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Effect of Pro-peptide Amino Acid Substitution in γ-Carboxylation, Activity and Expression of Recombinant Human Coagulation Factor IX

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Abstract

The production of recombinant K-proteins (VKD) for therapeutic purposes is an important challenge in the pharmaceutical industry. These proteins are primarily synthesized as precursor molecules and contain prepro-peptide sequences. The pro-peptide is connected to γ-carboxylase enzyme through the γ-carboxylase recognition site for the direct γ-carboxylation of VKD proteins that has a significant impact on their biological activity. Pro-peptides have different attitudes toward γ-carboxylase and certain amino acids in pro-peptide sequences are responsible for the differences in γ-carboxylase affinity. By aiming to replace amino acids in hFIX pro-peptide domain based on the prothrombin pro-peptide, pMT-hFIX-M14 expression cassette, containing cDNA of hFIX with substituted -14 residues (Asp to Ala) was made. After transfection of *Drosophila* S2 cells, expression of the active hFIX was analyzed by performing ELISA and coagulation test. A 1.4 fold increase in the mutant recombinant hFIX expression level was observed in comparison with that of a native recombinant hFIX. The enhanced hFIX activity and specific activity of the hFIXD-14A (2.2 and 1.6 times, respectively) were further confirmed by comparing coagulation activity levels of substituted and native hFIX. Enrichment for functional, fully γ-carboxylated hFIX species via barium citrate adsorption demonstrated 2-fold enhanced recovery in the S2-expressing hFIXD-14A relative to that expressed native hFIX. These results show that changing -14 residues leads to a decrease in the binding affinity to substrate, increase in γ-carboxylation and activity of recombinant hFIX.

Keywords**:** Factor IX, γ-carboxylase, pro-peptide, prothrombin

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Introduction

Human factor IX (hFIX) is a blood coagulation protein and its deficiency or dysfunction results in the bleeding disorder hemophilia B. The mature hFIX is a single chain glycoprotein comprising 415 amino acids which is derived from a 461 residue prepro-polypeptide precursor. This precursor protein is composed of a pre-peptide (signal peptide) and a pro-peptide that are N-terminally connected to the mature protein that consists of several domains including the gamma-carboxyglutamic acid-rich (GLA), epidermal growth factor-like (EGF) and activation and catalytic domain [1, 2]. The 46 amino acids sequence of pre and pro-peptide is known as the prepro-leader and contains Met -46 to Arg -1. Following cleavage and removal of the prepro-leader by signal peptidase and PACE/Furin endoprotease (paired basic amino acid cleaving enzyme) in the liver [3], hFIX is secreted into the bloodstream as an inactive precursor zymogen [4]. The zymogen of hFIX is proteolytically activated by factor XIa (of the contact pathway) or factor VIIa (of the tissue factor pathway) to produce a two-chain activated form [5].

Being a vitamin K-dependent protein (VKD), hFIX undergoes numerous post-translational modifications (PTMs), including γ-carboxylation which has a significant impact on its biological activity [6]. γ-carboxylation is performed by γ-carboxylase in the ER and it changes the specific glutamic acids in the GLA domain to γ-carboxyglutamic acid residues, which are mediated via the pro-peptide sequence in VKD proteins. The 18 amino acid pro-peptide contains the γ-carboxylase recognition site and plays a critical function in the VKD protein γ-carboxylation [7]. The pro-peptide is an intermediate in the recognition and/or binding of the γ-carboxylase to the substrate zymogen [8] and is involved in increasing the efficiency of γ-carboxylation [9].

The analysis of VKD plasma proteins (factor II, VII, IX, X, protein C, S, and Z) shows that all are primarily synthesized as precursor molecules and all contain hydrophobic prepro-peptide sequences. Although the pre-peptide sequence is different among these proteins, there is a significant homology in their pro-peptide domain. The pro-peptide has two recognition sites; the γ-carboxylase recognition site (γ-CRS) in the N-terminal part of the pro-peptide for connecting substrate to γ-carboxylase, and a peptidase recognition site in the C-terminal for cleaving by PACE/furin [10].

