# 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

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*The* Drosophila *γ-glutamyl carboxylase (dγC) has substrate recognition properties similar to that of the vertebrate γ–carboxylase (γC), and its carboxylated product yield, in vitro, was shown to be more than that obtained with the human enzyme. However, whether the* Drosophila *enzyme is able to γ-carboxylate the human vitamin K-dependent (VKD) proteins, such as the human coagulation factor IX (hFIX), as synthesized in cultured* Drosophila *cells was not known. To examine this possibility, the* Drosophila *Schnider (S2) cell line was transfected with a metallothionein promoter-regulated hFIX-expressing plasmid. After induction with copper ion, expression efficiency of the active hFIX was analyzed by performing enzyme-linked immunosorbent assey (ELISA) and coagulation test on the culture supernatant of the transfected S2 cells during 72 h of postinduction. In comparison with Chinese hamster ovary cell line, S2 cells showed higher (∼12-fold) expression level of the hFIX. The γ-carboxylation of the* Drosophila*-derived hFIX was confirmed by evaluation of the expressed protein, after being precipitated with barium citrate. The biological activity of the S2 cell-derived hFIX indicated the capability of S2 cells to fulfill the required γ-carboxylation of the expressed hFIX. Coexpression of the human γ–glutamyl carboxylases (hγC) was also shown to improve both expression and γ-carboxylation of the hFIX. This is the first in vivo data to describe the ability of the dγC to recognize the human-based propeptide as substrate, which is an essential step for production of biologically active γ-carboxylated VKD proteins.*
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## Introduction

Blood coagulation factor IX (FIX) participates in the middle phase of the intrinsic pathway of blood coagulation.[1] It circulates as an inactive precursor before activation by either factor XIa or tissue factor/factor VIIa during blood coagulation.[2] Deficiency in coagulation factor IX is the most common cause of hemophilia B (or Christmas disease), an X-linked recessive bleeding disorder which occurs in 1/30,000 males.[3–5] The current treatment for hemophilia B patients consists of replacement therapy with either pooled plasma concentrates or recombinant FIX.[6] γ-Carboxylation of specific glutamic acid residues in the so-called Gla domain of FIX is required for its biological activity of FIX.[7,8] The extra carboxyl group in these Gla residues results in a highly

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negatively charged side chain that allows for calcium to bind, thereby mediating the interaction of a properly folded Gla domain with the negatively charged phospholipid membranes.[9–11] γ-Carboxylation, carried out in the endoplasmic reticulum (ER), is mediated by two essential enzymes: VKD γC, which requires reduced vitamin K as a cofactor, and the warfarin sensitive enzyme vitamin K 2,3-epoxide reductase (VKOR), which produces the cofactor.[8] γ-Carboxylase recognizes its substrates through their propeptides, which in the case of FIX is an 18-amino acid sequence, N-terminal to the Gla domain, that is proteolytically removed in the Golgi following carboxylation.[10,12]

Over the past decades, a variety of cell lines, such as Chinese hamster ovary (CHO), baby hamster kidney (BHK), and human embryonic kidney-293 cells as well as hepatocytes have been examined for the production of FIX.[9,13–17] CHO cells have been previously reported to be the most suitable host for the production of FIX and several other recombinant therapeutics.[18] However, high-level expression of biologically active vitamin K-dependent proteins by these cells is hard to achieve due to inefficient γ-carboxylation.[17] This is exemplified by the observation that FIX expressed by CHO cells was incompletely γ-carboxylated.[19] Consequently, alternative expression systems with an efficient γ-carboxylation system may be exploited to increase the production of fully active FIX.

Genes encoding γC have been identified in vertebrates and two invertebrate species, Molluscs and *Drosophila melanogaster*. Although *D. melanogaster* carries genes necessary for both the γ-carboxylation and the recycling of vitamin K epoxide, no native substrate for dγC has been identified thus far.[20–22] It has previously been shown that the substrate recognition properties of dγC are remarkably similar to that of vertebrate γC. Aligning the propeptide binding sites of human hγC and dγC revealed that a considerable number of residues are conserved, suggesting that propeptides from human VKD proteins might be recognized by dγC.[20] Moreover, using equivalent amounts of dγC and hγC in vitro, the yield of γ-carboxylated products was about fivefold higher for dγC as relative to hγC.[20] In contrast, Li et al.[23] reported that dγC failed to γ-carboxylate, a peptide that was fused to the hFIX propeptide, suggesting that dγC may be incapable of γ-carboxylating hFIX.[23] Thus far, the potential of the *Drosophila* enzyme to γ-carboxylate, a human substrate such as FIX, synthesized in cultured *Drosophila* cells in vivo has not been established.

