PN32 construct contains the following key features:

-Human cytomegalovirus (hCMV) immediate-early promoter controlling transcription of human prothrombin cDNA followed by the Bovine Growth Hormone (BGH) polyadenylation signal for efficient transcription termination and polyadenylation of mRNA.

-SV40 early promoter controlling transcription of human γ-carboxylase cDNA (GGCX) without apparent polyadenylation site or signal.

-Other features are as shown in Figure 1 a).

For comparison the PText5 construct without GGCX was used (Figure 1b). PTEXT5 nucleotide sequence is shown in SEQ ID NO: 1. PN32 nucleotide sequence is shown in SEQ ID NO: 2

Prothrombin producing cell lines obtained

The two constructs in Figure 1 were transfected into CHO-S cells (Invitrogen). Stable transfectants were selected and screened for highly productive clones using a commercially available assay for prothrombin activity (Chromogenix). In this assay the prothrombin containing samples were first treated with snake venom toxin (Ecarin – available from Sigma) to generate thrombin. Thrombin activity was then assayed by addition of a chromogenic substrate (S-2238) - which generates colour when processed by thrombin. Transfection and selection of clones were done in parallel with both constructs. Cell culturing was done in DMEM medium containing 9% heat inactivated fetal bovine serum. Clones obtained were then adapted to growth in animal component free medium. The best producing clone obtained was from transfection with PN32 (FII + GGCX), which yielded up to 400mg/L human recombinant prothrombin when grown in animal component free chemically defined medium (far in excess of any published levels). Recombinantly produced rhFII was purified (according to the method disclosed in Josic et al., Journal of Chromatography B, 790:183-197, 2003), and fractionated by ion-exchange chromatography using a Q-Sepharose column according to standard techniques, to obtain pure fully-carboxylated rhFII. Of fermentor produced rhFII up to 78 mg/L was fully-carboxylated and had the same biological activity as prothrombin purified from human plasma. Carboxylation was analysed by N-terminal sequencing of the protein and by prothrombinase assay (Mao et al. JBC, 273:30086-30091, 1998). Thrombin generation was triggered in human platelet-poor plasma by the addition of tissue factor, and the endogenous thrombin potential was measured essentially as described by Stig et al., (Blood Coagulation and Fibrinolysis, 14:457-462, 2003).

The best clone obtained with the PText5 construct gave a productivity of up to 10 mg/L in animal component free chemically defined medium, which is in the same range reported in the literature. The share of fully-carboxylated prothrombin obtained from the PText5 clone was estimated at around 50%. The final recovery of fully active rhFII was thus at least ten times higher using the PN32 construct containing a low expression level arrangement of the γ-carboxylase. For each of the constructs several clones with similar expression levels were identified.

Example 2

Measurement of ggcx activity in CHO cell lines

Two rhFII producing CHO-S cell lines, obtained by transfection with the PN32 construct (co-expression of human GGCX) and PTEXT5 (no co-expression of GGCX), respectively, were grown in spinner bottles using protein free medium supplemented with 5 µg/ml vitamin K. One tenth of the growth medium was replaced daily. Cells were harvested after 7 days of culture and microsomes were prepared as described by Berkner et al., (Proc Natl Acad Sci USA **89**: 6242-6246 1992). Human recombinant FII was purified from the culture supernate of the harvested cells. GGCX activity was measured as described by Berkner and Pudota (Proc Natl Acad Sci USA **95**: 446-471 1998; and, Lingenfelter and Berkner (Biochemistry **35**: 8234-8243, 1996). Our measurements showed that the GGCX activity is 1.5 times higher in the human GGCX co-expressing CHO cell line compared to the CHO cell line expressing only rhFII, using the same growth conditions.

Example 3

Real Time Reverse Transcription Polymerase Chain Reaction (RT-PCR) analysis of mRNA expression of γ-Carboxylase and Prothrombin in CHO-S cell lines

Two CHO-S cell lines obtained by stable transfection with the PN32 (FII+GGCX) and Ptext5 (only FII) constructs respectively, were cultured in spinner bottles using protein free medium supplemented with vitamin K. Culture samples were withdrawn after 4, 5 and 6 days of culture to cover the estimated peak levels in mRNA production. RNA was isolated with Trizol™ according to the protocol supplied by the vendor, Invitrogen. The isolated RNA was DNaseI treated with the kit DNA-free™ from Ambion. cDNA synthesis was carried out using random hexamer primers and kit contents from Superscript™First-Strand Synthesis System for RT-PCR, Invitrogen.

