Enhanced γ-Carboxylation of Recombinant Factor X Using a Chimeric Construct Containing the Prothrombin Propeptide†

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Received May 11, 2000; Revised Manuscript Received August 24, 2000

ABSTRACT: Factor Xa is the serine protease component of prothrombinase, the enzymatic complex responsible for thrombin generation. Production of recombinant factor X/Xa has proven to be difficult because of inefficient γ-carboxylation, a critical post-translational modification. The affinities of the vitamin K-dependent propeptides for the γ-carboxylase vary over 2 logs, with the propeptide of factor X having the highest affinity followed by the propeptides of factor VII, protein S, factor IX, protein C, and prothrombin [Stanley, T. B. (1999) *J. Biol. Chem. 274,* 16940−16944]. On the basis of this observation, it was hypothesized that exchanging the propeptide of factor X with one that binds the γ-carboxylase with a reduced affinity would enhance γ-carboxylation by allowing greater substrate turnover. A chimeric cDNA consisting of the human prothrombin signal sequence and propeptide followed by mature human factor X was generated and stably transfected into HEK 293 cells, and modified factor X was purified from conditioned medium. The results indicate that on average 85% of the total factor X produced with the prothrombin propeptide was fully γ-carboxylated, representing a substantial improvement over a system that employs the native factor X propeptide, with which on average only 32% of the protein is fully γ-carboxylated. These results indicate that the affinity of the γ-carboxylase for the propeptide greatly influences the extent of γ-carboxylation. It was also observed that regardless of which propeptide sequence is directing γ-carboxylation (factor X or prothrombin), two pools of factor X are secreted; one is uncarboxylated and a second is fully γ-carboxylated, supporting the notion that the γ-carboxylase is a processive enzyme.

Human factor X (FX),[1] a vitamin K-dependent two-chain glycoprotein, is a substrate for both the extrinsic (tissue factor/FVIIa) and intrinsic (FVIIIa/FIXa) tenase complexes, thus linking these two pathways (*1*). The activated form of FX (FXa) is the serine protease component of the enzymatic complex termed prothrombinase, the only known physiological activator of prothrombin. Prothrombinase assembles through reversible interactions between FXa and the cofactor FVa on an appropriate membrane (i.e., platelet) surface in the presence of Ca^{2+} ions (*2*). Although FXa alone catalyzes prothrombin activation, the macromolecular interactions which stabilize prothrombinase lead to a substantial enhancement in catalytic efficiency (*3*), indicating that assembly of this complex is an important step for rapid and localized thrombin generation. Because FX/FXa occupies a central position in the coagulation pathway, there is considerable interest in its therapeutic modulation (*4*), highlighting the need to better understand structural determinants of function on FX/FXa.

Extensive progress has been made in delineating the structural determinants important for function on thrombin, FIXa, FVIIa, and APC, but less is known about FXa. One cause for this is the limited number of naturally occurring FXa mutations available for study (*5*). Another reason is the relative difficulty (compared to other vitamin K-dependent proteins) in producing functional recombinant FXa (rFXa). As with all vitamin K-dependent proteins, the biosynthesis of FX is complex, involving several co- and post-translational modifications (*6*), including removal of the signal sequence, formation of disulfide bonds, γ-carboxylation of 11 glutamic acid residues, modification of Asp^{63} to β-hydroxyaspartic acid, addition of N- and O-linked oligosaccharides, removal of an internal tripeptide, and removal of the propeptide.

Studies from our and other laboratories indicate that rFX produced in mammalian expression systems is heterogeneous

† This work was supported by NIH Grants NRSA HL07439 (to R.M.C), HL03240 (to P.J.L.), HL48322 (to K.A.H), and HL06350 (to D.W.S) and a grant from the National Blood Foundation (to P.J.L).

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[1] Abbreviations: FX, factor X; FXa, activated factor X; FVa, activated factor V; FVIIa, activated factor VII; FVIIIa, activated factor VIII; FIXa, activated factor IX; APC, activated protein C; rFXa, recombinant FXa; CHO, Chinese hamster ovary; HEK, human embryonic kidney; PACE, paired basic amino acid cleaving enzyme; Gla, γ-carboxyglutamic acid; RVV-X, FX activator from Russell's viper venom; pre-pro sequence, an amino acid sequence that contains the signal sequence and propeptide.

10.1021/bi001074q CCC: $19.00
Published on Web 10/25/2000

	-18 -16	-10	-6	-1	K_i (nM)
Factor X	S L F I	R R E Q A N N I	L A R V T	R	2.6
Factor VII	R V F V	T E E E A H G V	L H R R R	R	11.1
Protein S	A N F L	S K Q Q A S Q V	L V R K R	R	12.2
Factor IX	R V F L	D H E N A L K I	L N R P K	R	33.6
Protein C	S V F S	S S E R A H Q V	L R I R K	R	230
Prothrombin	H V F L	A P Q Q A R S L	L Q R V R	R	277