Different cell lines have been derived from *D. melanogaster*, including Schneider (S2) cells that have been developed as a plasmid-based, nonlytic integration system capable of stably expressing high levels of recombinant proteins.[24–26] S2 cells have the advantage of growing without requiring $CO_2$, being easily adapted to large-scale fermentors, and having the appropriate post-translational machinery.[25,26] The *Drosophila* metallothionein (Mtn) promoter has been shown to be tightly regulated and capable of driving high-level heterologous transcription upon induction by metals such as Cu or Cd ions.[24,27–29] On the basis of these advantages, we decided to evaluate this expression system for the production of hFIX. Our results presented here are the first to show that the *Drosophila* S2 cell line is able to express hFIX that is, at least in part, γ-carboxylated by dγC.

## Materials and Methods

### *Materials*

The plasmids pcDNA3 and pMT-V5-HisA were obtained from Invitrogen. Oligonucleotides were synthesized by Bioneer. Schneider's insect medium, penicillin G, and streptomycin were purchased from Sigma. RPMI-1640, fetal bovine serum (FBS), bovine serum albumin, L-glutamine, sodium pyruvate, and nonessential amino acids were obtained from Gibco-BRL Life Technology (Karlsruhe). All the enzymes used for the molecular biology in addition to the kits for polymerase chain reaction (PCR) purification, plasmid isolation, RNA preparation (Tripure), reverse transcriptase (RT; M-MuLV), and other chemicals, such as geneticin (G418) and protease inhibitors, were purchased from Roche. The InsT/Aclone kit was obtained from Fermentas. The ELISA kit specific for human FIX (Asserachrom, hFIX::Ag) and activated partial thromboplastin time (aPTT) reagents were purchased from Diagnostica Stago.

### *Construction of recombinant plasmids*

The cDNA of hFIX (~1.4 kb) was PCR-amplified from a human liver cDNA library (purchased from the MRC-Clinical Science Center at the Imperial College School of Medicine, London, UK) with pfu-DNA polymerase, using oligonucleotide pair, hFIX-KpnI (5′GGGGTAC CGCCACC ATGCAGCGCGTGAAC3′) and hFIX-XhoI (5′ CCGCTCGA GATCCATCTTTCATTAAGTGAGC 3′) as forward/reverse primers. The cDNA encoding hγC (~2.3 kb) was similarly amplified using oligonucleotide pair, hγC-KpnI (5′ GGGG TACCAGAGCAATGGCGGTGTCTG 3′) and hγC-XhoI (5′ CCGCTCGAGTTCAGAACTCTGAGTGGACAGG 3′) as forward/reverse primers. Following subcloning into T-vector, the KpnI–XhoI fragments were inserted into either the pMT-V5-HisA vector downstream of the *Drosophila* Mtn promoter or the pcDNA3 vector, 3′ to the CMV (cytomegalovirus) promoter. The resulting plasmids were designated pMT-hFIX, pMT-h*γC*, and pcDNA3-hFIX, respectively. The identity of the fragments was confirmed using restriction digestion followed by nucleotide sequence analysis.