Primers and Vic-labeled probes for Real-Time RT-PCR were selected using the software Primer Express™, Applied Biosystems.

Human γ-Carboxylase oligonucleotides

5´ACACCTCTGGTTCAGACCTTTCTT 3´	Forward primer (SEQ ID NO: 7)
5´ AATCGCTCATGGAAAGGAGTATTT 3´	Reverse primer (SEQ ID NO: 8)

5´ CAACAAAGGCTCCAGGAGATTGAACGC 3´ Probe (SEQ ID NO: 9)
Amplicon length 86 bp

Human Prothrombin oligonucleotides

5´ TGGAGGACAAAACCGAAAGAGA 3´ Forward primer (SEQ ID NO: 10)
5´ CATCCGAGCCCTCCACAA 3´ Reverse primer (SEQ ID NO: 11)
5´ CTCCTGGAATCCTACATCGACGGGC 3´ Probe (SEQ ID NO: 12)
Amplicon lenght 69 bp

Primers were manufactured by Operon/Qiagen and the probes were ordered from Applied Biosystems. Rodent GAPDH control primers and probe were also used (Applied Biosystems; ABI # 4308318 TaqMan® Rodent GAPDH Control Reagents Protocol)-Amplicon length 177 bp. The Real-Time RT-PCR reactions were performed on the ABI Prism™ 7700 Sequence detector, Applied Biosystems. The expected length of the amplified PCR products was confirmed on agarose gels. Dilution series to investigate the efficiency of the PCR reactions were carried out for all three genes. Expression levels of γ-Carboxylase and Prothrombin are presented relative to the expression of the control gene, rodent GAPDH.

Prothrombin

	CHO-S PText5 day 4	CHO-S PText5 day5	CHO-S PText5 day6	CHO-S PN32 day 4	CHO-S PN32 day 5	CHO-S PN32 day 6
2^-delta Ct	0.008014	0.076239	0.066677	0.204948	0.322343	0.364334

γ-Carboxylase

	CHO-S PText5 day 4	CHO-S PText5 day5	CHO-S PText5 day6	CHO-S PN32 day 4	CHO-S PN32 day 5	CHO-S PN32 day 6
2^-delta Ct	3.39E-07	0	0	0.000277	0.00159	0.001568

From the relative expression levels the of rhFII:GGCX detected, ratios of approximately 74-232:1 were calculated depending on day of sample collection. For the cell line transfected with PN32, co-expression rhFII and GGCX, the number of transcript per cell

were calculated to be approximately 8 for the GGCX mRNA and approximately 2000 for the rhFII mRNA, thus giving a rhFII:GGCX ratio of approximately 250:1. The GAPDH control mRNA transcripts/cell was for the same sample approximately 4000.

Example 4

Production of human FII

The human FII and GGCX cDNA cloned in Example 1 were inserted into pCDNA3.1 similarly as in Ex.1. In order to give higher GGCX levels, the polyadenylation signal from pZeoSV2+ was included in the pSV40-GGCX-pA fragment cloned into the blunted *Dra*III site of pCDNA3.1. A clone with the GGCX-containing fragment in the reverse order compared to Ex. 1 was selected. Cloning of the FII fragment was then done the same way as in Ex 1. The final construct PP6 is shown in Figure 2 and the PP6 nucleotide sequence is shown in SEQ ID NO: 13.

Two prothrombin producing cell lines, B2F4 and H3B10 were obtained by transfecting CHO-S as described in Ex 1. Prothrombin from these two cell lines was purified and characterized as in Ex 1. Cultures of B2F4 gave productivities ranging from 30-70 mg/L and the share of fully carboxylated from 55-87% (the more rhFII the less fully carboxylated). Addition of butyrate gave a somewhat higher productivity but decreased the share of fully carboxylated rhFII and was not considered to be beneficial. H3B10 is slow-growing and gave a productivity of about 50 mg/L, which was high relative to the amount of cells in the culture, and the share of fully carboxylated rhFII was around 60%. Compared to the cell line obtained in example 1, less fully carboxylated rhFII was produced using the PP6 construct for a CHO cell line. The production of fully active recombinant prothrombin is still, however, far above earlier published levels.

