Effect of prepropeptide replacement on γ-carboxylation and activity of recombinant coagulation factor IX

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Research Article

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Abstract

Based on observations indicating that γ-carboxylase enzyme has lesser affinity for propeptide of protein C (PC) and a higher net charge of γ-carboxylase region in PC propeptide, expression of recombinant chimeric Factor IX (FIX) equipped with PC propeptide was studied. The prepropeptide of FIX was replaced with PC by SOEing PCR and after cloning, recombinant pMT-prepro PC/FIX were transfected into the insect Drosophila S2 cells. The expression and activity of the active hFIX were analyzed by performing ELISA and coagulation test during 72 h of post-induction with copper ion. The results showed the higher secretion and activity level with an average of 1.2 and 1.6-fold, respectively, for chimeric prepro- PC/FIX construct in relation to normal FIX. Furthermore, after being precipitated with barium citrate, the evaluation of fully γ-carboxylated FIX indicated that more than 51% of the total FIX produced with the PC prepropeptide was fully γ-carboxylated, represents a substantial improvement (2 fold) over a system employing the native FIX propeptide, by which 25% of the protein is fully γ-carboxylated. The data illustrated that the expression of FIX using the PC propeptide led to much higher fully γ-carboxylated material, which is preferred to FIX constructs tolerating the sequence for the native FIX propeptide expressed in heterologous S2 systems.

Introduction

The bleeding disorder hemophilia B, which results from a defect in or deficiency of coagulation factor (F) IX, is considered to represent approximately 20–25% of all hemophilia disorders. Blood coagulation FIX is vitamin K-dependent (VKD) protein and is synthesized as a 461 amino acid precursor molecule, comprising a prepeptide (signal peptide) and a propeptide connected to the mature protein N-terminus (Mannucci 2008). The mature protein consists of a γ-carboxyglutamic acid-rich (GLA) domain, two epidermal growth factor (EGF) domains, the activation peptide, and catalytic domain (Brown 2002). The 46 amino acid pre- and propeptide sequence is removed during intracellular processing in the hepatocyte by signal peptidase and PACE/Furin endoprotease (paired basic amino acid cleaving enzyme), respectively (Jorgensen, Cantor et al. 1987).

Prior to hepatocellular secretion, FIX undergoes numerous posttranslational modifications, among which γ-carboxylation plays a significant role in the coagulation activity of FIX (Tie, Zheng et al. 2006). Non- γ-carboxylated FIX does not display coagulation activity, as a properly γ-carboxylated GLA domain mediates interaction with a negatively charged lipid surface, such as that of activated platelets or endothelial cells, thereby restricting coagulation to the site of injury. Hence, γ-carboxylation is an essential, and rate-limiting step during FIX processing (Blostein, Cuerquis et al. 2008). The γ-carboxylation is carried out by the enzyme γ-glutamyl carboxylase, which is able to change the specific glutamic acids to γ-carboxyglutamic acid residues following recognition of and interaction with the γ-carboxylase recognition site (γ-CRS) in the 18 residue FIX propeptide. Although there is significant homology between the propeptide sequences of VKD plasma proteins, their relative binding affinity for γ-carboxylase differs considerably, with the propeptide of FX displaying the highest binding affinity, followed by the propeptides of FVII, protein S, FIX, protein C (PC), and prothrombin (Camire, Larson et al. 2000, Blostein, Cuerquis et al. 2008). The weak interaction between the propeptide and γ-carboxylase is generally acknowledged to promote the rate of γ-carboxylation, as γ-carboxylase activity is lower for propeptides displaying high binding affinity relative to those with a low or moderate affinity (Camire, Larson et al. 2000, Blostein, Cuerquis et al. 2008, Rishavy and Berkner 2012). We have previously demonstrated that a higher net charge and net hydrophobicity of the γ-CRS resulting from specific point mutations increases the production of γ-carboxylated and functional FIX (Vatandoost and Bos 2019). Similar results were obtained following replacement of the FIX propeptide by that of prothrombin, thereby modifying the net charge and hydrophobicity of the propeptide as well (Rabiet, Jorgensen et al. 1987, Handford, Winship et al. 1991, Camire, Larson et al. 2000, Khorshidi, Zomorodipour et al. 2015). Since the impact of charge or

hydrophobicity on the γ-CRS region significantly affects the γ-carboxylation of FIX, investigating the effects of charge or hydrophobicity separately may provide novel insights into the requirements for γ-carboxylase binding. Here we studied how altered hydrophobicity of the propeptide affects FIX expression and function by replacing the FIX prepropeptide with that of PC.