### *Cell culture and transfection*

Drosophila Schneider (S2) cells (a kind gift from Professor M. Matsuda, Kyorin University School of Medicine, Tokyo, Japan)[26] were maintained at 28°C without $CO_2$ under normal atmosphere in Schneider's insect medium supplemented with 10% FBS, penicillin (50 U/mL), and streptomycin (50 μg/mL). The S2 cells were transfected with pMT-hFIX alone or together with pMT-hγC based on a calcium phosphate coprecipitation method with minor modifications.[30] For DNA transfection, $3 \times 10^6$ cells, seeded in 3 mL of medium in six-well plates 1 day before transfection, were allowed to loosely adhere. Every day, cells were counted, and cell viabilities were determined by trypan blue exclusion using a 0.4% (w/v) solution. For the expression of the transgene, the Mtn promoter was induced 1 day after transfection by adding 0.5 mM $CuSO_4$ to the medium. CHO cells (obtained from Pasteur Institute, Iran) were cultured and transfected as described previously.[31] Briefly, the CHO cells were grown in a 5% $CO_2$ atmosphere at 37°C subcultured at a density of $2 \times 10^5$ cells in 2 mL of medium in six-well plates. The cells were transfected with 2 μg of plasmid DNA, using FuGene-6. After transfection, the medium was harvested, and fresh rich medium (containing 6 μg/mL vitamin K1 and 10% (v/v) FBS) was added to the cells. On day 2 post-transfection, the cultured medium was collected for transient expression analysis, and fresh medium was added to the cells. For stable transfection, the transfectants were selected in media containing 450 μg/mL geneticin.

Individual colonies were expanded in the presence of geneticin as described previously.[14,32] Cells were screened for FIX production by collecting conditioned media from cells that were ~70% confluent.

### *Immunoassay of FIX*

FIX in conditioned media was quantified using an ELISA following the procedure provided by the manufacturer. Concentration of the expressed hFIX in culture media was calculated based on the standard curve and stated in ng/mL. The results obtained after the subtraction of nonspecific absorbance determined on cultured media from the nontransfected cells.

### *Analysis of intracellularly accumulated FIX*

Cells were pelleted by centrifugation at 100*g* for 5 min, on which they were resuspended in 500 $\mu$L of ice-cold lysis buffer (100 mM KCl, 2 mM $MgCl_2$, 10 mM, HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), pH 7.5, 0.5% Triton X100) containing an antiprotease mix (Roche). After 15–20 min, the lysate was centrifuged at 4°C at 12,000*g* for 10 min. Human FIX obtained supernatant was measured by ELISA as described earlier.

### *FIX clotting activity*

The biological activity of expressed FIX was examined using an aPTT assay, as described previously.[31] Briefly, plasma immunodepleted for FIX (100 $\mu$L) was mixed with conditioned media (100 $\mu$L) and 100 $\mu$L of the aPTT reagent. After 3 min of incubation at 37°C, 100 $\mu$L of a prewarmed $CaCl_2$ solution (25 mM) was added to the mixture, and the clotting time was measured. FIX activity was calculated using a standard curve of normal pooled plasma (kindly provided by Dr. N. Amirizadeh, Iranian National Transfusion Organization) following correction for nonspecific clotting activity determined from the conditioned media of nontransfected cells. By definition, 5 $\mu$g/mL of FIX in normal human plasma equals 1 U of FIX and is considered as 100% activity.[3]

### *RNA preparation and RT-PCR*

Total RNA from S2 cells ($10^6$ cells) expressing FIX was extracted according to the protocol provided by the manufacturer (Tripure Kit, Roche). First-strand cDNA synthesis was done using random primers and M-MuLV RT. The generated fragments were subsequently used as template for the PCR-amplification of the double-stranded cDNA, corresponding to a section of the hFIX coding sequence, using oligonucleotides hFIX-KpnI and hFIX-XhoI as forward and reverse primers, respectively.

### *Precipitation of γ-carboxylated FIX*

Barium citrate precipitation of $\gamma$-carboxylated FIX was adapted from methods described previously.[33,34] Briefly, sodium citrate (14 mg) and 1 M barium chloride (95 $\mu$L) were added to 2 mL of medium and incubated with gentle mixing at 4°C for 1 h. The mixture was then centrifuged at 100*g* for 5 min, and the supernatant was kept for analysis of unadsorbed FIX, lacking $\gamma$-carboxyglutamyl residues. The precipitated $\gamma$-carboxylated FIX was washed with ice-cold 5 mM $BaC1_2$ and recentrifuged, on which the supernatant was discarded. The precipitant was finally resuspended in 0.1 M sodium citrate (1 mL) and adjusted to 10% ammonium sulfate to dissolve the adsorbed FIX. After 30 min at 0°C and subsequent centrifugation at 100*g* for 5 min to remove the unwanted barium sulfate precipitant, the pellet was discarded, and the amount of the FIX in the supernatant was determined by ELISA.

