Successful synthesis of active human coagulation factor VII by co-expression of mammalian gamma-glutamyl carboxylase and modification of vit.K cycle in *Drosophila* Schneider S2 cells

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Abstract Mammalian gamma-glutamyl carboxylase and reduced vitamin K are indispensable for synthesis of mature mammalian vitamin K dependent proteins including some of blood coagulation factors (factors II, VII, IX, and X). It was well known that *Drosophila melanogaster* expressed gamma-glutamyl carboxylase and possessed a vit.K cycle although native substrates for them have not been identified yet. Despite the potential capability of gamma carboxylation in *D. melanogaster* derived cells such as S2 cells, *Drosophila* gamma-glutamyl carboxylase failed to gamma carboxylate a peptide fused to the human coagulation factor IX propeptide. Thus, it had been believed that the *Drosophila* system was not adequate to synthesize mammalian vit.K dependent proteins. Indeed, we previously attempted to synthesize biologically active factor VII in S2 cells although we were not able to obtain it. However, recently, a successful transient expression of biologically active human factor IX from S2 cells was reported. In the present study, several expression vectors which enable expressing mammalian *GGCX*, *VKORC1*, and/or *PDIA2* along with *F7* were developed. S2 cells transfected with pMKA85, pMAK86, and pMAK219

successfully synthesized active FVII. Thus, mammalian *GGCX* was indispensable to synthesize active FVII while mammalian *VKORC1* and *PDIA2* were not critical but supportive factors for S2 cells.

Keywords Vitamin K · S2 · *Drosophila* · Coagulation factor

Introduction

Mammalian gamma-glutamyl carboxylase (GGCX, encoded by *GGCX*) is indispensable for the synthesis of mature mammalian vitamin (vit.)K-dependent proteins; e.g., coagulation factors II, VII, IX, and X (FII, FVII, FIX, and FX, respectively) and anti coagulation factors such as protein (P)C and protein (P)S (Suttie 1980). These proteins have a so-called Gra domain at the N terminus; gamma-glutamyl carboxylation of glutamic acids in the Gra domain is necessary for protein maturation (Stenflo and Suttie 1977), a reaction that is catalyzed by GGCX with reduced vit.K acting as a cofactor. Reduced vit.K is produced by the vit.K cycle, in which vit.K 2,3-epoxide reductase (VKOR) plays a major role. Although the enzyme itself has not yet been homogeneously purified, the gene has been identified as vitamin K epoxide reductase complex, subunit 1 (*VKORC1*). *GGCX* and/or *VKORC1* deficiency result in the synthesis of proteins induced by vit.K absence or antagonist, which do not bind negatively charged phospholipids with

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calcium ions and are thus unable to function as mature proteins.

Recombinant activated FVII (rFVIIa) has been approved for the treatment of hemophilia and congenital FVII deficiency, and can also restore coagulopathy in traumatic or postoperative bleeding and postpartum hemorrhage (Dutta and Verma 2014). Several mammalian cell lines including HEK293 human embryonic kidney, Chinese hamster ovary, and baby hamster kidney cells have been used to synthesize recombinant FVII and other vit.K-dependent proteins (Bohm et al. 2015); however, as the yield is low, a more efficient large-scale culture system is needed.

The *Drosophila melanogaster* Schneider (S)2 cell line was established in 1972 (Schneider 1972). These cells grow rapidly at room temperature, without a need for CO₂. Many studies have demonstrated that recombinant proteins can be highly expressed in S2 cells (Bernard et al. 1994; Hill et al. 2001; Lehr et al. 2000; Li et al. 1996; Nilsen and Castellino 1999; Park et al. 2001). Our group has successfully expressed several human and murine proteins related to coagulation and fibrinolysis in S2 cells using the pMT-PURO2G expression vector, including plasminogen, urokinase-type plasminogen activator, FXII, high-molecular weight kininogen, and prekallikrein along with many variants of these proteins (Iwaki and Castellino 2008). We then subcloned the coding sequences of several human and murine vit.K-dependent proteins into the pMT-PURO2G vector and analyzed their expression. Although the proteins were secreted, they had no demonstrable activity (data not shown).