Methods

Construction of expression vectors

The pMT-hFIX vector (Vatandoost, Zomorodipour et al. 2012) that encodes for wild-type human FIX was used as expression vector and PCR template. To generate a fusion construct of FIX with the PC prepropeptide, a splicing by overlap extension PCR strategy was employed using the following primers:

1. F1-PC (5′ GGGGTACCTGTCATGGCGGCAGG 3′)

2. R1-PC/FIX (5′ TTTACCTGAATTATAAAGAGGAGCTGGTGT 3′)

3. F2-FIX/PC (5′ ACACCAGCTCCTCTTTATAATTCAGGTAAA 3′)

4. R2-FIX (5′ CCGCTCGAGATCCATCTTTCATTAAGTGAGC 3′).

In the first PCR, primers F1-PC and R1-PC/FIX with 56°C annealing temperature were used to amplify a 163 bp fragment including the pre- and propeptide of human PC. In the second PCR, F2-FIX/PC and R2-FIX with 45°C annealing temperature were separately used to amplify a FIX cDNA fragment without the prepro sequence (1284 bp). In the third PCR, the prepropeptide of PC was ligated to the FIX cDNA, where primer 2 and 3 (with 66°C annealing temperature) fused the fragments and primer 1 and 4 (with 52°C annealing temperature of) were the outside primers. Each PCR reaction was performed as follows: heating to 94°C for 3 min, 30 cycles of 94°C for 30 s, specific annealing temperature for 30 s and 72°C for 1 min, followed by a final extension for 5 min. The PC-FIX cDNA fragment (1417 bp) was digested by KpnI–XhoI enzymes and inserted into the pMT-V5-HisA vector downstream of the Drosophila Mtn promoter. The resulting plasmid was designated pMT-prepro-PC/FIX, and the identity of the fragment was confirmed by employing the restriction of digestion, followed by nucleotide sequence analysis.

Cell culture, transfection, and selection of stable FIX-expressing clones

Drosophila S2 (Invitrogen, Lot no; 769,915) cells were kept at 28ºC without CO2 under normal atmosphere in Schneider's insect medium, supplemented with penicillin G (50 units/ml) and streptomycin (50 µg/ml). One day before transfection, 3×10^6 cells were seeded in a volume of 3 ml in 6-well plates, through which the cells were allowed to loosely adhere. The pMT-prepro-PC/FIX construct and the pCoHygro plasmid were transfected into Drosophila S2 cells employing the calcium phosphate coprecipitation method with minor modifications [19]. After 48 h, the cells were cultured in 300 µg of hygromycin B/ml. The single-cell strategy was employed to select the clones stably expressing FIX [20]. In brief, approximately 150×10^6 parental S2 cells were treated for 4 h with mitomycin C to induce cell cycle arrest, upon which these feeder cells were plated in 24-well plates at 3×10^6 cells/ml [21]. Subsequently, the transfected cells were added at 1 cell/well. After approximately two weeks, the single cell-derived clones were screened for FIX expression by adding 0.5 mM $CuSO_4$ and 6 µg/ml vitamin K1 to the medium.