FIGURE 1: Sequence alignment of the vitamin K-dependent coagulation factor propeptides. The 18 amino acid propeptide sequence of the vitamin K-dependent coagulation factors constitutes the primary binding site for the carboxylase. The K_i values of propeptide/gla domain peptides (determined by inhibition of a FIX propeptide/gla domain substrate) are a relative measure of the affinity of the propeptide for the carboxylase (*19*).

with respect to removal of the internal tripeptide, propeptide cleavage, and γ-carboxylation. The efficiency of these required post-translational modifications appears to be less in CHO and COS-1 cells compared to HEK 293 cells (*7*−*11*). Studies indicate that some of the inefficiencies in post-translational modification can be overcome by modifying the FX propeptide at position −2 (Thr→Arg; henceforth referred to as native rwtFX) and by expressing rFX in HEK 293 cells that have been cotransfected with PACE/furin. However, inefficient γ-carboxylation remains a major problem (*9*, *11*). Although separation of uncarboxylated and fully γ-carboxylated rFX can be readily accomplished, the relatively low yield of fully γ-carboxylated material means that most of the secreted protein is of little use for functional studies. The heterogeneity in γ-carboxylation can be overcome completely by expressing Gla-domainless rFX (*12*); however, this modification of the full-length molecule precludes the study of the assembly of macromolecular complexes involving FX/FXa because these uniformly require binding to a membrane surface. Thus, an ideal expression system would direct high-level protein production (>2−5 μg of rFX/10^6 cells/24 h) while still allowing efficient execution of post-translational modifications essential to FX/FXa function.

The enzyme responsible for modification of glutamic acid residues to γ-carboxyglutamic acid in the amino-terminal portion of a number of blood coagulation proteins is the vitamin K-dependent γ-glutamyl carboxylase (*13*). The mechanism by which the γ-carboxylase recognizes its substrate is thought to be through initial binding to an 18 amino acid propeptide sequence on the vitamin K-dependent protein (for review see ref *14*). The importance of the propeptide sequence for γ-carboxylation is demonstrated by studies which show that disruption of this site in FIX, protein C, or prothrombin yields a mature protein either lacking or deficient in γ-carboxylation (*14*−*16*). This observation and analysis of naturally occurring mutations in this region support the conclusion that the propeptide is required for γ-carboxylation (*17*, *18*).

Although the vitamin K-dependent propeptides share sequence homology, their relative affinities for the γ-carboxylase vary considerably, with the propeptide of FX binding with the highest affinity followed by the propeptides of FVII, protein S, FIX, protein C, and prothrombin [Figure 1 (*19*)]. Recent studies indicate that specific amino acids within these propeptide sequences are responsible for differences in affinity for the γ-carboxylase (*18*). In the case of the protein C and prothrombin propeptides, a single amino acid change within this region substantially increases their affinity for the γ-carboxylase (*18*).

In an effort to optimize the expression of fully γ-carboxylated rFX and to better understand the significance of the differential binding affinities of propeptide sequences for the γ-carboxylase, a chimeric FX cDNA harboring the prothrombin propeptide was generated and stably transfected into HEK 293 cells. Expressed rFX was purified and analyzed for γ-carboxyglutamic acid content. Our data indicate that expression of rFX using the prothrombin propeptide results in much higher yields of fully γ-carboxylated material and is thus superior to FX constructs bearing the sequence for the native FX propeptide expressed in heterologous systems. In addition, we provide further evidence that the γ-carboxylase is a processive enzyme: regardless of which propeptide sequence is directing γ-carboxylation (FX or prothrombin), two pools of mature rFX are secreted, one that is completely *un*carboxylated and one that is *fully* γ-carboxylated. Results from this paper will facilitate the large-scale production of functional rFX/FXa for detailed structure/function studies and may also be adapted for the production of other vitamin K-dependent proteins.

MATERIALS AND METHODS

Materials. All restriction enzymes were obtained from New England Biolabs, Beverly, MA. *Pfu* DNA polymerase was obtained from Stratagene, La Jolla, CA. HEK 293 cells were obtained from ATCC, Rockville, MD. Lipofectamine, G418 (Geneticin), penicillin−streptomycin, trypsin−EDTA, L-glutamine, and DMEM F-12 were obtained from GIBCO-BRL, Gaithersburg, MD. Hydroxyapatite Bio-Gel HT was obtained from Bio-Rad Laboratories, Hercules, CA. Q-Sepharose and benzamidine-Sepharose were obtained from Pharmacia Biotech, Uppsala, Sweden. Rabbit polyclonal FX and FX−horseradish peroxidase antibodies for ELISA were obtained from Dako, Carpinteria, CA. The prothrombin cDNA was a generous gift from Dr. Sriram Krishnaswamy, The Joseph Stokes Jr. Research Institute, Philadelphia, PA. The calcium-dependent monoclonal human FX antibody [MoAb, 4G3 (*20*)] was obtained from Dr. Harold James, University of Texas, Tyler, TX. The factor X activator from Russell's viper venom (RVV-X) was isolated as described (*21*). Plasma-derived human prothrombin, FIX, FX, and thrombin were obtained from Haematologic Technologies Inc., Essex Junction, VT.

