Chapter 4 : Determination of the Kinetics and Mechanism of tg-FIX Activation by Factor XIa

4.1 Introduction

The biological activity of clotting factor IX (FIX) is dependent on its ability to be activated from zymogen FIX to active enzyme FIXa. Activation of FIX to FIXa occurs through cleavage of two peptide bonds: Arg^{145}-Ala^{146} and Arg^{180}-Val^{181}. Cleavage of these bonds releases the 10 kDa activation peptide and results in structural reorientation of the disulfide-linked FIXa light and heavy chains.[1] Two enzymatic mechanisms are responsible for FIX activation *in vivo*: the factor XIa pathway[2] (intrinsic pathway) and the tissue factor-factor VIIa (TF-FVIIa) pathway[3] (extrinsic pathway). Though both activation pathways are present in *in vivo* hemostasis, they are important in different aspects of the clotting process. TF-FVIIa activation of FIX is associated with initiation of clot formation at the site of injury, occurring when TF-bearing cells are exposed to FVII in plasma.[3] Because the TF-FVIIa complex is inhibited by tissue factor pathway inhibitor (TFPI)[4] and is located only on those cells expressing TF on their surface,[5] factor XIa activation of FIX becomes important under conditions where hemostasis is severely challenged. Consequently, it has been observed that factor XIa is important for converting FIX to FIXa in response to trauma and during surgery.[6] To determine the potential effectiveness of replacement FIX in response to these formidable bleeding challenges in hemophilia B patients, it is essential to characterize the structure and function of this FIX with respect to the factor XIa activation pathway.

Activation of FIX by factor XIa occurs by a mechanism distinct from TF-FVIIa activation of FIX. Wolberg *et al.*[7] reported that factor XIa-mediated activation of FIX is processive, with both the Arg^{145}-Ala^{146} and Arg^{180}-Val^{181} cleavages occurring in a single association of the enzyme and substrate. This is in contrast to TF-FVIIa which has been shown to activate FIX through the release of FIXα, an intermediate that possesses only the Arg^{145}-Ala^{146} cleavage. This inactive intermediate is subsequently converted by TF-FVIIa to FIXa by cleavage at Arg^{180}-Val^{181} in a separate association of enzyme and substrate.[8] Activation of FIX by both pathways is represented in Figure 4-1.

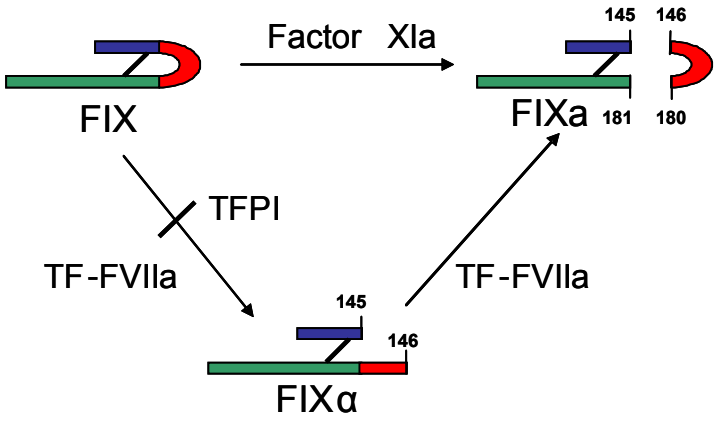

Figure 4-1. Activation of FIX by two pathways. FIX is activated *in vivo* by both factor IXa and the TF-FVIIa complex. Activation by factor XIa is processive, with both cleavages occurring during a single association of enzyme and substrate. In contrast, the TF-FVIIa activation pathway proceeds through the release of the FIXα intermediate. Activation by TF-FVIIa can be inhibited by tissue factor pathway inhibitor (TFPI). The FIX light chain is represented in blue, the activation peptide in red, and the heavy chain in green. The location of the proteolytic cleavages are indicated in by vertical lines with the amino acid numbers.

Association of FIX with factor XIa and its subsequent activation is dependent upon the post-translational processing of the FIX molecule. For efficient activation to occur, FIX must undergo a Ca^{2+}-induced structural conformation change in the Gla domain that exposes the factor XIa binding site. This conformation change is a result of the interaction of positively charged Ca^{2+} with negatively charged carboxylate groups of γ-carboxylated glutamic acid residues (Gla).[9] The Ca^{2+}-induced structure is stabilized by the presence of multiple Gla residues, though it is unknown which of the 12 Gla residues of the Gla domain are required for formation of the factor XIa binding site. Gillis *et al.*[10] showed that Gla's 36 and 40 are not necessary for FIX activity, though they did not explicitly investigate factor XIa binding or activation. In the only unequivocal study of the importance of an individual Gla residue, Wolberg *et al.*[11] found that Gla-21 is significant in factor XIa activation of FIX, as mutation of Glu-21 to Asp is accompanied by a 3-fold reduction of the FIX activation rate. It has been demonstrated that the N-terminal tyrosine of FIX is involved in a stabilizing interaction with the Ca^{2+}-induced conformation of the Gla domain. The presence of the FIX propeptide disrupts this interaction, and subsequently destabilizes the Ca^{2+}-induced conformation, even in FIX molecules with a fully carboxylated Gla domain.[12] As a result, proFIX has been shown

to be incapable of undergoing either of the two proteolytic cleavages catalyzed by factor XIa.[13]

Production of recombinant FIX in the milk of transgenic pigs (tg-FIX) shows promise as a cost-effective alternative source of recombinant human FIX. It has been demonstrated that biologically active tg-FIX can be produced in the porcine mammary gland when expressed at 1-3 mg/ml.[14] However, it was observed that this tg-FIX exists as a mixture of post-translational modification isoforms due to rate limitations and/or species-specific differences in the protein processing enzymes. It was the goal of this study to characterize tg-FIX with respect to the post-translational modifications (PTMs) required for factor XIa-mediated activation, γ-carboxylation and propeptide excision, and to determine how the PTM profile of tg-FIX affects the mechanism and rate by which it is activated. In order to accomplish this goal, tg-FIX was first fractionated into subpopulations based upon these PTMs and factor XIa activation analysis was then performed on the resulting tg-FIX fractions. The results from this study demonstrate the viability of tg-FIX for use in hemophilia B treatment under conditions of severe challenge to hemostasis and provide further insight into the structure/function relationships of FIX.

4.2 Materials and Methods

4.2.1 Transgenic Pig Milk

Transgenic pigs were generated and milk was collected as described previously.[14] The tg-FIX used in this study was purified from milk collected from pig K75 on day 32 of lactation, which contained approximately 1 mg tg-FIX per ml of milk.

4.2.2 Ion-Exchange Fractionation of tg-FIX

The total population of tg-FIX was purified from pig milk using heparin-Sepharose FF (Amersham Biosciences, Piscataway, NJ) chromatography as previously described.[14]

Pure tg-FIX was fractionated based upon molecular acidity using a 3.3 ml (1 cm I.D. x 4.2 cm L) Source 15Q anion-exchange column (15 μm porous particles) (Amersham Biosciences). The MiniQ HPLC method described previously[14] was adapted to the Source 15Q resin. The Source 15Q and MiniQ resins utilize the same quaternary amine functional group for anion-exchange but are composed of particles of different size and porosity. Two buffers were used in this fractionation: loading buffer: 20 mM Tris, pH 9; and elution buffer: 20 mM Tris 1 M ammonium acetate, pH 9. All steps were run at room temperature at a linear flow rate of 1.3 cm/min. The Source 15Q column was equilibrated with 90%:10% loading:elution buffer. Pure tg-FIX eluted from heparin-Sepharose was diluted 1:3 in loading buffer and then applied to 10 mg tg-FIX per ml of resin. After loading, the column was washed with 8 column volumes 90%:10% loading:elution buffer. A low-salt elution of tg-FIX was performed to remove more basic tg-FIX isomers from the column. This was achieved by a step elution with 8 column volumes of 53%:47% loading:elution buffer and, to ensure good resolution of inactive and active tg-FIX isomers, was followed by a shallow gradient from 53%:47% loading:elution buffer to 51%:49% loading:elution buffer over 3 column volumes . A high-salt elution of the most acidic tg-FIX isomers was then performed in a step to 30%:70% loading:elution buffer and was collected in 1.5 column volumes. The column was subsequently washed with 20 mM Tris 2 M NaCl pH 7.2 to remove strongly bound contaminants and to regenerate the column.