### *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 (nalysis of variance) program for analysis of variance followed by a Tukey post hoc test was performed to evaluate differences among the constructs. $P < 0.05$ was considered statistically significant. All statistical analyses were carried out with SPSS 16 (SPSS, Chicago, IL).

## Results

### *Expression of recombinant hFIX in* **Drosophila** *S2 cells*

Before transfection and induction, the *Drosophila* S2 cells were healthy, with over 99% viability. The cells were transfected with the pMT-hFIX plasmid based on a calcium phosphate coprecipitation procedure, as preliminary experiments showed this method to be more efficient than the lipofection-based method. On transfection, a temporary decrease in viability and growth rate of the cells occurred, which was followed by a normal cell growth in the following postinduction days.

Transfection of the S2 cells with FIX was confirmed by performing RT-PCR to demonstrate transcription of the transgene. Indeed, the electrophoresis pattern of the RT-PCR products indicated the presence of the hFIX mRNA within the mRNA-pool of the induced transfected cells (data not shown). To evaluate the transient expression of FIX in the S2 cells, both cultured media and cell lysates of the transfected cells were examined for FIX antigen levels at various postinduction time points. The obtained data indicate that while FIX was secreted at levels up to 670 ng/mL on day 3 postinduction, intracellular FIX levels remained minimal throughout this postinduction period (Figure 1).

Expression efficiency of the transfected S2 cells was compared with that of transiently transfected CHO cells up to 72 h post-transfection. Surprisingly, the S2 cells showed a maximum 12-fold higher FIX expression as compared with the CHO cells (Figure 2A). Moreover, S2 cells also expressed FIX at threefold higher levels as compared with CHO cells stably expressing FIX (Figure 2A).

### *Characterization of recombinant hFIX secreted from S2 cells*

The clotting activity of FIX was assessed in conditioned media from S2 cells at different time points (Figure 2B). Human FIX was secreted from S2 cells up to 157 mU/mL on day 3 of postinduction, which is sevenfold higher than the FIX activity levels obtained for transiently transfected CHO cells (Figure 2B). Consistent with the findings obtained on FIX antigen levels, the activity levels of S2-expressed FIX were also increased by fivefold in comparison with those of the stably expressing CHO cells. These data indicate that the S2 cells express biologically active FIX, thereby strongly suggesting that the S2 cells are capable of proper $\gamma$-carboxylation of hFIX. To confirm this, we took advantage

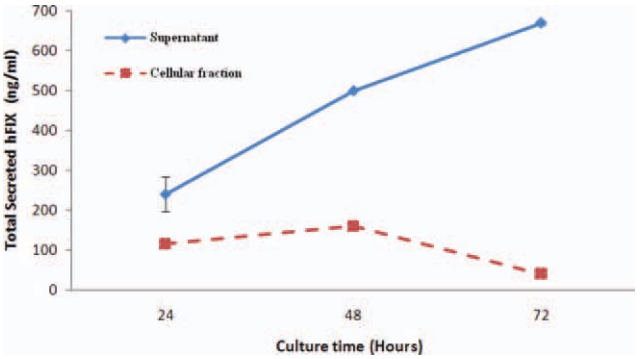


**Figure 1. Evaluation of transient hFIX expression in S2 cells at various postinduction times, based on ELISA performed on samples taken from both the culture supernatant and the cellular fraction at various post induction times.**

The average ± SD of three independent cultures is shown.

of the general characteristic of VKD proteins to adsorb to barium citrate via their γ-carboxylated glutamic residues.[35] During this process, the molecules which are poorly or not γ-carboxylated at all cannot be trapped by barium cation and remain in the soluble fraction. The barium-bound FIX can be subsequently eluted from the precipitate by buffer modification.[35] Quantification of FIX recovered from the barium citrate precipitate showed that 18–22% of the FIX secreted by S2 cells was adsorbed and was therefore most likely fully γ-carboxylated. Accordingly, around 80% of the FIX expressed was not precipitated, suggesting that this portion of the FIX synthesized was partially or not γ-carboxylated at all.