Analysis of recombinant FIX expression and activity

Recombinant human FIX expressed intracellularly and in conditioned media was quantified employing a FIX-specific ELISA based on the procedure provided by the manufacturer (Asserachrom, hFIX: Ag). The expressed FIX concentration (ng/ml) was calculated based on a reference curves as described below and corrected for background levels observed for nontransfected cells. The secretion efficiency was defined as the ratio of the secreted FIX fraction versus the total amount of FIX expressed. The FIX clotting activity was determined using a modified one-stage aPTT assay specific for FIX as described previously (Vatandoost, Zomorodipour et al. 2012). FIX activity was corrected for measurements obtained using conditioned media from nontransfected cells. For both measurements, reference curves of normal pooled plasma (provided by Hashemi Nezhad Hospital, Iran) were used to calculate the equivalent FIX concentration and clotting activity. Activity-based concentration or vice versa was calculated by defining one unit of FIX activity as corresponds to 5 µg of FIX in 1 ml of normal plasma.

Quantification of γ-carboxylated recombinant factor IX

To quantify γ-carboxylated FIX, the barium citrate method was used for adsorbing γ-carboxylated glutamic acid residues [9,17,18]. During this process, the proteins that are poorly or not γ-carboxylated at all cannot be trapped by barium caption and remain in the soluble fraction. The barium-bound FIX was subsequently eluted from the precipitate through buffer modification. FIX recovery was expressed as the percentage of FIX eluted over the FIX prior to barium citrate adsorption as determined by FIX-specific antigen analysis.

Data analysis

All the experiments for the expression analysis were carried out in duplicates or triplets, and the generated data were presented as the mean ± *SD*. One-way ANOVA, followed by a Tukey post hoc test, was used to evaluate the differences among the constructs. SPSS 16 (SPSS, Chicago, IL) was used to analyze all statistical data.

Results

Expression analysis of FIX variants

Following the hypothesis that VKD proteins contain a propeptide with a lower affinity for the γ-carboxylase are more efficiently γ-carboxylated, we here studied replacement of the FIX prepropeptide with that of PC that displays a low relative affinity for γ-carboxylase (Hallgren, Qian et al. 2006). Therefore, a chimeric molecule was constructed with a FIX protein and prepro-PC (pMT-prepro-PC/FIX). Expression of wild-type FIX and prepro-PC/FIX was assessed for both the intracellular compartment as well as for secreted FIX following 24, 48, and 72 h of expression (Fig. 1A). The obtained data indicate that although the total FIX expression (intracellular and conditioned media) was not altered following exchange of the FIX prepropeptide for that of PC (total FIX: 460 ng/ml/10^6 cells; total prepro-PC/FIX: 480 ng/ml/10^6 cells], prepro-PC/FIX was 1.2-fold more secreted after 72 h when compared to the FIX (444 vs. 366 ng/ml/10^6 cells, respectively; Table 1). The secretion efficiency of prepro-PC/FIX was also higher than that of FIX (91% vs 75% at 72 h, respectively) (Fig. 1B), indicating that less FIX is intracellularly retained when expressed with the prepropeptide of PC (40 vs. 91 ng/ml/10^6 cells at 72 h, respectively).

Functional activity of expressed FIX variants

As γ-carboxylation is essential for the functional clotting activity of FIX, the FIX expressed in conditioned media was assessed for both FIX-specific antigen and clotting activity. In theory, when the total FIX expressed is fully functional (1 ng = 0.2 mU), we would expect up to 73 and 89 mU/ml clotting activity for FIX and prepro-PC/FIX, respectively, after 72 h of expression (Table 1). However, comparison of the expected vs. the observed FIX activity indicated that the biological activity of the FIX expressed was 45 and 74 mU/ml for FIX and prepro-PC/FIX, respectively (Table 1). This indicates that not all secreted FIX is γ-carboxylated. It was also evident that the percentage of functionally active and as such γ-carboxylated FIX relative to the total amount of secreted FIX increased following exchange of the FIX prepropeptide for that of PC. In fact, approximately 80% of prepro-PC/FIX was functionally active, and as such γ-carboxylated, vs. ~60% of FIX (Table 1). These results suggest that the PC propeptide leads to higher γ-carboxylation of FIX and thus higher levels of functionally active FIX.

