

Chapter 2 : Background

2.1 Hemophilia

Hemophilia B is a debilitating congenital bleeding disorder that affects nearly one in every 25,000 male births.¹ It is caused by mutation of the gene encoding the plasma protein factor IX (FIX), located on the long arm of the X-chromosome,² that results in either reduced plasma levels of the protein or a disruption of its function. Though most cases of hemophilia result from inheritance of the defective gene from the female parent, 30% of cases result from a new gene mutation in patients with no family history of the disease. Hemophilia is characterized by a malfunction in the blood coagulation system, often resulting in severe joint damage or uncontrolled internal bleeding.¹ Unchecked, severe hemophilia inevitably results in disability or death. With optimal treatment, however, hemophiliacs can expect to have a near-normal quality of life and lifespan.

Treatment for hemophilia B involves intra-venous (IV) infusion of exogenous FIX either in response to a bleeding episode or prophylactically, with injections administered once or twice a week. On prophylaxis, adequate plasma levels of FIX for hemostasis are maintained, approximating a non-diseased state. When prophylaxis is started at a young age, many patients experience a complete lack of bleeding episodes, maintain undamaged joints, and can lead functionally normal lives.³ Thus, prophylaxis is preferred over on-demand therapy as it prevents most of the irreversible long-term effects brought about by uncontrolled bleeding. The standard prophylactic regimen requires that from 25-40 IU/kg be administered to a patient twice per week.³ For an 80 kg adult, this results in a clotting factor requirement of around 135,000 IU per year, approximately three times what is required for on-demand therapy. As an estimation, to treat all American hemophilia B patients on a prophylactic regimen, close to 300 million IU of FIX would be required yearly, and to treat all hemophilia B patients worldwide, close to 10 billion IU per year would be required^[kvc1].

FIX concentrates are currently derived from two sources. Plasma-derived FIX (pd-FIX) concentrates ^[kvc2]are prepared by purifying FIX from pools of donor human plasma. While introduction of pd-FIX concentrates in the 1970s revolutionized

hemophilia care, it is estimated that during the mid 1980s, 60-80% of hemophiliacs in Europe and the United States had contracted the HIV virus from contaminated blood products.⁴ Plasma-derived products are now much safer due to viral testing and inactivation. However, the discovery of any new human contagion leads to apprehension and fear within the community, as recently encountered with the emergence of West Nile virus, SARS, and Creutzfeldt-Jakob disease. The second source of FIX is a recombinant FIX (rFIX) produced in Chinese hamster ovary (CHO) cell cultures. This recombinant product, BeneFIX[®], has quickly become the preferred FIX source as its use eliminates exposure to any human-derived products and the dangers associated with them.

The major drawback of both current FIX sources is their low abundance. In plasma, FIX circulates at only 5 $\mu\text{g}/\text{ml}$ ⁵ and as a result, to extract enough FIX for the worldwide prophylactic treatment of hemophilia B, a minimum of 10 million liters of human plasma would be required yearly. Additionally, the highest reported expression level of active rFIX in CHO cell systems is 1.5 $\mu\text{g}/\text{ml}$.⁶ Approximately 27 million liters of CHO cell culture supernatant would be required to achieve the goal of worldwide prophylactic treatment. In contrast, transgenic pig bioreactors producing 200 $\mu\text{g}/\text{ml}$ of active FIX, the expression rate found in this study, could generate enough clotting factor in only 250,000 liters. For comparison, this data is presented in Figure 2-1.

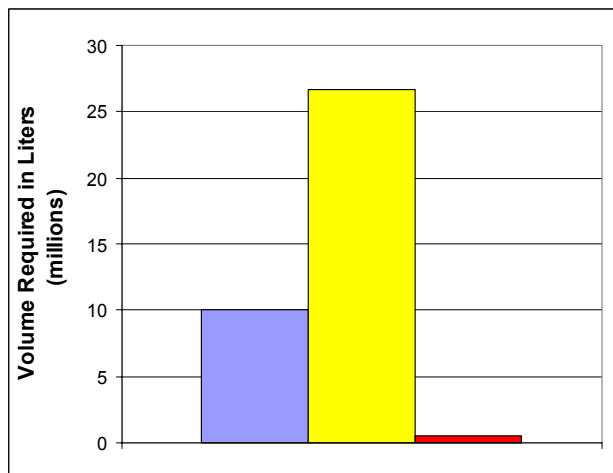


Figure 2-1. Minimum volumetric feedstock requirements for the worldwide prophylactic treatment of hemophilia B based on an estimate of 10 billion IU of FIX required. Human plasma (blue), CHO cell supernatant (yellow), and transgenic pig milk (red)

Though effective therapy is available, close to 80% of the world's hemophiliacs remain untreated.⁷ Due to the low availability of plasma for FIX isolation and the extreme complexity of the protein resulting in difficulty in achieving large-scale recombinant production, FIX concentrates are prohibitively expensive. The average yearly cost for clotting factor alone is approximately \$130,000⁸ per year for on-demand therapy and can be even higher for patients on the most efficacious prophylactic regimens. Even in developed countries, this burden proves to be too great for many patients. Only 13% of children receive full-dose prophylactic treatments in the United States despite its clear advantages over typical on-demand therapy.⁹ There is an obvious need for a more economical production method of FIX for treatment of hemophilia B.

2.2 FIX in Blood Coagulation

FIX plays a vital role in hemostasis as a protease in the blood coagulation cascade (Figure 2-2). As it is classically described, blood coagulation is regulated on a protein level by two interconnected pathways. The extrinsic pathway includes factors not normally found in the bloodstream, namely tissue factor (TF), which is present on the surface of cells associated with the extracellular matrix. The intrinsic pathway consists only of factors that can be isolated from plasma.¹⁰ The coagulation cascade is initiated by injury to the vasculature and subsequent exposure of TF found on the surface of extravascular cells to activated factor VII (FVIIa) present in the bloodstream.¹¹ This event initiates a complex sequence of proteolytic reactions involving multiple factors, cell types, and inhibitors that ultimately result in the formation of an insoluble cross-linked fibrin clot.