D. melanogaster expresses GGCX (Li et al. 2000; Walker et al. 2001) and has a vit.K cycle (Robertson 2004), although the endogenous substrates have yet to be identified. However, *Drosophila* GGCX failed to gamma carboxylate a peptide fused to human FIX propeptide. It was therefore concluded that the *Drosophila* system is unable to synthesize mammalian vit.K-dependent proteins.

A recent study reported the transient expression of biologically active human FIX in S2 cells; full-length human FIX cDNA (F9) was subcloned into the pMT-V5 HisA vector and protein expression was driven by metallothionein promoter (P_{MT}), while secretion was initiated by the F9 native signal peptide. Since these observations contradicted our unpublished data, in the

Fig. 1 Map of **a** pCoPURO, **b** pCoPURO2G, **c** pCoGGCX, **d** pCoVKORC1, **e** pCoPDIA2, **f** pCoPGE, **g** pCoPGKE, and **h** pCoVKE. PMT; Metallothionein promoter, PCOPIA; Copia promoter pA; SV40 late polyadenylation signal, AMP; ampicillin resistance gene, pUCori; pUC origin, PURO; puromycin N-acetyl-transferase (*pac*), PURO2G; enhanced green fluorescent protein fused to *pac* gene. Small red and blue arrows indicate primers for inverse PCRs (**a–b**) and primers for excision of an expression cassette for CoGGCX (**c**), CoVKORC1 (**d**), and CoPDIA2 (**e**). *AvrII*, *NotI*, and *AscI* are restriction enzyme sites (**f–h**)

present study we established a new expression system that enabled the generation of stable transformants in S2 cells with gamma carboxylation activity.

Materials and methods

Construction of pCoPGE, pCoPGKE, and pCoVKE

A co-expression vector (pCoPURO, Fig. 1a) (Iwaki et al. 2003) was used for an inverse polymerase chain reaction (PCR) using two primers: pMT-PURO2.tagF; 5'-GAGGCCACCGACTCTAGATCAAGC, and pMT-PURO2.KozakR; 5'-GGTGGCGGCGCAAGCTATCGAATTCCTGCAGCCCG (the Kozak sequence was underlined). The resulting amplicon was used as a backbone for pCoPURO2G, pCoGGCX, pCoVKORC1, and pCoPDIA2 described later. All PCRs in this study were carried out using PrimeSTAR[®] HS DNA Polymerase (TAKARA BIO, Shiga, Japan) according to manufacturer's instruction.

A coding sequence of a fusion protein of puromycin resistance marker (*pac*) and enhanced green fluorescent protein (EGFP) was taken from pMT-PURO2G (Iwaki and Castellino 2008) by a PCR using two primers: PURO.F; 5'-ATGACCGAGTACAAGCCACGGTG and 5'-EGFP.R; TTAATTGTACAGCTCGTCCATGC. The amplicon was phosphorylated by T4 polynucleotide kinase (NEB Japan, Tokyo, Japan), and then was ligated to the backbone using Quick Ligation Kit (NEB Japan). The resulting plasmid was named pCoPURO2G (Fig. 1b).

A coding sequence of murine gamma-glutamyl carboxylase (*Ggcx*) was taken from a FANTOM clone (ID#6820418L05, DANFORM, Yokohama, Japan) by a PCR using two primers: Ggcx.F; 5'-ATGGCTGTG CACCGCGGCTCCGC and Ggcx.R; 5'-TCAGAACT

