The N-terminal EGF Module of Coagulation factor IX. Studies of Calcium Binding and Module Interactions.

Persson, Kristina

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THE N-TERMINAL EGF MODULE OF COAGULATION FACTOR IX

Studies of calcium binding and module interactions

Kristina E. M. Persson

LUNDS UNIVERSITET

Department of Laboratory Medicine
Division of Clinical Chemistry
Lund University
University Hospital, Malmö
Sweden
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Fakultetsopponent: Professor Agneta Siegbahn,
Uppsala Universitet.

"When you have eliminated all which is impossible, then whatever remains, however improbable, must be the truth. It may well be that several explanations remain, in which case one tries test after test until one or other of them has a convincing amount of support."

Sherlock Holmes in "The Adventure of the Blanched Soldier", 1903.

Contents

List of papers

This thesis is based on studies reported in the following papers, which are referred to in the text by their Roman numerals:

I. Kristina E. M. Persson, Jan Astermark, Ingemar Björk and Johan Stenflo. 1998. Calcium binding to the first EGF-like module of human factor IX in a recombinant fragment containing residues 1-85. Mutations V46E and Q50E each manifest a negligible increase in calcium affinity. FEBS Lett. 421:100-104.

II. Kristina E. M. Persson, Karin E. Knobe and Johan Stenflo. 2001. An anti-EGF monoclonal antibody that detects intramolecular communication in factor IX. Biochem. Biophys. Res. Commun. 286:1039-1044.

III. Kristina E. M. Persson, Bruno O. Villoutreix, Ann-Marie Thämlitz, Karin E. Knobe and Johan Stenflo. 2002. The N-terminal EGF domain of coagulation factor IX: Probing its functions in the activation of factor IX and factor X with a monoclonal antibody. J. Biol. Chem. 277:35616-35624.

IV. Karin E. Knobe*, Kristina E. M. Persson*, Elsy Sjörin, Bruno O. Villoutreix, Johan Stenflo and Rolf C. R. Ljung. (*These authors contributed equally to this work). Functional studies of the EGF-like domain mutations Pro55Ser and Pro55Leu, which cause mild hemophilia B. Manuscript.

The coagulation system

Evolution of the coagulation system

The process of coagulation can be described as a protective system that has evolved to quickly stop a stream of life-supporting liquid through a damaged portion of a blood vessel. This complex entity developed from a simple by-product of the immune system, into what now comprises an advanced cascade of reactions (Krem and Di Cera 2002). The protein thrombin, which cleaves fibrinogen, is the ancestral clotting protein, but with the passing of the millennia, additional levels of regulation appeared upstream of thrombin. The efficiency of the coagulation system also generated the need for development of an anticoagulation system, to prevent uncontrolled clotting of the blood from spreading throughout the entire organism.

Initiation of coagulation

Coagulation is initiated when tissue factor (TF) is exposed on the surface of cells, for example fibroblasts (which are part of the vessel wall) or monocytes that have been activated by cytokines (Figure 1). TF binds both the zymogen factor VII (FVII) and the activated form of FVII (FVIIa), small amounts of which circulate in plasma. The bound FVII is rapidly converted to FVIIa, and the TF/FVIIa complex initiates activation of factor IX (FIX) and factor X (FX) to FIXa and FXa. Feedback amplification is achieved when TF/FVII is activated by FVIIa, FIXa, or FXa. The small amounts of FXa that are generated convert factor V (FV) to the active form FVa, and FXa and FVa together activate prothrombin to thrombin (Dahlbäck 2000).

Amplification of coagulation

Thrombin diffuses from the cell membrane of a TF-presenting cell and reaches the surface of platelets, where it binds to the thrombin receptor (PAR-1). Such interaction activates the platelets, which in turn change shape and expose phosphatidylserine on their surfaces. Phosphatidylserine

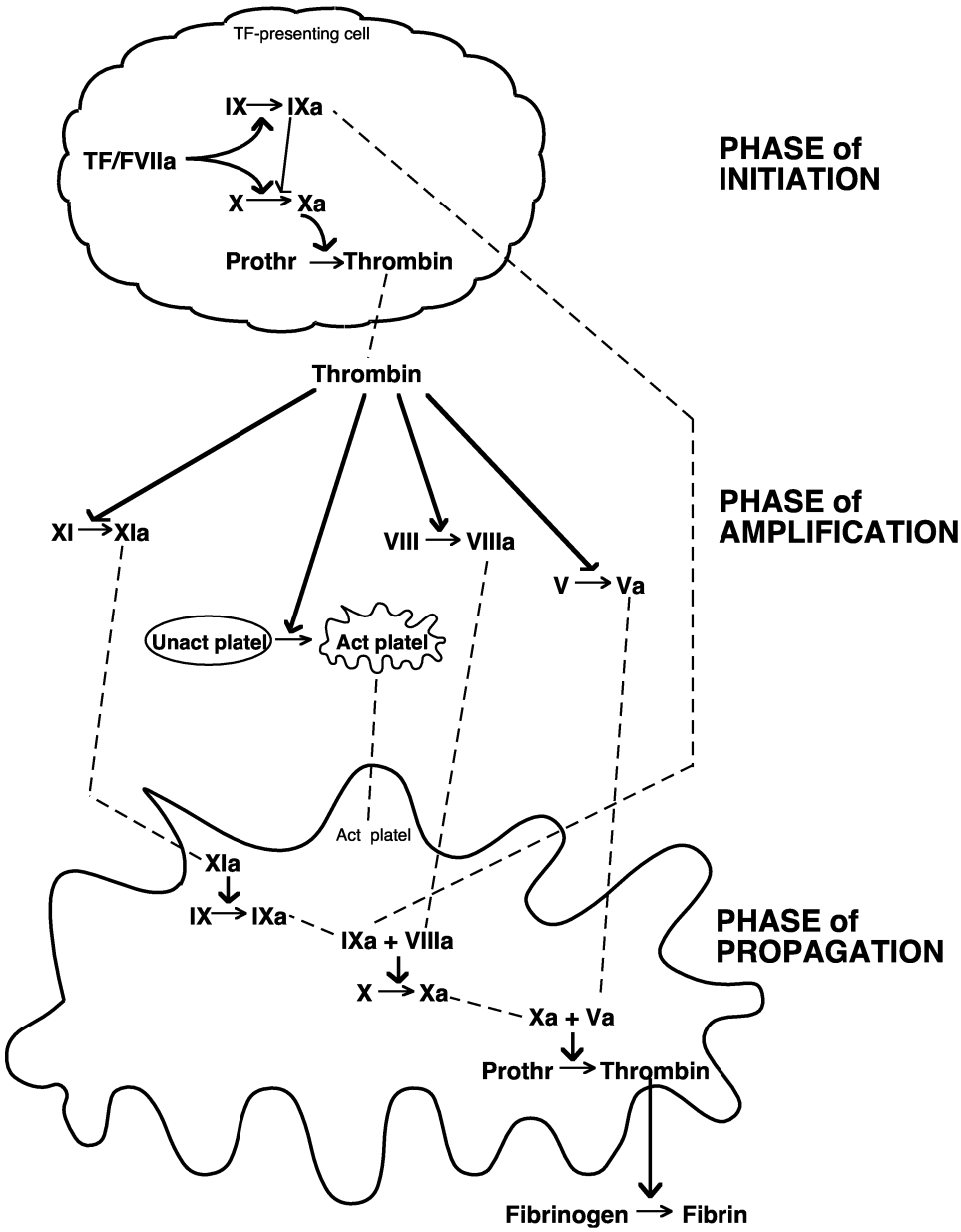


FIGURE 1. Overview of the coagulation system.

is a negatively charged phospholipid with a high potential for binding clotting factors, which results in a high local concentration of both enzymes and their substrates. When the platelet is activated, granulae containing Ca^{2+} and ADP, in addition to a number of proteins such as FV, von Willebrand factor, fibrinogen, thrombospondin and fibronectin, are exocytosed to facilitate platelet adhesion and aggregation. During amplification of the coagulation cascade, thrombin is responsible not only for activating platelets, but also for activating FV to FVa, factor VIII (FVIII) to FVIIIa, and factor XI (FXI) to FXIa (Stenflo and Dahlbäck 2001).

Propagation of coagulation

On the surface of an activated platelet, FXIa activates FIX to FIXa. Some FIXa can also diffuse directly from the TF-bearing cell to the activated platelet. FIXa binds its cofactor, FVIIIa, and activates FX to FXa. FXa can then bind its cofactor, FVa, and activate prothrombin to thrombin. Finally, thrombin cleaves fibrinogen to form fibrin. The levels of thrombin required for cleavage of fibrinogen are much higher than those required for amplification of the coagulation cascade. Thrombin also activates factor XIII (FXIII) to its active form, FXIIIa, which converts soluble fibrin to insoluble fibrin by creating covalent bonds between the different subunits of this protein. This results in a network of threads in which platelets and other cells can aggregate to plug a leaking blood vessel with a stable clot.

Regulation of the coagulation system

A number of anticoagulant mechanisms have evolved to prevent the coagulation cascade from proceeding in an uncontrolled fashion. One of the major inhibitors of coagulation is antithrombin, which circulates in plasma ready to attack free enzymes (complex-bound enzymes are less accessible to inhibition). Antithrombin inhibits thrombin, FIXa, FXa, and FXIa by binding to these molecules in a process that is catalyzed by the presence of heparan sulfate on the surface of endothelial cells. Another

inhibitor of coagulation is tissue factor pathway inhibitor (TFPI), which binds both FXa and the TF/FVIIa complex, resulting in inactivation of the TF/FVIIa pathway.