The role of activated enzyme FIX, FIXa, within the clotting process is to activate factor X (FX) to FXa through association with its cofactor, factor VIIIa (FVIIIa), and its substrate FX to form the tenase complex.¹⁰ This reaction requires both Ca^{2+} and phospholipids and occurs on the surface of both activated platelets and endothelial cells.¹² Activation of FX in the tenase complex represents the junction between the intrinsic and extrinsic clotting pathways. FIXa-dependent activation of FX is requisite for hemostasis as a deficiency in either FVIII or FIX results in the phenotypically indistinguishable

disorders hemophilia A and B respectively. As illustrated in Figure 2-2, FX is not only activated by FIXa but also by the TF-FVIIa complex. TF-FVIIa cannot activate sufficient FX to prevent the occurrence of hemophilia in individuals with FIX and FVIII deficiencies, and the reason for this is still up for debate. It is most likely due to the fact that FIXa is not readily inhibited by the plasma protease inhibitors antithrombin III (ATIII) and tissue factor pathway inhibitor (TFPI), while the TF-FVIIa complex is.¹³ It is reasoned then, that the inhibition of TF-FVIIa occurs too rapidly to allow for enough FXa to be formed or that it is formed on the wrong cell type.¹²

FIX circulates as an inactive zymogen and must first be converted to active enzyme, FIXa, before it will function as a FX activator. FIX is activated by either the TF-FVIIa complex¹⁴ or by factor XIa (FXIa).¹⁵ The TF-FVIIa FIX activation pathway is associated with initiation of the clotting process and occurs on the surface of TF-bearing cells.¹⁶ The TF-FVIIa mechanism of FIX activation results in only a small amount of FIXa. The FXIa pathway generates larger amounts of FIXa and occurs on the surface of activated platelets.¹² FIX activated through this mechanism is important for sustaining clot formation and integrity, especially in cases of severe hemostatic challenge or when clotting occurs in a highly fibrinolytic environment.¹⁷ Thus, FIX plays an important role in both the initiation of clot formation and in sustaining its integrity through activation of FX.

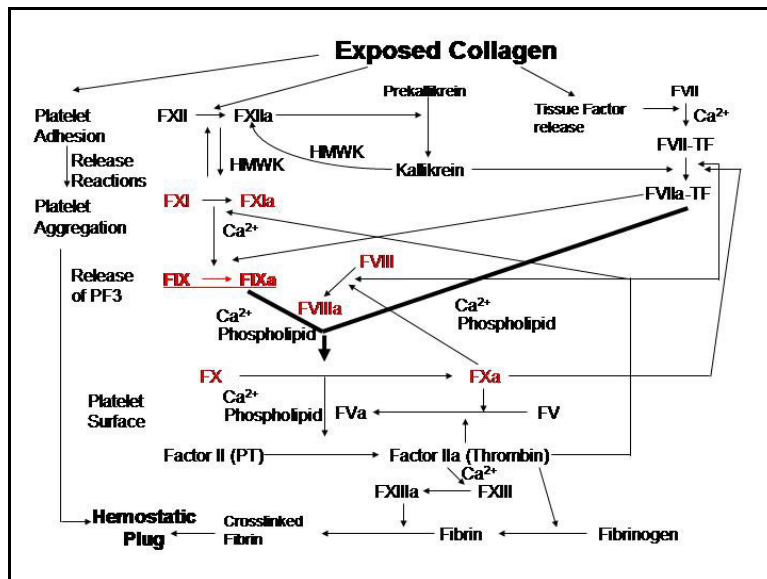


Figure 2-2. Schematic representation of the blood coagulation cascade. Reactions involving FIX are indicated in red.

2.3 Factor IX Structure and Post-translational Modification

2.3.1 General Structure

FIX is a 56 kDa serine protease made in the liver and secreted into the bloodstream as an inactive single-chain zymogen at level of 4-5 µg/ml. The FIX primary amino acid sequence and the mapped domains of the molecule are illustrated in Figure 2-3. As expressed, FIX consists of a signal peptide (SP), a propeptide (PP), the Gla domain, two domains that show homology to epidermal growth factor (EGF), an activation peptide (AP), and a catalytic domain containing the serine protease motif. In the FIX active form FIXa, the activation peptide is excised and the protein is converted to a two-chain molecule consisting of a 22 kDa non-enzymatic light chain (residues 1-145) and a 31 kDa heavy chain (residues 181-415) that contains the serine protease active site. These two chains are connected by a single disulfide bond.^{5,18} FIX is an extremely complex protein and is extensively post-translationally modified before its secretion by hepatocytes. Although not all functions of its post-translational modifications (PTMs) have been elucidated, it is known that propeptide removal¹⁹ and some level of γ -carboxylation¹⁰ are required for activity while glycosylation, phosphorylation, and sulfation may be related to the pharmacokinetic properties of recovery (the IU/dl FIX activity increase in plasma per the IU/kg injected) and half-life.²⁰ The fate of the FIX molecule within the secretory pathway of the cell with respect to PTMs is depicted in Figure 2-4.