CAGAGTGAACATGCTCAGAATCTGG. A coding sequence of murine vitamin K epoxide reductase complex, subunit 1 (*Vkorc1*) was taken from a FANTOM clone (ID#1110001K05, DANAFORM) by a PCR using two primers: *Vkorc1*.F; 5'-ATGGG CACCACCTGGAGGAGCCC and *Vkorc1*.R; 5'-TCAGTGCTTTTTGGTCTTGTGTTCTGGTACCTT CTG. A coding sequence of murine protein disulfide isomerase family A, member 2 (*Pdia2*) was taken from a FANTOM clone (ID#1810041F13, DANAFORM) by a PCR using two primers: *Pdia2*.F; 5'-ATGGA CAAGCAGCTTCTGCCAGTGTGCTGC and *Pdia2*.R; 5'-CTACAGCTCCTCCTTGGGACCCAAGG. Each amplicon was phosphorylated and ligated to the backbone. The resulting plasmids were named pCoGGCX, pCoVKORC1, and pCoPDAI2 (Fig. 1c–e, respectively).

The new co-expression vector (pCoPURO2G, Fig. 1b) was used for an inverse PCR using two primers VKBinvF; 5'-AGCTGCGGCCGCAAGGCGCGCC CCACAGAATCAGGGGATAACGCAGGAAAGAA C (The underlined parts; *NotI* recognition site and *AscI* recognition site, respectively) and VKB/MinvR; 5'-CCTTGCGGCCGCGAGCTTGTACATTCCTAGGCC GTATTACCGCCTTTGAGTGAGCTGATACC (The underlined parts; *NotI* recognition site and *AvrII* recognition site, respectively) to introduce new restriction enzyme recognition sites. The resulting amplicon was digested by *NotI* (NEB Japan) and *AvrII* (NEB Japan), and then the fragment was used to generate pCoPGE described in the next step.

An expression unit for murine *Ggcx* driven by Copia promoter (P_{COPIA}) and poly A signal (pA) was taken from pCoGGCX by a PCR using two primers: VKMinvF; 5'-GCGCACTAGTTTTCCCGAAAAG TGCCACCTGACGTC (The underlined part; *SpeI* recognition site) and VKB/MinvR. This amplicon was digested by *SpeI* (NEB Japan) and *NotI*, and then ligated to the previous fragment. The resulting plasmid was pCoPGE (Fig. 1f). An expression unit for murine *Vkorc1* driven by Copia promoter (P_{COPIA}) and poly A signal (pA) was taken from pCoGGCX by a PCR using two primers: VKMinvF and VKB/MinvR. This amplicon was digested by *SpeI* and *NotI*, and then ligated to pCoPGE digested by *NotI* and *AvrII*. The resulting plasmid was pCoPGKE (Fig. 1g). An expression unit for murine *Pdia2* driven by Copia promoter (P_{COPIA}) and poly A signal (pA) was taken from pCoGGCX by a PCR using two primers: VKMinvF and VKB/

Fig. 2 Map of **a** pMT-PURO2G, **b** pMAK80, **c** pMAK85, **d** pMAK86, **e** pMAK219, and **f** pMAK132. PMT; Metallothionein promoter, PCOPIA; Copia promoter pA; SV40 late polyadenylation signal, AMP; ampicillin resistance gene, pUCori; pUC origin, BiP; secretion signal, MCS; multiple cloning site

MinvR. This amplicon was digested by *SpeI* and *NotI*, and then ligated to pCoPGKE digested by *NotI* and *AvrII*. The resulting plasmid was pCoVKE (Fig. 1h).

Construction of pMAK80, pMAK85, pMAK86, pMAK132, and pMAK219

cDNA encoding human *F7*, which is the gene for human coagulation factor VII (FVII) was taken from a cDNA clone (MGC:163340, IMAGE:40146499) by PCR using the following primers: MAK80F; 5'-CTCGCTCGGGA GATCTGCAGTCTTCGTAACCCAGGAGGAAGCC (The underlined part; *BglIII* recognition site), and MAK 80R; 5'-CGAAGGGCCCTCTAGACTAGGGAAATG GGGCTCGCAGG (The underlined part; *XbaI* recognition site). This fragment was subcloned to pMT-PURO2G (Fig. 2a) (Iwaki and Castellino 2008) digested by *BglIII* (NEB Japan) and *XbaI* (NEB Japan) using In-Fusion® HD Cloning Kit (TAKARA BIO) according to manufacturer's instruction (pMAK80, Fig. 2b).