### *Coexpression of hFIX and hγC*

Bearing in mind the importance of γ-carboxylation for the biological function of FIX, we studied the coexpression of hγC in hFIX-expressing S2 cells. To do so, S2 cells were cotransfected with both the hγC-expressing plasmid (pMT-hγC) and the pMT-hFIX plasmid. This resulted in a twofold increase in FIX expression of both antigen and clotting activity levels (Figures 3A,B). These findings indicate that coexpression of hγC improved the secretion of human FIX in S2 cells. Barium citrate precipitation of conditioned media revealed that more of the total FIX was trapped (32%) as compared with that collected from S2 cells transfected with FIX alone. This observation suggests that coexpression with hγC enhances the fraction of properly γ-carboxylated FIX expressed. The highest level of the hFIX precipitation occurred 2 days after induction, for the two-plasmid-containing cells, with about 1.43-fold increase in comparison with that of the single-plasmid-containing cells.

### *The secretion efficiency of recombinant hFIX in* Drosophilia *S2 cells*

The secretion efficiency of a particular protein is defined as the ratio between the secreted fraction and its total amount.[36] On the basis of the data obtained, the secretion efficiency of FIX in S2 cells varied between 68 and 94% from 24 to 72 h postinduction (Figure 4). Therefore, the FIX trapped in the intracellular space reduced as the cell cultivation proceeded. A similar observation was made for the two-plasmid transfected S2 cells, as the secretion efficiency increased from 63 to 91% from 24 to 72 h postinduction (Figure 4).

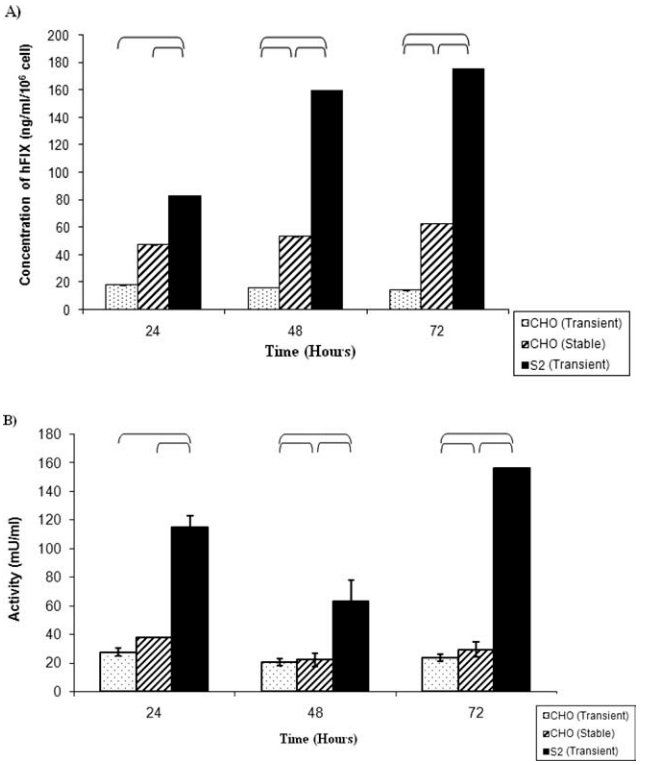


**Figure 2. Comparison of the efficiencies of the transfected S2 and CHO cells for the expression of FIX, assessed based on ELISA (A) and coagulation test (B).**

The hFIX secreted in cultured media was examined during 72 h of post-transfection. (A) Concentrations of the hFIX in culture media were determined based on ELISA and reported after the subtraction of nonspecific absorbance determined on cultured media from the nontransfected cells and finally stated in ng/mL. Horizontal brackets, on top, indicate samples that are significantly different ($P < 0.05$) compared with other samples, using analysis of variance. (B) 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. Normal pooled human plasma was used as a standard. Horizontal brackets, on top, indicate samples that are significantly different ($P < 0.05$) compared with other samples, using analysis of variance.

