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### Citation

Vatandoost, J., & Bos, M. H. A. (2022). Effect of prepropeptide replacement on gamma-carboxylation and activity of recombinant coagulation factor IX. *Biotechnology Letters*, 44(8), 975-984. doi:10.1007/s10529-022-03269-4

Version: Publisher's Version

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**Note:** To cite this publication please use the final published version (if applicable).



# Effect of propeptide replacement on $\gamma$ -carboxylation and activity of recombinant coagulation factor IX

Jafar Vatandoost · Mettine H. A. Bos

Received: 4 April 2022 / Accepted: 31 May 2022 / Published online: 22 June 2022  
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**Abstract** Based on observations indicating that the  $\gamma$ -carboxylase enzyme has a lower affinity for the protein C (PC) propeptide and that the  $\gamma$ -carboxylase region in the PC propeptide has a higher net charge, expression of recombinant chimeric factor IX (FIX) equipped with the PC propeptide was studied. The propeptide of FIX was replaced with that of PC by SOEing PCR and after cloning, recombinant pMT-prepro PC/FIX was transfected into insect *Drosophila* S2 cells. The expression and activity of expressed FIX were analyzed employing antigen and activity analyses 72 h of post-induction with copper. Higher secretion (1.2 fold) and activity (1.6 fold) levels were observed for chimeric prepro- PC/FIX in relation to

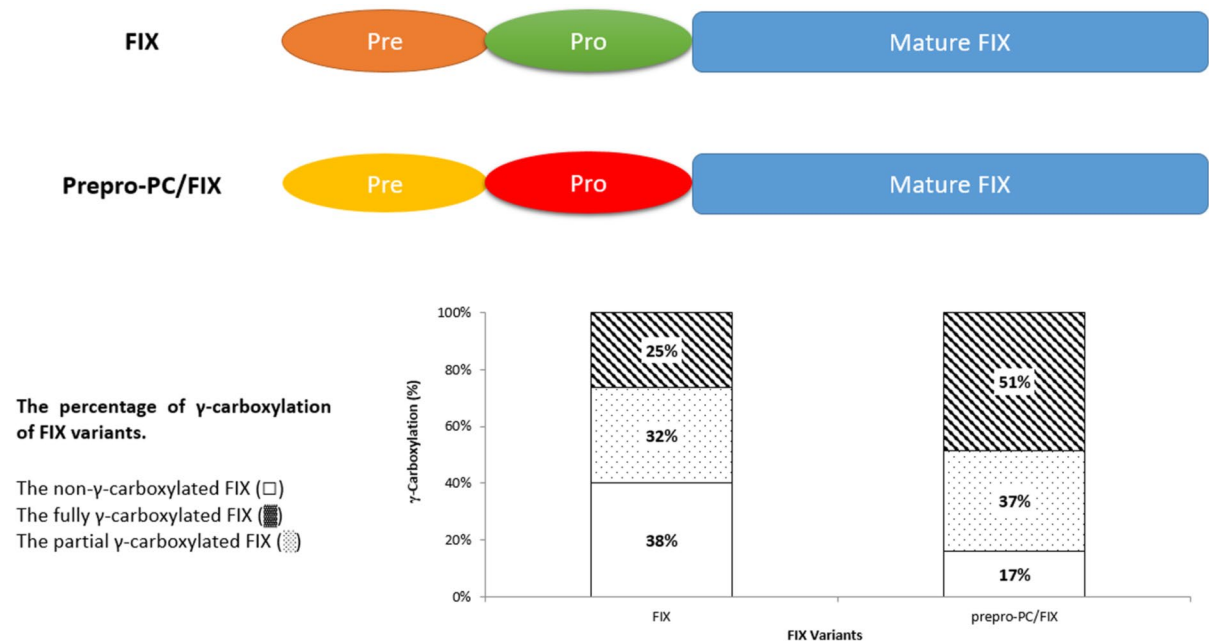
wild-type FIX. Furthermore, after barium citrate precipitation, the evaluation of fully  $\gamma$ -carboxylated FIX indicated that more than 51% of the total FIX produced with the PC propeptide was fully  $\gamma$ -carboxylated, representing a substantial improvement (twofold) over a system employing the native FIX propeptide in which 25% of the protein is fully  $\gamma$ -carboxylated. The data illustrated that the expression of FIX using the PC propeptide led to much higher fully  $\gamma$ -carboxylated material, which is preferred to FIX constructs tolerating the sequence for the native FIX propeptide expressed in heterologous S2 systems.