An expression unit for human *F7* driven by P_{MT} and poly A signal (pA) was taken from MAK80 by a PCR using two primers: MAK80F-LIC; 5'-GGTAATACG GCCTAGGCTGCAAGGCGATTAAGTTGGGTAAC GCCAG (The underlined part; *AvrII* recognition site) and MAK80R-LIC; 5'-GCGCGCCTTGCGGCCCGCC GCAGCGAGTCAGTGAGCGAGGAAG (The underlined part; *NotI* recognition site). The amplicon was subcloned to pCoPGE digested by *AvrII* and *NotI*, pCoVKE digested by *AvrII* and *NotI*, and pCoPGKE digested by *AvrII* and *NotI*, and then pMAK85 (Fig. 2c), pMAK86 (Fig. 2d), and pMAK219 (Fig. 2e) were generated, respectively. In order to replace BiP secretion signal in pMAK86 to native *F7* signal, a fragment containing *F7* signal sequence was taken from the cDNA clone (MGC:163340, IMAGE:40146499) by PCR using the following primers: MAK132F; 5'-ATGGTCTCC CAGGCCCTCAGGC (The underlined part; the initial codon), and MAK132R; 5'-GGCACCGACAGGAGCG CTTGG (The underlined part; *AfeI* recognition site). For an overlapped and extension PCR, an additional fragment was taken from pMAK80 by a PCR using the following primers: MAK80F-LIC and MAK132OLR;

5'-GGCCTGGGAGACCATATTGAGATCGGATCC CCCCTTTAG. These amplicons were subcloned to pMAK86 digested by *AvrII* and *AfeI* (NEB Japan) using In-Fusion® HD Cloning Kit (TAKARA BIO) (pMAK132, Fig. 2f).

Transfection of pMAK80, pMAK85, pMAK86, pMAK132, and pMAK219 to S2 cells

Non-transfected S2 cells (RIKEN BioResource Center, Tsukuba, Japan) were maintained at 27 °C in ExpressFive SFM (Life Technologies Japan, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS, Life Technologies Japan) and 1 × antibiotic antimycotic solution (Sigma-Aldrich Japan, Tokyo, Japan) (hereafter the medium were referred as FBS-supplemented medium). S2 cells (2×10^5) were seeded to each well of a 24-well plate containing 1 mL of FBS-supplemented medium, and the cells were grown for 16 h at 27 °C. Lipofectin was employed to produce stable transformants using HillyMax transfection reagent kit (Dojindo, Kumamoto, Japan). Briefly, 1 µg pMAK80, pMAK85, pMAK86, pMAK132, or pMAK219 was mixed with 30 µL ExpressFive SFM, and then 5 µL HillyMAX reagent was mixed. The mixture was incubated at RT for 15 min, and then added to each well. After 4 h, the medium was changed, and the cells were incubated for 48 h.

Puromycin selection

When the expanded cells in a 25 cm² flask were at 80% confluency, the medium was replaced with 5 mL FBS-supplemented medium containing 2.0 µg/mL puromycin, and the cells were continuously cultured for 72 h. These procedures were repeated twice.

Flow cytometric analysis

Flow cytometric analysis was performed to detect the fluorescent signals generated by enhanced green fluorescent protein (EGFP) in stably transformed cells. This analysis was performed using a FACS Aria cytometer (BD, Franklin Lakes, NJ, USA).

Conversion from FBS-supplemented medium to FBS-free medium

After selection, the cells were subcultured in ExpressFive SFM supplemented with 7.5% FBS and

1 × antibiotic antimycotic solution. The reduction of FBS was sequentially performed at each passage. After 5–6 passage, the medium was totally free of FBS with 1 × antibiotic antimycotic solution (hereafter the medium was referred as FBS-free medium).

