THE MOLECULAR MECHANISM OF FACTOR IX ACTIVATION BY FACTOR XIa

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LIST OF ABBREVIATIONS

Bovine serum albumin - BSA

Enzyme:substrate - E:S

Epidermal Growth Factor - EGF

Factor XIa catalytic domain (Factor XIa-CD, *a recombinant protein*)

γ-carboxyglutamic acid domain - Gla domain

Glycoprotein 1b, alpha subunit - Gp1bα;

High molecular weight kininogen - HMWK

Partial thromboplastin time - PTT

Prekallikrein - PK

Sodium dodecylsulfate - SDS

Tissue factor pathway inhibitor - TFPI

Tissue factor - TF

CHAPTER I

INTRODUCTION

Goal of the Research Project

Activation of coagulation factor IX, a protein found in blood, is a pivotal reaction in the formation and maintenance of a fibrin clot at a site of vascular injury. Hereditary deficiency of factor IX causes the severe bleeding disorder known as hemophilia B. Factor IX must be activated to participate in coagulation. This process is mediated by two plasma proteases, factor VIIa and factor XIa. While a considerable amount of information is available concerning factor IX activation by factor VIIa, relatively little is known about the factor XIa-mediated reaction. Recently, factor IX and factor XIa have been identified as contributors to pathologic blood coagulation in such disorders as arterial and venous thrombosis and disseminated intravascular coagulation, and there is substantial interest in therapeutic inhibition of these proteins. The goals of this project were to investigate the mechanism by which factor XIa activates factor IX, and to study the process by which factor XIa is generated from its inactive precursor, factor XI. The work resulted in the publication of two manuscripts with one manuscript in press and another in preparation:

Smith SB, Verhamme IM, Sun M-F, Bock PE, Gailani D**.** Characterization of novel forms of coagulation factor XIa: Independence of factor XIa subunits in factor IX activation. *J. Biol. Chem.* 2008;283:6696-6705.

Smith SB, Gailani D: Update on the physiology and pathology of factor IX activation by factor XIa. *Expert Review of Hematology* 2008;1:87-98.

Gailani D, **Smith SB**: Structural and functional features of factor XI. - *J. Thromb. Haemost.* (In press).

Smith SB, Agah S, Bajaj SP, Gailani D. An Analysis of Cleavage of the factor IX Activation Sites by Factor XIa. (Manuscript in preparation)

Introduction to Blood Coagulation

Bleeding is terminated at sites of injury by the process of blood coagulation, which involves the formation of a fibrin and platelet rich clot over a site of vascular damage. Clot formation is mediated by the interactions of a group of proteins (clotting factors) found in solution in blood plasma with specific receptors found on platelets and at the site of vascular injury (Davie *et al.*, 1991; Mann, 1999; Furie and Furie, 2009). A central concept underlying blood coagulation is that the blood borne coagulation factors, many of which are the zymogens of serine proteases, are incorporated into a self-amplifying proteolytic chain reaction (**Figure I-1),** only when presented with the