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## Graphical abstract



**Keywords** Coagulation Factor IX · Prepropeptide · Protein C ·  $\gamma$ -Carboxylase

## 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 a 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  $\gamma$ -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 et al. 1987).

Prior to hepatocellular secretion, FIX undergoes numerous posttranslational modifications, among which  $\gamma$ -carboxylation plays a significant role in the coagulation activity of FIX (Tie et al. 2006). Non- $\gamma$ -carboxylated FIX does not display coagulation activity, as a properly  $\gamma$ -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,  $\gamma$ -carboxylation is an essential, and rate-limiting step during FIX processing (Blostein et al. 2008). The  $\gamma$ -carboxylation is carried out by the enzyme  $\gamma$ -glutamyl carboxylase, which is able to change the specific glutamic acids to  $\gamma$ -carboxyglutamic acid residues following recognition of and interaction with the  $\gamma$ -carboxylase recognition site ( $\gamma$ -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  $\gamma$ -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

et al. 2000; Blostein et al. 2008). The weak interaction between the propeptide and  $\gamma$ -carboxylase is generally acknowledged to promote the rate of  $\gamma$ -carboxylation, as  $\gamma$ -carboxylase activity is lower for propeptides displaying high binding affinity relative to those with a low or moderate affinity (Camire et al. 2000; Blostein et al. 2008; Rishavy and Berkner 2012). We have previously demonstrated that a higher net charge and net hydrophobicity of the  $\gamma$ -CRS resulting from specific point mutations increases the production of  $\gamma$ -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 et al. 1987; Handford et al. 1991; Camire et al. 2000; Khorshidi et al. 2015). Since the impact of charge or hydrophobicity on the  $\gamma$ -CRS region significantly affects the  $\gamma$ -carboxylation of FIX, investigating the effects of charge or hydrophobicity separately may provide novel insights into the requirements for  $\gamma$ -carboxylase binding. Following our previous study in which we demonstrated that increasing both the net charge and hydrophobicity of the  $\gamma$ -CRS region improves active FIX expression (Vatandoost and Bos 2019), here we studied how a prepropeptide comprising an altered net charge in the  $\gamma$ -CRS relative to that of FIX affects FIX expression and function. Here we did so by replacing the FIX prepropeptide with that of PC, as while the net hydrophobicity of the PC prepropeptide is similar to that of FIX, its net charge is higher (+1 for PC vs -1 for FIX, (Vatandoost and Bos 2019)).

## Methods

### Construction of expression vectors

The pMT-hFIX vector (Vatandoost 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' TTTACCTGAATTATAAAG AGGAGCTGGTGT 3')
3. F2-FIX/PC (5' ACACCAGCTCCTCTTTATAAT TCAGGTA AAA 3')
4. R2-FIX (5' CCGCTCGAGATCCATCTTTCA TTAAGTGAGC 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 28°C without CO<sub>2</sub> 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<sup>6</sup> 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<sup>6</sup> 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  $3 \times 10^6$  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  $\text{CuSO}_4$  and 6  $\mu\text{g/ml}$  vitamin K1 (Konakion 10,125,641, Roche) to the medium.

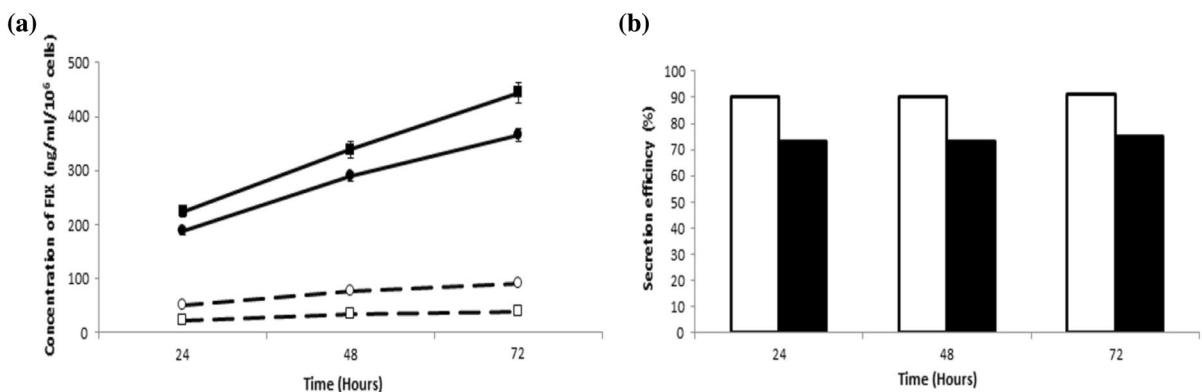
#### Analysis of recombinant FIX expression and activity