Table 1

Expression of functional and non-functional FIX in conditioned media. The expected activity was based on the FIX antigen expression, assuming all FIX expressed was fully functional (1 ng = 0.2 mU), and the observed FIX activity was based on FIX-specific clotting activity measurements. The amount of (non-)functional FIX was determined based on observed FIX activity (1 mU = 5 ng) and was also expressed as the percentage of functionally active secreted FIX with the total amount of secreted FIX set at 100%. The data are the means ± *SD* of three independent experiments.

FIX Variant	Expression Time *hours*	FIX Expression (Antigen) *ng/ml/10^6 cell*	Expected FIX Activity *mU/ml/10^6 cell*	Observed FIX Activity *mU/ml/10^6 cell*	*Functional FIX* *ng/ml/10^6 cell (%)*	*Non-functional FIX* *ng/ml/10^6 cell (%)*
FIX						
	24	188	38	23	113 (60)	75 (40)
	48	289	58	34	170 (59)	118 (41)
	72	366	73	45	227 (62)	139 (38)
prepro-PC/FIX						
	24	225	45	36	182 (81)	42 (19)
	48	339	68	54	271 (80)	68 (20)
	72	444	89	74	369 (83)	76 (17)

γ-carboxylation of FIX variants

The extent of full γ-carboxylation of the FIX expressed was assayed employing barium citrate adsorption, and assessment of FIX recovery showed a 2.5-fold enhanced recovery for prepro-PC/FIX relative to FIX after 48–72 h of expression (229 vs. 91 ng/ml at 72 h, respectively) (Table 2). Based on these observed FIX antigen values, we would expect FIX activity levels of 46 and 18 mU/ml at 72 h for prepro-PC/FIX and FIX, respectively, which in fact corresponds with FIX activity observed (Table 2). This indicates that the large majority of FIX adsorbed to the barium citrate is fully γ-carboxylated and displays clotting activity that is about 51% and 25% of the FIX in total secreted FIX by prepro-PC/FIX and FIX, respectively (Fig. 2).

Table 2

Barium citrate adsorption of FIX. Factor IX antigen and clotting activity was assessed in conditioned media following 72 h of expression prior or after barium citrate adsorption and elution (**+ Ba^{2+}**) in the precipitate and supernatant. The expected FIX activity was based on the FIX antigen expression, assuming all FIX was fully functional (1 ng = 0.2 mU) and the observed FIX activity was based on FIX-specific clotting activity measurements. The amount of full γ-carboxylated FIX was determined based on observed FIX activity (1 mU = 5 ng) and was also expressed as the percentage of full γ-carboxylated secreted FIX with the total amount of secreted FIX set at 100%. The partial γ-carboxylated FIX was calculated by subtracting the full γ-carboxylated FIX from functional FIX in Table 1. The data are the means ± *SD* of three independent experiments.

FIX Variant	**Expression Time** *hours*	**FIX – Ba^{2+}** *ng/ml/10^6 cell*	**FIX + Ba^{2+}** *ng/ml/10^6 cell*	**Expected FIX Activity** *mU/ml/10^6 cell*	**Observed FIX Activity** *mU/ml/10^6 cell*	**Full γ-*carboxylated FIX*** *ng/ml/10^6 cell (%)*	**Partially γ-*carboxylated FIX*** *ng/ml/10^6 cell (%)*
FIX							
	24	188	38	7.5	7	37 (20)	76 (40)
	48	289	64	13	13	63 (22)	107 (37)
	72	366	91	18	18	90 (25)	136 (37)
prepro-PC/FIX							
	24	225	101	20	20	100 (45)	82 (36)
	48	339	163	33	32	161 (47)	110 (32)
	72	444	229	46	45	226 (51)	142 (32)