Construction of Expression Vector. To exchange the signal sequence and propeptide of FX with that of prothrombin, the following specific oligonucleotide primers were constructed: *primer A*, 5′-GATCCAGATCTCCACCATGGCG-CACGTCCGA-3′, in which the underlined portion is a *Bgl*II restriction site and the last 15 bases correspond to prothrombin gene sequence coding for amino acid residues −43 to −39; *primer B*, 5′-AAGAAAGGAATTGGCTCGCCGGAC-CCGCTG-3′, in which the first 15 bases correspond to FX gene sequence coding for residues +5 to +1 and the last 15 bases correspond to prothrombin gene sequence coding for residues −1 to −5; *primer C*, 5′-GCCAATTCCTTTCT-TGAAGAGATG-3′, in which the 24 bases correspond to FX gene sequence coding for residues +1 to +8; and *primer D*, 5′-GAAACCCTCGTTTTCCTCATT-3′, in which the 21 bases correspond to FX gene sequence coding for residues +220 to +214. The prothrombin DNA sequence encoding the signal sequence and propeptide was ligated to the DNA

sequence of FX starting at position +1 by the technique of splicing by overlap extension or "geneSOEing", where primers B and C are the SOEing primers and primers A and D are the outside primers (*22*). The resulting 819 bp fragment was digested with *Bgl*II and *Sac*II, gel purified, and subcloned into pCMV4wt-FX, generating pCMV4-ss-pro-II-FX. To confirm the presence of the prothrombin signal sequence and propeptide and to ensure the absence of polymerase-induced errors, we carried out DNA sequencing of the entire chimeric prothrombin/FX insert.

Expression of rFX. HEK 293 cells were transfected with pCMV4-ss-pro-II-FX using Lipofectamine according to the manufacturer's instructions. Cotransfection with a plasmid containing the neomycin resistance gene and the PACE/furin gene (pcDNA3-PACE; pcDNA3 was obtained from Invitrogen and the PACE cDNA was a generous gift from Genetics Institute, Boston, MA) was performed at a 1:10 molar ratio (pcDNA3-PACE/pCMV4-ss-pro-II-FX). Transfectants were selected with the neomycin analogue G418, and resistant colonies were screened for FX production by sandwich ELISA as described (*11*). Expression levels of the clones varied but ranged from 0.5 to 4.0 μg of rFX/10^6 cells/24 h, which approximately corresponds to 0.5−4.0 μg/mL of rFX in a confluent T25 flask with 8 mL of medium. Selected clones were expanded into NUNC cell factories (1264 or 6320 cm^2; Nalge Nunc Int., Naperville, IL), and a total of 6−15 L of conditioned medium was collected over 14−21 days. Conditioned medium was filtered, and benzamidine was added to a final concentration of 10 mM prior to storage at −20 °C.

Purification of rFX. Recombinant FX was purified from conditioned media using a three-step chromatographic approach (Q-Sepharose, FX immunoaffinity, and hydroxyapatite chromatography) as described (*11*). The fully γ-carboxylated rFX eluting from the hydroxyapatite column was precipitated with ammonium sulfate, and the protein was stored at −20 °C in 50% glycerol/water. The concentration of rFX was determined by absorbance at 280 nm (M_r = 59000; $E^{1\%}_{280nm}$ = 11.6) (*23*).

Activation of FX to FXa and Purification on Benzamidine-Sepharose. Plasma-derived FX or rFX was activated using purified RVV-X (*24*) and subsequently purified using benzamidine-Sepharose as described (*25, 26*). The concentrations of plasma-derived FXa and rFXa were determined by absorbance at 280 nm (M_r = 46000; $E^{1\%}_{280}$ = 11.6) (*23*).

Characterization of rFX/Xa. Protein purity was assessed using NuPAGE 4−12% Bis-Tris gels (Novex, San Diego, CA) followed by staining with Coomassie Brilliant Blue R-250. γ-Carboxyglutamic acid analysis was carried out in our laboratory according to the modified method of Price (*27*) for alkaline hydrolysis, and separation of amino acids was accomplished using a DC-4A cation exchange column on a Waters LC-1 Plus HPLC as described by Przysiecki (*28*). Known amounts of L-γ-carboxyglutamic acid (250 pmol) and L-aspartic acid (500 pmol) were used as standards for peak areas as well as retention times. The Gla and Asp/Asn peak areas of the base-hydrolyzed plasma-derived and rFX samples were compared to the peak areas of the Gla and Asp standards; moles of Gla per mole of protein were calculated from these values. Amino-terminal sequence analysis of both the heavy and light chains of rFX was accomplished by transferring these fragments to PVDF membranes (*29*) followed by automatic Edman degradation on an Applied Biosystems 475A protein sequencing system in the laboratory of Dr. Alex Kurosky (University of Texas, Medical Branch at Galveston) (*30*).