4.2.3 Isolation of proFIX

Purification of proFIX was achieved through immuno-affinity chromatography using immobilized anti-proFIX antibodies. Anti-FIX propeptide antiserum was purchased from Sigma-Genosys (The Woodlands, TX) utilizing their custom peptide antisera service. Antiserum was produced by injection of the chemically synthesized human FIX propeptide (Sequence: TVFLDHENANKILNRPKR) conjugated to keyhole-limpet hemocyanin (KLH) into New Zealand white rabbits. FIX propeptide-specific antibodies were isolated from rabbit antiserum by affinity chromatography as follows. FIX propeptide was first immobilized onto CNBr-activated Sepharose 4B (Sigma, St. Louis, MO) according to the manufacturer's instructions. Rabbit antiserum was then

applied to the FIX propeptide-Sepharose column (1 cm I.D. x 2 cm H.; ~2 mg FIX propeptide/ml) equilibrated with phosphate buffered saline (PBS) pH 7.4. The column was subsequently washed with PBS pH 7.4 and FIX propeptide-specific antibodies were eluted with 0.1 M glycine pH 2.8. To every 2 ml of eluate, 100 µl of 1 M Tris pH 8 was added to neutralize the acidic pH of the glycine buffer. The glycine eluate was assayed for reactivity against proFIX by Western blot to determine the effectiveness of the separation. The anti-proFIX antibody eluate was then dialyzed against 0.1 M $NaHCO_3$ 0.5 M NaCl pH 8.3 to prepare for coupling onto CNBr-activated Sepharose 4B.

The isolated FIX propeptide-binding antibodies were immobilized onto CNBr-activated Sepharose 4B per the manufacturer's instructions. To purify proFIX, the proFIX-containing sample of interest was applied to the anti-proFIX Sepharose column (1 cm I.D. x 2 cm H.: ~ 2 mg anti-FIX antibodies/ml) equilibrated with PBS pH 7.4. The column was then washed with PBS pH 7.4 and proFIX was eluted with 0.1 M glycine pH 2.8. The eluted volume was neutralized with 100 µl of 1 M Tris pH 8 for every 2 ml of eluate. Multiple column tg-proFIX-deficient flow-throughs and tg-proFIX eluate fractions were pooled and were concentrated in Centricon YM-10 (Millipore, Billerica, MA) centrifugal concentrators at 4°C and then buffer exchanged with Tris-buffered saline (TBS). The efficiency of the separation was measured by Western blot of the column fractions using anti-FIX propeptide antisera as the probing antibody.

4.2.4 Determination of FIX Concentrations

FIX concentration in pure solutions were determined by measuring the UV absorbance at 280 nm. Protein concentration was estimated using a FIX extinction coefficient of 13.3 for a 1% solution. [15]

4.2.5 FIX Specific Activity Measurements

The specific activity of FIX was determined by the aPTT clotting assay as previously described.[14]

4.2.6 Gla Analysis

Total Gla content of the purified tg-FIX fractions were determined by amino acid analysis from basic hydrolysis. For comparison, plasma-derived FIX (Haematological Technologies, Inc., Essex Junction, VT) was also subjected to amino acid analysis. Prior, to submission, the pd-FIX solution was buffer exchanged with 50 mM Tris 150 mM NaCl pH 7.4 using Centricon YM-10 centrifugal concentrators. Analysis was performed at Commonwealth Biotechnologies (Richmond, VA). Briefly, 83 µl of 4N NaOH and 117 µl of water were added to 100 µl of sample. The tubes were sealed under vacuum and hydrolyzed for 20 hours at 100°C. The samples were then neutralized with 4 N acetic acid, diluted with a borate buffer, and then subjected to analysis. The molar ratio $R = Glu/Gla$ for each sample was obtained from the respective peak areas of the chromatogram. Mature FIX has 40 total Glx residues (28 Glu + 12 potential Gla). The (moles Gla)/(moles FIX) was obtained by solving the simple set of equations

$$Glu + Gla = 40$$

$$\frac{Glu}{Gla} = R$$

where R is the experimentally determined ratio of (moles Glu)/(moles Gla) for the sample.

Because the FIX propeptide contains a single glutamic acid, the above equations were altered for calculation of the proFIX Gla content.

$$Glu + Gla = 41$$

$$\frac{Glu}{Gla} = R$$

4.2.7 FIX Activation by Factor XIa – SDS-PAGE and Western Blot

Activation of FIX by factor XIa (Haematological Technologies, Inc.) was performed at 37°C in TBS pH 7.4 containing 5 mM $CaCl_2$ at the FIX and factor XIa concentrations designated in the associated figures[kvc1]. Aliquots of the reaction mixture were removed at the timepoints indicated in the associated figures and added to 1X SDS PAGE sample buffer to quench the reaction. Where indicated, the reducing agent 50 mM dithiothreitol (DTT) was added to reduce the disulfide bonds. Samples were then heated at 70°C for 10 minutes, loaded onto NuPAGE 10% Bis-Tris SDS-PAGE gels (Invitrogen, Carlsbad, CA), and then run in MES running buffer as suggested by the manufacturer. Gels were either stained using the Colloidal Coomassie Blue staining kit (Invitrogen) or transferred to Immuno-blot polyvinylidine fluoride (PVDF) membrane (Bio-Rad, Hercules, CA) for Western blots.

For Western blots, PVDF membranes containing the transferred protein were first blocked with TBS containing 0.1% Tween-20 and 0.5% casein (TBST-casein). These blots were then either probed with a 1:1000 dilution of polyclonal rabbit anti-FIX IgG (Dako, Carpenteria, CA) or a 1:500 dilution of anti-FIX propeptide antiserum in TBST-casein. The blots were then rinsed with TBST-casein and then probed with a 1:1000 dilution of horse radish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Sigma) in TBST-casein. The blots were subjected to another wash with TBST-casein and then developed by addition of metal enhanced 3,3'-diaminobenzidine (DAB) substrate (Pierce, Rockford, IL) per the manufacturer's instructions. Quantification of both Coomassie stained gels and Western blots were performed using a Bio-Rad GS-750 densitometer.

4.2.8 Integrated Michaelis-Menten Analysis

Activation of FIX to FIXa by factor XIa has been modeled by the enzyme (E)-catalyzed irreversible conversion of a single substrate, S, to product, P, as described by the following reaction:

$$S + E \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} SE \xrightarrow{k_2} E + P$$

The Michaelis-Menten equation of enzyme kinetics is defined for the above reaction mechanism as follows:

$$v = \frac{V_{\max} S}{K_m + S} \quad (1)$$

where

$$K_m = \frac{k_{-1} + k_2}{k_1} \quad (2)$$

$$V_{\max} = [E_o] k_2 \quad (3)$$

Equation 1 relates the rate of conversion of substrate to product, *v*, to the substrate concentration. In equation 3, k_2 is also often referred to as k_{cat}. Equation 2 can be analytically integrated and linearized to the following:

$$\frac{\ln \frac{[S_o]}{[S]}}{t} = \frac{[S_o] - [S]}{K_m t} + \frac{V_{\max}}{K_m} \quad (4)$$

The SDS PAGE densitometry data for HS-tg-FIX and pd-FIX zymogen were first converted to concentration, μM, versus time. The data were then plotted according to equation 4, with $\ln(S_o/S)/t$ plotted versus $(S_o-S)/t$. The resulting line has a slope of $-1/K_m$ and an x intercept of V_{max}/K_m.