Although some residues in pro-peptide of VKD proteins are conserved, there are non-conserved residues that may control the interaction with the γ-carboxylase and enzyme activity [10]. The Phe -16, Ala -10, Arg -1 and -4 are highly conserved and residue -2 is generally an Arg or Lys. Hydrophobic amino acids in -6, -7 and -17 residues are also conserved. The rest of the non-conserved residues such as -11 to -15 can affect the affinity of

the substrate to the enzyme [11]. In fact, certain amino acids in pro-peptide sequences are responsible for the differences in γ-carboxylase affinity. In pro-peptides that bind the enzyme with high affinity, γ-carboxylase activity is lower relative to those with a low or moderate affinity. In other words, increasing the inhibition constant (Ki) reduces the affinity for the enzyme and it more rapidly released after complete required carboxylation, therefore more proteins is γ-carboxylated. A lower Ki value, on the other hand, coincides with a high affinity for the enzyme, so lower proteins can be γ-carboxylated. In fact, there is substantial evidence that γ-carboxylase is a processive enzyme[12-14], so proteins remain bound to the γ-carboxylase for some time after all γ-carboxylation is completed. Thus reducing binding leads to rapidly release from substrate and rapid γ-carboxylation of other proteins.

The binding affinity of vitamin K-dependent proteins for γ-carboxylase from high to low binding affinity is as follows: Factor X, VII, protein S, Factor IX, protein C, prothrombin (II) [12]. Since the Ki of prothrombin (277 nM) is higher than that of hFIX (33 nM) [12], we have previously replaced the whole prepro-peptide of hFIX with that of human and pig prothrombin, resulting in more γ-carboxylation, activity, and expression [15]. As substitution of each residue may have a different effect on γ-carboxylation [16], our aim of this study is to substitute individual amino acids in hFIX pro-peptide based on the prothrombin propeptide, and to increase the amount of γ-carboxylated recombinant hFIX zymogen. In other hand, a new expression system, *Drosophila melanogaster* Schneider line 2 (S2) cells, was taken into consideration, which demonstrates the higher activity of Drosophila γ-carboxylase relative to human γ-carboxylase [9] and the hFIX expression of this alternative expression system is comparison with that of mammalian cells [17].

Materials and methods:

Materials

All the enzymes used for the molecular biology in addition to the kits for PCR purification and plasmid isolation were purchased from Cinaclon (Tehran, Iran). Oligonucleotides were synthesized by Denazist (Mashhad, Iran) and Bioneer (Daejeon, Republic of Korea). The pMT-V5-HisA plasmid and all cell culture reagents were from Thermo Fisher Scientific, except Schneider's insect medium, penicillin G and streptomycin (Sigma-Aldrich). The enzyme linked immunosorbent assay (ELISA) specific for human FIX (Asserachrom hFIX:Ag, Stago) and activated partial thromboplastin time (aPTT) reagents were purchased from Diagnostica Stago (Bern, Switzerland).

Hydrophobicity analysis

Since the hydrophobic amino acids in the pro-peptide domain play an important role in the affinity of pro-peptide to γ-carboxylase, online EXPASy-PROtscale program was used to analyze the hydrophobicity of amino acids in the pro-peptide domain. In fact the hydrophobicity of these amino acids is defined based on the kyte-Doolittle hydrophobicity scale [18]. A Kyte-Doolittle scale is widely used to detect hydrophobic domains in proteins and positive numbers are hydrophobic. Alignment of pro-peptide of VKD coagulation factors was also performed by online Blastp program and Clustal Omega alignment program.

Construction of hFIXD-14A cDNA and pMT-hFIX.M14 plasmid

Splicing by Overlap Extension or SOEing PCR was used to exchange Asp to Ala (D to A) in residue -14 of the pro-peptide of hFIX cDNA. To do this, substituted prepro-peptide sequence of hFIX (133 bp) and the rest of hFIX encoding sequence (1292 bp) were synthesized separately from a human liver cDNA library (a kind gift from Dr AR. Zomorodipour, NIGEB, Iran) using KF1/R1-M14 (5'-GGGGTACC CAAACAGCGCGTGAACATG-3' / 5'-CCGCTCGAGAATCCATCTTTCATTAAGTGAGC-3') and F2/XR2 (5'-ACGCCAACAAAATTCTGAATC-3' / 5'-ACGCCAACAAAATTCTGAATC-3') oligonucleotide primers, respectively. The modified prepro-peptide DNA sequence was ligated to the DNA sequence of FIX by SOEing PCR technique, where primers R1-M14 and F2 fused the fragments and primers KF1 and XR2 were the outside primers. Following PCR purification, the KpnI-XhoI fragments were inserted into the pMT-V5-HisA vector downstream of the *Drosophila* Mtn promoter. The resulting plasmid was designated pMT-hFIX.M14. The identity of the fragments was confirmed employing restriction digestion and the substitution of -14 D to A was also confirmed using nucleotide sequence analysis.