RESULTS

Construction of a Prothrombin/FX Chimera. On the basis of the observations that (1) high-level expression of prothrombin (in CHO cells) and protein C (in HEK 293 cells) yields fully γ-carboxylated protein (*31*−*33*) and (2) the affinity of the FX propeptide for the γ-carboxylase is much higher than that of other vitamin K-dependent proteins (*19*), we set out to determine if we could enhance γ-carboxylation of rFX by exchanging the FX propeptide with one that binds the γ-carboxylase with a reduced affinity. We hypothesized that having a propeptide sequence which binds the γ-carboxylase with a very high affinity may reduce that rate of γ-carboxylation by decreasing substrate turnover. Because it has the most reduced affinity for the γ-carboxylase, we chose to exchange the propeptide of FX with that of prothrombin. The signal sequence and propeptide of prothrombin were substituted for the native sequence in the FX cDNA starting at position +1 (pCMV4-ss-pro-II-wtFX). It should be noted that the signal sequence of prothrombin was included simply to facilitate PCR and subcloning of the prothrombin propeptide; its substitution should not influence the extent of γ-carboxylation.

Expression, Purification, and Characterization of a Recombinant Prothrombin Propeptide/FX Chimeric Protein. The chimeric expression vector, pCMV4-ss-pro-II-wtFX, was used to transfect HEK 293 cells, and clones positive for rFX by ELISA were selected and subsequently expanded to establish cell lines. Two clones bearing the prothrombin pre-pro sequence, B5 and A1, as well as native rwtFX (clone D3; described previously in ref *11*)) are described in detail. Each clone expressed rFX at levels comparable to that seen with native rwtFX (B5, 4.0 μg/10^6 cells/24 h; A1, 2.3 μg/10^6 cells/24 h; D3, 4.0 μg/10^6 cells/24 h), indicating that the prothrombin pre-pro sequence did not alter expression of rFX in this cell system. Following expansion into cell factories and collection of conditioned media over 14−21 days, rFX was purified as described (*11*). Specifically, following Q-Sepharose chromatography for initial capture, rFX was purified by immunoaffinity chromatography using a monoclonal antibody [MoAb, 4G3 (*20*)] that binds rFX but does not discriminate between uncarboxylated and fully γ-carboxylated protein. To separate these two forms of rFX, phosphate elution from hydroxyapatite was employed. We have previously demonstrated that at low phosphate concentrations (~150 mM; peak 1) uncarboxylated rFX elutes, whereas at high phosphate concentrations (~275 mM; peak 2) fully γ-carboxylated material elutes (*11*). Thus, elution of rFX from hydroxyapatite not only provides a useful method for isolating fully γ-carboxylated protein but also permits quantitation of the fraction of total rFX that is fully γ-carboxylated for any given clone.

The detailed purification table for clone B5 (rFX with the prothrombin pre-pro sequence) is presented in Table 1. Similar results were obtained with clone A1. Chromatograms of the hydroxyapatite eluates for both of the prothrombin

Table 1: Purification of rwtFX Clone B5

purification step	vol (mL)	rFX (mg/mL)	total FX (mg)	% recovery
conditioned media[a]	9660	0.003	27.5	100
Q-Sepharose[a]	150	0.18	27.0	98.2
immunoaffinity (4G3)[b]	46.0	0.49	22.5	82.0
hydroxyapatite peak 1[b]	30.0	0.09	2.7	9.8
hydroxyapatite peak 2[b]	52.0	0.34	17.7	64.3
final rFX uncarboxylated[b,c]	0.6	3.25	2.0	7.1
final rFX carboxylated[b,c]	2.0	8.62	17.2	62.7

[a] As determined by FX specific ELISA. [b] As determined by absorbance at 280 nm. [c] Final purified material following ammonium sulfate precipitation.

propeptide/rFX chimeras, clones B5 and A1 (panels B and C of Figure 2, respectively), show that ~90% of the total rFX applied to the column eluted in peak 2. This indicates that the majority of the starting material is fully γ-carboxylated. This finding is in marked contrast to the results observed with native rwtFX (Figure 2A), with which only ~20% of the rFX is fully γ-carboxylated. We have now expressed and purified a total of seven rFX clones with the prothrombin pre-pro sequence and seven clones containing the native FX pre-pro sequence. Collectively, our results indicate that ~85% of the total rFX expressed with the prothrombin pre-pro sequence is fully γ-carboxylated, compared to ~32% of rFX that is expressed from constructs containing the native pre-pro sequence (Table 2). In addition, rFX expressed from clone B5 has been purified on three separate occasions, with similar levels of fully γ-carboxylated rFX (~90% of total rFX protein) obtained each time. These results demonstrate that in this system, expression of rFX from a cDNA bearing the sequence for a propeptide with a reduced affinity for the γ-carboxylase markedly improves γ-carboxylation when compared to constructs bearing the native FX propeptide sequence.

As shown in Table 2, the protein expression levels varied for each of the FX clones (+ FX propeptide and + prothrombin propeptide) and ranged from 0.5 to 4.0 μg of FX/10^6 cells/24 h. There was no apparent correlation between the expression levels of pre-pro-prothrombin-rFX and the percent of fully γ-carboxylated protein, indicating that expression of pre-pro-prothrombin rFX at levels up to 4.0 μg of rFX/10^6 cells/24 h in HEK 293 cells does not saturate the endogenous γ-carboxylation machinery. However, when the native FX propeptide is employed, the percentage of fully γ-carboxylated protein is substantially reduced even at expression levels comparable to that of rFX expressed with the prothrombin propeptide. These results indicate that the native FX propeptide is far less efficient in this expression system in directing the γ-carboxylation reaction and may in some way retard this reaction.