4.2.9 FIX Activation in the Presence of 4-aminobenzamidine

FIX was activated by factor XIa in the presence of the FIXa active site binding 4-aminobenzamidine (Sigma) to monitor the extent of activation, as outlined by Monroe *et al.*[16] 4-aminobenzamidine binds to the active site of FIXa, resulting in a measurable increase in the 4-aminobenzamidine intrinsic fluorescence.[17] FIX (2 μm) was activated by factor XIa (9 nM) at room temperature in TBS pH 7.4 containing 150 mM 4-aminobenzamidine and 5 mM $CaCl_2$. Fluorescence measurements were recorded over the course of the reaction until the fluorescence stabilized. Plots of the fluorescence

versus time data were presented as the percentage change in fluorescence, as defined by equation 5.

$$\%\ \text{change in fluorescence} = \frac{(f - f_o)}{(f_{final} - f_o)} * 100\% \quad (5)$$

where f_o is the initial fluorescence, f_{final} is the fluorescence measured at the end of the reaction, and f is the fluorescence measured at time t during the reaction. Fluorescence was recorded on a Hitachi F-4500 spectrofluorometer using an excitation wavelength of 333 nm and an emission wavelength of 376 nm.

4.3 Results

4.3.1 Fractionation of tg-FIX

The heparin-Sepharose tg-FIX product, representing the total population of tg-FIX isoforms isolated from transgenic pig milk, was first fractionated by molecular acidity utilizing Source 15Q anion-exchange chromatography. Two populations of tg-FIX were collected from this column: a high-salt (HS) elution fraction (more acidic) and a low-salt (LS) elution fraction (more basic). It was previously demonstrated that the more acidic the tg-FIX population, the higher its Gla content and subsequent biological activity.[14] For this study, the criterion for successful fractionation on the Source 15Q column was collection of tg-FIX with wild-type biological specific activity (~200 IU/mg) in the high-salt isocratic elution step. This high-salt elution fraction constitutes approximately 10% of the peak area.

From previous N-terminal sequence analysis of tg-FIX, it was discovered that at a 1 mg/ml expression level, the porcine mammary epithelial cells do not process all proFIX to mature FIX. As a result, a significant amount of proFIX is present in the Source 15Q fractions. To isolate the effect of the propeptide on tg-FIX activation by factor XIa, proFIX was extracted from both the Source 15Q HS and LS tg-FIX elution fractions by immuno-affinity chromatography. From a mass balance over the affinity columns, it was

observed proFIX constitutes a larger percentage of the tg-FIX in the Source 15Q HS elution fraction than the LS fraction (20% compared to 10%).

The tg-FIX fractionation procedure is presented in Figure 4-2. SDS PAGE analysis of the four isolated tg-FIX populations, HS-tg-FIX, HS-tg-proFIX, LS-tg-FIX, and LS-tg-proFIX, is shown in Figure 4-3a. All populations are at least 95% pure as determined by densitometry. Mature tg-FIX migrates faster on SDS PAGE than pd-FIX. Since the primary amino acid sequence as encoded by the FIX transgene should be identical, the difference in apparent molecular weight is most likely due to differences in post-translational processing. As confirmation of the proFIX isolation procedure, it was observed that the tg-proFIX populations migrate at a higher apparent molecular weight than the mature tg-FIX populations, corresponding to the presence of the propeptide, and react with anti-proFIX antisera (see Figure 4-1 a and b).

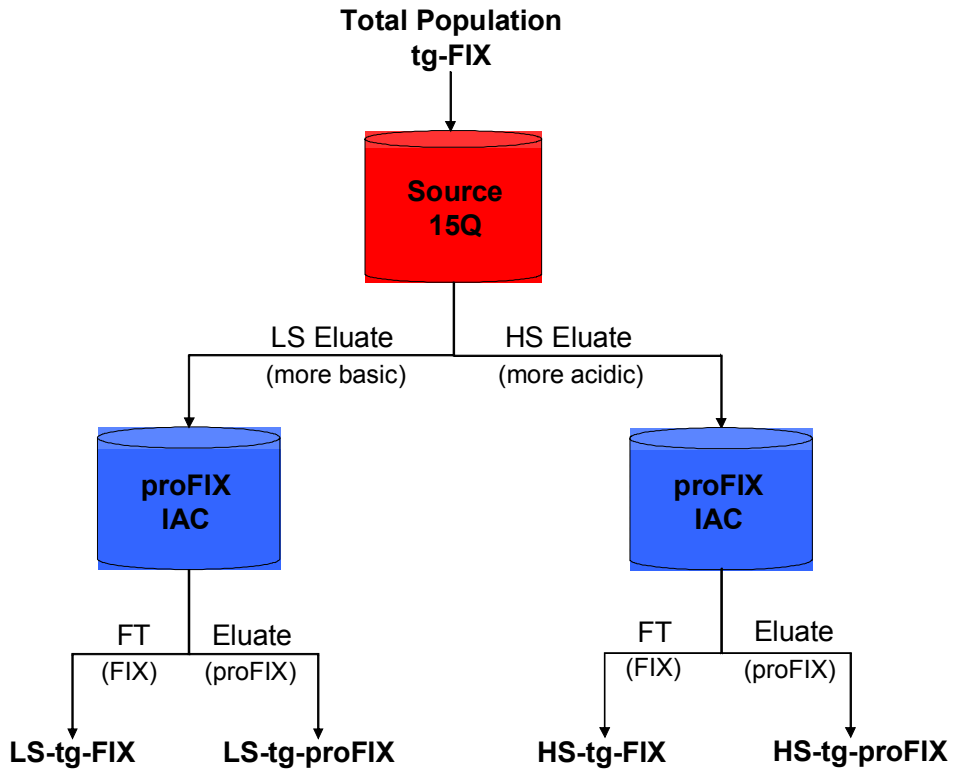

Figure 4-2. tg-FIX fractionation procedure. Total population tg-FIX purified by heparin-Sepharose chromatography was loaded onto Source 15Q. Tg-FIX was eluted in two steps, a low-salt elution (LS Eluate) and a high-salt elution (HS Eluate) as described in the Materials and Methods section. These two fractions were subsequently separated into mature FIX and proFIX populations by immuno-affinity chromatography (IAC). The non-binding material (FT) corresponds to mature tg-FIX

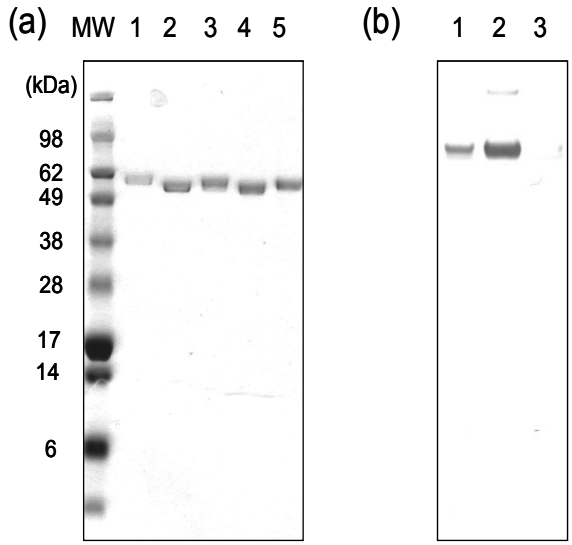

Figure 4-3. SDS PAGE and Western blot of tg-FIX and tg-proFIX purification products.