Example 5

Real Time RT-PCR analyses of the expression of γ-Carboxylase and Prothrombin in CHO-S cell lines by measuring amount of mRNA

The B2F4 and H3B10 cell lines from example 4 were analysed by real-time PCR analyses by the same method and the same primers as in Ex 3. Culture samples of 10 ml were collected at peak productivity in order to be equivalent to samples in Ex 3. For clone

H3B10 samples were from day 10 due to the slow growth of this clone, and for clone B2F4 samples were from day 6.

Table 4. Results from Real time RT-PCR analyses of prothrombin producing cell lines co-expressing GGCX. Two independent 100 ml spinner cultures for each B2F4 and H3B10 were sampled for Real-Time RT-PCR analyses.

Transcript	Total # cells	Resulting amount of total RNA	Total RNA used for RT-PCR	Amount mRNA in RT-PCR	Number of cells in RT-PCR	Ct	Copies mRNA	Copies mRNA/cell
P1E2 * Day 6								
PT	2.00E+07	2.39E-04	1.25E-08	2.50E-09	1.05E+03	19	2.10E+06	2005
GGCX	2.00E+07	2.39E-04	1.25E-08	2.50E-09	1.05E+03	27	8.19E+03	8
GAPDH	2.00E+07	2.39E-04	1.25E-08	2.50E-09	1.05E+03	18	4.19E+06	4010
B2F4-1 Day 6								
PT	1.30E+07	2.20E-04	1.25E-08	2.50E-09	7.39E+02	19.2	1.83E+06	2472
GGCX	1.30E+07	2.20E-04	1.25E-08	2.50E-09	7.39E+02	24.1	6.11E+04	83
GAPDH	1.30E+07	2.20E-04	1.25E-08	2.50E-09	7.39E+02	19.8	1.20E+06	1631
B2F4-2 Day 6								
PT	1.10E+07	1.40E-04	1.25E-08	2.50E-09	9.82E+02	19.2	1.83E+06	1859
GGCX	1.10E+07	1.40E-04	1.25E-08	2.50E-09	9.82E+02	24.1	6.11E+04	62
GAPDH	1.10E+07	1.40E-04	1.25E-08	2.50E-09	9.82E+02	19	2.10E+06	2135
H3B10-1 Day10								
PT	1.10E+07	2.90E-04	1.25E-08	2.50E-09	4.74E+02	17.77	4.92E+06	10375
GGCX	1.10E+07	2.90E-04	1.25E-08	2.50E-09	4.74E+02	23.4	9.93E+04	210
GAPDH	1.10E+07	2.90E-04	1.25E-08	2.50E-09	4.74E+02	17.96	4.31E+06	9095
H3B10-2. Day10								
PT	8.90E+06	3.10E-04	1.25E-08	2.50E-09	3.59E+02	19.2	1.83E+06	5087
GGCX	8.90E+06	3.10E-04	1.25E-08	2.50E-09	3.59E+02	25.3	2.66E+04	74
GAPDH	8.90E+06	3.10E-04	1.25E-08	2.50E-09	3.59E+02	18.9	2.25E+06	6263

* P1E2 data from example 3 for comparison.

The calculated ratio rhFII mRNA: GGCX mRNA was approximately 30:1 for clone H3B10, approximately 50:1 for clone B2F4 and approximately 250:1 for clone P1E2.

Example 6

Production of human coagulation factor IX (FIX)

Human coagulation factor IX cDNA was amplified from human Gene pool liver cDNA purchased from Invitrogen. Oligonucleotide primers were for

the 5'-end; F9f.ampl.: 5´-CACCATGCAGCGCGTGAACATGAT-3´ (SEQ ID NO: 16), and the 3'-end; F9r.ampl.: 5´- CCTTGGAAATCCATCTTTCATTA -3´ (SEQ ID NO: 17).