To determine the γ-carboxylation state of the recombinant proteins eluting from the hydroxyapatite column, direct chemical γ-carboxyglutamic acid analysis of the alkaline hydrolysate of both clones (B5 and A1) harboring the prothrombin pre-pro sequence was performed (Table 3). rFX eluting in peak 1 from the hydroxyapatite column is essentially uncarboxylated, and material eluting in peak 2 is fully γ-carboxylated. γ-Carboxyglutamic acid analysis of plasma-derived coagulation factors (used as controls) were consistent with known values. We have previously demonstrated that rFX synthesized with the native FX propeptide results in secreted protein which is either uncarboxylated or fully γ-carboxylated (*11*). Results presented in Tables 2 and 3 support this observation and also demonstrate that rFX synthesized with the prothrombin propeptide also yields

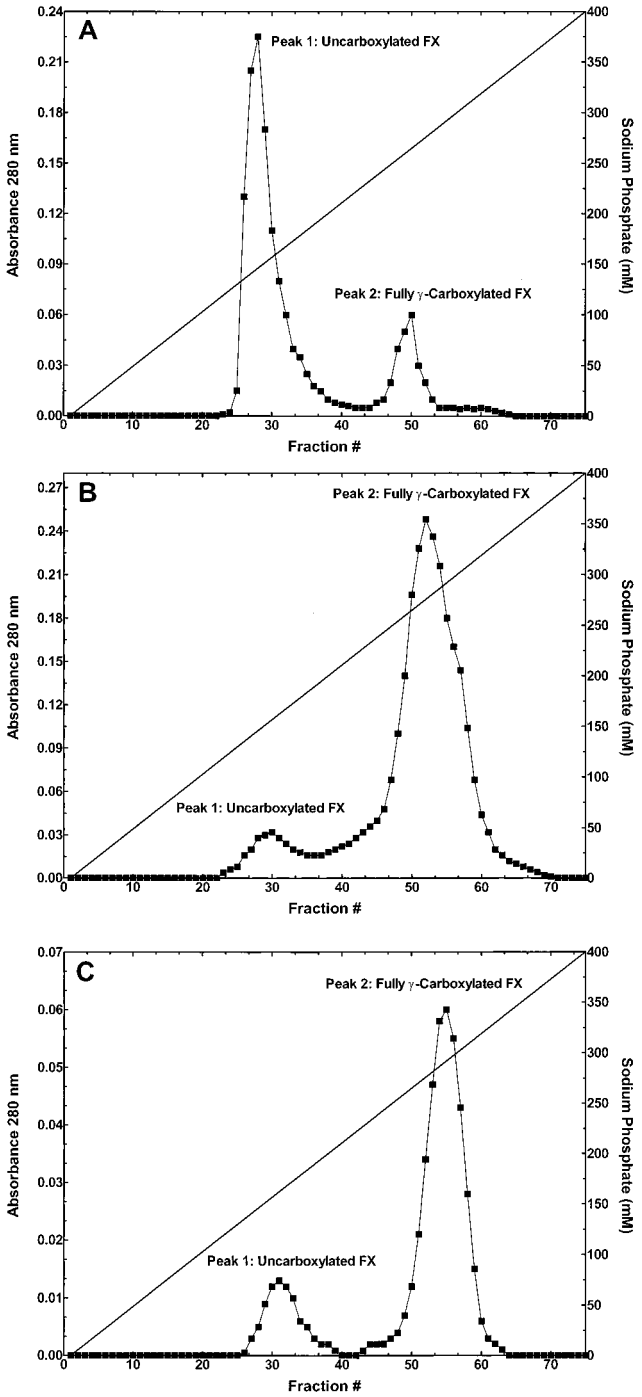

FIGURE 2: Separation of γ-carboxylated and uncarboxylated rFX by hydroxyapatite chromatography. Hydroxyapatite (HA) chromatography was used to separate uncarboxylated and fully γ-carboxylated rFX. Approximately 80% of the rwtFX eluted in the first peak and represents uncarboxylated protein, and the remaining 20% eluted in the second peak and represents fully γ-carboxylated protein (A). rFX expressed with the prothrombin pre-pro sequence (B, clone B5; C, clone A1) also separated on HA into two peaks, with ~10% of the protein eluting in peak 1 (uncarboxylated protein) and the remaining 90% of the protein eluting in peak 2 (γ-carboxylated protein). Elution of each protein was monitored by absorbance at 280 nm (left axis).