(a) Reduced (50 mM DTT) Coomassie blue stained gel of FIX products used in this study. Mass of molecular weight standards (MW) are indicated on the left of the figure. pd-FIX (lane 1), HS-tg-FIX (lane 2), HS-tg-proFIX (lane 3), LS-tg-FIX (lane 4), and LS-tg-proFIX (lane 5). 2 μg of protein was loaded per lane.

(b) Reduced (50 mM DTT) Western blot probed with anti-proFIX antisera. Source 15Q HS elution fraction (lane 1), FIX propeptide affinity purification tg-proFIX eluate (lane 2), and FIX propeptide affinity purification tg-FIX flow-through (lane 3). 0.2 μg of protein was loaded in each lane.

4.3.2 FIX In Vitro Activity and Gla Content

The *in vitro* clotting activity, calculated as a percentage of pd-FIX activity, and the Gla content of each tg-FIX population are reported in Table 4-1. The specific activity of HS-tg-FIX was found to be equal to pd-FIX, while no other tg-FIX population exhibited more than 2% of the clotting activity of pd-FIX. The mechanism of the anion-exchange chromatographic fractionation of tg-FIX is confirmed by the higher Gla content of the high-salt eluate fractions. HS-tg-FIX was found to posses on average 7 Gla residues per molecule compared to the 5 Gla residues per molecule for LS-tg-FIX. Interestingly, both HS-tg-proFIX and LS-tg-proFIX possessed a higher Gla content, 9 and 8 Gla's respectively, than HS-tg-FIX and LS-tg-FIX. The presence of propeptide in each population as well as the percentage of total tg-FIX comprised by each population are also summarized in Table 4-1.

	Specific Activity (% pd-FIX Activity)	Number of Gla's	proFIX	Percentage of tg-FIX (%)
Plasma-derived FIX	100	11.2	No	-
HS-tg-FIX	112	7	No	9
LS-tg-FIX	1.1	5	No	72
HS-tg-proFIX	2.3	9.3	Yes	1
LS-tg-proFIX	N/D	8.1	Yes	18

Table 4-1. Table of properties of FIX preparations. All FIX preparations used in this study were subjected to *in vitro* clotting activity measurement and Gla analysis. The specific activity was calculated relative to the pd-FIX standard (assumed to be 100% activity). The activity of LS-tg-proFIX was below the detection limit of the assay and is reported as not detectible (N/D). The Gla and propeptide content of each of the FIX preparations are reported, as well as the percentage that each fraction comprises of the total tg-FIX population.

4.3.3 Activation of HS-tg-FIX by Factor XIa

The HS-tg-FIX fraction represents the tg-FIX population with properties most similar to pd-FIX. To investigate the mechanism of HS-tg-FIX activation by factor XIa, the pattern of zymogen cleavage was examined by SDS PAGE analysis. HS-tg-FIX and pd-FIX were subjected to activation by factor XIa at a 1:100 enzyme:substrate ratio (w/w) in the presence of 5 mM Ca^{2+}. Reducing SDS PAGE analysis of the time-course activations are presented in 4-4a. Upon activation, FIX is cleaved to two major fragments, the heavy chain which migrates at approximately 28 kDa and the light chain which migrates at approximately 22 kDa. Under reducing conditions, the release of free FIX heavy chain is indicative of FIXa generation since the second of the two proteolytic cleavages of FIX activation, Arg^{180}-Val^{181}, has occurred. Another major fragment is observed upon activation, heavy chain plus activation peptide, that corresponds to the FIXα intermediate cleaved at only Arg^{145}-Ala^{146}. For both HS-tg-FIX and pd-FIX, the amount of FIX heavy chain increases during the first 10 minutes of the reaction without significant accumulation of FIXα. By comparison, TF-FVIIa activation of FIX under similar conditions has been reported to proceed through an accumulation of FIXα

intermediate before *any* FIXa formation is observed.[8] After 30 minutes, however, there appears to be an increase in the relative amount of the FIXα intermediate present in the HS-tg-FIX activation reaction. FIXα generation is also observable, though to a lesser extent, later in the pd-FIX activation reaction.

Densitometric analysis of the gels of the time-course FIX activations are shown in Figures 4-4b and 4-4c, with the relative amounts of single-chain zymogen FIX and the FIX heavy chain represented as a percentage of the total protein in each lane. As shown in Figure 4-4b, the concentration versus time profile of both tg-FIX and pd-FIX zymogen appears to be nearly indistinguishable. However, differences in the activation of tg-FIX and pd-FIX can be observed in Figure 4-4c. In this plot, it can be seen that, though generation of free FIX heavy chain occurs at the same rate initially, after 1 minute, a percentage of tg-FIX molecules in the HS-tg-FIX population undergoes the Arg^{180}-Val^{181} cleavage at a reduced rate. By the end of the 60 minute reaction, the amount of HS-tg-FIX that is activated to FIXa approaches that observed for pd-FIX. Data represent the average of two experiments.