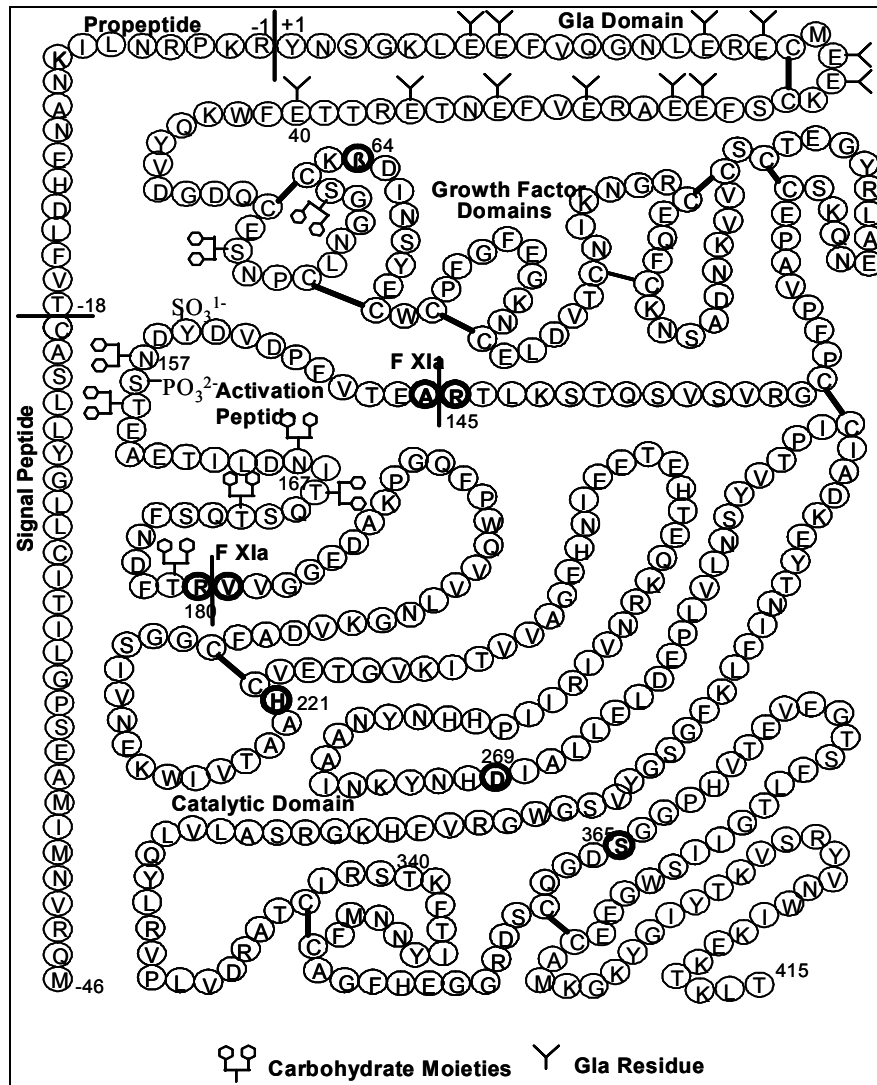


Figure 2-3. FIX primary sequence and post-translational modifications. The FIX molecule contains several domains, including the signal peptide, propeptide, Gla domain, growth factor domains, activation peptide, and catalytic domain. Sites of peptide bond excision are represented by solid lines between the corresponding amino acids. The serine protease catalytic triad is indicated by thick circles on the heavy chain. Disulfide bonds are signified by dark lines connecting cysteines (C). Sulfation and phosphorylation sites are indicated on the activation peptide while Gla and carbohydrate moieties are symbolized by indicated structures.

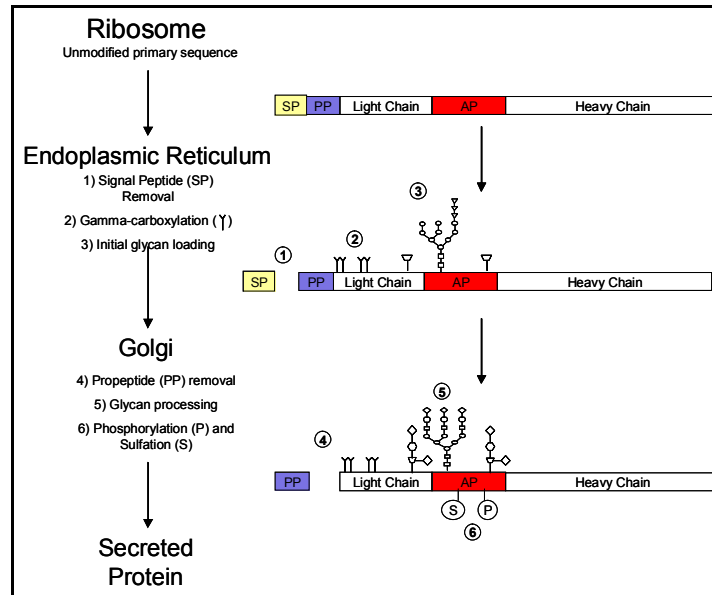


Figure 2-4. Post-translational modifications of FIX in the secretory pathway. The FIX molecule as it makes its way through the cell is highly modified. In the ER, the signal peptide is cleaved, γ -carboxylation occurs, and the initial glycan structure is loaded. In the Golgi, the propeptide is removed, the glycan structure is processed, and sulfation and phosphorylation occurs.

2.3.2 γ -Carboxylation

FIX is a member of the vitamin K-dependent (VKD) proteins, a group that contains coagulation proteins factors VII, IX, and X, prothrombin, protein C, and protein S, as well as the bone protein osteocalcin. All of these proteins contain γ -carboxy glutamic acid (Gla), a glutamic acid residue that has been modified via the addition of a carboxylate group to the γ -carbon of the amino acid. This modification is carried out by the enzyme γ -glutamyl carboxylase, an integral membrane protein located in the endoplasmic reticulum of hepatocytes.²¹ The carboxylation reaction (Figure 2-5) requires both vitamin K and CO_2 . During the reaction, vitamin K is first oxidized to vitamin K 2,3-epoxide by γ -glutamyl carboxylase and is later recycled back to its reduced form by vitamin K reductase.²² The anticoagulant Warfarin blocks the reduction of vitamin K, and thus interrupts the formation of Gla residues in VKD proteins that are required for them to properly function.²³