Table 2: Characterization of Various rFX Clones

+ FX propeptide[a]			+ prothrombin propeptide		
FX clone[b]	expression (μg/10^6 cells/24 h)	% of total rFX fully γ-carboxylated[c]	FX clone[b]	expression (μg/10^6 cells/24 h)	% of total rFX fully γ-carboxylated[c]
rFX-1 D3	4.0	20	rFX-1 B5	4.0	90
rFX-2 B5	3.0	30	rFX-2 A1	2.3	91
rFX-3 D1	4.0	40	rFX-3 C5	4.0	89
rFX-4 C4	2.4	30	rFX-4 E2	2.3	82
rFX-5 A3	4.0	25	rFX-5 A4	1.0	81
rFX-6 B2	3.2	35	rFX-6 A6	1.2	73
rFX-7 C3	3.0	45	rFX-7 C1	0.5	91

[a] The FX propeptide is modified at position −2 (Thr→Arg) to allow for efficient propeptide cleavage in HEK 293 cells. [b] The FX clones presented represent wtrFX or rFX with various mutations in the catalytic domain. No mutations were introduced in the Gla domain. [c] The percentage of total rFX fully γ-carboxylated is calculated as follows: the total amount of rFX eluting in peak 2 from hydroxyapatite chromatography is divided by the total amount of rFX loaded on the column. γ-Carboxyglutamic acid analysis was performed on each sample listed above to confirm full γ-carboxylation (10.0−11.2 mol of Gla/mol of rFX).

Table 3: γ-Carboxyglutamic Acid Analysis

sample	av ± SD, mol of Gla/mol of protein	theoretical
PD-h prothrombin[a]	10.1 ± 0.6	10.0
PD-h factor IX[a]	12.3 ± 0.3	12.0
PD-h factor X[a]	10.8 ± 0.1	11.0
PD-h thrombin[a]	ND[b]	0
rwtFX-ss-pro-II (B5) peak 1	0.1 ± 0.02	0
rwtFX-ss-pro-II (B5) peak 2	10.7 ± 0.10	11.0
rwtFX-ss-pro-II (A1) peak 1	0.2 ± 0.04	0
rwtFX-ss-pro-II (A1) peak 2	10.3 ± 0.10	11.0

[a] Plasma-derived human coagulation factors used as standards. See Methods for determination of Gla values. Values are the average of three separate determinations ± SD. [b] ND, no Gla peak was detectable.

protein which is either uncarboxylated or fully γ-carboxylated, although the percentage of fully γ-carboxylated rFX synthesized with the prothrombin pre-pro sequence is much higher. These results are consistent with the notion that the γ-carboxylase is a processive enzyme.

Amino-terminal sequence analysis of clone B5 (similar results obtained with clone A1, data not shown) indicate that the prothrombin pre-pro sequence was removed from the rFX light chain irrespective of its γ-carboxyglutamic acid content (Table 4). In addition, blanks were observed at positions 6, 7, 14, and 16 for rFX eluting in peak 2, suggesting the presence of γ-carboxyglutamic acid, which is not resolved by Edman degradation. Glutamic acid was observed at these sites for uncarboxylated rFX eluting in peak 1. These results are consistent with the γ-carboxyglutamic acid analyses. The rFX heavy chain from both peaks was also sequenced and confirmed the proper cleavage at amino acids Arg^{139}−Arg^{142} generating the heavy and light chains from single-chain precursor (data not shown).

Following purification of fully γ-carboxylated protein from hydroxyapatite chromatography, rFX derived from clone B5 and plasma-derived FX were subjected to SDS−PAGE. Each protein was judged to be >95% pure and migrated at the expected molecular weight under both reducing and nonreducing conditions (Figure 3). The amount of single-chain rFX was minimal, indicating almost complete removal of the internal tripeptide. Following activation by RVV-X and purification on benzamidine-Sepharose, rFXa migrated in an identical fashion to its plasma-derived counterpart on both reducing and nonreducing SDS−PAGE.

Table 4: Amino-Terminal Sequence Analysis of the rwtFX Clone B5 Light Chain

peak 1 from hydroxyapatite column			peak 2 from hydroxyapatite column		
cycle	amino acid	pmol	cycle	amino acid	pmol
1	Ala	85.8	1	Ala	75.2
2	Asn	36.5	2	Asn	38.0
3	Ser	25.7	3	Ser	21.8
4	Phe	69.0	4	Phe	52.6
5	Leu	69.4	5	Leu	43.4
6	Glu	30.3	6	(Gla)	
7	Glu	51.4	7	(Gla)	
8	Met	54.6	8	Met	34.3
9	Lys	51.8	9	Lys	15.4
10	Lys	86.1	10	Lys	50.9
11	Gly	52.3	11	Gly	24.8
12	His	5.8	12	His	9.90
13	Leu	34.9	13	Leu	17.8
14	Glu	4.8	14	(Gla)	
15	Arg	25.0	15	Arg	13.8
16	Glu	12.2	16	(Gla)	
17	Cys	10.1	17	Cys	10.1
18	Met	10.2	18	Met	6.70

DISCUSSION

The development of highly potent and selective inhibitors of FXa (*4*) and the elucidation of its structure by X-ray crystallography (*34*−*36*) have led to an increasing interest in the modulation of this serine protease as a therapeutic strategy to reduce thrombin generation in order to accomplish anticoagulation. However, identification of the structural determinants important for FX/FXa function has been difficult, due to the limited number of naturally occurring homozygous FX mutations available for study (*5*) and the relative difficulty in expressing recombinant FX/FXa. A major obstacle to the production of large quantities of recombinant FX/FXa has been the inefficient γ-carboxylation of the first 11 glutamic acid residues of FX by heterologous mammalian expression systems.