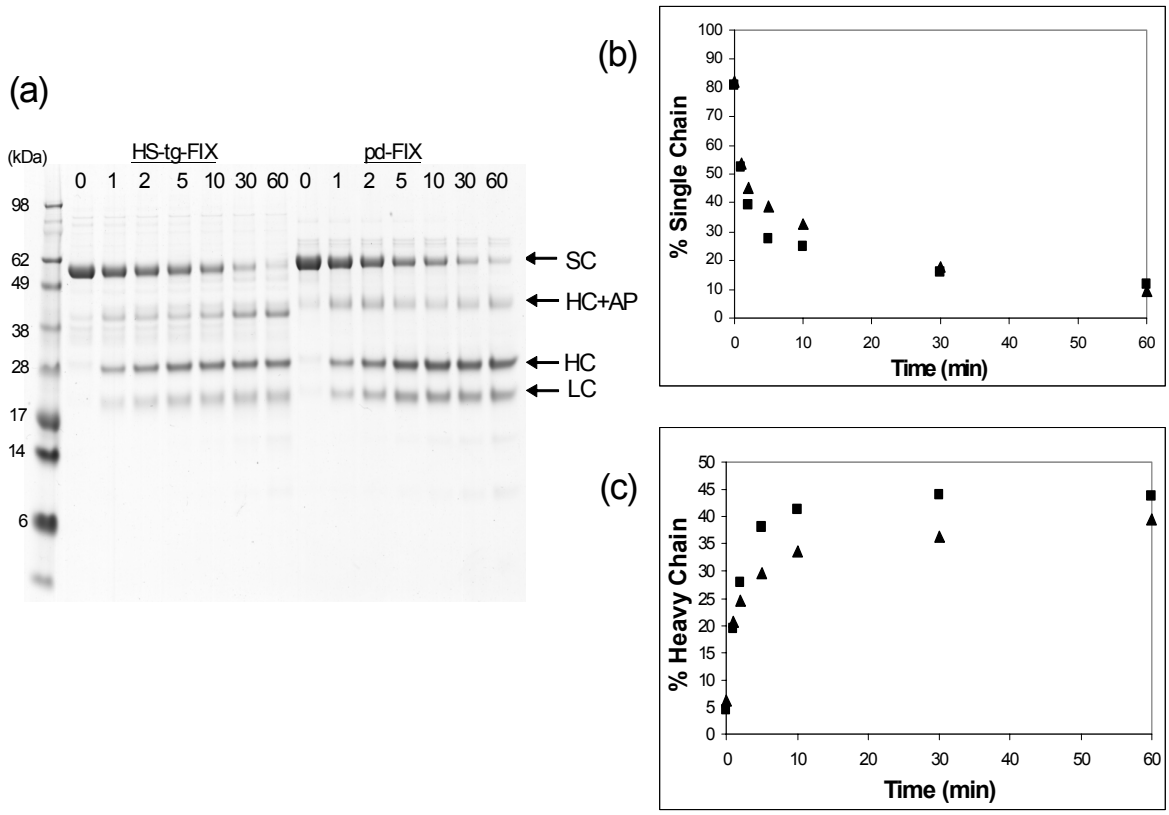

Figure 4-4. SDS PAGE analysis of FIX activation by factor XIa

(a) HS-tg-FIX and pd-FIX were activated by factor XIa. FIX (5 µM) was activated with factor XIa (17 nM) in TBS pH 7.4 containing 5 mM Ca^{2+} at 37°C. Aliquots of the HS-tg-FIX and pd-FIX activation reactions were taken at 0, 1, 2, 5, 10, 30, and 60 minutes, quenched with reducing (50 mM DTT) SDS PAGE sample buffer, run on an SDS PAGE gel, and then stained with Coomassie blue. Molecular weight markers are indicated on the left. The location of zymogen FIX (SC), the heavy chain plus activation peptide intermediate (HC+AP), the FIX heavy chain (HC) and light chain (LC) are indicated by arrows on the right.

(b and c) Coomassie stained gels of time-course activation of FIX were quantified by densitometry. The percentage of total protein represented by single-chain zymogen FIX (non-activated) (b) or heavy chain (activated) (c). pd-FIX is represented by squares (■) and HS-tg-FIX is represented by triangles (▲). Data from (b) and (c) represent the average of two independent experiments.

4.3.4 Integrated Michaelis-Menten Analysis

To generate a more quantitative analysis from the SDS PAGE data, the denstitometric data for single-chain zymogen FIX from Figure 4-4a was plotted according to the linearized integrated form of the Michaelis-Menten equation (Figure 4-5). This plot was expected to yield straight lines of slope $-1/K_m$ and intercept V_{max}. However, for both HS-tg-FIX and pd-FIX, these plots generated lines of with a positive

slope. As a result, negative values for K_m were derived. Consequently, meaningful quantities for the Michalis-Menten constants K_m and k_{cat} could not be achieved from this single progress curve analysis.

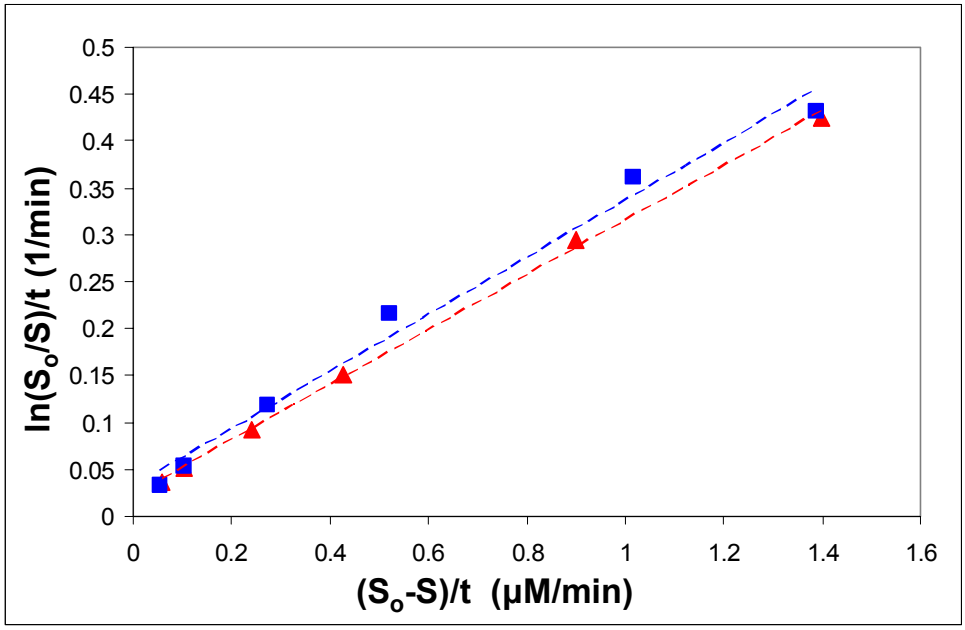

Figure 4-5. Analysis of SDS PAGE FIX activation data by the linearized integrated Michaelis-Menten equation. The collected densitometric SDS PAGE data for the activation of HS-tg-FIX (▲) and pd-FIX (■) was plotted according to the linearized integrated Michaelis-Menten equation for substrate (S). Linear regression of the data is represented by dashed lines. S_o (µM) is the initial zymogen FIX concentration and S (µM) is the concentration of zymogen FIX at time t (min) of activation.

4.3.5 Activation of HS-tg-FIX in the Presence of Benzamidine

To provide an alternate means of quantifying FIX activation by factor XIa, the time-course activation of both HS-tg-FIX and pd-FIX by factor XIa was also monitored in the presence of 4-aminobenzamidine. The increase in fluorescence over time during FIX activation by factor XIa was measured and the results are presented in Figure 4-6. Because fluorescence measurements are arbitrary, fluorescence is reported as the relative percent change in fluorescence. The initial rates of the reaction, 9.4 %/min for pd-FIX and 11.9 %/min for HS-tg-FIX, were derived from linear regression of the curves at the beginning of the reaction (up to 5 min) where the curves are linear. Data represent the average of two experiments.

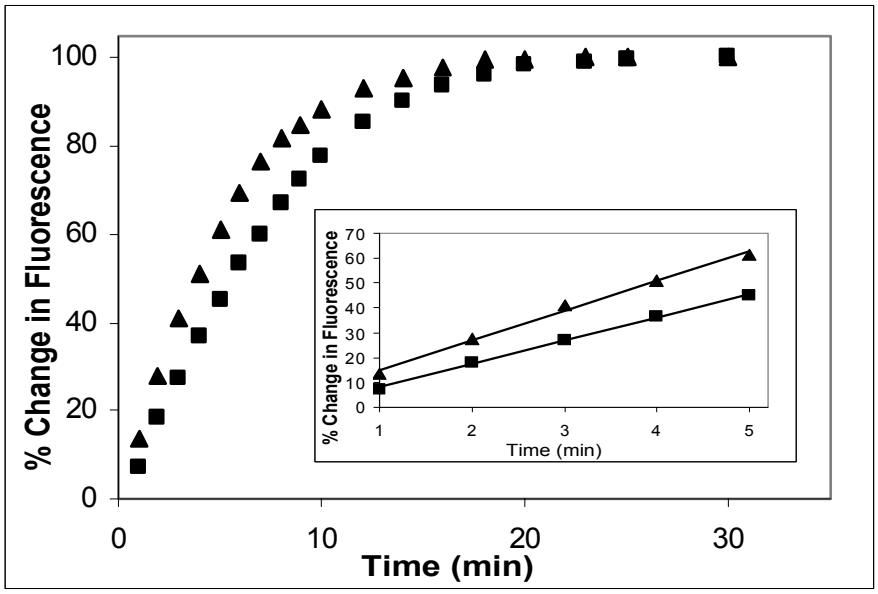

Figure 4-6. FIX activation in the presence of 4-aminobenzamidine. FIX (2 μM) was activated by factor XIa (6.9 nM) in the presence of 4-aminobenzamidine. Fluorescence was measured over time for pd-FIX (■) and HS-tg-FIX (▲). Percentage fluorescence was calculated as indicated in the Materials and Methods section. Initial rates were determined by linear regression of the first 5 minutes of the of the progress curve (inset). Data represent the average of two experiments.