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). To assess intracellular FIX, cells were pelleted by centrifugation at  $100 \times g$  for 5 min, upon which they were resuspended in 500  $\mu\text{l}$  of ice-cold lysis buffer (100 mM KCl, 2 mM  $\text{MgCl}_2$ , 10 mM HEPES pH 7.5, 0.5% Triton X100) containing an antiprotease mix (Completed Protease Inhibitor, Roche). Subsequently, the lysate was centrifuged at  $12,000 \times g$  for 10 min at 4°C and the supernatant was assessed for FIX by ELISA. The FIX concentration (ng/ml) was calculated based on a reference curve 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 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  $\mu\text{g}$  of FIX in 1 ml of normal plasma.

#### Quantification of $\gamma$ -carboxylated recombinant factor IX

To quantify  $\gamma$ -carboxylated FIX, the barium citrate method was used for adsorbing  $\gamma$ -carboxylated glutamic acid residues [9,17,18]. During this process, the proteins that are poorly or not  $\gamma$ -carboxylated at all cannot be trapped by barium cation 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.



**Fig. 1** Evaluation of human FIX variants expression (A) and secretion efficiency (B) in stably transfected S2 cells. The FIX expression was assessed both in the cellular fraction and the conditioned media by assaying the FIX antigen levels at the indicated points. The symbols represent the following: • FIX

in conditioned media, ○ FIX in the cellular fraction, ■ prepro-PC/FIX in conditioned media, □ prepro-PC/FIX in the cellular fraction. B; Assessment of secretion efficiency from the FIX (■) and prepro-PC/FIX (□) cells. The data are the means  $\pm$  SD of three individual experiments

**Table 1** Expression of functional and non-functional FIX in conditioned media

FIX Variant	Expression time (Hours)	FIX expression (antigen) (ng/ml/10 <sup>6</sup> cell)	Expected FIX activity (mU/ml/10 <sup>6</sup> cell)	Observed FIX activity (mU/ml/10 <sup>6</sup> cell)	Functional FIX (ng/ml/10 <sup>6</sup> cell) (%)	Non-functional FIX (ng/ml/10 <sup>6</sup> 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)

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  $\pm$  SD of three independent experiments

## 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  $\pm$  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 containing a propeptide with a lower affinity for the  $\gamma$ -carboxylase are more efficiently  $\gamma$ -carboxylated, we here studied replacement of the FIX prepropeptide with that of PC that displays a low relative affinity for  $\gamma$ -carboxylase (Hallgren et al. 2006). Therefore, a

**Table 2** Barium citrate adsorption of FIX

FIX Variant	Expression time (Hours)	FIX -Ba <sup>2+</sup> (ng/ml/10 <sup>6</sup> cell)	FIX + Ba <sup>2+</sup> (ng/ml/10 <sup>6</sup> cell)	Expected FIX activity (mU/ml/10 <sup>6</sup> cell)	Observed FIX Activity (mU/ml/10 <sup>6</sup> cell)	Full $\gamma$ -carboxylated FIX (ng/ml/10 <sup>6</sup> cell) (%)	Partially $\gamma$ -carboxylated FIX (ng/ml/10 <sup>6</sup> 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)

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<sup>2+</sup>) 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  $\gamma$ -carboxylated FIX was determined based on observed FIX activity (1 mU=5 ng) and was also expressed as the percentage of full  $\gamma$ -carboxylated secreted FIX with the total amount of secreted FIX set at 100%. The partial  $\gamma$ -carboxylated FIX was calculated by subtracting the full  $\gamma$ -carboxylated FIX from functional FIX in Table 1. The data are the means  $\pm$  SD of three independent experiments

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<sup>6</sup> cells; total prepro-PC/FIX: 480 ng/ml/10<sup>6</sup> cells), prepro-PC/FIX was 1.2-fold more secreted after 72 h when compared to the FIX (444 vs. 366 ng/ml/10<sup>6</sup> 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<sup>6</sup> cells at 72 h, respectively).