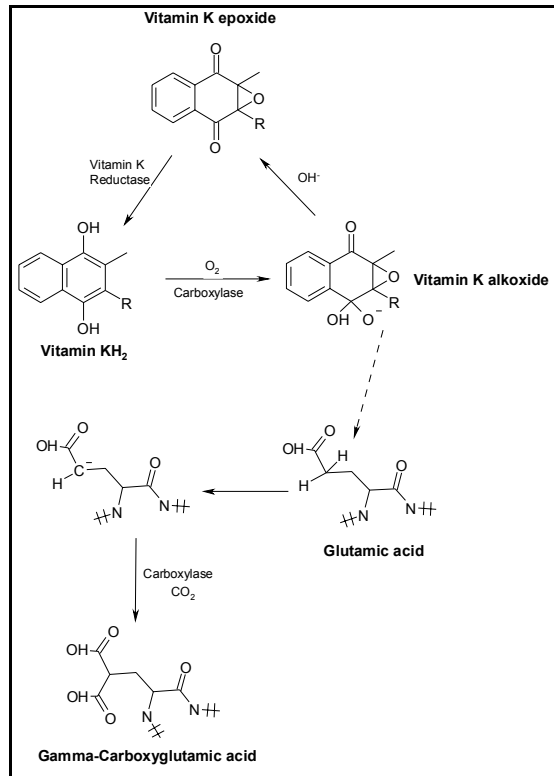


Figure 2-5. γ -Carboxylation reaction. Glutamic acid in a VKD protein is converted in the endoplasmic reticulum to γ -carboxy glutamic acid in a reaction involving vitamin K, the γ -glutamyl carboxylase, and CO_2 .

Carboxylation of human FIX by human γ - glutamyl carboxylase occurs via a tethered-processive mechanism, with the carboxylase binding to the propeptide and subsequently “scanning” the amino acid sequence for Glu residues to modify.²⁴ It is reasoned that this is why Gla residues are sequestered to the N-terminus, assuming that the carboxylase only modifies those Gla residues that are located near its binding site, the propeptide.²⁵ All carboxylation occurs in a single enzyme/substrate binding event, as opposed to each Gla formation resulting from a separate association of enzyme and substrate. As a consequence, there is an observed all-or-nothing behavior usually associated with carboxylation in the liver. The putative tethered-processive mechanism is unique, as most processive enzymes move along the substrate, binding at repetitive sequences that represent the sites of catalysis.²⁶

Evidence for the tethered-processive mechanism has been documented *in vitro* in two studies of FIX carboxylation. Morris *et al.*²⁷ studied carboxylation of truncated FIX

molecules containing only the propeptide and Gla domain. In this study, the authors found that despite the presence of only a small amount of carboxylated material in their samples, all the material that was carboxylated was multiply carboxylated, with the large majority containing 6-12 Gla residues. From a statistical standpoint, this cannot happen unless either the reaction is processive or that partially carboxylated molecules are a better substrate for the carboxylase than non-carboxylated molecules.

More proof of the tethered-processive mechanism was uncovered in research by Stenina *et al.*²⁴ In their study, native FIX bound to γ -glutamyl carboxylase was formed by co-expressing the two proteins in baby hamster kidney (BHK) cells in a vitamin K-deficient environment. After purification of the complex and addition of vitamin K, the carboxylation of FIX within the complex was measured by the complex alone and in the presence of an excess of a distinguishable variant of FIX. The rate and extent of carboxylation of FIX in the complex was unaffected by the presence of challenge protein. To rule out the possibility that partially carboxylated forms of FIX are better substrates for further carboxylation than non-carboxylated forms, it was shown that carboxylated challenge protein is generated early within the reaction but its presence does not interfere with FIX carboxylation within the complex. From this study, it is also inferred that the rate-limiting steps in the reaction are the substrate binding and release steps, and not the actual carboxylation reactions themselves^[kvc3].

The most recent data on FIX carboxylation show that the Gla domain has an allosteric effect on carboxylase binding to the FIX propeptide.²⁸ This study demonstrated that the dissociation rate of the carboxylase from the uncarboxylated substrate proGla41, which contains the FIX propeptide coupled to the first forty-one residues of the Gla domain, is 30-fold lower than for the propeptide alone. However, the dissociation rate is increased by a factor of four after carboxylation of proGla41, promoting release of carboxylated FIX product from the carboxylase. These findings support the notion of processive carboxylation, where the carboxylase binds the FIX propeptide of uncarboxylated FIX with increased affinity, the Gla domain is carboxylated, and then FIX is released rapidly once the carboxylated state has been achieved.

In its native form, FIX contains 12 Gla residues, all which are located within a segment on the N-terminal end of the molecule known as the Gla domain. These Gla

residues are responsible for a Ca^{2+} -induced change in conformation of the FIX light chain recognizable by conformation-specific antibodies.²⁹ This structural change results in exposure of hydrophobic amino acids within the Gla domain and elicits FIX binding to phospholipids. A hydrophobic patch, including Leu-6, Phe-9, and Val-10, on the FIX light chain, which is normally buried within the protein, is exposed to the solvent after Ca^{2+} binding. Concomitantly, the first 9 Gla residues of the Gla domain are internalized.³⁰ The Gla domain binds 8-12 Ca^{2+} , of which 2-3 are cooperatively bound by more than one Gla residue,³¹ via electrostatic interactions of the negatively charged carboxylate groups with the positively charged Ca^{2+} . The NMR structure of the Ca^{2+} -bound Gla domain of FIX is shown in Figure 2-6. Via phospholipid binding on the surfaces of cells, FIX is brought into close proximity to other members of the coagulation cascade, thus greatly increasing the rates of the reactions in which it is involved.