4.3.6 Activation of HS-tg-proFIX

To determine the effect of the FIX propeptide on activation of tg-FIX, activation analysis of proFIX isolated from the Source 15Q high-salt elution fraction, HS-tg-proFIX, was performed. HS-tg-proFIX was much less efficiently activated than HS-tg-FIX. At a 1:100 enzyme:substrate (w/w) ratio, almost no HS-tg-proFIX was activated to FIXa, even after 12 hours (results not shown). However, as demonstrated in Figure 4-7, at a 1:3.3 enzyme:substrate ratio (w/w), HS-tg-proFIX undergoes nearly complete activation within 8 hours. A Western blot of the reduced time-course activation products probed with anti-FIX antisera, Figure 4-7a, reveals that HS-tg-proFIX activation occurs in two steps. Within the first 30 minutes, single-chain zymogen FIX is converted to the FIXα intermediate with little or no generation of FIXa. Complete conversion of the FIXα intermediate to FIXa, however, requires at least 7 hours to complete. In this blot, the FIX light chain is not efficiently visualized.

To ensure that the propeptide is not being cleaved prior to activation as previously reported,[12] a non-reduced Western blot of the reaction products was probed with antiserum specific for the FIX propeptide. As shown in Figure 4-7b, both FIX and FIXa react with anti-proFIX antisera, indicating the propeptide has not been cleaved during activation. The higher molecular weight band on this blot represents the sum of both FIX and FIXα. As the reaction progresses, the apparent molecular weight of this band increases. This is most likely a consequence of the conversion of FIX to FIXα and an associated altered migration rate caused by the cleavage of the Arg^{145}-Ala^{146} bond. The lower molecular weight band on this blot corresponds to FIXa, with a difference in molecular weight equal to the size of the activation peptide, ~10 kDa. A decrease in signal intensity is observed over the course of the reaction. The cause for this is unclear. However, it appears that it occurs equally in both blots, indicating that it is not a consequence of the cleavage of the propeptide.

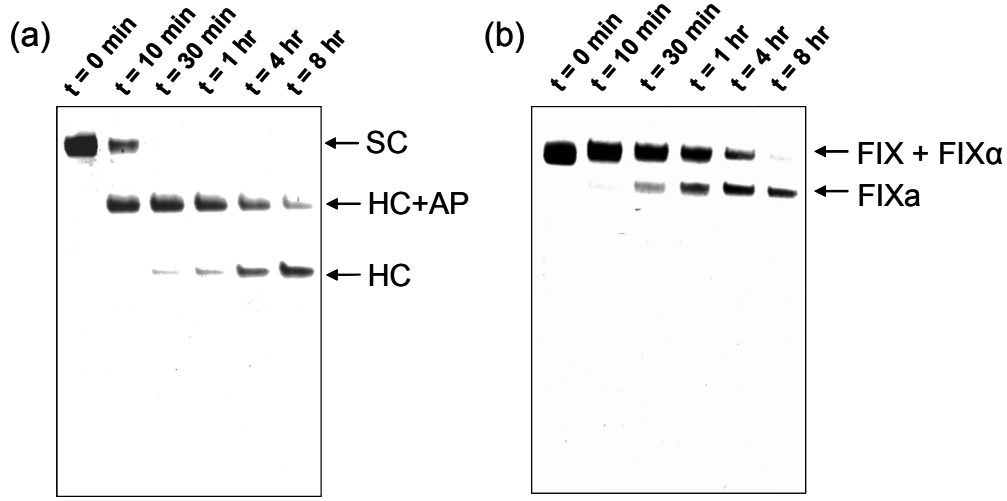

Figure 4-7. Western blot of the activation products of the time-course activation of HS-tg-proFIX by factor XIa. HS-tg-proFIX (0.2μM) was activated by factor XIa (0.02 μM) in TBS pH 7.4 in the presence of 5 mM Ca^{2+} at 37°C. Aliquots were removed at 0 min, 10 min, 30 min, 1 hr, 4 hr, and 8 hr and quenched in reducing (a) and non-reducing (b) SDS PAGE sample buffer. Western blot of activation products probed with total anti-FIX Ab (a) and anti-proFIX antiserum (b).

4.3.7 Activation of LS-tg-FIX and LS-tg-proFIX

To determine the effect of Gla content on activation of tg-FIX, activation analysis of both LS-tg-FIX and LS-tg-proFIX by factor XIa was investigated. These two

populations were not efficiently activated by factor XIa at a 1:100 enzyme:substrate ratio. However, at a 1:3 (w/w) enzyme:substrate ratio, nearly all single-chain LS-tg-FIX and LS-tg-proFIX is converted to FIXa within the 8 hour reaction, as demonstrated by Western blot analysis in Figure 4-8. As with HS-tg-proFIX, activation occurs through the release of the intermediate FIXα. Under these reaction conditions, the Arg^{145}-Ala^{146} cleavage occurs quickly, with most FIX converted to FIXα within the first 30 minutes. The FIXα intermediate is subsequently processed to FIXa slowly over the course of the remainder of the reaction. It is interesting to note that it appears that the conversion rate and mechanism of FIX to FIXa for LS-tg-FIX, HS-tg-proFIX, and LS-tg-proFIX are similar. As was observed for HS-tg-proFIX activation, an unexplained reduction in signal intensity is observed over the course of the reaction.

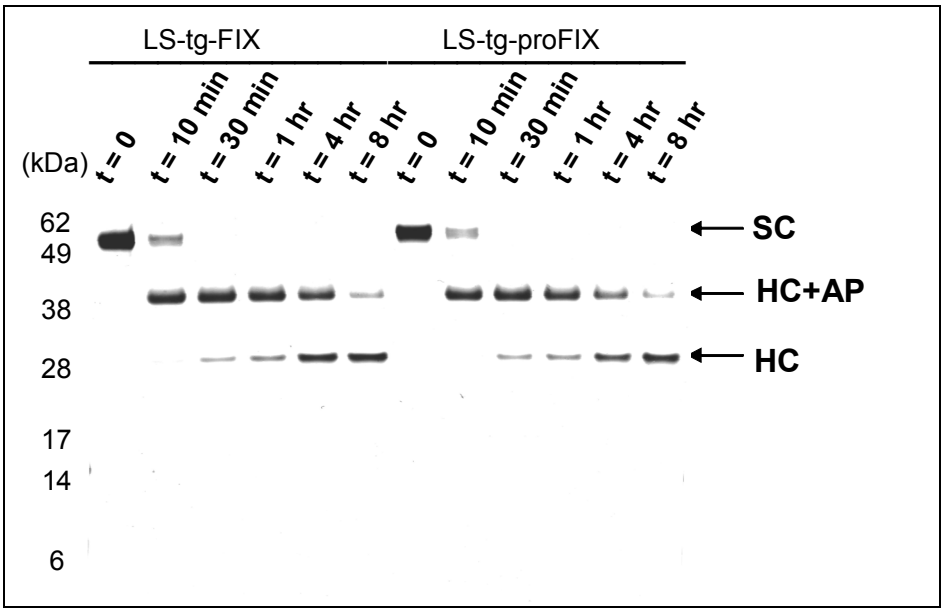

Figure 4-8. Western blot of the activation products of the time-course activation of LS-tg-FIX and LS-tg-proFIX by factor XIa. LS-tg-FIX and LS-tg-proFIX (0.2 μM) were activated by factor XIa (0.02 μM) in TBS pH 7.4 in the presence of 5 mM Ca^{2+} at 37°C. Aliquots were removed at 0 min, 10 min, 30 min, 1 hr, 4 hr, and 8 hr, quenched in reducing SDS PAGE sample buffer, and subjected to Western blot analysis. Activation products were probed with total anti-FIX antisera. Position of molecular weight markers are indicated on the left. Proteolytic products are identified on the right, with FIX zymogen (SC), heavy chain plus activation peptide (HC+AP), and heavy chain (HC) indicated by arrows.