Although thrombin is an integral part of the coagulation cascade, it turns into an anticoagulant when it binds to thrombomodulin that is attached to the membrane of intact endothelial cells. When bound to thrombomodulin, thrombin can no longer cleave fibrinogen, but instead activates protein C, which, supported by its cofactor protein S, inactivates FVa and FVIIIa, resulting in inhibition of the coagulation cascade (Dahlbäck and Stenflo 2001).

The balance between coagulation and anticoagulation is carefully regulated. Inherited or acquired factors can disturb this balance, resulting in a predisposition for thrombosis when there is a deficiency of anticoagulant proteins, and hemophilia when there is a lack of coagulation factors such as FVIII or FIX.

FIX in hemophilia and thrombosis

Deficiency of FIX causes hemophilia B, the second-most common form of inherited hemophilia. Hemophilia B and hemophilia A were distinguished as two separate conditions in 1947, when it was found that mixing blood from a patient suffering from severe hemophilia with blood from another hemophilia patient resulted in normal clotting time (Pavlovsky 1947). Hemophilia B is an X-chromosome-linked disorder with a prevalence of one per 70 000 males, and only isolated cases of hemophilia have been described in women (Sadler 2001). Almost 400 different amino acid substitutions in FIX have been reported to cause hemophilia B (Giannelli et al. 1998). The symptoms of this disease range from lethal bleeds to nothing more than prolonged clotting time. Hemophilia B is classified as severe (<1%), moderate (1–5%), or mild (6–25%), according to the percent of FIX activity relative to that found in normal plasma. More than 50% of hemophilia B patients have no detectable FIX antigen.

One unit of FIX activity is defined as the amount detected in 1 mL of pooled normal plasma. Clearance of FIX has been investigated using FIX injected into hemophilia B patients; the half-life was found to be

approximately 18 hours, with a slightly decreased initial recovery of recombinant FIX compared to plasma-derived FIX (Sadler 2001). The half-life of free FIXa in plasma is very short: about two minutes after injection, more than half of this protein is bound to antithrombin. Over the past few decades, treatment with daily injections of recombinant FIX has been a panacea to patients with hemophilia B. However, a few percent of the treated patients develop antibodies against FIX and cannot be given replacement therapy. These individuals bleed severely following trauma, and they can also suffer from recurrent spontaneous bleeding that can lead to lethal intra-cranial hemorrhage or complications such as crippling joint deformities.

The risk of thrombosis has been found to be increased two- to three-fold in patients with elevated levels of FIX (van Hylckama et al. 2000). Active-site inhibited FIXa has been shown to prevent thrombosis in experimental animal models (Benedict et al. 1991). In addition, it has been reported that an anti-FIX/FIXa monoclonal antibody can be used to treat thrombotic stroke in rats, and this might also prove to be effective in humans (Toomey et al. 2002).

FIX — the molecule

FIX is a 57-kDa glycoprotein composed of 415 amino acid residues, and it is synthesized mainly in the liver and circulates in plasma at a concentration of 4 mg/L (Figure 2) (Stenflo and Dahlbäck 2001). A signal peptide directs the protein for secretion, and a propeptide functions as a recognition element for a vitamin K-dependent carboxylase. Both the signal peptide and the propeptide are cleaved off the FIX molecule before it enters the plasma. The carboxylase catalyzes the conversion of 12 glutamic acid residues to γ-carboxyglutamic acid (Gla) in the N-terminal part of FIX, resulting in the formation of the Gla module (residues 1–47), which is required for the Ca^{2+}-dependent binding of FIX and FIXa to phospholipid membranes. Not only FIX, but also FVII, FX, prothrombin, protein C, and protein S contain Gla modules, which direct the coagulation cascade to the surface of membranes.

The Gla module in FIX is followed by two epidermal growth factor (EGF)-like modules, the N-terminal (EGF1) of which binds one Ca^{2+} ion. The C-terminal part of EGF1 seems to interact with the C-terminal EGF module (EGF2) via hydrophobic interactions and a salt bridge (Brandstetter et al. 1995; Christophe et al. 1998; Celie et al. 2000). EGF2 is in close contact with, and is probably required for proper orientation and folding of, the serine protease region. FVII, FX, and protein C are homologous to FIX and have the same modular construction, comprising one Gla module, two EGF modules, and a serine protease region.

During conversion of FIX to FIXa, a 35-amino-acid activation peptide that precedes the serine protease region is cleaved off, and the resultant heterodimer is held together by a disulfide bond. FIX is first cleaved at Arg145 (when activation is effected by TF/FVIIa or FXIa), which generates a catalytically inactive two-chain form called FIXα (Lindquist et al. 1978). A second cleavage at Arg180 is rate-limiting and results in the active form of FIX, designated FIXaβ (commonly referred to as FIXa). If activation is instead achieved using RVV-X, a protease from the venom of Russell's viper, FIX is first cleaved at Arg180, generating FIXaα, which has only 50% of the activity of FIXaβ. The active site of the serine protease

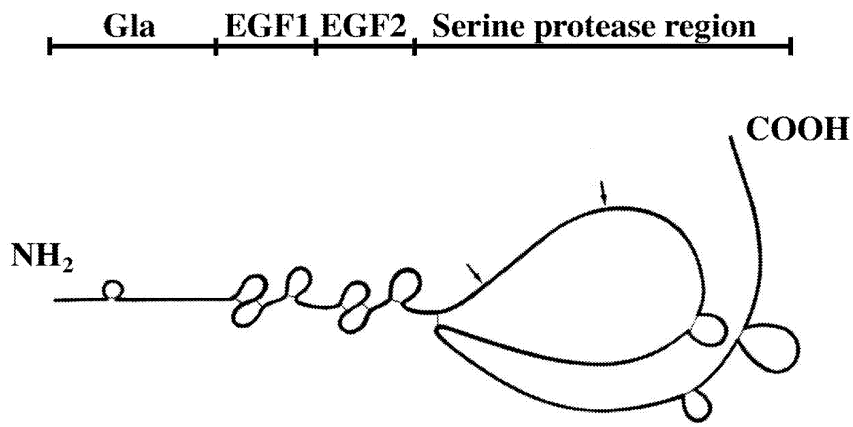


FIGURE 2. Schematic drawing of FIX. The arrows indicate the sites that are cleaved upon activation of FIX to FIXa.

FIXa comprises the catalytic triad His221, Asp269 and Ser365. Cleavage of the Arg180–Val181 peptide bond allows the new amino-terminal Val residue to form a salt bridge with Asp364, which is adjacent to the active site Ser. After FIX is activated, it can interact with its cofactor, FVIIIa, to form a membrane-bound macromolecular complex called Xase, which activates FX to FXa.

About 20% of the mass of FIX is carbohydrate (Sadler 2001). The activation peptide contains two N-glycosylation sites, one at Asn157 and the other at Asn167, and, in 35% of FIX molecules, O-linked oligosaccharides are attached to Thr159, Thr169, and Thr172. In EGF1, there is an O-linked oligosaccharide (Xyl-Glc) bound to Ser53 and a tetrasaccharide (Gal-Fuc-GlcNAc-NeuAc) linked to Ser61 (Nishimura et al. 1989, 1992). Other posttranslational modifications of FIX include sulfation at Tyr155 and phosphorylation at Ser158. The functions of these modifications are to a large extent unknown, although they have been reported to affect the recovery of FIX and may also influence the half-life of FIX (Arruda et al. 2001).

The structure of porcine FIXa has been determined by X-ray crystallography, which revealed that FIXa is an extended molecule, with the Gla module at one end and the serine protease region at the other (Brandstetter et al. 1995). In agreement with that, the active site of human FIXa has been found to be located more than 70 Å above the phospholipid surface (Mutucumarana et al. 1992). Binding of FVIIIa to membrane-bound FIXa does not alter the location of the active site relative to the membrane surface, but it does appear to induce a conformational change in the active site. The structure of part of the human FIXa molecule has also been determined (EGF2 and the serine protease region), as well as the isolated Gla and isolated EGF1 modules, and these structures were observed to be similar to their porcine counterparts (Hopfner et al. 1999; Freedman et al. 1995; Rao et al. 1995). The structure of FVIIa (in complex with TF) has also been determined and as for FIXa, it was found to be elongated, with the serine protease region remote from the membrane surface (Banner et al. 1996). Positioning of the active sites of FVIIa and FIX at the same distance above the membrane, and also the active sites of FIXa and FX equidistant to the membrane, probably makes it easier for the proteases to interact with each other. Computer models of the structures of the zymogens of FIX, FX

and FVII have been constructed, and they also predict extended structures (Perera et al. 2001, 2002; Venkateswarlu et al. 2002).

FIX binds to thrombin-activated platelets, and it has been shown that EGF1 is not directly involved in this interaction (Ahmad et al. 1992). FIX has also been shown to bind to collagen 4 on endothelial cells and this interaction, which is reversible, is mediated by the Gla module (Cheung et al. 1992, 1996; Wolberg et al. 1997). The physiological implication of this binding is not yet known, but it has been suggested that it may have a function in the assembly of activated coagulation factors since FIXa binds to the endothelial surface with a 20-fold higher affinity than FIX does (Stern et al. 1985).