Cloning of the correct sequence was confirmed by DNA sequencing. The human FIX fragment was PCR amplified using *Pfx* polymerase (Invitrogen) and the cloning primers to produce a blunt ended fragment. The blunt-ended fragment was phosphorylated using T4 polynucleotide kinase, and cloned into the EcoRV digested and de-phosphorylated pCDNA-GGCX vectors from Ex. 1 and Ex 4. In this way constructs for co-expression of human FIX and GGCX analogous to the co-expression constructs used for production of human prothrombin (Ex.1 and 4) were obtained. Cloning of the correct sequences was confirmed by DNA sequencing and transient expression in COS-7 cells. The vector construct F9NopA can be seen in Fig. 3a and the vector construct F9hglx is shown in Fig. 3b. The difference between the vectors F9NopA and F9hglx is the transcription direction of the GGCX gene. The F9NopA nucleotide sequence is shown in SEQ ID NO: 14 and the F9hglx nucleotide sequence is shown in SEQ ID NO: 15

Establishment of cell lines producing rhFIX

The rhFIX constructs were transfected to CHO-S cells using the procedure described in Ex1. For each FIX construct approximately 3000 clones were screened for rhFIX expression by ELISA of cell supernates. Antibodies used were from Haemathology Technology Inc. and DakoCytomation. Clones were selected and adapted to growth in protein free chemically defined CHO medium. Cells were grown either in T-flasks at 37 °C or in spinner bottles at 32-37 °C. CO_2 concentration was 5% for both types of cultures. The rhFIX produced was purified to homogeneity by Q-Sepharose anion exchange

chromathography at pH 7.0. Recombinant hFIX activity was determined by Clotting assay using FIX deficient plasma (Precision Biologic). The best producing rhFIX clone obtained was N4D5, which was obtained using the F9NopA construct, produced up to 4 µg/ml active rhFIX grown in protein free chemically defined medium in T-flask. Grown in spinner bottle the same clone produced up to 7.1 µg/ml rhFIX. The overall productivity, also including incompletely carboxylated, non-active rhFIX, was estimated by Western blotting to be at least 30 µg/ml. The best producing clone obtained with the rhFIX construct F9hglx was P1G9 that produced 0.7 (T-flask) – 1.3 (spinner) µg/ml rhFIX under similar conditions. The results indicate that rhFIX productivity improved by co-expression of GGCX at a low level using the F9NopA construct, but that co-expression of GGCX using construct F9hglx, was less beneficial. It was also noted that the F9NopA construct, giving rise to the N4D5 clone, generally gave higher ELISA signals than the F9hglx construct, giving rise to the P1G9 clone, in simultaneous screens for productivity during the cell line development procedure.

The productivity of the N4D5 cell line is approximately 4-6 better than previously published levels obtained under comparable conditions, wherein IC4, IG8, r-FIX BHK and r-FIX 293 is the name of the clones mentioned in the references (Table 5).

Table 5. Comparison of productivity from human FIX producing cell lines.

Cell line/construct	**Amount of active rhFIX produced**		**Total productivity (µg/ml)**	**Reference**
	T-flask (µg/ml)	**Spinner (µg/ml)**		
N4D5/F9NopA CHO, low GGXC co-expr.	4	7.1	>30	Example 6
P1G9/F9hglx CHO, medium GGCX co-expr.	0.7	1.3	nd	Example 6
IC4	0.9	nd	30	Rehemtulla 1993.

CHO, HA (control) co-expr.				
IC4 CHO. High GGCX co-expr.	1	nd	29	Rehemtulla 1993.
IC4	0.9	nd	20	US 5,460,950
1G8 CHO	1.5	nd	43	Kaufman, RJ et al 1986 JBC 261:9622-9628
r-FIX BHK	0.004/24h	nd	0.004/24h	Hallgren 2002
r-FIX 293	0.004/24h	nd	0.004/24h	Hallgren 2002

Example 7

Real-Time RT-PCR analyses of the expression of γ-Carboxylase and Factor IX in CHO-S cell lines by measuring amount of mRNA

Recombinant hFIX-producing clones were grown in spinner bottles at 32-37 °C, in 100 ml protein free chemically defined medium supplemented with Vitamin K. Samples of 5-10 ml were collected at peak rhFIX concentration and analysed for content of human FIX and GGCX transcripts, as well as transcripts of the GAPDH control (house-keeping) gene. Procedure was as in example 3. Primers for rhFIX were as follows:

Human Factor IX primers

5´ AATAGTGCTGATAACAAGGTGGTTTG 3´ Forward primer (SEQ ID NO: 18)

5´ CACTGCTGGTTCACAGGACTTCT 3´ Reverse primer (SEQ ID NO: 19)

5´ TCCTGTACTGAGGGATATCGACTTGCAGAAAAC 3´ Probe (SEQ ID NO: 20)

Amplicon length 84 bp

Messenger RNA levels were found to peak at different days depending on culture temperature and culture inoculum size. Peak levels of mRNA were found to correspond well with peak concentration of rhFIX in the culture medium.