## Discussion

When production of proteins with pharmaceutical importance is aimed for, mammalian cells are generally considered to be the most suitable host, as they are known to be able to properly post-translationally modify the proteins produced. However, being overproduced in mammalian expression systems, mammalian VKD proteins are most often inefficiently γ-carboxylated.[17] This is exemplified by the observation that hFIX secreted from CHO cells is incompletely γ-carboxylated.[19] Partial γ-carboxylation of synthesized FIX[18] and incomplete removal of the propeptide by the PACE (paired basic amino acid cleaving enzyme)/furin proteases in the Golgi apparatus[37] have been reported as two major causes of the poor yield of fully processed FIX. As such, this is a major difficulty that needs to be overcome for the efficient

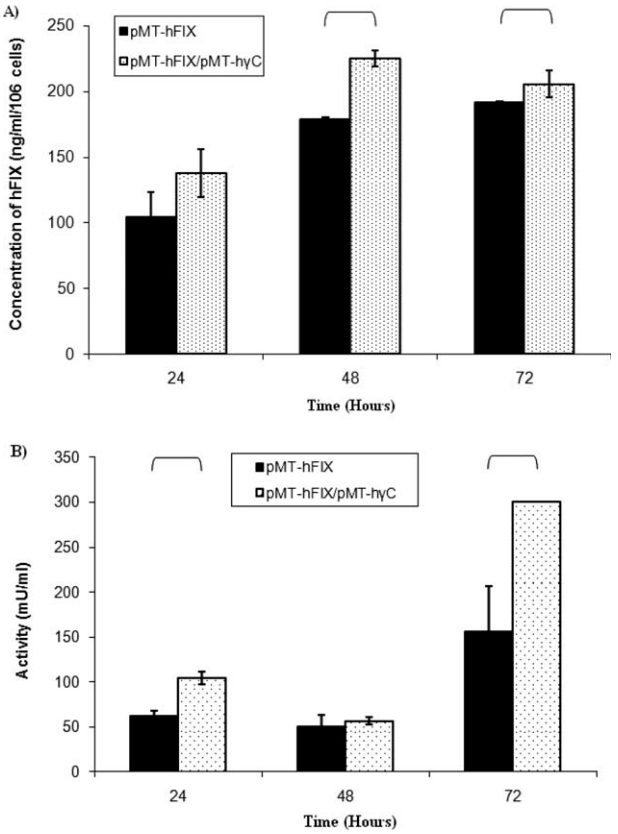


**Figure 3. Studying the effect of on the expression of FIX by the *Drosophila* S2 cells, by performing ELISA (A) and clotting test (B) on the cultured media.**

(For experimental and analytical details see legend to Figure 2 and "Materials and Methods" section.)

expression of mammalian vitamin K-dependent coagulation factors.[13,16,18]

Consistent with previous in vitro findings,[36,38] our results establish that *Drosophila* S2 cells have the potential of efficiently producing vitamin K-dependent human FIX. The secretion efficiency of human FIX by the S2 cells reached up to 94%, with an estimation of 670 ng/mL, whereas the intracellular FIX content remained minimal throughout the experiment.

In this study, a 12-fold increase in the FIX expression level was observed for the S2 cells in comparison with that of the transiently transfected CHO. The enhanced FIX productivity of the S2 cells was further confirmed by comparing their levels with those of CHO cells stably expressing FIX.

The coagulation activity of S2 cell-derived FIX provided convincing evidence that *Drosophila* S2 cells fulfill the required γ-carboxylation, which is essential for a normal clotting activity of FIX. As the propeptide confers binding affinity to the γ-carboxylase,[10,12] our in vivo results indicate that dγC is able to recognize the human FIX propeptide as substrate, which is in accordance with the in vitro data provided by Bandyopadhyay et al.,[20] thereby opposing those provided by Li et al.[23].

Both the coagulation activity and barium citrate precipitation indicated that a major portion of the *Drosophila*-derived hFIX is properly γ-carboxylated. Several different methods have been used to fractionate highly carboxylated, active rFIX from inactive rFIX, and they are all based on the