Cell culture and transfection of S2 cells

Drosophila Schneider (S2) cells were maintained at 28ºC without CO_2 under normal atmosphere in Schneider's insect medium supplemented with penicillin (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, upon which the cells were allowed to loosely adhere. The S2 cells were transfected with pMT-hFIX.M14 plasmid employing the calcium phosphate co-precipitation method with minor modifications [19]. Cell density and viability were monitored prior to and following transfection by trypan blue exclusion using a 0.4% (w/v) solution. For the expression of the transgene, the Mtn promoter was induced one day after transfection by adding 0.5 mM $CuSO_4$ to the medium.

Immunoassay and clotting activity of recombinant hFIXD-14A

Human FIX was quantified in conditioned media employing an ELISA (Asserachrom IX:Ag, Stago, France) following the procedure provided by the manufacturer. In addition, intracellular accumulated FIX was assessed, for which the cells were pelleted by centrifugation at 100 g for 5 min, upon which they were resuspended in 500 µL of ice-cold lysis buffer (100 mM KCl, 2 mM MgCl2, 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 g for 10 min at 4 ºC, the supernatant was assessed for FIX.

The functional activity of recombinant hFIX was examined using an activated partial thromboplastin time (aPTT)assay as described previously [17]. Briefly, human plasma immuno-depleted of FIX (100 µL) was mixed with conditioned media (100 µL) and aPTT reagent (100 µL). After 3 min of incubation at 37 ºC, 100 µL of a prewarmed CaCl2 solution (25 mM) was added to the mixture, and the clotting time was recorded. The activity of expressed hFIX was calculated based on the standard curve of normal human plasma (Iranian blood transfusion organization), and one unit of FIX activity corresponded to the amount of FIX in 1 ml of normal plasma (~ 5 µg/ml).

Quantification of γ-carboxylated recombinant hFIX

The barium citrate precipitation of γ-carboxylated hFIX was adapted [20] from previously described methods [21]. Briefly, sodium citrate (14 mg) and 1M barium chloride (95 µL) were added to 2 ml of conditioned media, which were incubated for 1 hour at 4°C with gentle mixing. The mixture was centrifuged at 100g for 5 min, and the supernatant was kept for the analysis of unabsorbed FIX lacking γ-carboxyglutamyl residues. The pelleted γ-carboxylated FIX was washed with ice-cold 5mM $BaC1_2$, subsequently centrifuged at 100g for 5 min, resuspended in 0.1 M sodium citrate (1 ml), and adjusted to 10% ammonium sulphate to dissolve the adsorbed hFIX. After 30 min at 0 °C and subsequent centrifugation at 100g for 5 min, the barium sulphate pellet was discarded and hFIX was determined in the supernatant by ELISA as described.

Western Blotting

The prepared protein samples were subjected to electrophoresis on a 13% polyacrylamide, and gels were stained with Comassie Brilliant Blue. Electroblotting of proteins onto PVDF membrane (Amersham-Pharmacia Biotech, Germany) was performed using semidry procedure in a transfer buffer (25 mM Tris base, 192 mM

glycine, 20% methanol) at 86 mA for overnight. Presence of rhFIX in the cultured media was confirmed by western blotting experiment, during which the electroblotted proteins were probed with a polyclonal antiserum (ICN, USA) prepared against the hFIX. The hFIX-antibody complex was then treated with horseradish peroxidase-conjugated antirabbit immunoglobulin and visualized using a solution of 4-chloronaphtol with hydrogen peroxidase as enzyme substrate.

Statistical analysis

All expression analysis experiments were carried out in duplicates or triplets, and the generated data were presented as the mean ± SD. The ANOVA (analysis of variance) program for the analysis of variance followed by a Tukey post-hoc test were performed for evaluation; $P<0.05$ was considered statistically significant. All statistical analyses were carried out with SPSS 16 (SPSS Inc., Chicago, IL, USA).