4.4 Discussion

Activation of FIX via the factor XIa pathway is vital in cases of severe hemostatic challenge. As a result, it is important to demonstrate that a FIX product intended for replacement therapy in hemophiliacs can efficiently undergo factor XIa-mediated activation. Structure/function analysis of FIX has shown that two post-translational modifications (PTMs) are required for efficient activation of FIX by factor XIa: cleavage of the N-terminal propeptide and γ-carboxylation of glutamic acids in the Gla domain. The formation of the factor XIa binding site on the FIX light chain is dependent on these PTMs. When FIX is expressed at 1 mg/ml, the porcine mammary gland is unable to completely effect the correct post-translational processing. The resulting tg-FIX is a heterogeneous mixture of FIX isoforms, representing a spectrum of molecules of varying Gla and propeptide content. A great deal of effort has been exerted toward the goal of isolating tg-FIX species possessing a PTM profile conducive to biological activity. In this research, a fractionation process consisting of Source 15Q anion-exchange and anti-proFIX immuno-affinity chromatography has been developed to separate tg-FIX into subpopulations based upon their PTM character. These populations were subjected to factor XIa activation analysis to determine their usefulness as potential replacement FIX in hemophilia B therapy.

The kinetics of factor XIa-mediated activation of FIX has been the subject of much research focused at elucidating FIX and factor XIa structure/function relationships as well as determining the role that both these enzymes play in hemostasis. Factor XIa-mediated activation of FIX has normally been associated with the Michaelis-Menten enzyme kinetic model for a single enzyme and substrate and as a result, emphasis has been placed on determining the two Michaelis-Menten constants, K_m and k_{cat}.[18,20,23] Typically, these constants are calculated from linear regression of double reciprocal plots of initial rates of FIX conversion to FIXa at varying FIX concentrations. For several zymogens in the coagulation cascade, chromagenic substrates are available that make studying the conversion of zymogen to active enzyme relatively straightforward.[19,20] However, no chromagenic substrate is available for FIX with the specificity and sensitivity needed to make this analysis feasible. There are currently two methods

utilized for obtaining the required initial reaction rates for factor XIa-mediated activation of FIX. Walsh *et al*.[21] described a radiolabeling method, where FIX substrate was tritiated and the release of free ^{3}H, proportional to the amount of FIX activation peptide cleaved, was measured over the course of the activation reaction. The second method involves the use of a coupled tenase assay, a FIXa functional assay, where the level FIXa generated in an activation reaction is measured by conversion of the FIXa substrate factor X (FX) to its activated form, FXa. The quantity of FXa is then estimated by the cleavage of a chromogenic substrate specific for this enzyme.[22]

Both these kinetic methods have major drawbacks. With the tritiated method, there is the need to work with radioactive material and more importantly, there is potential for modifying the activity and interactions of FIX with factor XIa as a result of the radiolabeling process. The coupled assay is very complex with several sources of potential error, including multiple calcification and decalcification steps that may result in additional activation of FIX after the initial reaction has been stopped for analysis. Underscoring these problems is the fact that the values of the Michaelis-Menten constants for FIX activation by factor XIa derived from these two methods differ significantly. Sun *et al*.[23] reported a value of 0.16 μM for K_m and 11.0 min^{-1} for k_{cat} using the coupled assay while Walsh *et al*.[21] calculated a K_m of 0.49 μM and a k_{cat} of 7.7 s^{-1} via the radiolabeling method. The problems with these assays, as well as the expense associated with them, have resulted in most studies of FIX activation to be derived from comparative analysis of progress curves. The most common of these is the use of SDS PAGE to monitor the extent of the activation reaction over time.

In this research, a combination progress curve approach was used to investigate the factor XIa-mediated activation rate and mechanism of tg-FIX. Of the four isolated tg-FIX populations, only HS-tg-FIX, which represents approximately 9% of the total tg-FIX, was efficiently activated by factor XIa at a rate comparable to pd-FIX. Densitometry of Coomassie blue stained SDS PAGE gels yielded a qualitative comparison of the activation of HS-tg-FIX versus pd-FIX. In this analysis, it was shown that initial FIXa generation occurred without accumulation of the FIXα intermediate for both pd-FIX and HS-tg-FIX. This profile would only be expected to arise under two conditions: processive activation of FIX or a mechanism by which FIXα is rapidly

converted to FIXa. It has been demonstrated that factor XIa-mediated conversion of both zymogen FIX and FIXα to FIXa occurs at the same rate.[7] As a result, the efficiency enhanced mechanism where FIXα is quickly converted to FIXa is not viable. Thus, it is reasoned that a significant percentage of tg-FIX in the HS-tg-FIX fraction is activated by factor XIa via a processive mechanism, identically to pd-FIX.

The activation profile of HS-tg-FIX, however, does differ from pd-FIX. At later reaction times, the FIXα intermediate accumulates to a higher level than observed with pd-FIX. The most likely explanation for this is the presence of tg-FIX with a lower Gla content in the HS-tg-FIX fraction that is activated less efficiently through the release of the FIXα intermediate. In this reaction, the Arg^{145}-Ala^{146} cleavage would occur at the same rate as the processive cleavage of both bonds in pd-FIX. These results are likely a consequence of incomplete resolution of the most active tg-FIX isoforms by the Source 15Q column.

An attempt was made to extract a quantitative comparison from the SDS PAGE analysis through the use of the integrated form of the Michaelis-Menten equation for substrate, as described by Wolberg *et al*.[7] The integrated form of the Michaelis-Menten equation can yield values for the kinetic constants from a single progress curve.[24] This analysis, however, was problematic. A plot of the linearized equation was expected to yield a straight line with a slope of $-1/K_m$. However, when the FIX zymogen concentration data for both pd-FIX and HS-tg-FIX were plotted, a line of positive slope resulted, yielding a negative value for K_m. Bisswanger[25] argued that enzymatic reactions that undergo competitive product inhibition can result in a positive slope of the linearized integrated Michaelis-Menten equation. The equation for the linearized integrated Michaelis-Menten equation with competitive product inhibition is defined in equation 6 as follows:

$$S + E \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} SE \xrightarrow{k_2} SP \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} P + E$$

$$\frac{[S_o]-[S]}{t} = \frac{V_{\max} K_p}{K_p - K_S} - \frac{K_m (K_p + [S_o])}{K_p - K_m} \cdot \frac{\ln \frac{[S_o]}{[S]}}{t} \quad (6)$$

where,

$$K_p = \frac{k_3}{k_{-3}} \quad (7)$$

It can be seen from equation 6 that if $K_m > K_p$, then the plot of $(S_o-S)/t$ versus $\ln(S_o/S)/t$ would yield a line of positive slope, as observed in Figure 4-3. Though the values for the kinetic constants could not be derived from this analysis, the plots imply that the product, FIXa, likely competes strongly with zymogen FIX for factor XIa. Though this does not directly impact the goals of this study, this is interesting to note since this behavior has not been previously reported. This observation is not surprising, however, since it has been noted that both FIX and FIXa associate with factor XIa with equal affinity.[9]

A second progress curve comparison method using the FIXa active site probe 4-aminobenzamidine to measure the formation of FIXa over the course of the activation reaction was implemented to provide an alternate quantitative analysis. This method offers the advantage of generating more frequent data points, making the determination of the initial rate from the linear segment of the progress curve less difficult. Another characteristic of this method is that it measures only conversion of FIX to FIXa, and not the intermediate FIXα, since benzamidine does not recognize the active site of this intermediate.[16] According to this analysis, HS-tg-FIX is activated by factor XIa at a rate that is similar to that observed for pd-FIX. These results are consistent with the measured similarity of the *in vitro* clotting activity of this fraction and pd-FIX measured by the aPTT coagulation assay. The aPTT assay measures the effectiveness of the intrinsic clotting pathway, which includes factor XIa-mediated activation of FIX.[26] As a result, it was expected that tg-FIX displaying wild-type activity in the aPTT clotting assay would also exhibit normal activation by factor XIa.