Western blot for recombinant human FVII

After establishing stable transformants with FBS-free medium, the cells (1×10^7) were seeded onto a 6-well plate containing 3 mL FBS-free medium. In order to induce the expression of recombinant FVII, 3 µL of 500 mM CuSO₄ with or without 3.0 µL of 10 mg/mL phytonadione (vit.K; Vitamin K1 injection, Isei-Pharma, Yamagata, Japan) was added to each well, and the cells were incubated for 48 h at 27 °C. The culture supernatants (5 µL) were electrophoresed on sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE). The gels were used to transfer the recombinant proteins to a PVDF membrane (Immobilized Western, Nihon Millipore, Tokyo, Japan) for western blots. The membranes were exposed to a rabbit anti-human FVII polyclonal antibody (Cat.# ab97614; Abcam, Tokyo, Japan) and a goat anti-rabbit IgG-HRP conjugate (Cell Signaling Technology Japan, Tokyo, Japan), and visualized with a SuperSignal West Pico kit (Thermo Fisher Scientific, Yokohama, Japan) according to manufacturer's instructions.

Quantification of recombinant human FVII

The quantity of recombinant human FVII in the culture supernatant ($N = 4$) was measured by Enzyme-Linked Immuno Sorbent Assay (ELISA) using F7 (Human) ELISA kit (Abnova, Taipei City, Taiwan). Briefly, the culture supernatant (from 0.5 to 2.0 µL) was applied to a well of 96-well plate coated with polyclonal anti human FVII antibody, and then incubated for 2 h at RT. After washing, biotinylated polyclonal anti human FVII antibody was added to each well, and the mixture was incubated for 1 h at RT. After washing, streptavidin-peroxidase conjugate was added to each well, and then chromogenic substrate was added to determine the concentration of FVII.

Activity assay for recombinant human FVII

The FVII activity in all culture supernatant was evaluated by a clotting-time based method. In the assays, FVII deficient plasma and the supernatant was

mixed and the clotting time was measured in a clinical laboratory center (SRL, Tokyo, Japan). The procoagulant was observed in the supernatant from S2/85, S2/86, or S2/219 cultured with vit.K in the medium. Then, the recombinant human FVII activity in the culture supernatant ($N = 4$) was measured by F7 (Human) Chromogenic Activity kit (Abnova). The assay measured the ability of lipoprotein tissue factor (TF)/FVIIa complex to activate factor X (FX) to FXa. The amidolytic activity of the TF/FVIIa complex was quantified by the amount of FXa produced using a highly specific FXa substrate releasing a para-nitroaniline (pNA) chromophore. The culture supernatant (0.5 to 2.0 μ L) was applied to a well of 96-well plate coated by monoclonal antibody specific for human FVII. After washing, recombinant human TF and FX was added to each well, and then a highly specific substrate for FXa was added. The FVII activity was measured via the change in absorbance of a yellow para-nitroaniline (pNA) chromophore released from the FXa substrate.

Statistical analyses

Data are expressed as mean $\pm$ SD. FVII antigen levels with and without vit.K were compared by two-way factorial analysis of variance (ANOVA). FVII antigen and activity levels with vit.K were evaluated by one-way factorial ANOVA followed by Tukey's post hoc test. Analyses were performed using EZR (Kanda 2013), and the null hypothesis was voided at $P < 0.05$.

Results

Construction of pCoPGE, pCoPGKE, and pCoVKE

We were able to successfully assemble pCoPGE, pCoPGKE, and pCoVKE (Fig. 1f–h, respectively). All of the vital parts, such as the coding sequences for promoter (P_{COPIA}), poly A signal (pA), and each gene (PURO2G, *Ggcx*, *Vkorc1*, *Pdia2*) were verified by DNA sequencing (data not shown).

Construction of pMAK80, pMAK85, pMAK86, pMAK132, and pMAK219

pMAK80 was generated by insertion of cDNA encoding human *F7* without native signal sequence (hF7 δ SIg) into

*Bgl*III and *Xba*I sites in the multiple cloning sites (MCS) of pMT-PURO2G (Fig. 2b). The signal sequence in pMAK80 was BiP, which enables to secrete the recombinant protein from S2 cells efficiently. The expression units were successfully transferred into pCoPGE (pMAK85, Fig. 2c), pCoVKE (pMAK86, Fig. 2d), and pCoPGKE (pMAK219, Fig. 2e). The replacement of BiP signal sequence to *F7* native signal sequence was successfully performed (pMAK132, Fig. 2f).