Results

Hydrophobicity analysis

Hydrophobicity results of all VKD coagulation factors such as X, VII, IX, and prothrombin factors showed that their pro-peptides have high homology, and some residues are conserved (Fig 1). In some residues, such as those in positions -1, -4, -6, -10, -11, -12, -16 and -17, hydrophobicity of the VKD proteins is identical, but there is not a certain order in the remaining amino acids according to VKD proteins hydrophobicity graph. In the γ-CRS region, there is a substantial difference between the hydrophobicity of hFIX and prothrombin for the residues at position -14 (-3.5 vs. 1.8). These data indicate that the prothrombin pro-peptide, having the lowest affinity for γ-carboxylase, is more hydrophobic than the hFIX pro-peptide.

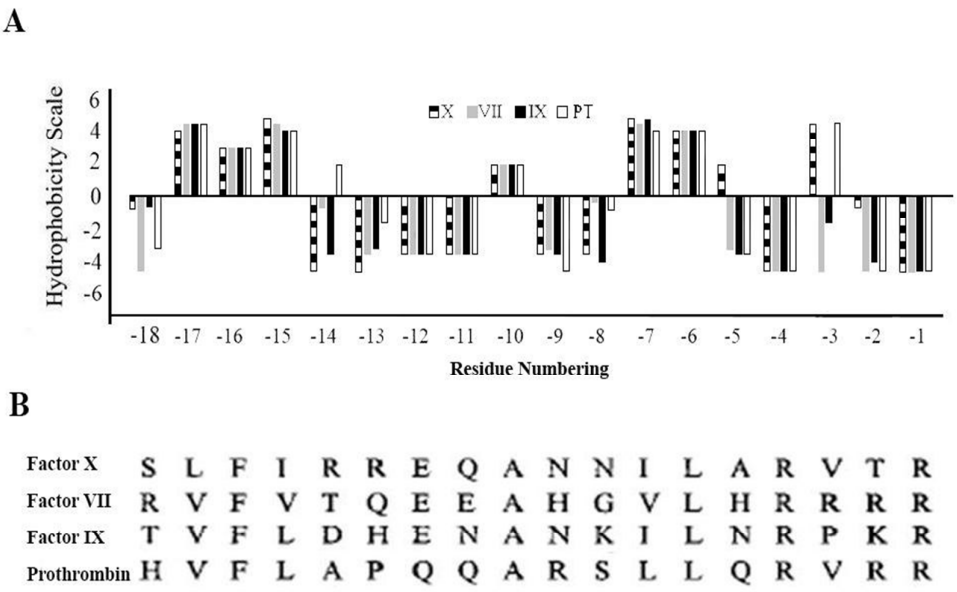


Fig. 1 Hydropathy plots (A) and alignment (B) of the amino acids in the pro-peptide of VKD coagulation factors. (A) Online EXPASy-PROtscale program was used to analyze the hydrophobicity of amino acids in the pro-peptide domain. Plots were made using hydropathy values for amino acids taken from kyte-Doolittle scale and positive numbers are hydrophobic. (B) Alignment of pro-peptide of VKD coagulation factors performed by online Blastp program and Clustal Omega alignment program.

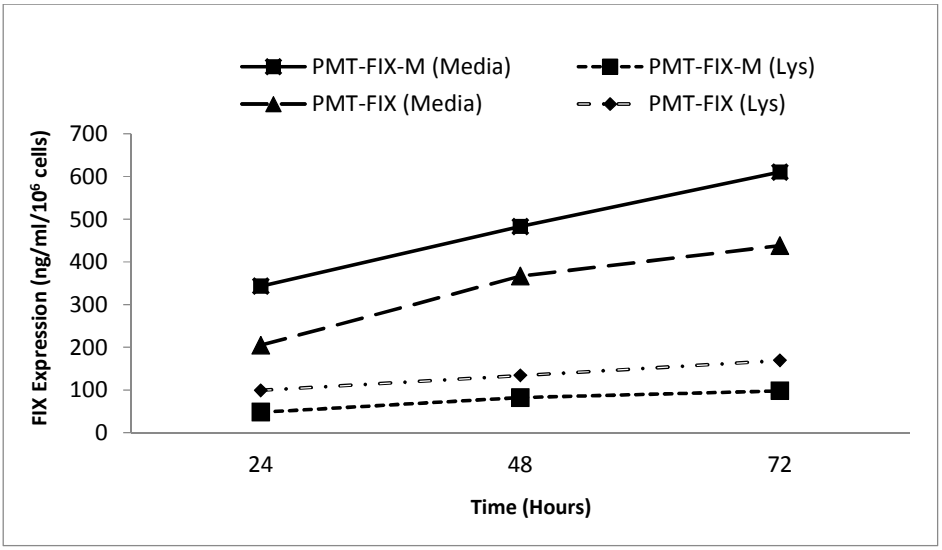
Expression and activity of recombinant hFIXD-14A