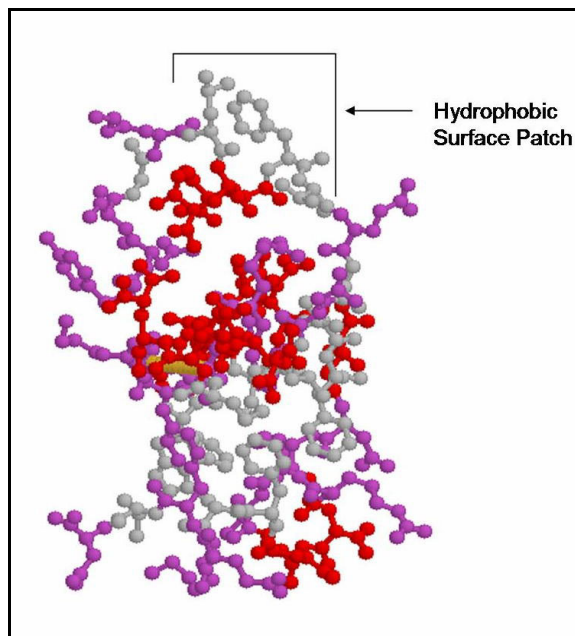


Figure 2-6. Ca^{2+} -bound FIX Gla domain 3-D structure. Red: Gla residue; Gray: Hydrophobic amino acids. Image created in Protein Explorer Version 2.411³² using the NMR-determined structure of the Ca^{2+} -bound FIX Gla domain.³⁰

Though the FIX Gla domain can bind other divalent metal cations, only Ca^{2+} can induce the formation of the phospholipid-binding conformation FIX*. Another conformation, FIX', can be induced by metal ion non-specific binding of the FIX Gla

domain to a number of metal ions, including Ca^{2+} , Mg^{2+} , Mn^{2+} , and Ba^{2+} . This change in structure also results in an antigenic determinant that can be recognized by conformation-specific antibodies.²⁹ The formation of either metal-induced conformation is accompanied by quenching of the intrinsic fluorescence of Trp-42, occurring as a result of translocation of Trp-42 adjacent to the disulfide loop.³⁰

The N-terminal ten Gla residues within the Gla domain are widely conserved amongst the VKD plasma proteins, while the last two are unique to FIX. Gillis *et al.*³³ determined that these two Gla residues, 36 and 40, are not necessary for normal FIX function, as *in vitro* specific activity, phospholipid binding, and Ca^{2+} -induced fluorescence quenching are unaffected by their absence. Interestingly, conformation-specific antibodies that recognize the Mg^{2+} -bound form of 12 Gla FIX have a much lower affinity for the 10 or 11 Gla forms. For FIX, limited research on the effects of mutations of other Gla residues has been undertaken. Gla-21 is presently the only Gla residue that has been studied in detail. Wohlberg *et al.*³⁴ probed the necessity of Gla-21 in FIX by exchanging the Gla for an Asp. The resulting molecule displayed only 9% normal activity in the *in vitro* aPTT assay, slower activation kinetics by FXIa, and a reduced affinity for phospholipids. This molecule did, however, display normal quenching of the intrinsic fluorescence when exposed to Ca^{2+} . Numerous point mutations of individual Gla residues have been discovered in hemophilia B patients with varying degrees of severity.³⁵ However, no detailed studies of these variants have been performed.

Beyond this, which Gla residues in the FIX Gla domain are needed for proper function are unknown. However, studies involving point mutations made in other VKD proteins with homologous Gla domains have provided insight into the necessity of each Gla residue in FIX. In prothrombin (factor II) each Gla has been individually replaced with an Asp residue and the resulting activity and phospholipid-binding properties measured. Mutations in Gla residues 16, 26, and 29, analogous to residues 17, 27, and 30 in FIX, were found to completely abolish activity, while mutation in Gla residues 7, 14, 19, 20, 25, or 32, analogous to residues 8, 15, 20, 21, 26 and 33 in FIX, yielded a protein with a reduced activity. Only a mutation in Gla-6, Gla-7 in FIX, was tolerable in terms of retaining a fully active prothrombin molecule.³⁶ There are discrepancies, however, in the relative importance of Gla residues in prothrombin as compared to

protein C. For example, replacing Gla-14 and 19 in prothrombin brings about a decrease in activity of prothrombin but has no effect on the activity of protein C. On the other hand, mutations of Gla-7 and 20 in protein C completely abolish protein C functionality³⁷ but only reduce the activity of prothrombin. These observations underscore the necessity of determining how essential each Gla residue is for each of the VKD proteins.

2.3.3 Glycosylation

Plasma-derived FIX contains four sites with O-linked oligosaccharide attachment and two sites with N-linked oligosaccharide attachment. Two of the O-linked sites, Ser-53 and Ser-61, are uniformly glycosylated and are found within the first EGF domain.²⁰ The remaining two O-linked sites, Thr-159 and Thr-169, are only partially glycosylated and are located on the activation peptide.³⁸ The activation peptide also contains two sites of N-linked oligosaccharide attachment, Asp-157 and Asp-164.³⁹ The N-linked glycans represent a mixture of complex tri- and tetra-antennary, sialylated structures.⁴⁰ FIX contains a total of 10-12 sialic acid residues, up to three of which are thought to reside on the O-linked sugars with the remaining 8-10 located within the N-linked sugars of the activation peptide.¹⁵ Aside from potentially playing a role in protein folding, secretion, or circulation half-life, little is known about the function of the FIX glycans.

2.3.4 Propeptide Cleavage

In FIX, the proteolytic removal of the propeptide is vital for activity. Propeptide removal occurs in the trans-Golgi, just prior to secretion from the cell.⁴¹ Though the enzyme responsible for propeptide excision has not been unequivocally determined, it is hypothesized that the enzyme PACE (paired basic amino acid cleavage enzyme)/furin, which cleaves after paired basic residues and is known to be capable of FIX propeptide excision and is found in hepatocytes, is responsible.²⁰ In fact, PACE/furin has been successfully co-expressed with FIX in CHO cells and resulted in a higher level of propeptide removal from the co-expressed FIX.⁴¹ For FIX molecules containing the propeptide (proFIX), it is thought that the presence of the propeptide interferes with the formation of the necessary Ca²⁺-induced secondary and tertiary structure of the Gla

domain required for FIX binding to phospholipids or to its cofactor or activators. The presence of the propeptide greatly reduces the ability of the molecule to be activated.^{42,43} Hence, individuals who have a mutation in their FIX gene that prevents the removal of the propeptide before secretion exhibit severe clotting deficiency.⁴⁴