Establishment of stable transformants by puromycin selection

The positive cells were around 2–5% at the initial transfection. The S2 cells transformed with pMAK80, pMAK85, pMAK86, pMAK132, or pMAK219 were selected by puromycin. After selection, almost 100% of these cells were expressing EGFP (Fig. 3). The viability and growing rates of these transformed cells were similar to those of non-transfected S2 cells, which was similar to previous reports (Iwaki et al. 2003). Hereafter, these cells were referred as S2/80, S2/85, S2/86, S2/132, and S2/219.

Western blots for recombinant human FVII

After induction of the stable transformants by addition of CuSO_4 with or without vit.K, the recombinant FVII in the culture supernatant was detected by western blots. The

Fig. 3 Fluorescent signals from the stably transformed cells. The graph shows fluorescent intensity of S2/86. Non-transfected cells (black line) and the cells after puromycin selection (red line). (Color figure online)

Fig. 4 Western blotting of FVII antigen in S2/80, S2/85, and S2/86 (a), S2/86 and S2/219 (b), and S2/86 and S2/132 (c)

expression of recombinant FVII in the culture supernatant with or without vit.K from S2/80, S2/85, S2/86, and S2/219 was clearly detected (Fig. 4a, b). On the other hand, no signals were detected in the culture supernatant from S2/132 (Fig. 4c).

Antigen and activity assay for recombinant human FVII

There were no significant differences in FVII antigen levels by two-way factorial ANOVA when vit.K+/- and type of vector were used as independent variables. However, a significant difference was detected by one-way factorial ANOVA when vector type was an independent variable. The Tukey post hoc analysis revealed that FVII antigen levels were lower in S2/132 than in S2/80, S2/85, S2/86, and S2/219 (Fig. 5a). Thus, vit.K supplementation did not affect the synthesis of FVII antigen in S2 cells; however, the native FVII signal peptide compromised FVII secretion from these cells. FVII activity was detected in the culture supernatant of S2/85, S2/86, and S2/219 with vit.K but not in that of cells cultured without vit.K; a significant difference was detected by one-way factorial ANOVA when vector type was an independent variable, and the Tukey post hoc analysis revealed that FVII activity level was higher in S2/86 than in S2/219, which was in turn higher than that in S2/85 (Fig. 5b).

Discussion

We successfully expressed several human and murine proteins related to coagulation and fibrinolysis using

the pMT-PURO2G vector (Iwaki and Castellino 2008). Gene expression was induced by addition of CuSO_4 to the culture medium, and secretion of recombinant proteins was controlled by means of the BiP signal peptide.

In a recent study, biologically active human FIX was expressed in S2 cells by transient transfection of the pMT-hFIX expression vector without addition of vit.K. Moreover, co-expression of human *GGCX* further enhanced human FIX expression (Vatandoost et al. 2012). However, some caveats of their study were the presence of FBS in the culture supernatant, transient expression, and absence of vit.K in the medium. To address these issues, we developed a new expression system that enabled the generation of stable transformants without FBS.

S2/80 generated and secreted recombinant human FVII with or without vit.K; however, no activity was detected under either condition, indicating that *Drosophila Ggcx* and *Vkor* do not contribute to the gamma carboxylation of recombinant human FVII. Additionally, S2/85 and S2/86 generated and secreted recombinant human FVII with or without vit.K; however, the absence of vit.K was critical for the synthesis of active recombinant human FVII in S2 cells. On the other hand, S2/85, S2/86, and S2/219 produced active recombinant human FVII in the presence of vit.K. These results indicate that co-expression of mammalian *Ggcx* and vit.K supplementation are indispensable for the synthesis of active FVII in S2 cells. *Drosophila Vkor* in S2 cells was functional, but co-expression of mammalian *Vkorc1* and *Pdia2* synergistically enhanced FVII activity, while the native signal peptide inhibited FVII

Fig. 5 **a** FVII antigen levels in S2, S2/80, S2/85, S2/86, S2/132, and S2/219. **b** FVII activity levels in S2, S2/80, S2/85, S2/86, S2/132, and S2/219

secretion in S2 cells (summarized in Fig. 6). On the other hand, these were unnecessary for the synthesis of biologically active recombinant human FIX (Vatandoost et al. 2012).