FIX — the gene

The gene for human FIX is 33.5 kb in length and is located near the terminus of the long arm of the X-chromosome (Yoshitake et al. 1985; Sadler 2001). Eight exons (I–VIII) are separated by seven introns A–G (Figure 3) and the positions of the introns in the FIX gene are similar to those of the homologous genes encoding FVII, FX, and protein C. The introns divide the FIX gene into sections that correspond to the different structural regions of the FIX protein. In FIX, exon I encodes the signal peptide, exon II codes for the propeptide and Gla module, exon III for the helix region, exon IV for EGF1 (amino acids 47–84), exon V for EGF2 (amino acids 85–127), and exons VI–VIII encode the serine protease region.

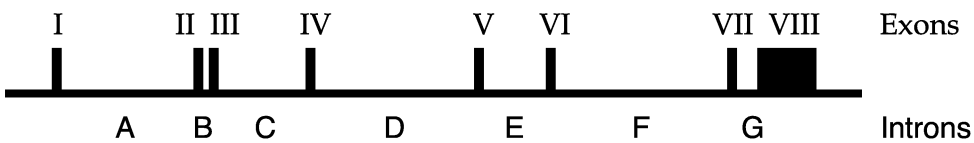


FIGURE 3. Map of the FIX gene. The part of the X-chromosome that contains the gene for FIX is illustrated, with the exons numbered I–VIII and introns designated A–G.

The structure, function, and evolution of EGF modules

The EGF modules of FIX are homologous to EGF itself, which is cleaved off from a membrane-bound precursor containing nine EGF modules. EGF itself contains 53 amino acids, it is located in the C-terminal end of the precursor, and it can bind to the EGF receptor and induce an intracellular tyrosine kinase-mediated response (Carpenter and Cohen 1990). EGF modules, which in most cases do not bind to the EGF receptor, are widely distributed in extracellular and membrane proteins that are associated with blood coagulation, fibrinolysis, neural development and cell adhesion. They have been found not only in humans, but also in animals, viruses and protozoa (Table 1). Sometimes they occur as single modules, sometimes there are several in a row, and sometimes other modules are inserted between the EGF modules. The grouping of multiple consecutive copies of a module has also been observed for other types of modules, and suggests a preference for further duplications of the module. The term "module" was proposed by Patthy to describe a type of genetically autonomous domain that has been shuffled and rearranged in several ways during evolution (Patthy 1985). The exons that have been shuffled in the vitamin K-dependent proteins, as well as in related proteins, are surrounded by introns of phase class 1 (i.e., those situated between the first and second nucleotide of a codon) (Patthy 1987). It has been suggested that the observed preference for phase 1 introns between modules arose in the ancestral protease when a phase 1 intron of the exon encoding the signal peptide was separated from that encoding the serine protease region and the phase 1 intron may have served as the recipient for incoming exons with class 1 splice junctions on either side.

An EGF module usually contains around 40–50 amino acids, including six cysteine residues with the same characteristic pairing as in EGF itself: 1-3, 2-4, and 5-6. The structure of the EGF modules is dominated by β-sheets and β-turns, and, owing to the disulfide bonds, the EGF modules are quite stable as long as they are not exposed to reducing agents. FIX and the homologues FVII, FX and protein C each contain two EGF modules, whereas protein S contains four. The structures of the EGF1 modules of FIX and FX have been determined by NMR spectroscopy and

TABLE 1. Examples of proteins containing EGF modules.

	Number of EGF modules	*Reference*
Human proteins:		
Factor IX	2	(Yoshitake et al. 1985)
Factor VII	2	(O'Hara et al. 1987)
Factor X	2	(Leytus et al. 1986)
Protein C	2	(Foster et al. 1985)
Protein Z	2	(Fujimaki et al. 1998)
Protein S	4	(Edenbrandt et al. 1990; Ploos van Amstel et al. 1990; Schmidel et al. 1990)
Gas6	4	(Manfioletti et al. 1993)
EGF precursor	9	(Bell et al. 1986)
TGF-α	1	(Derynck et al. 1984)
Heparin-binding growth factor	1	(Higashiyama et al. 1991)
Amphiregulin	1	(Plowman et al. 1990)
Urokinase-type plasminogen activator	1	(Riccio et al. 1985)
Tissue-type plasminogen activator	1	(Ny et al. 1984)
C1r	1	(Arlaud et al. 1987)
C1s	1	(Tosi et al. 1987)
Low density lipoprotein (LDL) receptor	3	(Yamamoto et al. 1984)
LDL receptor-related protein (LRP)	22	(Herz et al. 1988)
Thrombomodulin	6	(Jackman et al. 1987)
TAN-1	36	(Ellisen et al. 1991)
Nidogen	6	(Nagayoshi et al. 1989)
Tenascin	14	(Nies et al. 1991)
Thrombospondin	3	(Lawler and Hynes 1986)
Granule membrane protein 140	1	(Johnston et al. 1989)

Fibrillin	47	(Maslen et al. 1991)
Fibulin-2	10	(Zhang et al. 1994)
Uromodulin	3	(Pennica et al. 1987)
Core protein of large cartilage-specific proteoglycan	1	(Baldwin et al. 1989)
Lymph node homing receptor core protein	1	(Siegelman and Weissman 1989)
Endothelial leucocyte adhesion molecule 1 (ELAM 1)	1	(Bevilacqua et al. 1989)
Tie (endothelial cell surface receptor tyrosine kinase)	3	(Partanen et al. 1992)
FXII	2	(Cool et al. 1985)
Non-human proteins:		
Vaccinia virus growth factor	1	(Brown et al. 1985)
Shope fibroma virus growth factor	1	(Chang et al. 1987)
Myxoma virus growth factor	1	(Upton et al. 1987)
Notch (*Drosophila melanogaster*)	36	(Wharton et al. 1985)
Delta (*D. melanogaster*)	9	(Vässin et al. 1987)
Slit (*D. melanogaster*)	7	(Rothberg et al. 1988)
Crums (*D. melanogaster*)	29	(Tepass et al. 1990)
Serrate (*D. melanogaster*)	14	(Fleming et al. 1990)
Spitz (*D. melanogaster*)	1	(Rutledge et al. 1992)
Lin-12 (*Caenorhabditis elegans*)	13	(Yochem et al. 1988)
Lin-3 (*C. elegans*)	1	(Hill and Sternberg 1992)
Factor C (*Limulus*)	1	(Muta et al. 1991)
Merozoite surface protein 1 (*Plasmodium falciparum*)	2	(Hall et al. 1984)
Merozoite surface protein 4 (*P. falciparum*)	1	(Marshall et al. 1997)
Merozoite surface protein 8 (*P. falciparum*)	2	(Black et al. 2001)
Pypag-2 (*P. yoelii*)	2	(Burns et al. 2000)

X-ray crystallography; overall the structures resemble EGF itself (Baron et al. 1992; Selander Sunnerhagen et al. 1992; Rao et al. 1995; Cooke et al. 1987; Campbell and Bork 1993).

Hundreds of different EGF modules have been described in the literature, but the function or functions of many of them are still unknown. In some cases, the modules have been reported to participate in protein-protein interactions, for example, in the EGF module-mediated interaction between urokinase and its receptor, and in the interaction between FVIIa and TF, and that between thrombomodulin and thrombin (Appella et al. 1987; Banner et al. 1996; Suzuki et al. 1989). It has also been proposed that EGF modules function as spacers that position the active site of a serine protease at a distance above the phospholipid membrane that will allow interaction with cofactors and substrates. Some EGF modules bind Ca^{2+}, and such metal ion binding may be a means of orienting an adjacent module to render it biologically active. Several kinds of post-translational modifications are apparent in EGF modules, β-hydroxylation (see below) and glycosylations, the functions of which have not been clarified.

The significance of Ca^{2+}

Regulation of levels of ionic Ca^{2+} is fundamental for the existence of the human organism. The concentration of ionic Ca^{2+} in extracellular fluid is 1.15–1.35 mM, which is at least 10 000-fold higher than the resting level of intracellular Ca^{2+} in most cells. In adult humans, more than 99% of total Ca^{2+} is found in bone and teeth, 0.1% occurs in extracellular fluids and blood, and only a small fraction is present in the cytosol of cells (Maurer et al. 1996). The concentration of extracellular Ca^{2+} is carefully controlled, and deviations can result in serious conditions, such as convulsive seizures and arrhythmia of the heart. Since the Ca^{2+} concentration is relatively high in extracellular fluids, once secreted, the coagulation proteins would contain bound Ca^{2+} at a constant degree of saturation, as dictated by the equilibrium constants of the proteins, and Ca^{2+} could be assumed to play only a static role. However, local decreases in Ca^{2+} concentration have been shown in, for example, cerebrospinal fluid, where the concentration can drop to around 0.1 mM during normal neuronal activities and in connection with

pathological conditions such as chronic epilepsy and trauma. Also, experiments *in vivo* have shown that, during the formation of a thrombus, platelet masses become anoxic and depolarize to yield interstitial cation conditions characteristic of the more voluminous platelet cytosol, with extracellular levels of Ca^{2+} falling below 0.2 mM (Owen et al. 1995). In experiments *in vitro*, the cited investigators found that disintegration of a thrombus caused levels of Ca^{2+} to rise to those seen under normal extracellular conditions. This capability of changing the levels of extracellular Ca^{2+} in the microenvironment of platelets provides a physiological basis for the evolution of a hemostatic system that is regulated by the concentration of Ca^{2+}, with a possibility of activation and inactivation of coagulation factors in response to alteration of local levels of Ca^{2+}.