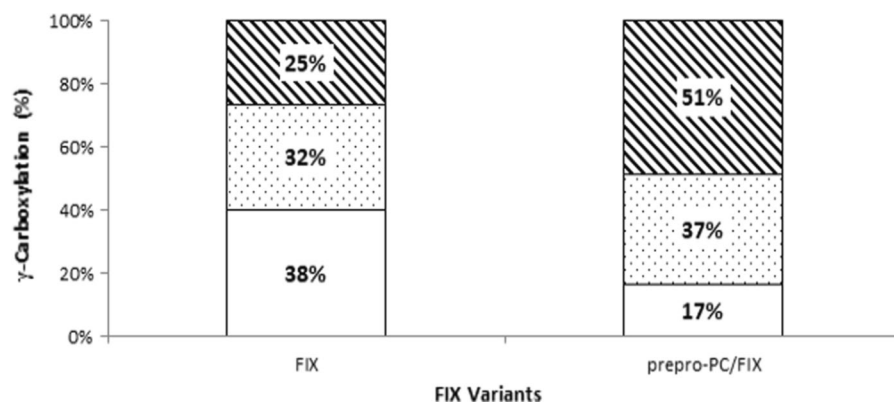
#### Functional activity of expressed FIX variants

As  $\gamma$ -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  $\gamma$ -carboxylated. It was also evident that the percentage of functionally active and as such  $\gamma$ -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  $\gamma$ -carboxylated, vs. ~60% of FIX (Table 1). These results suggest that the PC propeptide leads to higher  $\gamma$ -carboxylation of FIX and thus higher levels of functionally active FIX.

#### $\gamma$ -carboxylation of FIX variants

The extent of full  $\gamma$ -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  $\gamma$ -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).



**Fig. 2**  $\gamma$ -carboxylation of FIX variants. The percentage of non- $\gamma$ -carboxylated FIX (□) was defined as the calculated by subtracting the  $\gamma$ -carboxylated FIX from the secreted FIX. The fully  $\gamma$ -carboxylated FIX (▨) was based on FIX-specific

clotting activity measurements (1 mU = 5 ng). The partial  $\gamma$ -carboxylated FIX (▤) was calculated by subtracting the full  $\gamma$ -carboxylated FIX from  $\gamma$ -carboxylated FIX



## Discussion

Poor production and inefficient  $\gamma$ -carboxylation of synthesized FIX by heterologous expression systems are regarded as major obstacles to the production of large quantities of functional recombinant FIX (Kaufman et al. 1986; Baghani and Vatandoost 2016). There are different strategies to increase the expression of functional FIX when targeting the prepropeptide. Previously, we have exchanged the prepropeptide of human FIX for that of human prothrombin, resulting in an improvement in activity (tenfold) (Khorshidi et al. 2015). The prepeptides have different degrees of total hydrophobicity, which may influence protein synthesis and secretion (Bird et al. 1990; Hatsuzawa et al. 1997; Zhang et al. 2005; Knappskog et al. 2007; Khorshidi et al. 2015). Since the prepeptide of prothrombin has a higher total hydrophobicity than that of FIX (0.93 vs. 0.79) (Khorshidi 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  $\gamma$ -CRS, the FIX propeptide was substituted at positions -12, -13, and -14 based on the prothrombin propeptide. By increasing both the net charge and hydrophobicity of the  $\gamma$ -CRS in the propeptide region of FIX, this replacement led to high fully  $\gamma$ -carboxylated material (2.3 fold) and activity (3.4 fold) (Vatandoost and Bos 2019). The effect of hydrophobicity alone by 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). These results indicated that the hydrophobicity of the propeptide sequence is inversely correlated with the binding affinity of the VKD proteins for  $\gamma$ -carboxylase. Consistent with this, the FIX activity increased tenfold when the prepropeptide of FIX was replaced by that of prothrombin (Khorshidi et al. 2015), which is a considerably higher increase than that observed for replacement with the PC prepropeptide (1.6 fold). Although these results demonstrate the role of net hydrophobicity in  $\gamma$ -carboxylation, the  $\gamma$ -CRS region's charge is also important for the rate of  $\gamma$ -carboxylation. Interestingly, although the