Table 6. Results from Real-Time RT-PCR analyses of rhFIX-producing clones.

Cell line-batch	Day of culture	2^-delta Ct FIX	2^-delta Ct GGCX	mRNA ratio FIX:GGCX
N4D5-100	11	0.255253	0.005461	47:1
N4D5-2	14	0.264866	0.006201	43:1
P1G9-A	6	0.022982	0.005601	4:1
P1G9-B	8	0.04181	0.007687	5:1

From Real-Time RT-PCR analyses we also found that, although 2^-delta Ct-values varied with culture time and conditions, the FIX:GGCX mRNA ratios were approximately the same for each clone. For the best rhFIX-producing clone N4D5 the ratio was approximately 45:1. Analyses of another clone, P1G9, gave a lower ratio of approximately 4.5:1. The P1G9 clone produced only 20% of the amount rhFIX produced by N4D5.

Claims:

1. A host cell comprising an expression vector comprising a nucleic acid molecule encoding a protein requiring gamma-carboxylation and associated expression control sequences comprising a first promoter and a nucleic acid molecule encoding a γ-glutamyl carboxylase and associated expression control sequences comprising a second promoter, wherein the first promoter is sufficiently stronger than the second promoter so that the protein requiring gamma-carboxylation and the γ-glutamyl carboxylase are expressed in a ratio of at least 10:1.

2. The host cell as claimed in claim 1, wherein the nucleic acid molecules are within a single expression vector.

3. The host cell as claimed in claim 1 or 2, wherein the first promoter is human cytomegalovirus (hCMV) immediate-early promoter and the second promoter is SV40 early promoter.

4. A cell which is engineered to express (i) a protein which requires gamma-carboxylation, and (ii) a γ-glutamyl carboxylase, wherein the proteins (i) and (ii) are expressed in a ratio between 10:1 and 500:1.

5. A cell as claimed in any of claims 1 to 4, wherein the protein which requires gamma-carboxylation is selected from the group consisting of: coagulation factor VII, coagulation FVII, coagulation factor IX, coagulation FIX, prothrombin, coagulation factor II, coagulation FII, coagulation factor X, coagulation FX, Protein C, Protein S, Protein Z, Bone Gla protein, Matrix Gla protein, Growth arrest-specific protein 6 and Acanthophiinae FXa-like protein.

6. A cell as claimed in any of claims 1 to 4, wherein the protein which requires gamma-carboxylation is a vitamin K dependent coagulation factor.

7. A cell as claimed in any of claims 1 to 6, wherein the protein which requires gamma-carboxylation is Factor IX.

8. A cell as claimed in any of claims 1 to 6, wherein the protein which requires gamma-carboxylation is Factor X.

9. A cell as claimed in any of claims 1 to 6, wherein the protein which requires gamma-carboxylation is prothrombin.

10. A cell as claimed in any of claims to 1 to 9, wherein the protein which requires gamma-carboxylation is a human protein.

11. A cell as claimed in any of claims to 1 to 10, wherein γ-glutamyl carboxylase is a human protein.

12. A cell as claimed in any of claims 1 to 11, wherein the cell is selected from the group consisting of: a mammalian cell, yeast cell or insect cell.

13. A cell as claimed in claim 12, wherein the cell is a mammalian cell.

14. A genetically modified eukaryotic host cell comprising:

(i) a polynucleotide sequence encoding γ-glutamyl carboxylase protein wherein said γ-glutamyl carboxylase protein encoding sequence is operably linked to expression control sequences permitting expression of γ-glutamyl carboxylase protein by said cell; and

(ii) a polynucleotide encoding a protein requiring carboxylation by the γ-glutamyl carboxylase protein operably linked to expression control sequences permitting expression of said protein requiring carboxylation by said cell;

wherein the cell is expressing the γ-glutamyl carboxylase protein and the protein requiring carboxylation in the ratio of at least 1:10.