Discussion

Poor production and inefficient γ-carboxylation of synthesized FIX by heterologous expression systems are regarded as major obstacles to the production of large quantities of functional recombinant FIX (Kaufman, Wasley et al. 1986, Baghani and Vatandoost 2016). There are different strategies to increase the expression of functional FIX. Previously, we have exchanged the prepropeptide of human FIX for that of human prothrombin, resulting in an improvement in activity (10 fold) (Khorshidi, Zomorodipour et al. 2015). The prepeptides have different degrees of total hydrophobicity, which may influence protein synthesis and secretion (Bird, Gething et al. 1990, Hatsuzawa, Tagaya et al. 1997, Zhang, Leng et al. 2005, Knappskog, Ravneberg et al. 2007, Khorshidi, Zomorodipour et al. 2015). Since the prepeptide of PT has higher total hydrophobicity than that of the FIX (0.93 vs. 0.79) (Khorshidi, Zomorodipour et al. 2015), it can result in higher production and secretion of FIX, which affects the number of active FIXs. So to eliminate the effect of the prepeptide and study the effect of net hydrophobicity and charge in propeptide γ-CRS, the FIX propeptide was substituted at positions − 12, -13, and − 14 based on the PT propeptide. By increasing both the net charge and hydrophobicity of the γ-CRS in the propeptide region of FIX, this replacement lead to high fully γ-carboxylated material (2.3 fold) and activity (3.4 fold) (Vatandoost and Bos 2019). The effect of hydrophobicity alone by double substituting amino acids at positions E-12Q/H-13P and H-13P/D-14A was also investigated. The net charge was constant in these mutants, but the hydrophobicity increased from − 5.3 in wild FIX to − 3.7 in double E-12Q / H-13P and + 1.6 in double H-13P/ D-14A rFIX mutants, which resulted in 1.4 and 2.9 fold enhanced activity, respectively (Vatandoost and Bos 2019).

Although these results demonstrate the role of net hydrophobicity in γ-carboxylation, the γ-CRS region's charge is also important in the rate of γ-carboxylation. Interestingly, although the PC and FIX γ-CRS sequences display a similar net hydrophobicity, they have a substantial difference in apparent affinity for PC and FIX (230 vs. 33.6 nM), leading to a 7-fold decrease in binding affinity to γ-carboxylase (Camire, Larson et al. 2000, Hallgren, Qian et al. 2006). Hence, hydrophobicity is not the single affecting factor of γ-carboxylase binding. Since a rise in the net charge of the γ-CRS region of PC relative to FIX was observed, the net charge of this region may contribute to γ-carboxylase binding as well. The latter is supported by our previous work, in which substitution of E-12Q with a change of net charge from − 1 to 0 led to 1.2 fold enhanced fully γ-carboxylated material and 1.4 fold activity (Vatandoost and Bos 2019). The second strategy was used to examine and confirm the effect of γ-CRS net charge on γ-carboxylase binding. Specifically, FIX prepropeptide was replaced with that of PC, leading to 1.6 more γ-carboxylation and activity (Table 1). It is speculated that by increasing the FIX γ-CRS region's net charge, the γ-carboxylase turnover and, therefore, γ-carboxylated FIXs may be enhanced. Support for the effect of the net charge of the γ-CRS region on γ-carboxylation comes from several studies. The − 9N to K substitution in FIX, while not affecting the hydrophobicity (from − 5.3 to -5.7), did affect the net charge of the γ-CRS region (from − 1 to 0). This substitution resulted in an increase in apparent affinity from 33 to 370 nM and enhanced γ-carboxylation of FIX (Stanley, Jin et al. 1999). It was also shown that charged side chains at position − 10 as well as the residues − 9 and − 8 in γ-CRS region have an effect on γ-carboxylation (Vermeer 1990). Moreover, charged amino acids that are present close to the predicted α-helical domain in several of the propeptides may affect γ-carboxylation (Czerwiec, Kalume et al. 2006). As such, these examples further point to a role for the charge of the FIX γ-CRS region in the functional interaction with γ-carboxylase, which is essential for an efficient turnover and γ-carboxylation. Since γ-carboxylation is crucial for a normal clotting activity of FIX, an increase in γ-carboxylation leads to more active FIX and a significant increase in activity as observed here (74 vs 45 mU/ml, 1.6-fold).

Furthermore, the FIX recovery assessment following barium citrate adsorption demonstrated that using PC prepropeptide result in 2-fold enhancement in fully γ-carboxylated FIX. In line with the findings of others, it was inferred that factors such as using altered propeptides (Presnell and Stafford 2002, Khorshidi, Zomorodipour et al. 2015), γ-carboxylase (Hallgren, Hommema et al. 2002), and VKOR overexpression (Hallgren, Qian et al. 2006) that can enhance γ-carboxylase turnover and decrease binding affinity may lead to full γ-carboxylation.