Binding of Ca^{2+} to the Gla module and serine protease region of FIX

The Gla module of FIX binds about nine Ca^{2+} ions (Freedman et al. 1995; Li et al. 1997). Considering FX, the Gla module has a structure similar to that of the Gla of FIX, and it has been shown that the Gla residues are exposed to solvent in the Ca^{2+}-free form of FX, whereas, in Ca^{2+}-loaded FX, the remote Gla residues are interlinked and the N-terminal part of the module is turned inside out so that the hydrophobic residues are exposed (Sunnerhagen et al. 1995, 1996; Stenflo 1999). Binding of Ca^{2+} to the Gla module is necessary for interaction with phospholipid membranes. In the presence of vitamin K antagonists such as warfarin, there is decreased γ-carboxylation of the Glu residues, and the proteins cannot bind Ca^{2+} and they have no biological activity (Stenflo and Dahlbäck 2001). For most of the coagulation factors, the average K_D for binding of Ca^{2+} to the Gla modules is 0.5–0.7 mM (Stenflo 1999).

In the serine protease region of FIX, only one Ca^{2+} is bound with a K_D of around 300 μM (Bajaj et al. 1992). The Ca^{2+}-binding site is probably formed by the carboxyl groups of glutamates 235 and 245 and the main chain carbonyl oxygen atoms of residues 237 and 240 (Hamaguchi and Stafford 1994). A similar binding site for Ca^{2+} exists in other serine

proteases involved in coagulation, such as FVII and FX (Stenflo and Dahlbäck 2001).

Binding of Ca^{2+} to EGF modules

One Ca^{2+} is bound to the N-terminal part of about 25% of the EGF modules that have been described thus far. The Ca^{2+}-binding EGF modules have the same general fold and pairing of disulfide bonds as seen in the non-Ca^{2+}-binding modules. Ca^{2+} has been shown to be important for stabilizing the orientation of the surrounding EGF modules; an example of this is fibrillin (which contains numerous EGF modules), which is shaped like a rod in the Ca^{2+}-loaded state, but is coiled like a snake when it is free of Ca^{2+} (Cardy and Handford 1998). Mutations in the EGF modules of fibrillin cause a degenerative connective tissue disorder known as Marfan syndrome.

The consensus sequence for binding of Ca^{2+} to EGF modules is Asp/Asn-Xxx-Asp/Asn-Glu/Gln-Xxx_m-Asp/Asn*-Xxx_n-Tyr/Phe (where m and n are variables and * indicates possible β-hydroxylation) (Downing et al. 2000). The Ca^{2+} ion is coordinated by a pentagonal bipyramidal arrangement of oxygen atoms, six of which are provided by intra-module ligands. In FVII, FIX, FX, and protein C, the beginning of the sequence is Asp-Gly-Asp-Gln, and conservation of the Gly residue seems to be important, because exchange of Gly for Val in FIX results in hemophilia B (Giannelli et al. 1998). The EGF1 module in FVII, FIX, FX, and protein C, and the EGF modules 2, 3, and 4 in protein S each bind one Ca^{2+}. These modules have Asp or Asn at the residue corresponding to position 64 in FIX (Figure 4), and this residue is β-hydroxylated to form *erythro*-β-hydroxyaspartic acid (Hya; in EGF1 of FIX, FX, protein C, and protein S) or *erythro*-β-hydroxyasparagine (Hyn; in EGFs 2, 3, and 4 in protein S). The consensus sequence for β-hydroxylation is Cys-Xxx-Asp/Asn-Xxx-Xxx-Xxx-Xxx-Tyr/Phe-Xxx-Cys-Xxx-Cys (Stenflo et al. 1998). The extent of β-hydroxylation is often partial (30% in FIX), and in FVII, hydroxylation does not occur despite the presence of the consensus sequence. This indicates that the consensus sequence alone is insufficient to direct hydroxylation (Stenflo and Dahlbäck 2001). β-hydroxylation is not necessary for binding of Ca^{2+}, and its function in the coagulation factors is

unclear (Selander Sunnerhagen et al. 1993). The gene for β-hydroxylase has been knocked out in mice, which resulted in developmental defects, presumably due to an effect on members of the Notch gene family members, but did not lead to any apparent defects in the coagulation system (Dinchuk et al. 2002). However, mutation of residue 64 in FIX results in a decreased affinity for Ca^{2+} and hemophilia, and there are also reports of other mutations in the Ca^{2+}-binding area of EGF1 that result in hemophilia (Giannelli et al. 1998).

In EGF1 of FIX, incorporation of the single Ca^{2+} occurs through backbone binding to residues Gly48 and Asp65, and side-chain binding to residues Asp47, Gln50, and Hya64 (Rao et al. 1995). The carboxyl group of Hya64 contributes two ligands for binding of Ca^{2+}, whereas the β-hydroxyl group does not participate in this binding. Instead, binding of Ca^{2+} causes the hydroxyl group to point away from the Ca^{2+} ion. In addition to these six Ca^{2+} ligands, in the crystal structure, a seventh ligand is contributed from an

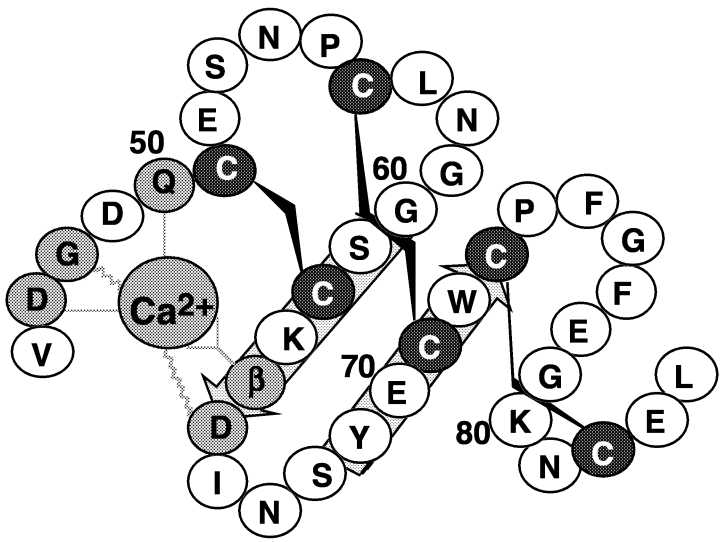


FIGURE 4. Schematic drawing of EGF1 of FIX. Ca^{2+}-binding residues are shaded, the straight lines represent binding of side-chain ligands to the Ca^{2+} ion, and the zigzag lines represent backbone binding.

adjacent EGF module (Asn58). Packing of the modules during formation of the crystal structure probably leads to replacement of a water molecule to allow interaction with the adjacent module, and it has been speculated that this kind of displacement of water from Ca^{2+}-binding EGF modules might play a general role in protein-protein interactions (Downing et al. 2000). It is plausible that the water molecule is replaced by other coagulation proteins during the formation of complexes on the surface of phospholipid.

The Ca^{2+}-binding site in EGF1 of FIX is specific for Ca^{2+}, hence it cannot bind ions such as Mg^{2+} (Astermark et al. 1991). Isolated, synthetic EGF1 of FIX binds Ca^{2+} with a K_D of 1.8 mM, which is comparable to corresponding values for other single EGF modules (Handford et al. 1991). The affinity for Ca^{2+} often increases when an EGF module is attached to other EGF modules; this is the case for EGF4 of protein S, which has a K_D of 9 mM for the single module, and 20 nM when it is connected to EGFs 1–3 (Stenberg et al. 1997a, 1997b). Sunnerhagen et al. (1996) have determined the structure of the Gla-EGF1 module pair from bovine FX (Sunnerhagen et al. 1996). Although the structures of the individual modules were well defined, the structure of the module pair was poorly defined in the absence of Ca^{2+}, due to variation in the orientation of one module relative to the other; the inter-module region appeared to function as a flexible hinge. Isolated EGF1 bound Ca^{2+} with a K_D of 10^{-3} M, but, when linked to the Gla module, the affinity increased 10-fold. Also, binding of Ca^{2+} to EGF1 fixed the two modules in a position in which they were folded towards one another.

The function of EGF1 in FIX

The importance of EGF1 in FIX is obvious when considering that a large number of mutations spread throughout this module are known to cause from mild to severe hemophilia (Giannelli et al. 1998). Nevertheless, it is not clear whether EGF1 is directly involved in protein-protein interactions. One approach to study the function of EGF1 has been to construct hybrids of different coagulation proteins. Zhong and coworkers (1994) replaced the EGF1 of FIX with EGF1 from protein C and found that this exchange did not affect activation of FIX by FXIa, but it did impair the

activation of FIX induced by TF/FVIIa. In line with this, it has been suggested that EGF1 of FIX interacts with TF during activation of FIX (Zhong et al. 2002). However, earlier experiments had shown that exchange of EGF1 in FIX for EGF1 from FX or FVII did not affect activation by either FXIa or TF/FVIIa (Lin et al. 1990; Chang et al. 1997). Moreover, contradictory results have been obtained by two groups studying the effect of the mutation Gln50Pro in EGF1 of FIX: FXIa-induced activation of FIX Gln50Pro was found to be slow by Lozier et al. (1990) but occurred at a normal pace in experiments by Zhong et al. (1994). In summary, results in the literature are inconclusive as to whether EGF1 of FIX is directly involved in interactions with FXIa or TF/FVIIa.