Secretion of recombinant proteins is an important aspect of expression systems. Addition of BiP instead of native signal peptide enhanced secretion efficiency in S2 cells (Kirkpatrick et al. 1995). On the contrary, it was reported that native signal peptide of tissue plasminogen activator—which was functional in S2 cells—was highly effective (Ivey-Hoyle et al. 1991a, b) whereas BiP reduced secretion of rabies virus glycoprotein (Dos Santos et al. 2009). Protein disulfide isomerase (PDI) is a thioredoxin-like oxidoreductase and chaperone that is present at high concentrations in the endoplasmic reticulum (Wajih

et al. 2008) and provides electrons for reduction of the CXXC center in VKORC1 to enable the reduction of vit.K epoxide to vit.K hydroquinone by VKOR (Garcia and Reitsma 2008). In this study, mammalian VKORC1 enhanced active FVII synthesis; thus, exogenous PDIA2 facilitated vit.K cycling in S2 cells. The signal peptide and Gra region of FVII differed from those of FIX; such differences may complicate gamma carboxylation and secretion. To evaluate the functional significance of these differences, we are currently carrying out experiments in which other vit.K-dependent proteins are expressed from the pCoVKE vector.

Precise determination of the number of Glu residues that are changed to Gra residues in the Gra domain is important. We performed barium citrate

Fig. 6 Schema of recombinant FVII produced in S2/80, S2/85, S2/86, S2/132, and S2/219 with various parameters

precipitation of recombinant FVII in preliminary experiments and confirmed the expression of FVII by western blotting; however, these experiments revealed only some Glu were converted to Gra residues. We have cloned the cDNAs for all vit.K-dependent proteins such that a hexa-histidine tag and C3 protease recognition site flank the Gra domain. We intend to analyze the expressed proteins by matrix-assisted laser desorption ionization–time-of-flight spectroscopy to determine the levels of gamma-carboxylation.

Bioactive recombinant FVII was expressed in HEK293 cells—which endogenously express human *GGCX*—by co-transfecting rat *Vkorc1* (Wajih et al. 2008); it was also expressed in Sf9 cells (another insect cell line) by co-transfecting human *GGCX* (Masroori et al. 2010). Mammalian cells are used to express bioactive vit.K-dependent proteins as therapeutic compounds, including recombinant FVII (Novo Nordisk, Princeton, NJ, USA), recombinant FIX (CSL Behring, King of Prussia, PA, USA), and recombinant activated protein C (Eli Lilly and Company, Indianapolis, IN, USA). These cells have a vit.K cycle and are capable of gamma carboxylation. However, given that the yield of recombinants proteins is relatively low, cost is a major concern when using mammalian cell-based expression systems. Sf9 cells are useful for

expressing various types of recombinant protein, although they require baculovirus infection and die during protein generation, precluding continuous production. In contrast, S2 cells can be maintained as stable transformants once properly selected; this is an important consideration in large-scale culture systems, for instance when producing large amounts of recombinant protein using bioreactors.

The new recombinant mammalian vit.K-dependent protein expression system described herein yielded reliable and reproducible results. FVII is an essential factor for the coagulation cascade; our system can be easily expanded for mass production so that recombinant FVII can be produced in large quantities for clinical applications, for instance disseminated intravascular coagulation in trauma control units. The pMAK80, pMAK85, pMAK86, pAMK132, and pMAK219 vectors will be made available from the Riken BioResource Center (<http://www.brc.riken.jp/lab/dna/>).

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