In this paper we present a successful strategy to overcome the problem of inefficient γ-carboxylation in the production of fully functional rFX. Our data indicate that by using a propeptide sequence that binds the γ-carboxylase with a relatively weak affinity, enhanced γ-carboxylation of rFX can be achieved in HEK 293 cells. The rationale for the use of the prothrombin propeptide was based on two observations. First, recent studies indicate that the relative affinities of the vitamin K-dependent propeptides for the γ-carboxylase

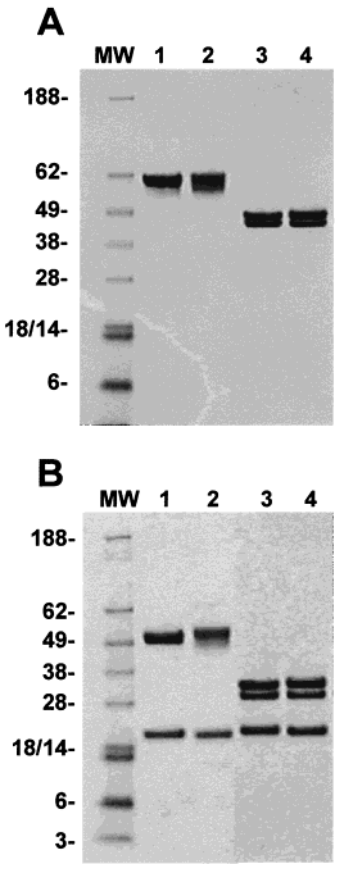

FIGURE 3: SDS−PAGE of plasma-derived and recombinant wild-type factor X/Xa. Plasma-derived and recombinant wild-type FX/Xa (5 μg of protein/lane) were analyzed by 4−12% linear gradient NOVEX NuPAGE Bis-Tris gels using the MES buffer system followed by staining with Coomassie Brilliant Blue R-250. The molecular weight markers are indicated at the left of the gel. In panel A, samples were nonreduced and in panel B samples were reduced with DTT: lanes 1, plasma-derived factor X; lanes 2, recombinant wild-type FX (derived from clone B5); lanes 3, plasma-derived FXa; lanes 4, recombinant wild-type FXa (derived from clone B5). In panels A and B, lanes 3 and 4, the doublet represents the α- and β-forms of FX and FXa, respectively.

vary by >2 orders of magnitude, with the propeptide of factor X having the highest affinity and prothrombin having the lowest [Figure 1 (*19*)]. Second, in contrast to the production of rFX, the expression of vitamin K-dependent proteins with a reduced affinity for the γ-carboxylase, such as recombinant prothrombin (CHO cells) and protein C (HEK 293 cells), results in fully γ-carboxylated protein (*31*−*33*). On the basis of these observations, we hypothesized that the rate of substrate turnover by the γ-carboxylase may be influenced by the affinity of the propeptide, with high-affinity binding propeptides having a decreased off-rate compared to moderate to low affinity propeptides.

Early experience with the expression of recombinant prothrombin and FIX showed that a greater percentage of prothrombin was fully γ-carboxylated when compared to the synthesis of recombinant factor IX when both proteins were expressed at comparable levels in CHO cells (*31*). When the amounts of these proteins were amplified, the levels of fully γ-carboxylated prothrombin are ~5-fold higher than the levels of fully γ-carboxylated factor IX (*31*, *37*). It was hypothesized that pre-pro-prothrombin was a better substrate for γ-carboxylation than pre-pro-factor IX due to elements present in the prothrombin propeptide. Data from previous results (*18*, *19*) and the results presented in this paper suggest that the relative affinities of these proteins for the γ-carboxylase dictate the efficiency of γ-carboxylation, especially in heterologous expression systems (i.e., CHO, HEK 293) that may have limiting γ-carboxylation machinery and are not thought to perform this post-translational modification or synthesize vitamin K-dependent proteins to any significant degree in the basal (untransfected) state.