2.3.5 Phosphorylation and Sulfation

Greater than 90% of plasma-derived human FIX contains a phosphorylated serine residue, Ser-158, located within the activation peptide.²⁰ The purpose of the phosphorylated serine has yet to be determined. It is known that recombinant FIX produced in CHO cells contains less than 1% of the phosphorylation of pd-FIX, but still maintains a similar specific activity.⁴⁵ This fact indicates that phosphorylation is not a necessary modification for activity. Other purposes of the modification, such as effect on half-life, have been proposed, but not definitively demonstrated. There are multiple locations on the FIX light and heavy chains that represent potential phosphorylation sites that remain unphosphorylated in the native molecule.⁴⁶ FIX also contains a single sulfated residue, Asp-157. The presence of the sulfation site is also thought to be related to both *in vivo* recovery, defined as the measured plasma increase in FIX activity per the administered dose, and half-life.²⁰

2.3.6 β -hydroxylation

FIX contains a single β -hydroxylation of Asp-64 in the first EGF domain.⁴⁷ The function of this PTM is currently not determined. However, it is known that hydroxylation is not required for *in vitro* activity, as demonstrated by the normal clotting activity exhibited by recombinant FIX expressed in a β -hydroxylation inhibited environment.⁴⁸ Also, EGF domain-dependent high-affinity Ca^{2+} binding is not dependent upon the presence of hydroxylated Asp-64.⁴⁹

2.4 FIX Activation

For FIX to become a fully functioning protease in the coagulation cascade, the activation peptide must be excised from the single-chain zymogen. Activation is performed *in vivo* by either TF-FVIIa or FXIa. Activation of FIX by TF-FVIIa occurs as part of the extrinsic pathway and requires a negatively charged phospholipid membrane and Ca^{2+} .⁵⁰ This reaction occurs on the surface of TF-bearing cells, and also requires that FIX contain a functioning Gla domain. In this mechanism, FIX is first cleaved between residues Arg¹⁴⁵-Ala¹⁴⁶, yielding the FIX α intermediate, and then is again cut between residues Arg¹⁸⁰-Val¹⁸¹ in the rate-limiting step of activation, reactions 1 and 2 in Figure 2-7.⁵¹ It has been shown that FIX α is active in amidolytic assays, but not efficient at activating FX, the normal FIX substrate.⁵² Unlike the zymogen, however, FIX α is fully capable of binding to its cofactor, FVIIIa.⁵³ Interaction of FIX with TF-FVIIa requires both an intact Gla domain⁵⁴ and the EFG-1 domain.⁵⁵

The exact mechanism by which FIX is activated by FXIa has been the source of much debate. It is known that the reaction can and does occur in the fluid phase and requires Ca^{2+} , a FIX molecule with a functioning Gla domain and excised propeptide, but *not* phospholipid *in vitro*.⁵⁶ To further this point, FXI is the only coagulation protease lacking a Gla domain, which implies that it is not localized to phospholipid membranes in the presence of calcium. This evidence proved to be counterintuitive in that it suggests that the reaction occurs in the fluid phase of blood and not on cell surfaces as the other reactions in the coagulation cascade. Recent evidence, however, suggests that the reaction occurs on the surface of activated platelets, likely involving a specific platelet surface protein and not merely a generic phospholipid membrane.⁵⁷ Recent data also proves that the reaction involving FXIa is mechanistically different than the one involving TF-FVIIa. This data shows that both enzymatic cleavages occur processively without the release of a FIX α intermediate as in activation by TF-FVIIa,⁵⁸ reaction 5 in Figure 2-7. Though the FIX structural elements necessary for FXIa binding and subsequent activation have not been unambiguously identified, most evidence points to a light chain binding site, potentially involving the Gla domain or EFG domains.⁵⁹ The presence of the activation peptide itself, however, is not required for proper activation by FXIa.⁶⁰

FIX can also be activated by a protease in Russell's viper venom (RVV). This protease primarily cleaves FIX between residues Arg¹⁸⁰-Val¹⁸¹, reaction 3 in Figure 2-7, yielding factor IXa α . This intermediate possesses approximately 20% of the activity of the fully activated molecule.⁶¹ However, RVV is also capable of fully activating FIX to FIXa by further cutting factor IXa α between residues Arg¹⁴⁵-Ala¹⁴⁶ via a less efficient reaction, reaction 4 in Figure 2-7. Though activation of FIX by RVV has no application in normal hemostasis, RVV activation is widely utilized in studies of FIX.

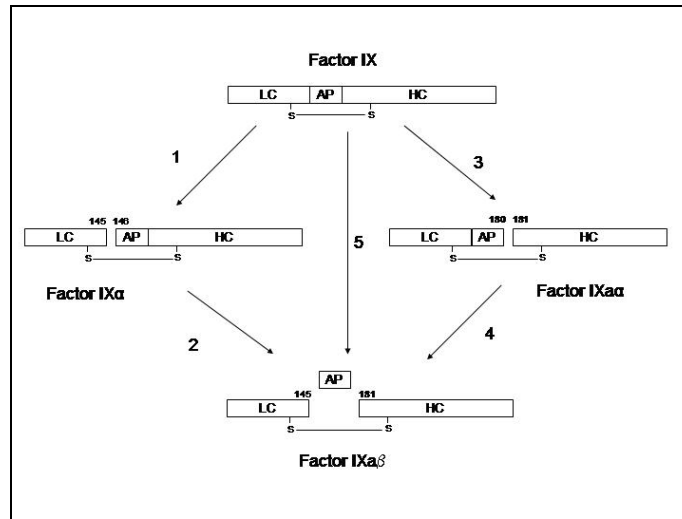


Figure 2-7. FIX activation pathways. FIX is activated by FXIa (reaction 5), TF-FVIIa (reactions 1 and 2), and RVV (reactions 3 and 4). After cleavage of the activation peptide (AP), the light chain (LC) and heavy chain (HC) are connected by a single disulfide bond.

Since the mechanism of FIX activation by its two natural activators differ, it is not surprising that the kinetics of activation also differ. Reported enzyme kinetics studies on FIX activation assume normal Michaelis-Menten kinetics, as described by equation (1).