Concerning the role of FIX EGF1 in the activation of FX, it has been debated whether EGF1 interacts directly with FVIIIa in the Xase complex, or if it merely positions the serine protease region at the correct distance above the membrane surface (Lenting et al. 1996; Celie et al. 2000). Three-dimensional structures of FIXa and molecular models of FVIIIa (based on its homology with ceruloplasmin) have allowed preliminary models of the FIXa-FVIIIa complex to be constructed (Stoilova-McPhie et al. 2002; Celie et al. 2002). These models take into consideration the well established findings that the Gla module of FIXa and the C2 domain of FVIIIa interact with biological membranes, and they also incorporate evidence from site-directed mutagenesis indicating that the helix formed by residues 330–338 of the serine protease region interacts with the A2 domain of FVIIIa (Mathur et al. 1999; Kolkman et al. 1999). However, according to data in the literature, it is not clear whether EGF1 takes part in this interaction. For instance, Lin et al. (1990) found that recombinant mosaic FIXa, with EGF1 substituted for the corresponding module of FX, possessed full biological activity, whereas Lenting and coworkers (1996) observed that binding of Ca^{2+} to EGF1 of FIX promoted association with the FVIIIa light chain and also enhanced amidolytic activity and activation of FX. Moreover, other researchers have found that replacing the EGF1 of FIX with the N-terminal EGF module of FVII increased the affinity for FVIIIa, as judged by measuring activation of FX (Chang et al. 1997). In conclusion, the role of EGF1 of FIX in the activation of FX is unclear.

FIX is cleaved by chymotrypsin and granulocyte elastase but the physiological significance of this event is not known (Enfield et al. 1984;

Takaki et al. 1983). It is yet to be elucidated whether EGF1 of FIX takes a direct part in interactions with these enzymes.

Low-density lipoprotein receptor-related protein (LRP) contains 22 EGF modules and has been shown to contribute to binding and transport of FVIIIa and FIXa to the intracellular degradation pathway (Neels et al. 2000). FIX and vWF-bound FVIII do not bind to LRP. Multiple sites within FVIIIa allow this coagulation factor to interact with LRP, but studies have not yet been performed to ascertain what parts of FIXa are important in this context. It can only be speculated that the EGF modules of FIXa may play a part in this interaction.

The present investigations

Studies of binding of Ca^{2+} to EGF1 in FIX (Paper I)

Several mutations in the Ca^{2+}-binding area of EGF1 have been shown to cause hemophilia B in patients and therefore it is of interest to study this region. The Ca^{2+} affinity of the isolated EGF module is low both for FIX (human; $K_D \approx 1.8$ mM) and FX (bovine; $K_D \approx 1$ mM) (Handford et al. 1991; Persson et al. 1989). In bovine FX, the affinity for Ca^{2+} is augmented approximately 20-fold when the EGF module is linked to the Gla module and this increase does not require the Glu residues to be carboxylated to Gla (Valcarce et al. 1993). The objective of the study reported in Paper I was to investigate whether the Gla module has the same effect on the Ca^{2+}-affinity of the EGF module in human FIX as in bovine FX. We therefore expressed the Gla*-EGF fragment (the asterisk indicates that Glu is present instead of Gla) fragment containing residues 1–85 of FIX in a baculovirus system and then used fluorescence spectroscopy to measure the affinity for Ca^{2+} of the site in the EGF module. Due to the presence of three tryptophan residues in the fragment, the intrinsic fluorescence of the protein could be used to detect binding of Ca^{2+}. Ca^{2+} -titration of the fragment revealed enhanced fluorescence at micromolar concentrations of Ca^{2+} and a K_D of 160 (± 40) μM for the wild-type fragment, which indicates an approximately 10-fold increase in the affinity for Ca^{2+} compared to the isolated EGF1 module. This means that the site is essentially saturated at the free Ca^{2+} concentrations found in extracellular fluids such as blood plasma. Thus it appears that human FIX does behave like bovine FX in this respect. Other investigators have found that, in the absence of Ca^{2+}, both the Gla module and the EGF module in FX are folded to characteristic structures (Sunnerhagen et al. 1996). However, these two modules are very mobile in relation to each other. In both FIX and FX, the hinge between the Gla and EGF1 modules is probably locked into position by the binding of Ca^{2+} to EGF1. Thus, the low biological activity exhibited by FIX molecules bearing mutations that affect the Ca^{2+} ligands in the EGF module is presumably due to increased mobility between the Gla and EGF modules.

Both FIX and FX have a Gln residue N-terminal to the first Cys in EGF1 (position 50 in human FIX), whereas most other EGF modules have a

Glu residue in that position. The fourth EGF module in protein S, which binds Ca^{2+} with very high affinity (a nanomolar K_D at physiological ionic strength), also has a Glu residue instead of Val at position 46 as in FIX. To investigate whether the affinity of FIX EGF1 for Ca^{2+} could be increased (and in that way explain the high affinity of protein S for Ca^{2+}), we expressed Gla*-EGF fragments with either a Gln50Glu or a Val46Glu mutation and measured the Ca^{2+} affinity of the site in the EGF module. This also served as a preparatory step for subsequent studies of the effect of such mutations on the biological activity of FIX. The two mutated fragments showed a negligible increase in affinity of Ca^{2+}, with K_D values of 90 (± 40) μM for the Gln50Glu mutation and 90 (± 40) μM for the Val46Glu mutation. The experimental error in these measurements was quite large due to the small change in fluorescence, but the increase in fluorescence was nevertheless clearly reproducible. Interestingly, substitution of Val46 for Glu also seemed to produce an increase in the affinity for Ca^{2+}, even though this residue is not considered to be a direct ligand for Ca^{2+}. The maximum fractional increase in fluorescence intensity at Ca^{2+} saturation was larger for the Gln50Glu fragment (0.027 ± 0.01) than for the wild-type (0.016 ± 0.009) or Val46Glu (0.016 ± 0.006) fragments, though the reason for this was not apparent. Inasmuch as the mutated FIX fragments showed such small changes in affinity for Ca^{2+}, factors other than the nature of the amino acid at the mutated positions (possibly the influence of neighboring EGF modules) may explain the very high affinity for Ca^{2+} displayed by the EGF modules of protein S.

Characterization of an anti-EGF1 monoclonal antibody and detection of intramolecular communication between EGF1 and the serine protease region in FIX (Papers II and III)

It has been suggested that the two EGF modules in FIX are interlinked via hydrophobic interactions and a salt bridge (Christophe et al. 1998; Celie et al. 2000). EGF2 has a large interface oriented towards the serine protease region and this EGF module is probably needed for correct folding of the serine protease region. The tight inter-module contacts in FIX are presumably a prerequisite for the existence of allosteric linkage, as

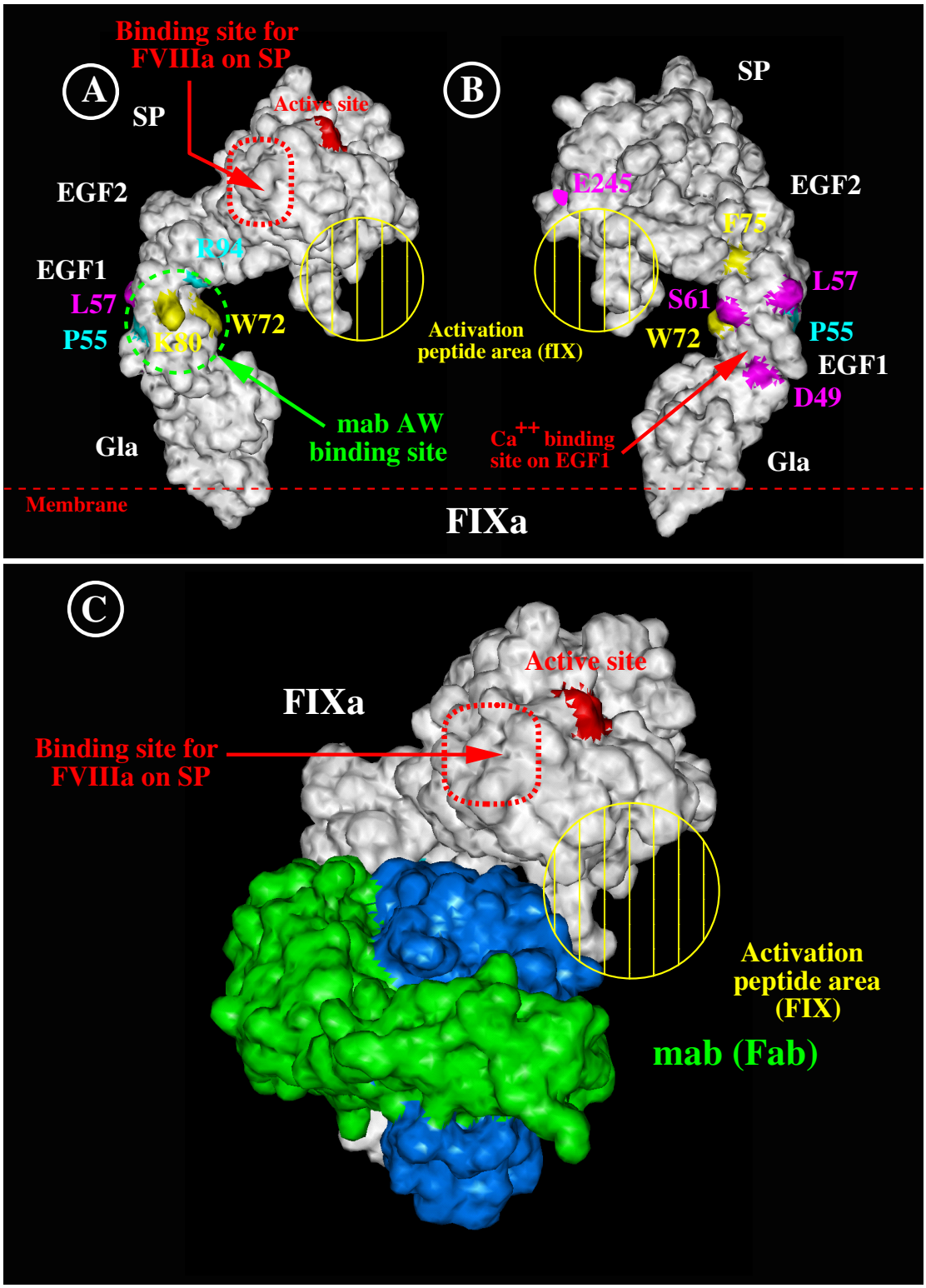
suggested by the observation that binding of Ca^{2+} to the N-terminal EGF module affects the amidolytic activity of FIXa. Such linkage between the serine protease region and the N-terminal EGF module has also been found in FVII (Leonard et al. 2000). We have now characterized a monoclonal antibody (mab), designated AW, that recognizes an epitope in EGF1 of FIX and used it to study intramolecular communication in FIX.