Following substitution of Asp to Ala (D to A) in residue -14 of the pro-peptide of hFIX, the expression of hFIXD-14A was evaluated in S2 cells. Both cultured media and cell lysates of the transfected cells were examined for hFIX antigen levels at various post induction periods. While the S2 cell line expressed up to 438 ng/ml/10^6 cells of native hFIX after 72 hours, the hFIXD-14A expression increased over time up to 1.4 fold and accumulated to 611 ng/ml/10^6 cells (Fig. 2A). Furthermore, the intracellular levels of hFIXD-14A were lower throughout the post-induction period in comparison with those of hFIX (Fig. 2A). These results suggest that replacing hFIXD-14A results in more γ-carboxylation, higher FIX secretion and reduces the amount of intracellular FIX.

The specificity of the expressed recombinant hFIX was also examined by Western blotting (Fig 2B). The provided data proves that the mature mutant FIX form with ~55 kDa is correctly produced and, therefore, there remain no alternative or partially-processed FIX molecules. Moreover, it demonstrated molecular weight of the

S2 expressed mutant FIX molecules are equally the same with that of the S2 expressed nature FIX and plasma-derived factor IX as an external control.

A



B

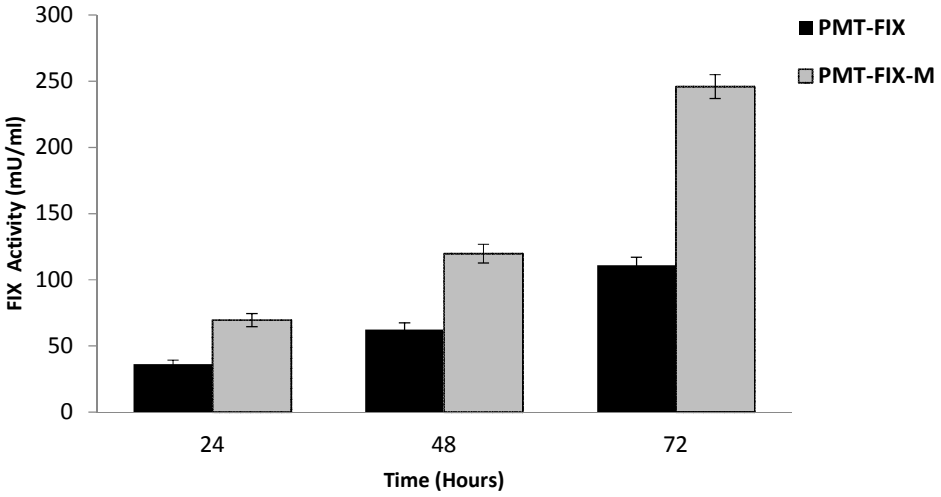
Fig 2 Evaluation of hFIX expression with Elisa (A) and Western bloting (B) in S2 cells transfected with PMT-FIX or PMT-FIX-M14

(A) Following transfection of S2 cells with PMT-FIX or PMT-FIX-M14 constructs separately, FIX expression was induced with 0.5 mM CuSO4 in the presence of 6 μg/ml vitamin K_1. At various post induction times, hFIX expression was assessed in the conditioned media and cell lysate by ELISA. The data are the means ± S.D. of 3 similar experiments. (B) The specificity of the expressed rFIX by Western blotting. Lanes 1, normal plasma-derived hFIX. Lanes 2, untransfected cells. Lanes 3, S2 cells transfected with pMT-hFIX-M14. Lanes 4, S2 cells transfected with pMT-hFIX. The apparent molecular weights of the standards are indicated on the left.