Since prepeptide (signal peptide) of PC has the same total hydrophobicity of FIX prepeptide, as anticipated, no difference was observed in total FIX expression (intracellular and conditioned media) when using a PC prepropeptide for the FIX, which is consistent with previous findings (Knappskog, Ravneberg et al. 2007, Trösße, Ravneberg et al. 2007, Trösße, Ravneberg et al. 2007, Vatandoost and Bos 2019). Nonetheless, we observed an improved secretion efficiency for the altered propeptide FIX variant (91% vs. 75%)(Fig. 1B). From this we speculatethat the increased γ-carboxylation leads to higher secretion efficiency and a decrease in trapped FIX in the intracellular space, indicating that secretory machinery is affected by γ-carboxylation. It is in light of other studies that show poor secretion of FIX following γ-carboxylase overexpression (Rehemtulla, Roth et al. 1993, Hallgren, Hommema et al. 2002, Wajih, Hutson et al. 2005) or increased secretion of FIX in overexpressed VKOR cells (Wajih, Hutson et al. 2005, Wajih, Sane et al. 2005, Hallgren, Qian et al. 2006, Pakdaman, Vatandoost et al. 2019), implying that γ-carboxylase acts as an essential factor during protein secretion (McClure, Walls et al. 1992). Moreover, it was shown that γ-carboxylation is part of the protein secretion pathway (Rishavy and Berkner 2012) and was required for both efficient secretion and anticoagulant activity (McClure, Walls et al. 1992). The following hypothesis is also particularly attractive: Glu γ-carboxylation may result in an increasing negative charge on protein substrates, and subsequent interaction with positively charged amino acids could neutralize the resulting charge density or contribute to triggering an active protein release mechanism (Rishavy and Berkner 2012).

Recently, Hao et al. demonstrated the carboxylation efficiency of a reporter-protein as directed by different propeptides (Hao, Jin et al. 2019). Fusion of FIX or PC propeptides to the N-terminus of the chimeric reporter-protein FIXgla-PC indicated that the FIX propeptide is the most efficient propeptide for reporter protein γ-carboxylation, with a 2.5-fold higher level of γ-carboxylation relative to the PC propeptide. Interestingly, these findings are in contrast with earlier observations on the apparent affinity for γ-carboxylase, which led to a 7-fold decrease in binding affinity to γ-carboxylase (Camire, Larson et al. 2000). Since in these studies a chimeric VKD-protein variant was studied in which the FIX GLA domain was fused to mature PC, we speculate that VKD protein domains such as EGF may differentially impact carboxylation in an indirect manner. For instance, the EGF-like region could have subtly altered the global folding of the chimeric protein, leading to a reduction in γ-carboxylation (Yu, Zhang et al. 1994). Furthermore, of note the S2 expression system employed in the current study has been reported to display a higher γ-carboxylation efficiency relative to mammalian cells (Vatandoost, Zomorodipour et al. 2012).

In conclusion, a leader peptide replacement approach is possible to improve the expression and activity of interest protein. Increasing the FIX γ-CRS region's net charge increased the turnover by γ-carboxylase, thereby enhancing the production of fully γ-carboxylated and functional FIX.

Declarations

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Compliance with Ethical Standards

Conflict of Interest:

J. Vatandoost and M. Bos declares that has no conflict of interest.

Ethical approval:

This article does not contain any studies with human participants or animals performed by any of the authors.”

Author Contribution Statement

JV an MB conceived and designed research. JV performed research and analyzed data. JV an MB wrote the paper. All authors read and approved the manuscript.

Data availability

All data generated or analyzed during this study are included in this manuscript.