To begin with, we identified the epitope recognized by the mab. This non-Ca^{2+}-dependent antibody did not bind to bovine FIX, the EGF1 of which differs from human FIX EGF1 at only four positions (the bovine residues are Met61, Gln74, Ala75, and Thr80). By using mutated recombinant full-length FIX or synthetic EGF modules, we were able to more precisely pinpoint the epitope. We chose mutations that are either known to cause hemophilia B or could be useful to probe the different sides of EGF1. The following residues were substituted: in recombinant FIX, Asp49Glu and Pro55Ser; in the synthetic EGF modules, Leu57Gln, Ser61Met, Trp72Phe, Phe75Ala, and Lys80Thr. We also produced a recombinant Arg94Asp mutation, since binding of the mab would give an indication of the importance of the area of interaction between EGF1 and EGF2. In addition, we generated a Glu245Lys mutation in the Ca^{2+}-binding loop of the serine protease region because we wanted to investigate whether the mab could interact with an area close to the activation peptide. We used surface plasmon resonance and ELISA to investigate the affinities of the mutated proteins for the mab, and the results showed that Asp49Glu, Leu57Gln, Ser61Met, and Glu245Lys had the same affinity for the mab as the wild-type protein did. The mutants Pro55Ser and Arg94Asp exhibited slightly lower affinity and Trp72Phe, Phe75Ala, and Lys80Thr showed very low affinity for the mab. Taken together, these findings demonstrate that the epitope recognized by the mab is located in the C-terminal part of EGF1, which agrees with the fact that the antibody is not dependent on Ca^{2+}. Since it has been postulated that an essential salt bridge exists between Glu78 and Arg94, we expected the interaction between FIX and the mab to be markedly impaired by the Arg94Asp mutation, which is next to the area containing the epitope recognized by the antibody. However, as mentioned, the mab showed only slightly reduced affinity for the Arg94Asp protein compared to wild-type FIX, and this mutation has no effect on clotting, therefore we believe that the salt bridge between Glu78 and Arg94 is not

required for proper orientation of the EGF modules. Indeed, other interactions in this area are probably sufficient to maintain the structure of FIX. The finding that the Glu245Lys protein had the same affinity for the mab as the wild-type protein also indicates that this antibody can bind to FIX without being in direct contact with the activation peptide.

To visualize binding of the mab, we created a model of FIXa bound to a Fab fragment of the mab (Figure 5). In isolated EGF1, residues 72, 75, and 80 were part of the epitope recognized by the mab. However, in the model of full-length FIXa, it can be seen that residue 75 is located on the same side of EGF1 as residues 49, 57, and 61 (the Ca^{2+}-binding area), whereas residues 55, 72, 80, and 94 are located on the opposite side of this module. The reduced binding of EGF1 Phe75Ala may have occurred because Phe75 is fully exposed in the isolated module but is partially covered by EGF2 in full-length FIX. Along this line of reasoning, it should be noted that Ser53 and Ser61 are O-glycosylated in FIX, whereas the synthetic EGF1 is not (Nishimura et al. 1989; Nishimura et al. 1992). Since mab AW exhibited the same affinity for synthetic EGF1 and FIXa,

FIGURE 5. Model of the FIXa-mabAW (Fab fragment) complex. FIXa is shown in white and its active site in red. In **A** and **B**, the molecule has been rotated 180°. The yellow residues constitute the major epitope recognized by mab AW and the blue residues play a minor role in forming the epitope. The pink residues are not involved in binding of mab AW. The striped yellow area indicates the space that may be occupied by the activation peptide of FIX. The binding site of FVIIIa in the serine protease region is indicated in red. In **C**, a Fab fragment (green and blue residues respectively depict heavy and light chains) is centered on the residues Trp72 and Lys80 in FIXa.


Binding site for FVIIIa on SP
A
SP
Active site
EGF2
EGF1
R94
L57
P55
K30
W72
Gla
mab AW binding site
Membrane
B
SP
E245
EGF2
F75
L57
S61
W72
P55
EGF1
D49
Activation peptide area (fIX)
Ca++ binding site on EGF1
Gla
FIXa
C
Active site
FIXa
Binding site for FVIIIa on SP
Activation peptide area (FIX)
mab (Fab)

the glycosylated residues cannot be part of the epitope. The glycosylated residues are located on the same face as residues 49 and 57, which are not part of the epitope recognized by mab AW. Therefore, Phe75, which is also on this face, cannot be a direct binding site for mab AW in full-length FIX.

We employed surface plasmon resonance to study the interaction of mab AW with FIX and FIXa, and we also performed a competition experiment using Eu^{3+}-labeled FIXa. The mab (or its Fab fragment) showed about 10-fold higher affinity for FIXa ($K_D = 7.6 \times 10^{-10}$ M) than for FIX ($K_D = 6.2 \times 10^{-9}$ M). Binding to FIXa was not affected by active-site inhibition with chloromethylketone EGR. The difference in affinity for FIX and FIXa indicates the existence of intramolecular communication between the serine protease region and the N-terminal EGF module.

Elucidating the role of EGF1 of FIX in activation of FIX and FX (Papers II and III)

Activation of FIX by TF/FVIIa or FXIa and activation of FX by FIXa-FVIIIa involve a large number of functionally important protein-protein interactions. However, it is not known whether EGF1 is directly involved in these interactions. We used the above-mentioned, well-characterized mab AW to study activation of FIX and FX as a means of elucidating the function of EGF1 in these reactions.

We utilized the synthetic substrate CH_3SO_2-LGR-pNA to investigate the amidolytic activity of FIXa and found that mab AW did not block such activity, nor did it affect the ability of antithrombin to inhibit FIXa. Accordingly, it was possible to use this antibody to study the activation of FIX in a plasma clotting system. Mab AW (or its Fab fragment) prolonged the clotting time threefold when coagulation was initiated by an APTT reagent (i.e., via the FXIa pathway), but it had no effect when coagulation was induced by a thromboplastin reagent (via the TF pathway). We also studied the ability of TF/FVIIa or FXIa to activate FIX in a system comprising purified components. In this case, the mab (or its Fab fragment) only partially inhibited the activation of FIX caused by TF/FVIIa, whereas it almost completely blocked activation effected by FXIa (Figure 6), and these findings agree with the results of the plasma clotting

assays. The mab AW may prevent putative direct interaction of FIX EGF1 with FXIa, or the FIX-bound mab may interfere sterically with the bulky FXIa molecule. In the activation by TF/FVIIa, it was recently shown that EGF1 in FIX is important for binding to TF (Zhong et al., 2002). We conclude that the binding site for TF/FVIIa in FIX is not located in the C-terminal part of EGF1 (around residues 72 and 80), since this region contains the binding site for the mab AW.

To investigate the role of EGF1 of FIX in activation of FX, we added mab AW to FX activation reactions in the presence and absence of phospholipid and/or rate-limiting concentrations of FVIIIa. When FIXa was bound in the Xase complex, the mab caused a marginal reduction in the apparent k_{cat} ($k_{cat,app}$) for FX, and such a decrease was also observed in the absence of FVIIIa. Only a minor change in the apparent K_m ($K_{m,app}$) was seen. If FVIIIa had been in direct contact with FIXa in the area containing the epitope recognized by AW, the effect of the mab on activation of FX in the presence of phospholipid would have been much larger than we actually

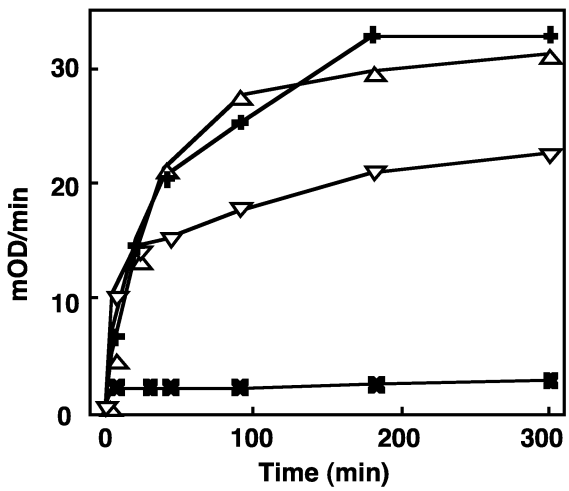


FIGURE. 6. Effect of the mab AW (Fab fragment) on activation of FIX by TF/FVIIa or FXIa. FIX was activated either by TF/FVIIa with (▽) or without (+) Fab AW, or by FXIa with (×) or without (Δ) Fab AW, and the amidolytic activity of FIXa was monitored using a synthetic substrate.

observed. Addition of the mab in the presence of FVIIIa, but in the absence of phospholipid, completely blocked activation of FX. In the absence of both phospholipid and FVIIIa, the mab had a major inhibitory effect that could not be accurately determined due to the low absorbance values. In solution, the molecules are not pre-oriented for optimal interaction, and during formation of an encounter complex the contacts between the molecules are weak. Therefore, any direct or indirect disturbance created by the mab would inhibit evolution toward the formation of a normal, stable complex. It is also plausible that AW can slightly modify the positioning of the FIXa active site with respect to the interaction with FX in such a way that the catalytic machinery does not function properly. This is also consistent with the inhibitory effect we observed when the mab AW was added to assays including only FIXa and FX.