Consistent with these findings, the activity levels of S2-expressed hFIXD-14A were also increased by 2.2-fold in comparison with those of S2-expressed hFIX (246 vs. 111 mU/ml) (Fig 3A). Overall, the S2-expressed

hFIXD-14A displayed a 1.6-fold enhanced specific activity higher to that expressed native FIX (0.4 vs. 0.25 U/mg; Fig 3B). Taken together, these findings indicate that the substitution in residue -14 results in the production of higher levels of functional human FIX.

A



B

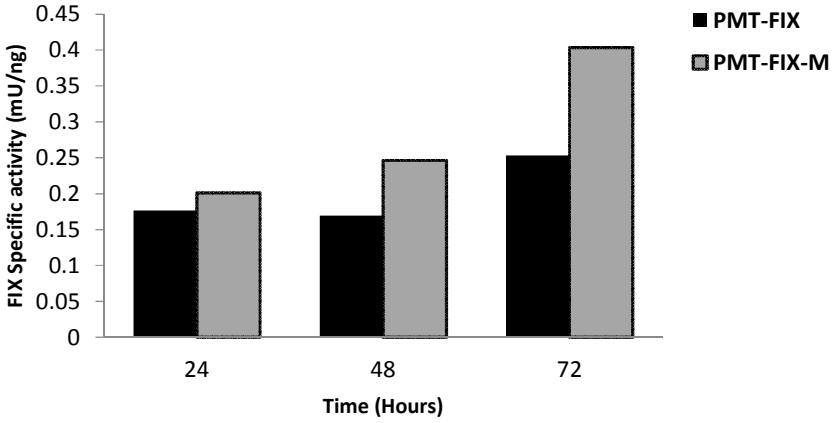


Fig 3 Comparison of coagulation activity (A) and specific activity (B) of hFIX in S2 cells transfected with PMT-FIX or PMT-FIX-M14. The hFIX coagulation activity of the cultured media was examined by performing clotting test, using immunodepleted plasma for the hFIX and aPTT reagent. The hFIX activity was calculated after exclusion of nonspecific clotting activity, determined from the cultured media of nontransfected S2 cells. The data are the means ± S.D. of 3 similar experiments (A). For assessment of specific activity, FIX expression and activity levels were assessed in the conditioned media to determine the specific FIX activity. Specific hFIX activity was determined as the ratio of activity per hFIX antigen (B).

To confirm this, the general characteristic of VKD proteins to adsorb to barium citrate via their γ-carboxylated glutamic residues was used to check whether -14 exchanges would enhance the proper γ-carboxylation of recombinant hFIX. Here, we used barium citrate adsorption to fractionate active γ-carboxylated protein from inactive subpopulations. During this process, neither poorly γ-carboxylated molecules nor non- γ-carboxylated are trapped by barium action and remain in the soluble fraction[22, 23]; the barium-bound hFIX can be subsequently eluted from the precipitate.

The assessment of the hFIX recovery following barium citrate adsorption showed a 2-fold enhanced recovery in the S2-expressed hFIXD-14A relative to native hFIX. Up to 40% of the hFIXD-14A and up to 22% of the native hFIX were recovered employing barium citrate adsorption, indicating that -14 substitution results in more γ-carboxylation.

Discussion

Since the pro-peptide is the to be recognized by γ-carboxylase, and different pro-peptides have different binding characteristics toward γ-carboxylase, replacing the amino acids in the pro-peptide domain based on the pro-peptides of factors which have less affinity for γ-carboxylase enzyme, is a way to increase the expression of FIX. In this regard, we have previously proven that replacing signal sequence and pro-peptide of VKD proteins with those having a better reaction with the γ-carboxylase enzyme has a increasing effect on the expression of the recombinant factor IX [15]. It has also been demonstrated that when the prothrombin pro-peptide is introduced into factor X, this resulted in an almost 3-fold increase in γ-carboxylation [12]. This is also confirmed by other studies demonstrating that the replacement of the prothrombin pro-peptide enhances in γ-carboxylation results in more γ-carboxylated products [10]. So it is hypothesized that prepro-prothrombin is a better substrate for the γ-carboxylase enzyme.