References

1. Baghani A, Vatandoost J (2016) Comparison of recombinant FIX expression in stable CHO-FIX cells in small and large-scale. Islamic Azad University-Sabzevar Branch
2. Bird P, Gething M, Sambrook J (1990) "The functional efficiency of a mammalian signal peptide is directly related to its hydrophobicity". J Biol Chem 265(15):8420–8425

3. Blostein M, Cuerquis J, Landry S, Galipeau J (2008) The carboxylation efficiency of the vitamin K-dependent clotting factors: studies with factor IX. Haemophilia 14(5):1063–1068
4. Brown D (2002) Haemophilia A and haemophilia B: molecular insights. J Clin Pathol 55(1):1
5. Camire RM, Larson PJ, Stafford DW, High KA (2000) Enhanced γ-carboxylation of recombinant factor X using a chimeric construct containing the prothrombin propeptide. Biochem 39(46):14322–14329
6. Czerwiec E, Kalume DE, Roepstorff P, Hambe B, Furie B, Furie BC, Stenflo J (2006) "Novel γ-carboxyglutamic acid-containing peptides from the venom of Conus textile". FEBS J 273(12):2779–2788
7. Hallgren KW, Hommema EL, McNally BA, Berkner KL (2002) Carboxylase overexpression effects full carboxylation but poor release and secretion of factor IX: implications for the release of vitamin K-dependent proteins. Biochem 41(50):15045–15055
8. Hallgren KW, Qian W, Yakubenko AV, Runge KW, Berkner KL (2006) r-VKORC1 expression in factor IX BHK cells increases the extent of factor IX carboxylation but is limited by saturation of another carboxylation component or by a shift in the rate-limiting step. Biochem 45(17):5587–5598
9. Handford PA, Winship PR, Brownlee GG (1991) Protein engineering of the propeptide of human factor IX. Protein Eng Des Sel 4(3):319–323
10. Hao Z, Jin D-Y, Stafford DW, Tie J-K (2019) "Vitamin K-dependent carboxylation of coagulation factors: insights from a cell-based functional study." Haematologica: haematol. 2019.229047
11. Hatsuzawa K, Tagaya M, Mizushima S (1997) "The hydrophobic region of signal peptides is a determinant for SRP recognition and protein translocation across the ER membrane". J Biochem 121(2):270–277
12. Jorgensen MJ, Cantor AB, Furie BC, Brown CL, Shoemaker CB, Furie B (1987) "Recognition site directing vitamin K-dependent γ-carboxylation resides on the propeptide of factor. IX " Cell 48(2):185–191
13. Kaufman RJ, Wasley LC, Furie BC, Furie B, Shoemaker CB (1986) "Expression, purification, and characterization of recombinant gamma-carboxylated factor IX synthesized in Chinese hamster ovary cells". J Biol Chem 261(21):9622–9628
14. Khorshidi S, Zomorodipour A, Behmanesh M, Vatandoost J, Bos MH (2015) Functional expression of the human coagulation factor IX using heterologous signal peptide and propeptide sequences in mammalian cell line. Biotechnol Lett 37(9):1773–1781
15. Knappskog S, Ravneberg H, Gjerdrum C, Tröβe C, Stern B, Pryme IF (2007) "The level of synthesis and secretion of Gaussia princeps luciferase in transfected CHO cells is heavily dependent on the choice of signal peptide". J Biotechnol 128(4):705–715
16. Knappskog S, Ravneberg H, Gjerdrum C, Tröβe C, Stern B, Pryme IF (2007) "The level of synthesis and secretion of Gaussia princeps luciferase in transfected CHO cells is heavily dependent on the choice of signal peptide". J Biotechnol 128(4):705–715
17. Mannucci PM (2008) Back to the future: a recent history of haemophilia treatment. Haemophilia 14(s3):10–18
18. McClure D, Walls J, Grinnell B (1992) "Post-translational processing events in the secretion pathway of human protein C, a complex vitamin K-dependent antithrombotic factor". J Biol Chem 267(27):19710–19717
19. Pakdaman SF, Vatandoost J, Bos MH (2019) "Enhanced functional recombinant factor IX production by human embryonic kidney cells engineered to overexpress VKORC1." Biotechnol. Prog: e2938
20. Presnell SR, Stafford DW (2002) The vitamin K-dependent carboxylase. Thromb Haemost 87(06):937–946
21. Rabiet M-J, Jorgensen MJ, Furie B, Furie BC (1987) "Effect of propeptide mutations on post-translational processing of factor IX. Evidence that beta-hydroxylation and gamma-carboxylation are independent events". J