15. A vector comprising a nucleic acid molecule encoding a protein requiring gamma-carboxylation and associated expression control sequences comprising a first promoter and

a nucleic acid molecule encoding a γ-glutamyl carboxylase and associated expression control sequences comprising a second promoter, wherein the first promoter is sufficiently stronger than the second promoter so that the protein requiring gamma-carboxylation and the γ-glutamyl carboxylase are expressed in a ratio of at least 10:1

16. A vector as claimed in claim 15, wherein the first promoter is human cytomegalovirus (hCMV) immediate-early promoter and the second promoter is SV40 early promoter.

17. A vector as claimed in claim 15 or 16, wherein the protein which requires gamma-carboxylation is selected from the group consisting of: coagulation factor VII, coagulation FVII, coagulation factor IX, coagulation FIX, prothrombin, coagulation factor II, coagulation FII, coagulation factor X, coagulation FX, Protein C, Protein S, Protein Z, Bone Gla protein, Matrix Gla protein, Growth arrest-specific protein 6 and Acanthophiinae FXa-like protein.

18. A vector as claimed in any of claims 15 to 17, wherein the protein which requires gamma-carboxylation is Factor IX.

19. A vector as claimed in any of claims 15 to 17 wherein the protein which requires gamma-carboxylation is Factor X.

20. A vector as claimed in any of claims 15 to 17, wherein the protein which requires gamma-carboxylation is prothrombin.

21. A method for producing gamma-carboxylated protein comprising: (i) culturing a cell adapted to express a protein which requires gamma-carboxylation and γ-glutamyl carboxylase in a ratio of at least 10:1, under conditions suitable for expression of both proteins, and (ii) isolating gamma-carboxylated protein.

22. A method for production of a gamma-carboxylated protein in a mammalian cell line, comprising the step of co-expressing with said protein requiring gamma-carboxylation in the mammalian cell line a γ-glutamyl carboxylase, wherein the amount of expressed

protein requiring gamma-carboxylation is at least 10-fold greater than the amount of expressed γ-glutamyl carboxylase, and (ii) isolating gamma-carboxylated protein.

23. Isolated gamma-carboxylated protein produced according to the method claimed in claim 21 or 22.

24. Use of isolated carboxylated protein produced according to claim 21 or 22 in the manufacture of a medicament for use in coagulation therapy.

25. A method of producing a pharmaceutical composition suitable for inducing blood clotting or promoting increased or decreased coagulation, comprising purifying active carboxylated protein expressed from a host cell adapted to express a protein requiring gamma-carboxylation and γ-glutamyl carboxylase in a ratio of between 10:1 and 500:1 and admixing the purified carboxylated protein with one or more pharmaceutically acceptable carriers or excipients.

26. A pharmaceutical composition obtained from the method of claim ~~26~~ 25.

27. A method for producing gamma-carboxylated human Factor X comprising: (i) culturing a cell adapted to express human Factor X and human γ-glutamyl carboxylase in a ratio of at least 10:1, under conditions suitable for expression of both proteins, and (ii) isolating gamma-carboxylated Factor X.

28. Use of isolated Factor X produced according to claim 27 in the manufacture of a medicament for use in coagulation therapy.

29. A pharmaceutical composition comprising gamma-carboxylated human Factor X obtained from the method of claim 27.

30. A method for producing gamma-carboxylated human Factor IX comprising: (i) culturing a cell adapted to express human Factor IX and human γ-glutamyl carboxylase in a ratio of at least 10:1, under conditions suitable for expression of both proteins, and (ii) isolating gamma-carboxylated Factor IX.

31. Use of isolated Factor IX produced according to claim 30 in the manufacture of a medicament for use in coagulation therapy.

32. A pharmaceutical composition comprising gamma-carboxylated human Factor IX obtained from the method of claim 30.

33. A method for producing gamma-carboxylated human prothrombin comprising: (i) culturing a cell adapted to express human prothrombin and human γ-glutamyl carboxylase in a ratio of at least 10:1, under conditions suitable for expression of both proteins, and (ii) isolating gamma-carboxylated prothrombin.

34. Use of isolated prothrombin produced according to claim 33 in the manufacture of a medicament for use in coagulation therapy.

35. A pharmaceutical composition comprising gamma-carboxylated human prothrombin obtainable from the method of claim 33.