It was indicated that the pro-peptide with an 18 residue length has a peptidase site in the C-terminal and a γ-carboxylase recognition site (γ-CRS) in the N-terminal [10]. It was shown that substitutions of the Arg at positions -1 or -4 inhibit propeptide processing and result in nonfunctional FIX [24]. Lind et al also defines that the Arg -1 to Arg -4 are the basic amino acids of the propeptidase recognition sequence [25]. On the other hand, It was found that -11 to -18 amino acids play an important role in γ-carboxylation, and the loss of these domains results in the loss of γ-carboxylation and hence the inactivity of hFIX [26]. Czerwiec et al (2006) have also shown that although adding -1 to -8 amino acids (the high charged portion) decreased Km (nM) almost 3 times, adding -1 to -14 amino acids containing hydrophobic amino acids between -8 and -14 positions decreased Km to 45 times [27].

Therefore, the hydrophobic amino acids in the pro-peptide domain are one of the important structural elements in the detection of γ-carboxylation site and affects γ-carboxylation [27]. In addition, Handford et al indicated that residues between -10 and -18 define the γ-carboxylation recognition site [28]. Huber et al also identified amino acids in the γ-carboxylation recognition site on the propeptide of prothrombin and have shown these residues are between positions -10 and -18 [29]. Our results based on the Kyte-Doolittle comparative hydrophobicity graph (Fig. 1) shows that between positions -11 to -18, the residues -11, -12,-15,-16, -17 in hFIX and prothrombin are similar in terms of hydrophobicity. But there is not a certain order in the remaining three amino acids (-13, -14, and -18) according to the hydrophobicity graph. Given that the hydrophobicity of the residue at position -14 differs most between FIX and prothrombin, we replaced the Aspartic amino acid (D) in FIX with the Alanine (A) of prothrombin. By doing so, we anticipated reducing the affinity of the γ-carboxylase for the FIX pro-peptide, it more rapidly released after complete required carboxylation, thereby increasing the γ-carboxylation of more FIXs.

The hFIX-D-14A showed a significant increase in S2-expression (1.4 fold increased), activity (2.2 times higher) and specific FIX activity (1.6 fold enhanced) relative to the native hFIX. Similar results were shown when the CHO cells were transfected with 0.8, 1.5 and 0.9 fold increases in expression, activity and specific activity, respectively (data not shown). This indicates that the substitution of -14 amino acid (D to A), while increasing hydrophobicity from -3.5 to 1.8 [16, 18], leads to a decrease in binding affinity to substrate. So, the consumption of substrate will be increased over time, resulting in more protein expression, which is consistent with the antigen results. Furthermore, reducing binding affinity leads to an increase in enzyme turnover after complete γ-carboxylation and so increased γ-carboxylation and activity of more rFIXs. These results are in contrast to Huber et al. (1990), who have shown that -14 residues do not play a role in γ-carboxylation. In fact, they demonstrated that the conversion of Ala -14 in prothrombin to Ser does not inhibit the γ-carboxylation [16]. This result is expected since conversion of Ala to Ser decreases the hydrophobicity from 1.8 to - 0.8, thereby decrease in γ-carboxylation and not inhibition are also expected. On the other hand, since in -14 position protein S and C exhibit serine or threonine in factor VII with similar hydrophobicity as serine (-0.7), modification of this amino acid in prothrombin to another amino acid in other coagulation factors can not specify the importance of this position in γ-carboxylation. But there is an aspartic acid in -14 position of factor IX which hydrophobicity of this polar acidic amino acid is -3.5, so it is expected that changing 14D to A in factor IX will increase γ-carboxylation and protein production.

Conclusion

Both our studies as well as those of others demonstrate that substitutions at different positions throughout the pro-peptide of FIX have different effects. For instance, the FIXH-13A mutation results in higher γ-carboxylation [30], while substitutions at positions -10, -16 and -17 reduce γ-carboxylation [11]. The substitution of -14D to A leads to an increase in Ki and this will lead to more γ-carboxylation and activity of FIX. Future studies on this expression system are warranted to clarify that increased carboxylation is not due to increased activity of the enzymes in the carboxylation cycle. Moreover, it needs to confirm increasing in Ki and exact γ-carboxylation. The increase of activity and expression of the produced FIX in this research can help the improvements in production methods and it can decrease the production costs in S2 cells. It is also important to mention that this mutation was not described in hemophiliac patients.

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