PC and FIX  $\gamma$ -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 sevenfold decrease in binding affinity to  $\gamma$ -carboxylase (Camire et al. 2000; Hallgren et al. 2006). Hence, hydrophobicity is not the single factor directing  $\gamma$ -carboxylase binding. Since a rise in the net charge of the  $\gamma$ -CRS region of PC relative to FIX was observed, the net charge of this region may contribute to  $\gamma$ -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  $\gamma$ -carboxylated material and 1.4 fold activity (Vatandoost and Bos 2019). The second strategy was used to examine and confirm the effect of  $\gamma$ -CRS net charge on  $\gamma$ -carboxylase binding. Specifically, the FIX prepropeptide was replaced with that of PC, leading to 1.6 more  $\gamma$ -carboxylation and activity (Table 1). It is speculated that by increasing the FIX  $\gamma$ -CRS region's net charge, the  $\gamma$ -carboxylase turnover and, therefore,  $\gamma$ -carboxylated FIXs may be enhanced. Support for the effect of the net charge of the  $\gamma$ -CRS region on  $\gamma$ -carboxylation comes from several studies. The  $-9$  N to K substitution in FIX, while not affecting the hydrophobicity (from  $-5.3$  to  $-5.7$ ), did affect the net charge of the  $\gamma$ -CRS region (from  $-1$  to  $0$ ). This substitution resulted in an increase in apparent affinity from 33 to 370 nM and enhanced  $\gamma$ -carboxylation of FIX (Stanley et al. 1999). It was also shown that charged side chains at position  $-10$  as well as the residues  $-9$  and  $-8$  in  $\gamma$ -CRS region have an effect on  $\gamma$ -carboxylation (Vermeer 1990). Moreover, charged amino acids that are present close to the predicted  $\alpha$ -helical domain in several of the prepeptides may affect  $\gamma$ -carboxylation (Czerwiec et al. 2006). As such, these examples further point to a role for the charge of the FIX  $\gamma$ -CRS region in the functional interaction with  $\gamma$ -carboxylase, which is essential for an efficient turnover and  $\gamma$ -carboxylation. Since  $\gamma$ -carboxylation is crucial for a normal clotting activity of FIX, an increase in  $\gamma$ -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 the PC prepropeptide resulted in a twofold enhancement of fully  $\gamma$ -carboxylated FIX. In line with the findings of others, it was inferred that factors such as using altered prepeptides (1.8 to 2.7 fold increase



in  $\gamma$ -carboxylated FIX) (Camire et al. 2000; Khorshidi et al. 2015),  $\gamma$ -carboxylase (threefold increase in  $\gamma$ -carboxylated FIX) (Hallgren et al. 2002), and VKOR overexpression (two to threefold increase in  $\gamma$ -carboxylated FIX) (Hallgren et al. 2006) enhance  $\gamma$ -carboxylase turnover and decrease binding affinity thereby improving full  $\gamma$ -carboxylation.

Since the prepeptide (signal peptide) of PC has the same total hydrophobicity as that of FIX prepeptide, as anticipated, no difference was observed in total FIX expression (intracellular and conditioned media) when using a PC prepropeptide for FIX, which is consistent with previous findings (Knappskog et al. 2007, Tröbe 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 speculate that the increase in  $\gamma$ -carboxylation leads to higher secretion efficiency and a decrease in trapped FIX in the intracellular space, indicating that the secretory machinery is affected by  $\gamma$ -carboxylation. It is in light of other studies that show poor secretion of FIX following  $\gamma$ -carboxylase overexpression (Rehemtulla et al. 1993; Hallgren et al. 2002; Wajih et al. 2005a, 2005b) or increased secretion of FIX in overexpressed VKOR cells (Wajih et al. 2005a, 2005b; Hallgren et al. 2006, Pakdaman et al. 2019), implying that  $\gamma$ -carboxylase acts as an essential factor during protein secretion (McClure et al. 1992). Moreover, it was shown that  $\gamma$ -carboxylation is part of the protein secretion pathway (Rishavy and Berkner 2012) and was required for both efficient secretion and anticoagulant activity (McClure et al. 1992). The following hypothesis is also particularly attractive: Glu  $\gamma$ -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 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  $\gamma$ -carboxylation, with a 2.5 fold higher level of  $\gamma$ -carboxylation relative to the PC propeptide. Interestingly, these findings are in contrast with earlier observations on the apparent affinity for  $\gamma$ -carboxylase, which led to a sevenfold decrease in binding affinity to  $\gamma$ -carboxylase (Camire 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  $\gamma$ -carboxylation (Yu et al. 1994). Furthermore, selection of an expression system with desired features may affect the results of functional FIX production. The S2 expression system employed in the current study has been reported to display a higher  $\gamma$ -carboxylation efficiency relative to mammalian cells (Vatandoost et al. 2012).

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

**Acknowledgements** We thank Hakim Sabzevari University for its support in the postdoctoral fellowship and conducting this research

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

**Funding** This study was supported by the Hakim Sabzevari University (Grant No. 1400) to Jafar Vatandoost.

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

**Declarations**

**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.

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