Based on our results and the large body of data on the Xase complex accumulated in the literature, we generated a computer model of the FIXa-FVIIIa-AW complex (Figure 7). In this model, only minor re-orientations are necessary to align the key interacting regions of FIXa and FVIIIa in the manner reported by other investigators (Bajaj et al. 2001; Stoilova-McPhie et al. 2002). Studies have shown that the serine protease region of FIXa interacts with the A2 domain of FVIIIa, and it has been suggested that residues 84–91 and/or 89–93 of FIXa are involved in binding to FVIIIa (Lenting et al. 1996; Olsen et al. 2001; Chang et al. 2002). In the model of FIXa-FVIIIa-AW illustrated in Figure 7, a region around residues 85–90 (but not 78 or 94) in the linker area between EGF1 and EGF2 of FIXa is in contact with FVIIIa, but there is no direct contact between FVIIIa and EGF1 of FIXa. A Fab fragment mimicking AW can be centered on the epitope defined within EGF1 of FIXa, and the mab-FIXa complex can accommodate the expected contacts with FVIIIa without interference from the mab. Accordingly, we propose a model in which EGF1 of FIXa is not in direct contact with FVIIIa in the Xase complex.

In summary, the main function of EGF1 seems to be to bind one Ca^{2+} ion that stabilizes the structure of FIX. During activation of FIX and FX, EGF1 of FIX seems to act primarily as a spacer to position the serine protease region at a proper distance above the phospholipid surface. The possibility of direct contact with EGF1 of FIX exists only when this coagulation factor is activated by FXIa.

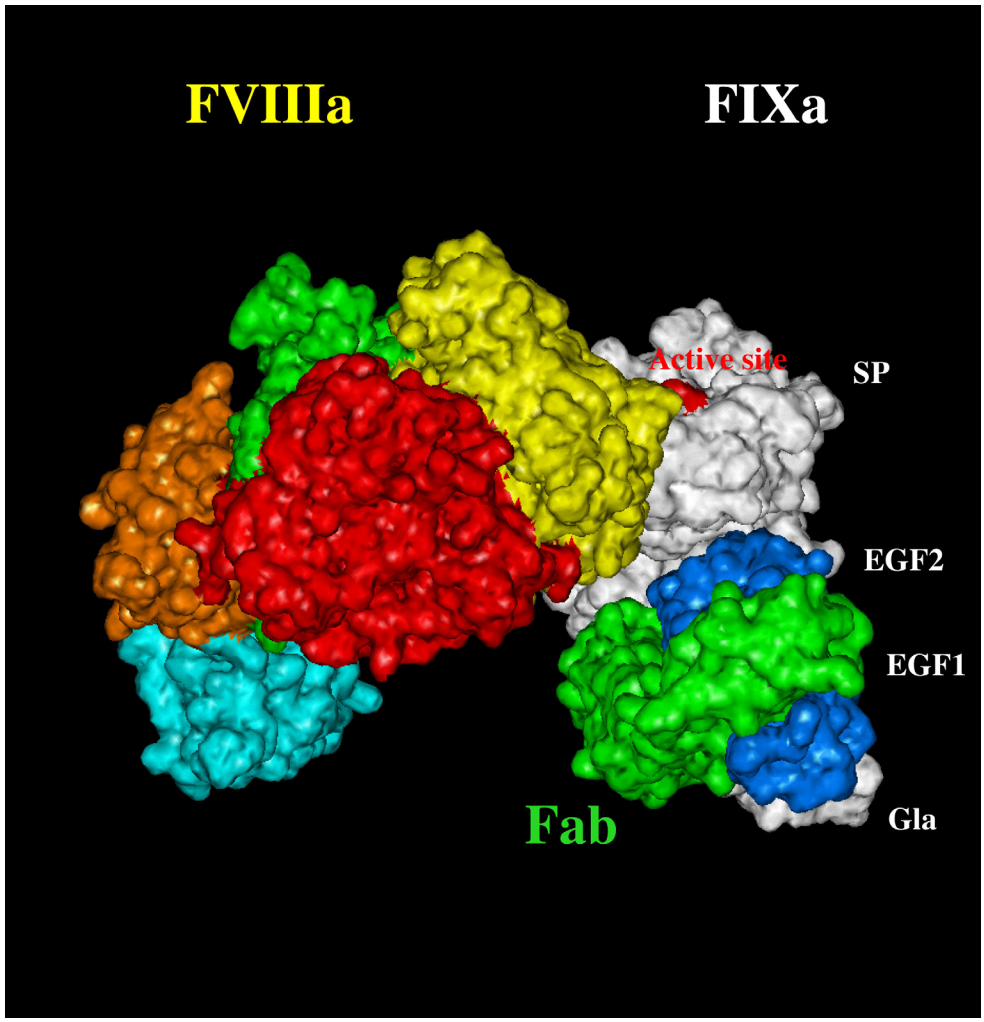


FIGURE 7. Model of the FIXa-FVIIIa-mabAW (Fab fragment) complex. FIXa is shown in white and its active site in red. A Fab fragment of mab AW (green and blue) has been positioned on the epitope it recognizes on FIXa. The subunits of FVIIIa are colour-coded as follows: A1, green; A2, yellow; A3, red; C1, orange; C2, blue. The mab does not interfere with contact between the key binding area of the serine protease region of FIXa and the A2 subunit of FVIIIa.

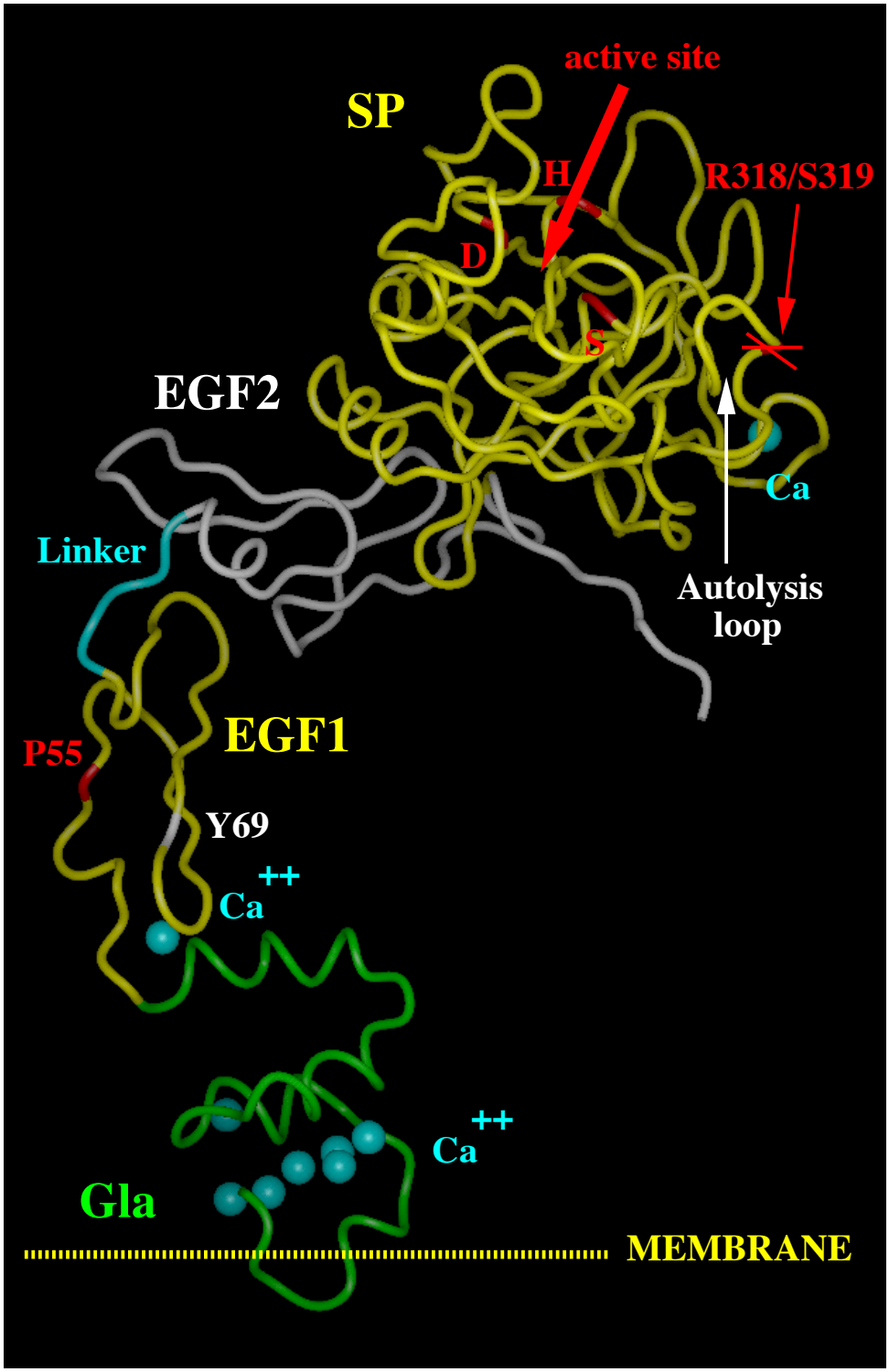

active site
SP
H
R318/S319
D
S
EGF2
Ca
Linker
Autolysis
loop
P55
EGF1
Y69
++
Ca
++
Ca
Gla
MEMBRANE