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

The values for the constants, K_m and V_{\max} , have been determined from linearized plots of initial rates, v , versus the substrate concentrations, S . Reported values for the Michaelis constant, K_m , which is a measure of the affinity of the enzyme for its substrate, vary for

FXIa-mediated activation from $0.16 \mu\text{M}$ ⁵⁷ to $0.49 \mu\text{M}$.⁶² For TF-FVIIa activation of FIX, a K_m value of $0.54 \mu\text{M}$ has been reported,⁶³ indicating that the two enzymes have a similar affinity for the FIX substrate. However, k_{cat} , related to V_{max} , for FXIa-mediated activation is much higher, ranging from 7.7 s^{-1} to 11 s^{-1} ,^{57,62} compared to 0.55 s^{-1} for TF-FVIIa,⁶³ indicating that FIXa-mediated activation of FIX occurs at a significantly faster rate than the TF-FVIIa activation of FIX.

2.5 Current Recombinant Technologies

The ideal treatment for hemophilia B is prophylactic infusion of efficacious FIX into the bloodstream of patients starting as soon as possible after diagnosis, ideally while the patient is still an infant. However, for this treatment regimen to be practical, large quantities of safe and biologically active FIX must be available. The advent of recombinant DNA technology heralded a new era in treatment of diseases associated in deficiencies of proteins. For instance, diabetic patients unable to produce functional insulin are administered insulin produced in genetically engineered yeast.⁶⁴ The use of prokaryotic and simple eukaryotic expression strains to produce recombinant proteins often provides a cheap and abundant source of material. However, a severe limitation of these expression systems is their lack of ability to perform PTMs often associated with more complicated human proteins. Bacterial systems, like *E. coli*, are unable to glycosylate or carboxylate recombinant mammalian proteins.⁶⁵ Yeast cells have the capacity to glycosylate proteins, but the resulting glycans consist of high-mannose structures that are quickly eliminated from circulation if injected in humans.⁶⁵ As such, bacterial and yeast expression systems are of little or no use for producing recombinant FIX.

Cultured mammalian cells have been utilized to produce complex human proteins for therapeutic use. As a result, this was most obvious source for production of recombinant FIX. Several cell types, including CHO cells, were shown to be capable of producing detectable levels of active rFIX.^{66,67} CHO cells are preferable to the other mammalian cell types since they possess qualities that make them amenable to large-scale culture and industrial production. Kaufman *et al.*⁶ reported a line of CHO cells

secreting 100 µg/ml of FIX into the media, of which only 1.5 µg/ml possessed the post-translational processing required to bind to antibodies that recognize the Ca²⁺-induced conformation state. These data indicate the rate limitations inherent to this recombinant production method as both γ-carboxylation and propeptide cleavage appeared to occur at a low rate in these cells. With some engineering, however, the problem of propeptide processing was overcome by co-expressing a soluble form of PACE, a protease that cleaves at dibasic amino acid sequences.⁶⁸ The rate of γ-carboxylation, however, still appears to be limited, unaffected by co-expression of the γ-glutamyl carboxylase. It was determined that at high concentrations of carboxylase, the FIX that was being produced was completely carboxylated but that it was not being released from the γ-carboxylase/FIX enzyme complex efficiently.⁶⁹ Therefore, attempts at making mammalian cells that are more efficient at γ-carboxylating FIX have thus far been unsuccessful. Despite this limitation, rFIX produced in CHO cells was approved by the FDA in 1997 under the trade name BeneFIX[®]. This FIX concentrate contains fully active FIX, having a specific activity of approximately 200-250 U/mg, similar to FIX purified from human plasma.

Comparisons between BeneFIX[®] and pd-FIX on a molecular level have yielded interesting results. Though pd-FIX contains 12 Gla residues per molecule, BeneFIX[®] molecules contain from 10-12 Gla residues, but still retain full activity.⁷⁰ Consistent with this finding, it has been demonstrated that only the first 10 Gla residues are required for full FIX activity.³ BeneFIX[®] is properly proteolytically processed with complete removal of the propeptide. O-linked oligosaccharide content of the FIX from the two sources is similar in both number and in structure. There is some variation in terms of N-linked oligosaccharide content, however, as BeneFIX[®] displays low heterogeneity and less than full sialation. Where the two molecules differ most greatly, however, is in serine phosphorylation and tyrosine sulfation. In pd-FIX, Ser-158 is nearly fully phosphorylated and Tyr-155 is nearly fully sulfated. In BeneFIX[®], however, only a low level of sulfation exists and phosphorylation is almost completely unobserved.⁷¹ The differences in the extent and nature of the PTMs of BeneFIX[®] are hypothesized to play a role in its diminished plasma recovery, as rFIX has been shown to have a 28% lower recovery than pd-FIX upon administration to patients, though the circulation half-life is

approximately the same.⁷² This means that 28% more BeneFIX[®] is required to achieve the same level of FIX in plasma than pd-FIX, leading to a higher cost of treatment. A summary of the properties of BeneFIX[®] and pd-FIX is presented in Table 2-1.

	pd-FIX	BeneFIX[®]
PTM		
Number of Gla	12	10-12
Glycosylation		
N-linked Asn-157,167	Complete occupancy High heterogeneity	Complete occupancy Low heterogeneity
O-linked Ser-53,61; Thr-159,169,172	Partial occupancy	Partial occupancy
Phosphorylation – Ser-158	>90%	<1%
Sulfation – Tyr-155	>90%	<1%
β-hydroxylation – Asp-64	37%	46%
Pharmacology		
Specific Activity	200-250 U/mg	250 U/mg
Recovery	48%	34%
Half-Life	22.6 hrs	19 hrs

Table 2-1. Comparison of post-translational modifications and pharmacological properties of pd-FIX and BeneFIX[®]