At first glance, our observation that a high-affinity binding propeptide sequence (i.e., FX) is less efficient than a weaker affinity propeptide sequence (i.e., prothrombin) in directing the γ-carboxylation reaction appears to be puzzling. This apparently paradoxical effect may be related to, in part, low substrate turnover as a result of the very high affinity of the FX propeptide for the γ-carboxylase. The notion that the FX propeptide has a high affinity for the γ-carboxylase is supported by several studies. In vitro studies using a competitive inhibition assay with propeptide/Gla domain substrates demonstrate that γ-carboxylase binding of a FX propeptide/Gla domain substrate exceeds that of the prothrombin and protein C propeptide/Gla domain substrates by 2 orders of magnitude (*19*). In addition, others have shown that warfarin treatment of cultured human or rat primary hepatocytes, or the hepatocellular carcinoma derived HepG2 cells, causes an increase in the prothrombin and FX concentrations in the ER. In these experiments, an increase in the level of FX associated with the ER membrane fractions (presumably FX bound to the γ-carboxylase) was observed with warfarin treatment along with a concomitant decrease in the amount of prothrombin associated with the membrane fraction (*38*−*40*). Although this difference could be accounted for by a higher turnover rate of prothrombin compared to FX in the ER, the authors attributed their findings to a reduced affinity of the prothrombin precursor compared to the FX precursor, resulting in competition for the γ-carboxylase. In addition, analysis of purified bovine carboxylase from cattle that had been pretreated with warfarin showed that the majority of the bovine hepatocyte-derived γ-carboxylase was complexed with the FX precursor rather than with prothrombin or FIX precursors (*41*). Taken together, these observations suggest that vitamin K-dependent precursors with high affinities for the γ-carboxylase effectively compete with other precursors for γ-carboxylase binding during warfarin-induced accumulation of vitamin K-dependent precursors in the ER. It remains unclear, however, whether this high affinity translates into greater substrate turnover and subsequently higher levels of γ-carboxylated product. Support for the notion that high-affinity propeptide binding to the γ-carboxylase does not translate into greater substrate turnover comes from in vitro studies of liver microsomes derived from the ER and which contain membrane-associated γ-carboxylase. Wallin et al. found that although both prothrombin and FX precursors were tightly bound to microsomal membrane fragments, in vitro γ-carboxylation of these substrates resulted in the release of prothrombin precursors from the membrane fragments, but not FX precursors (*38*). These observations strongly suggest that although the FX precursor binds with high affinity to the γ-carboxylase, turnover of this substrate by this enzyme is greatly diminished.

Using purified γ-carboxylase and a FIX peptide substrate (residues −18 to 41), Morris et al. have demonstrated that

vitamin K-dependent carboxylation is a processive post-translational modification (*42*). In that study, it was shown that purified γ-carboxylase can convert as many as 12 glutamate residues in the substrate peptide. Results from the current study demonstrate that regardless of which propeptide sequence is directing the γ-carboxylation reaction (FX or prothrombin), two pools of rFX are secreted, one which is uncarboxylated and one which is fully γ-carboxylated. This confirms our earlier experience that production of rFX in HEK 293 cells does not result in partially γ-carboxylated material (*11*). These results are consistent with the notion that the γ-carboxylase is a processive enzyme; that is, multiple modifications of the substrate molecule occur during a single association between enzyme and substrate.

Although the results of the present study clearly demonstrate that enhanced γ-carboxylation of rFX is achieved by using the prothrombin propeptide, we were never able to attain complete γ-carboxylation of the recombinant protein. For example, expression of rFX with the prothrombin propeptide consistently yielded 10−30% of *un*carboxylated material. This is in contrast to recombinant prothrombin and protein C, for which it has been reported that complete γ-carboxylation of these proteins can be achieved at expression levels which do not overwhelm the γ-carboxylase machinery (*31*−*33*). Although these differences could be related to cell type, expression levels, amounts of γ-carboxylase, or amounts of available vitamin K, it is also possible that substrate components other than the propeptide sequence modulate the efficiency of the γ-carboxylation reaction. For example, it has been proposed that a second recognition site for the γ-carboxylase, in addition to the propeptide, is present in the precursors of vitamin K-dependent proteins (*43*). Disruption by site-specific mutagenesis of a region within the mature sequence of the Gla domain of protein C (hexapeptide disulfide loop; $E^{16}XXXE^{20}$-XC^{22}) resulted in the expression of incompletely γ-carboxylated protein (*44*). However, similar mutations when introduced into recombinant prothrombin resulted in fully γ-carboxylated protein (*45*). These results may relate to differences in the heterologous expression system, but it is also possible that factors specific to the interaction of the γ-carboxylase with the different vitamin K-dependent proteins are responsible. It is clear that the propeptide sequence provides the major initial docking and recognition site for the γ-carboxylase (*14*); however, other structural determinants present in the various vitamin K-dependent proteins may be important in the γ-carboxylation reaction.

In summary, our data indicate that expression of rFX using the prothrombin propeptide is superior to that using the native FX propeptide and results in much higher yields of fully γ-carboxylated protein. We also provide additional evidence that the γ-carboxylase is a processive enzyme; regardless of which propeptide sequence is directing γ-carboxylation, FX or prothrombin, two pools of mature rFX are secreted, one that is *un*carboxylated and one that is *fully* γ-carboxylated. Employment of such a strategy should greatly enhance the large-scale production of wild-type and mutant rFX/FXa molecules, allowing for detailed structure/function studies that should ultimately facilitate the identification of structural determinants on FX/FXa important to its function through the use of site-directed mutagenesis. Our observations should also be applicable to the production of other vitamin K-dependent proteins, especially in a cellular setting or in expression systems where γ-carboxylation of such proteins is not optimal. For example, the use of the prothrombin propeptide in gene therapy strategies for FIX deficiency that rely on expression of the transgene in cell types other than the hepatocyte (*46*) may result in a higher yield of fully functional protein.

ACKNOWLEDGMENT

We express our gratitude to Dr. Alex Kurosky and Steve Smith from the University of Texas, Medical Branch at Galveston, for amino-terminal sequencing from PVDF membranes. We thank George A. Day III for technical assistance.

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BI001074Q