FIGURE 8. Overview of FIXa. Residues forming the catalytic triad (H, D, and S) are shown in red, Ca^{2+} ions are blue spheres, and an arrow indicates the autolysis loop in the serine protease region. The various regions of FIXa (Gla module, EGF1, linker, EGF2, serine protease region) are illustrated in different colours to facilitate interpretation of the figure. Pro55 is shown in red, and the nearby Tyr69 residue in grey.

Studies of the hemophilia B mutations Pro55Ser and Pro55Leu (Paper IV)

We have in this work investigated FIX variants with the mutations Pro55Ser and Pro55Leu in EGF1, respectively found in two patients with hemophilia B. Both patients persistently showed FIX coagulant activity that was 10–12% of normal, and they had 50% FIX antigen levels, suggesting dysfunctional proteins. Although Pro55 is not a ligand for Ca^{2+}, we wanted to investigate whether the hemophilia in the two patients could be caused by a decreased affinity for Ca^{2+}, since residue 55 is normally a Pro, and any change at that position might affect the folding of EGF1 in the Ca^{2+}-binding area. We performed Ca^{2+} titrations of synthetic EGF modules and monitored the intrinsic fluorescence of the peptide. Dissociation constants were 1.3 mM for the wild-type EGF1 module, 0.7 mM for the Pro55Ser substitution, and 2.2 mM for the Pro55Leu substitution, showing that the affinity of Ca^{2+} was slightly increased when residue 55 was exchanged for a Ser and slightly decreased when it was replaced by a Leu. These changes are quite small, and we assumed that they could not explain the hemophilia in the two patients, hence we continued our investigation by examining the activation of FIX. FIX Pro55Leu was degraded soon after it was activated, with accompanying cleavage at Arg318 and concomitant loss of amidolytic activity. In contrast, the wild-type and Pro55Ser protein showed no such degradation. This was further investigated by use of the mab AW. Activated

FIX Pro55Ser behaved like the wild-type protein and showed a higher affinity for the antibody compared to the non-activated protein, but the affinity of AW for FIXa Pro55Leu was very low. The cleavage that occured at Arg318 when the protein was activated could induce a conformational change that propagates from the serine protease region to EGF1, and this allosteric linkage may be detected by mab AW. An alternative explanation is that the new C-terminus at Arg318 sterically hindered antibody binding, but we do not favor this theory, since the distance from Arg318 to EGF1 is quite large (Figure 8). It is possible that a Ser at position 55 would be better tolerated structurally, and, although it might be slightly destabilizing, a Leu at the same position could have an impact on Tyr69 and induce larger conformational changes.

The degradation of FIX Pro55Leu led to a concomitant loss of amidolytic activity, indicating intramolecular communication between EGF1 and the serine protease region. Yet we did not observe such proteolysis in the FIX Pro55Ser mutant, which appeared to have normal amidolytic activity. The two patients had essentially the same clinical symptoms and coagulation activity, and the differences we noted may have been the result of the longer activation times needed in experiments *in vitro* compared to the situation *in vivo*. On the other hand, the clinical observations are based on only two patients.

Since the FIX Pro55Leu protein was degraded upon activation, it could not be used for further studies. The FIXa Pro55Ser, on the other hand, could be used to study activation of FX, and we found an approximately threefold reduction in $k_{cat,app}$ when FVIIIa and phospholipid were present in the assays. The reduction was approximately the same regardless of whether FVIIIa or phospholipid was omitted from the assay, indicating that Pro55 in EGF1 of FIXa does not interact directly with FVIIIa.

In conclusion, the main reason the mutation Pro55Ser causes hemophilia may be that it impairs the ability of FIXa to activate FX. In the case of the Pro55Leu variant, the major problem seems to be that improper cleavage of FIX occurs during the activation process. It remains to be determined whether these assumptions apply *in vivo*.

Summary

— The present results show that binding of Ca^{2+} to EGF1 of FIX was 10-fold stronger when the Gla module was added to EGF1 (K_D = 160 μM), indicating that binding of Ca^{2+} is important for stabilizing the structure of FIX. The mutations Val46Glu and Gln50Glu caused only a marginal increase in the affinity of Ca^{2+}.

— The mab AW was carefully characterized, and its non-Ca^{2+}-dependent epitope was found to be in the C-terminal part of EGF1 of FIX. This mab has a 10-fold higher affinity for FIXa than for FIX, a difference that is maintained when residue 55 is mutated, indicating the existence of intramolecular communication between the serine protease region and EGF1.

— The mab AW did not affect the amidolytic activity of FIXa, and it was used to study activation of FIX. The antibody had very little effect on activation induced by TF/FVIIa, whereas it almost completely blocked activation caused by FXIa, indicating that the C-terminal part of EGF1 (where the epitope recognized by the mab is located) can interact with FXIa, but not with TF/FVIIa.

— The mab AW caused a marginal reduction in the $k_{cat,app}$ for activation of FX, both in the presence and absence of FVIIIa. This information was used in the production of a computer model of the FIXa-FVIIIa complex, in which it can be seen that EGF1 of FIXa does not interact directly with FVIIIa. The model also shows that the salt bridge proposed to link Glu78 and Arg94 is not important for maintenance of the structure of FIX, which is supported by normal behavior of FIX Arg94Asp in clotting assays.

— Two patients with different mutations in EGF1 of FIX (Pro55Ser and Pro55Leu) that gave rise to mild hemophilia were studied. These patients exhibited 10–12% of FIX coagulant activity and 50% of FIX antigen levels compared to normal subjects. Binding of Ca^{2+} to the isolated mutated EGF modules was investigated, and only minor changes were found in the affinity for this ion. When FIX Pro55Leu was activated to FIXa, it was degraded soon after activation, with cleavage at Arg318 and a concomitant loss of amidolytic activity. These findings indicate the existence of intramolecular communication between EGF1 and the serine

protease region. In contrast, wild-type FIX and the Pro55Ser variant showed no such degradation. In FX activation reactions, there was a small decrease in $k_{cat,app}$ for FIXa Pro55Ser, and this reduction was in the same range regardless of whether FVIIIa was included in the assay. These results support the above-mentioned computer model in which EGF1 of FIXa does not interact directly with FVIIIa in the activation of FX.

Populärvetenskaplig sammanfattning på svenska

Faktor IX (FIX) är en molekyl som behövs för att blodet ska koagulera. Brist på FIX leder till blödarsjuka. För att öka den grundläggande förståelsen av hur FIX fungerar har vi gjort studier av en bit av FIX, som kallas för EGF1. EGF1 är en av två EGFmoduler som finns i FIX. De kallas EGFmoduler för att de liknar en annan molekyl, Epidermal Growth Factor. I denna avhandling har studier genomförts angående hur EGF1 i FIX binder kalcium, hur bindningen påverkas av mutationer och av närheten till omgivande moduler samt hur olika delar av FIX kan kommunicera med EGF1 (så kallad "intramolekylär kommunikation"). För att FIX ska kunna fungera i koagulationen måste den aktiveras, antingen av faktor XI eller av faktor VII. Vi har studerat funktionen hos EGF1 i aktiveringen av FIX samt i aktiveringen av en annan koagulationsmolekyl, faktor X, och en modell av hur komplexet mellan FIX och faktor VIII (en hjälpmolekyl till FIX i aktiveringen av faktor X) ser ut har producerats. Resultaten visar att EGF1 är viktigt för bindningen av kalcium och därmed för bibehållandet av strukturen hos FIX, men EGF1 deltar inte i någon direkt interaktion med FVIII i aktiveringen av FX.

Tack

Jag vill tacka alla som hjälpt mig på vägen under avhandlingsarbetets gång. Först på listan är givetvis Johan Stenflo, som har varit till ovärderlig hjälp när det gällt att få min hjärna att vecka sig i fåror lämpade för vetenskapligt tänkande och för skrivande av vetenskapliga artiklar. Han har också beredvilligt delat med sig av sina rika kunskaper om proteiner och deras egenskaper. *"Hans vishet överträffade all Österlandets och all Egyptens vishet." 1 Kung 4:30.*

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Per Fernlund vill jag tacka för att han lärt mig praktisk klinisk kemi och för att han tagit sig tid och ork att gå igenom tjocka kemiböcker med mig varje vecka. *"Genom vishet varder ett hus uppbyggt, och genom förstånd hålles det vid makt." Ordspr 24:3.*

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