2.6 Transgenic Animal Bioreactors

The limitations of current FIX production technologies are reviewed above. Safety, availability, and cost are the most pressing problems associated with current FIX production methods. Transgenic animals may provide an alternative, high production source of rFIX. The mammary gland is proficient at expressing highly concentrated proteins and large volumes of milk. This high expression capacity is a result of a high cell density in the mammary gland, on the order of 10^9 cells per ml of tissue, 2-3 orders of magnitude higher than is possible in cultured mammalian cells.⁷³ In pigs, up to 50 mg/ml of protein is expressed with a total milk yield of approximately 100-300 liters per

year.⁷⁴ To date, expression levels of recombinant proteins in pig milk, driven by efficient promoters, have reached very high levels. Human protein C, for example, has been expressed at up to 2 mg/ml in pig milk.⁷⁵

The key to production of a complex molecule like FIX is, as is the case in mammalian cell culture, the rate at which the gland is capable of effecting the necessary PTMs. Transgenic mice, sheep, goats, and pigs have all been generated capable of producing human FIX in their milk.^{76,77,78,79} Not all of these species, however, have displayed the same capability of producing the *active* molecule. Of those studied the pig has shown the most promise, able to produce up to 200 µg/ml of active, γ -carboxylated FIX.⁸⁰ Since all FIX generated is active, one could hypothesize that the capacity of the porcine mammary gland to γ -carboxylate and cleave propeptide on the FIX molecule is not saturated at this rate of expression. Additionally, in contrast to ruminant milk proteins that contain high-mannose glycans, the porcine mammary gland is known to express milk glycoproteins with a human-like N-linked glycosylation pattern, complex glycans with terminal sialic acid residues.⁸¹ Utilizing a different promoter system, much higher levels of FIX can be produced, and from this higher expression level lineage, the rate limitations of post-translational processing can be studied.

As mentioned, there is precedent, beyond FIX, for the expression of vitamin K-dependent proteins in the mammary gland of transgenic pigs. Recombinant human protein C (rhPC), an anticoagulant, has also been produced in transgenic pigs. The rate of post-translational modification of rhPC appears to be limited in the porcine mammary epithelium at higher rates of expression. It was found that at expression levels of 0.1-1 mg/ml, only 30-60% of rhPC was sufficiently γ -carboxylated while at these same expression levels, 10-20% still contained propeptide and 30-40% was in the single-chain form.⁷⁵ The limitations of propeptide processing and γ -carboxylation experienced by rhPC may be an indicator that rFIX expressed at higher levels are rate limited as well. However, the protein-specific nature of the modifying enzymes may yield different results.

Given the mammary gland's high capacity for phosphorylation, of special interest in this study is the ability of the porcine mammary gland to phosphorylate the rFIX molecule. The mammary gland is especially proficient at serine phosphorylation, as the

caseins, expressed at levels approaching 25 mg/ml in milk, contain numerous phosphoserine residues. A single casein molecule may contain 1-13 phosphorous atoms, depending on the casein subtype.⁸² *In vivo*, the caseins are phosphorylated in the Golgi apparatus by the protein Golgi-enriched fraction casein kinase (GEF-casein kinase). This kinase specifically recognizes and phosphorylates serines within the sequence S-X-E/D, where S is serine, X is any amino acid, and E/D represents glutamate and, to a lesser extent, aspartate.⁸³ The FIX light chain contains three GEF-casein kinase phosphorylation sites, corresponding to Ser-24, 68, and 123, and the activation peptide contains one that corresponds to the naturally occurring phosphoserine residue, Ser-158. A query of the NetPhos phosphorylation prediction database⁴⁶ predicts numerous sites of potential phosphorylation of the FIX molecule at serine, tyrosine, and threonine residues, as shown in Figure 2-8. According to the calculation algorithm, there are 24 sites on the FIX molecule whose phosphorylation score exceeds the threshold: 10 on the light chain, 4 on the activation peptide, and 10 on the heavy chain. Interestingly, all three of the GEF-casein kinase serine phosphorylation sites have phosphorylation scores over 0.9 (threshold is 0.5), while the normally phosphorylated Ser-153 has a score of less than 0.2. It is not known what the effect of additional phosphorylated amino acid residues would have on the function of tg-FIX. However, the high phosphorylation capacity exhibited by the mammary gland as well as the numerous potential phosphorylation sites on FIX may provide a unique opportunity for study.

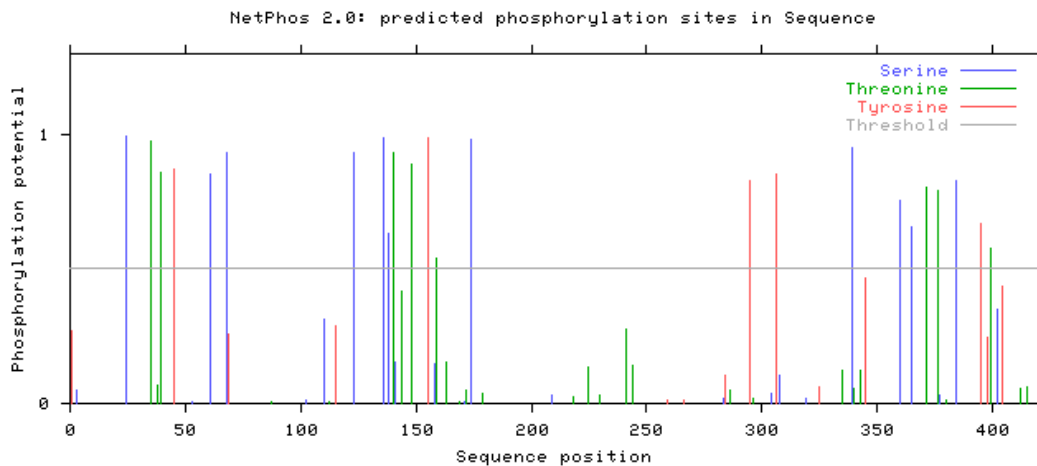


Figure 2-8. NetPhos predicted phosphorylation sites on human FIX. The threshold score used for creation of this figure is 0.5. Colored lines represent potentially phosphorylated amino acid, as indicated in the legend.

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