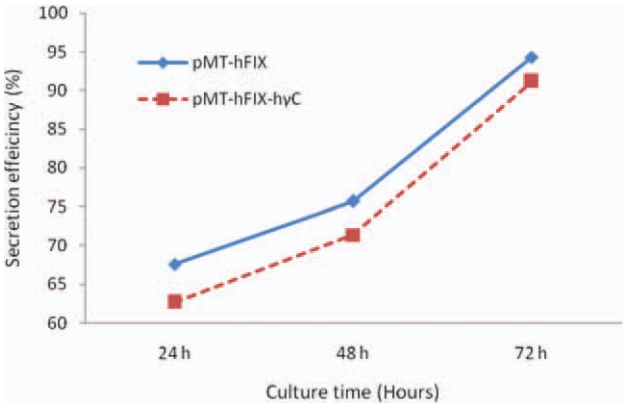


**Figure 4. Assessment of the secretion efficiency of FIX from the *Drosophila* S2 cells, after coexpression of hγC.**

metal-dependent conformational changes of the Gla domain.[39,40] Barium citrate adsorption is one of the earliest methods used and considered as a moderately successful method in fractionating active carboxylated protein from inactive subpopulations.[39] Quantification of this fraction of FIX showed that 18–22% of the secreted FIX was most likely fully γ-carboxylated. As such, the remaining ~80% of the secreted FIX is expected to be either poorly or not γ-carboxylated at all. No clear definition has been presented for proper γ-carboxylation of a vitamin K-dependent protein so far. However, as it has been reported that only five out of the 12 Glu residues in the Gla domain of human FIX are functionally important,[41] we may not rule out that partially γ-carboxylated, but functionally active FIX was present in the nonprecipitated fraction.

Coexpression of hγC in FIX-expressing S2 cells increased FIX expression nearly twofold. Although the secretion efficiency of FIX was slightly reduced on coexpression of hγC, overall our findings indicated that hγC enhances the total expression of γ-carboxylated hFIX. This was supported by comparing both total and secreted FIX levels of the two-plasmid-containing cells with those of the single-plasmid transfected cells 72-h postinduction. During this period, the FIX secretion efficiency of two plasmid cells was approximately 91%. Therefore, less than 10% of the expressed FIX might have been trapped in the intracellular space. Our result is comparable with the results reported by Hallgren et al.,[12] who showed an 80% intracellular accumulation of human FIX expressed in mammalian cells, when a γC gene is coexpressed. The failure of γC overexpression in mammalian cells to improve secretion of hFIX was also reported by other investigators. Rehemtulla et al.[17,37] showed that the overexpression of γ-carboxylase in stably transfected mammalian cells inhibits synthesis of functional hFIX. In a separate study, it was shown that the FIX secretion by BHK and 293 cells was greatly reduced after γC over expression.[12] VKORC1,[42] which is an 18 kDa ER membrane protein and putative subunit of VKOR,[43] was suggested to play a role in the inhibited expression. Hallgren et al.[12] have suggested that an excess of γ-carboxylase in the ER inhibits release of γ-carboxylated proteins by forming intracellular complexes with the precursors of VKD proteins. This difference in the effect of γC overexpression in mammalian vs. *Drosophila* cells is not unexpected due to evolutionary distance between the two examined cell types. The positive effect of the hγC overexpression on FIX expression in the *Drosophila* cells

may be explained by differences in either the γ-carboxylation or the secretion process in these cells, which should be elucidated. For instance, the episomally encoded γ-carboxylase that exists in the ER requires sufficient supplies of its cofactor, which may be more efficiently delivered in the S2 cells. Taken together, the data obtained in this study provide convincing evidence that the *Drosophila* cells have the potential to efficiently secrete and properly γ-carboxylate hFIX, an archetypic VKD protein.

The *Drosophila*-specific human FIX-expressing plasmid, that was constructed, was shown to be capable of overproduction and secretion of active hFIX in an S2 expression system. Further optimization of the culture media as well as growth and induction conditions of stably transfected S2 cells is necessary to achieve an efficient expression of hFIX. Having now identified some of the limitations in an S2 expression technology, the recombinant hFIX expression system developed in this study has provided ample ground to further develop bioengineering strategies to examine various important factors that affect the expression efficiency of FIX, in particular *cis*-acting elements, and to explore large-scale production of hFIX in the *Drosophila* S2 cells.

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## Notation

hFIX = human coagulation factor IX
hγC = human γ-carboxylase
dγC = Drosophila γ-carboxylase
VKD = viamin K dependent
CHO = Chinese hamster ovary
PCR = polymerase chain reaction
VKOR = vitamin K 2,3-epoxide reductase
RT-PCR = reverse transcriptase PCR
Mtn = metallothionein
hFIX antigen = hFIX::Ag
ELISA = enzyme-linked immunosorbent assay
ER = endoplasmic reticulum
γC = γ-carboxylase
CMV = Cytomegalovirus
HEPES = 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
PACE = Paired basic Amino acid Cleaving Enzyme
ANOVA = analysis of variance

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