Biol Chem 262(31):14895–14898

22. Rehemtulla A, Roth DA, Wasley LC, Kuliopulos A, Walsh CT, Furie B, Furie BC, Kaufman RJ (1993) In vitro and in vivo functional characterization of bovine vitamin K-dependent gamma-carboxylase expressed in Chinese hamster ovary cells. Proc Natl Acad Sci 90(10):4611–4615
23. Rishavy MA, Berkner KL (2012) Vitamin K oxygenation, glutamate carboxylation, and processivity: defining the three critical facets of catalysis by the vitamin K–dependent carboxylase. Adv Nutr 3(2):135–148
24. Stanley TB, Jin D-Y, Lin P-J, Stafford DW (1999) The propeptides of the vitamin K-dependent proteins possess different affinities for the vitamin K-dependent carboxylase. J Biol Chem 274(24):16940–16944
25. Stanley TB, Jin DY, Lin PJ, Stafford DW (1999) The propeptides of the vitamin K-dependent proteins possess different affinities for the vitamin K-dependent carboxylase. J Biol Chem 274(24):16940–16944
26. Tie J-K, Zheng M-Y, Pope RM, Straight DL, Stafford DW (2006) "Identification of the N-linked glycosylation sites of vitamin K-dependent carboxylase and effect of glycosylation on carboxylase function." Biochem 45(49): 14755–14763
27. Tröße C, Ravneberg H, Stern B, Pryme IF (2007) "Vectors encoding seven oikosin signal peptides transfected into CHO cells differ greatly in mediating Gaussia luciferase and human endostatin production although mRNA levels are largely unaffected."Gene Regul Syst Bio1
28. Tröße C, Ravneberg H, Stern B, Pryme IF (2007) "Vectors encoding seven oikosin signal peptides transfected into CHO cells differ greatly in mediating Gaussia luciferase and human endostatin production although mRNA levels are largely unaffected."Gene Regul. Syst. Biol.1
29. Vatandoost J, Bos MH (2019) "Improved activity and expression of recombinant human factor IX by propeptide engineering". DARU J Pharm Sci 27(2):653–660
30. Vatandoost J, Zomorodipour A, Sadeghizadeh M, Aliyari R, Bos MH, Ataei F (2012) Expression of biologically active human clotting factor IX in Drosophila S2 cells: γ-carboxylation of a human vitamin K-dependent protein by the insect enzyme. Biotechnol Progr 28(1):45–51
31. Vermeer C (1990) Gamma-carboxyglutamate-containing proteins and the vitamin K-dependent carboxylase. Biochem J 266(3):625
32. Wajih N, Hutson SM, Owen J, Wallin R (2005) "Increased production of functional recombinant human clotting factor IX by baby hamster kidney cells engineered to overexpress VKORC1, the vitamin K 2, 3-epoxide-reducing enzyme of the vitamin K cycle". J Biol Chem 280(36):31603–31607
33. Wajih N, Sane DC, Hutson SM, Wallin R (2005) "Engineering of a recombinant vitamin K-dependent γ-carboxylation system with enhanced γ-carboxyglutamic acid forming capacity evidence for a functional cxxc redox center in the system". J Biol Chem 280(11):10540–10547
34. Yu S, Zhang L, Jhingan A, Christiansen WT, Castellino FJ (1994) "Construction, expression, and properties of a recombinant chimeric human protein C with replacement of its growth factor-like domains by those of human coagulation factor IX. " Biochem 33(3):823–831
35. Zhang L, Leng Q, Mixson AJ (2005) "Alteration in the IL-2 signal peptide affects secretion of proteins in vitro and in vivo". J Gene Med 7(3):354–365

Figures

Figure 1

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Figure 2

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