In contrast, tg-FIX not isolated in the HS-tg-FIX fraction, approximately 91% of tg-FIX expressed, was not efficiently activated by factor XIa. Western blot analysis of the activation of the three other isolated tg-FIX fractions indicated that the processive

factor XIa activation mechanism was not retained for these tg-FIX populations. These tg-FIX fractions required a low enzyme:substrate ratio for sufficient activation to FIXa. Each population's activation profile was nearly identical, with all tg-FIX zymogen converted to FIXα within the first 30 minutes, followed by slow cleavage of this intermediate to FIXa. It is evident from these blots that the rate-limiting step in the activation of these tg-FIX products to FIXa is cleavage of the Arg^{180}-Val^{181} bond. However, Woldberg *et al*.[7] demonstrated that conversion of plasma-derived zymogen FIX, FIXα, and FIX cleaved at Arg^{180}-Val^{181} (FIXaα) to FIX all occur at the same rate, indicating a shared rate-limiting step. This discrepancy indicates that there is a clear mechanistic difference in the association of these FIX isoforms with factor XIa than is normally exhibited.

Post-translational modification analysis of the tg-FIX fractions revealed that HS-tg-FIX contains an average of only 7 Gla residues per molecule, despite its wild-type biological activity and efficient activation by factor XIa. These results suggest, for the first time, that normal factor XIa-mediated activation of FIX can occur for Gla-deficient FIX species containing less than 10 Gla residues. Which of the N-terminal 12 glutamic acids of the HS-tg-FIX Gla domain have undergone γ-carboxylation is unknown. It may be assumed that, due to the processivity of the carboxylation process,[27] glutamic acid residues 36 and 40 are not carboxylated. These residues have been shown to be the last glutamic acids in the Gla domain to undergo carboxylation and are not required for factor XIa-mediated activation or biological activity.[10] However, though carboxylation is processive, it is not generally sequential from N-to C-terminus. For example, in prothrombin, carboxylation occurs three-dimensionally from glutamic acids structurally inside to outside the Gla domain of the molecule.[28] It is tempting to assume structural homology between the FIX and prothrombin Gla domains and subsequently assign the order of carboxylation for FIX by analogy to prothrombin. While the Gla domains of these two molecules are very similar, significant structural deviation occurs within the first 11 amino acids.[29] Additionally, there may be species-specific differences in carboxylation between the human and porcine carboxylase, making this process even more subjective to the system in question. As a result, the location of the 7 Gla residues cannot be determined without further analysis.

In contrast, the post-translational processing of the lower Gla mature tg-FIX population, LS-tg-FIX, was insufficient to support full biological activity and factor XIa-mediated activation. Complete activation required over 8 hours, a low enzyme:substrate ratio, and proceeded by rapid cleavage of the Arg^{145}-Ala^{146} relative to the slow conversion of FIXα to FIXa. LS-tg-FIX contains on average 5 Gla residues per molecule, only two fewer Gla residues than the average of 7 for HS-tg-FIX. It is likely, then, that the loss of these two carboxylations result in destabilization of the Gla domain-dependent factor XIa binding site. It appears that the Gla content required for normal FIX activity and factor XIa-mediated activation lies within the range of 6-7 carboxylations. This is somewhat surprising since most naturally occurring point mutations to glutamic residues in the FIX Gla domain give rise to clotting deficiency.[30] However, all of these mutations result in the substitution of glutamic acids for other, often very different, amino acids. Thus, for the mutation of most Gla residues, it is not known if the lack in clotting activity results from the absence of the carboxylate group on the glutamic acid or the presence of a destabilizing amino acid.

Activation of both tg-proFIX populations was similar to that observed for LS-tg-FIX. The Gla content of these proFIX fractions was found to be greater than that required by HS-tg-FIX for efficient activation by factor XIa. Consequently, these results support previous observations that the propeptide abolishes FIX biological activity and the ability of FIX to be efficiently activated by factor XIa despite possessing an otherwise functional Gla domain.[11] However, contrary to previously published work by Wojcik *et al.*[12] for proFIX, tg-proFIX was capable of being activated to proFIXa at a 1:3.3 (w/w) enzyme:subustrate ratio. This difference in observed behavior may be a result of incomplete carboxylation of the tg-proFIX Gla domain. It is possible that the destabilizing effect of the propeptide is reduced as a consequence of the non-native Gla domain. Because of the similarity of the activation profiles of LS-tg-FIX and tg-proFIX, it appears that both low Gla content and the presence of the propeptide result in comparable reduction of the FIX/factor XIa binding affinity.

It is evident that limited association between both LS-tg-FIX and the pro-tgFIX fractions with factor XIa still occurs due to the relative efficiency of the Arg^{145}-Ala^{146} cleavage. For these tg-FIX populations, the FIX/factor XIa interaction is strong enough

to facilitate the conversion of FIX to FIXα, but not strong enough to allow for effective progression of the rate-limiting step of the reaction, the conversion of FIXα to FIXa. It is not known if the PTM profile of these tg-FIX populations results in complete alleviation the factor XIa binding properties of the FIX Gla domain, or if they are merely reduced in magnitude. The FIX/factor XIa interaction has yet to be fully characterized. There are structural elements outside the FIX Gla domain that contribute to this interaction, most notably the EGF1 domain, which has been shown to be involved in FIX/factor XIa binding.[31] It is unknown if the EGF1 domain of FIX acts only to stabilize the factor XIa Gla domain binding site or if it represents a distinct site of interaction. If the latter is the case, then it would not be surprising to find that interaction between FIX and factor XIa still occurs despite an altered Gla domain binding site.

4.5 Summary and Conclusions

This study has shown that a fraction of the tg-FIX produced in the porcine mammary gland undergoes factor XIa-mediated activation similarly to pd-FIX. This tg-FIX represents approximately 9% of the tg-FIX expressed, and can be isolated by a combination of Q anion-exchange chromatography and immuno-affinity chromatography. The ability of tg-FIX to be activated by factor XIa was demonstrated to be dependent upon its level of γ-carboxylation and propeptide excision, consistent with literature. However, this study has shown, for the first time,that FIX molecules containing less than 10 Gla residues can undergo normal factor XIa-mediated activation. It was also determined that low Gla tg-FIX isoforms, with average of 5 Gla's per molecule, as well as highly carboxylated tg-proFIX can be activated to FIXa, but that this activation does not occur through the normal factor XIa processive mechanism. Because the Ca^{2+}-induced factor XIa binding site on these tg-FIX populations is likely not properly formed, the FIX/factor XIa association is weak, and the rate-limiting Arg^{180}-Val^{181} cleavage is inefficient. Ultimately, these experiments have shown that the porcine mammary gland can effect the post-translational modifications required for activity and efficient activation by factor XIa. As a result, tg-FIX shows potential for use in replacement therapy for hemophiliac B patients, even in cases of severe hemostatic challenge.

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