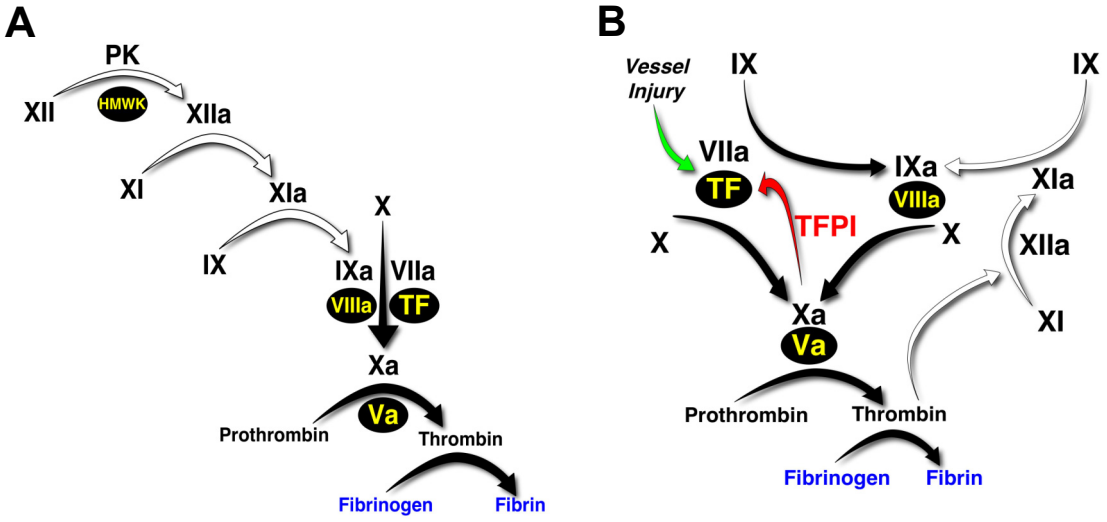

Figure I-1. Models of fibrin generation. (A) Cascade model. Fibrin generation is initiated by a series of proteolyic reactions involving plasma proteases (black lettering). Activated factors have a lower case "a" following the Roman numeral. Coagulation is triggered by activation of factor (F) XII to FXIIa on a charged surface or by exposure of FVIIa to tissue factor (TF). **(B) Current Model.** TF binds to FVIIa at a site of vascular injury. The FVIIa/TF complex activates FX, which converts prothrombin to α-thrombin to initiate fibrin formation. FVIIa/TF is inhibited by tissue factor pathway inhibitor (TFPI) in a FXa-dependent manner. Sustained FX activation requires FIXaβ (IXa), which is activated by FVIIa/TF or FXIa. Once FVIIa/TF is inhibited, FXIa may continue to activate FIX to ensure adequate FX and prothrombin activation. In this model, factor XI is activated by α-thrombin or factor XIIa. For both models, cofactors are shown in black circles. FV and FVIII are activated by α-thrombin, and enhance the catalytic efficiency of FXa and FIXaβ, respectively. Black and white arrows indicate protease-mediated reactions, with white arrows indicating reactions involved in factor XI activation and factor XIa activity. The red arrow indicates an inhibitory process.

appropriate triggering molecules that are exposed by vascular damage. Thus stimulated, coagulation factors in association with the triggering molecules introduced to blood during injury, catalyze the conversion of soluble fibrinogen into insoluble fibrin and contribute to the activation of platelets. Because inappropriate or excessive clot formation can occlude blood vessels, a delicate balance exists between damage recognition, and clot formation, maintenance, and breakdown.

To facilitate the formation of a blood clot, as well as to localize clot formation to the site of injury, vertebrates have evolved an elaborate, multi-step vascular damage recognition mechanism that was originally termed the coagulation cascade (**Figure I-1A**), (Macfarlane 1964; Davie and Ratnoff 1964). First proposed in 1964, the cascade or waterfall model provided a conceptual framework for incorporating known plasma coagulation factors into a fibrin generating mechanism. Most coagulation factors were assigned Roman numerals by an international committee between 1957 and 1963. The recurring theme in this model is that a plasma protease activates the next zymogen (the inactive precursor of a protease) in the cascade, to propagate the process. A limitation of this model is that it is not easy to reconcile with the bleeding patterns associated with congenital deficiencies of the individual clotting factors. For example, factor IX deficiency is associated with a severe hemorrhagic phenotype, while factor XII deficiency is not associated with excessive bleeding, despite the fact they both appear to be components of a linear pathway of reactions that trigger clot formation.

Over the past forty years, the coagulation factors have been purified from plasma, their genes and cDNAs have been cloned, and three-dimensional structures are available for most of them. With advances in our understanding of structure-function relationships

for these molecules, newer models of fibrin formation and maintenance have been proposed that have replaced the original cascade, and emphasize reactions that are more likely to occur *in vivo* (**Figure I-1B**) (Broze *et al.*, 1990; Gailani and Broze, 1991; Davie *et al.*, 1991). It is the goal of this thesis proposal to investigate the process by which the plasma coagulation factor, factor XI, is converted to an active protease (factor XIa), and the mechanism by which factor XIa contributes to coagulation through proteolytic activation of factor IX.

Coagulation Proteases

The coagulation factors depicted in the schemes in **Figure I** fall into three general categories. Fibrinogen (factor I) is converted to fibrin, the structural component of the clot. Factors II (prothrombin), VII, IX, X, XI, XII and prekallikrein (PK) are the zymogens of serine proteases. Finally, tissue factor (TF - also considered factor III), factors Va and VIIIa, and high molecular weight kininogen (HMWK) are protein cofactors that facilitate assembly of coagulation proteases on surfaces (Davie *et al.*, 1991; Furie and Furie, 2009). The work in this thesis primarily is concerned with the activation and activity of certain coagulation proteases. All coagulation proteases contain a C-terminal catalytic domain that is homologous to the digestive enzyme trypsin, and a series of N-terminal auxiliary domains homologous to portions of other proteins that have been added to the catalytic domain by the processes of gene duplication and translocation (Tordai *et al.*, 1999; Jiang and Doolittle, 2003; Davidson *et al.*, 2003; Ponczek *et al.*, 2008) (**Figure I-2**). Auxiliary domains generally contain one or more binding sites that determine or modify the specificity of enzyme binding to substrates, cofactors, and

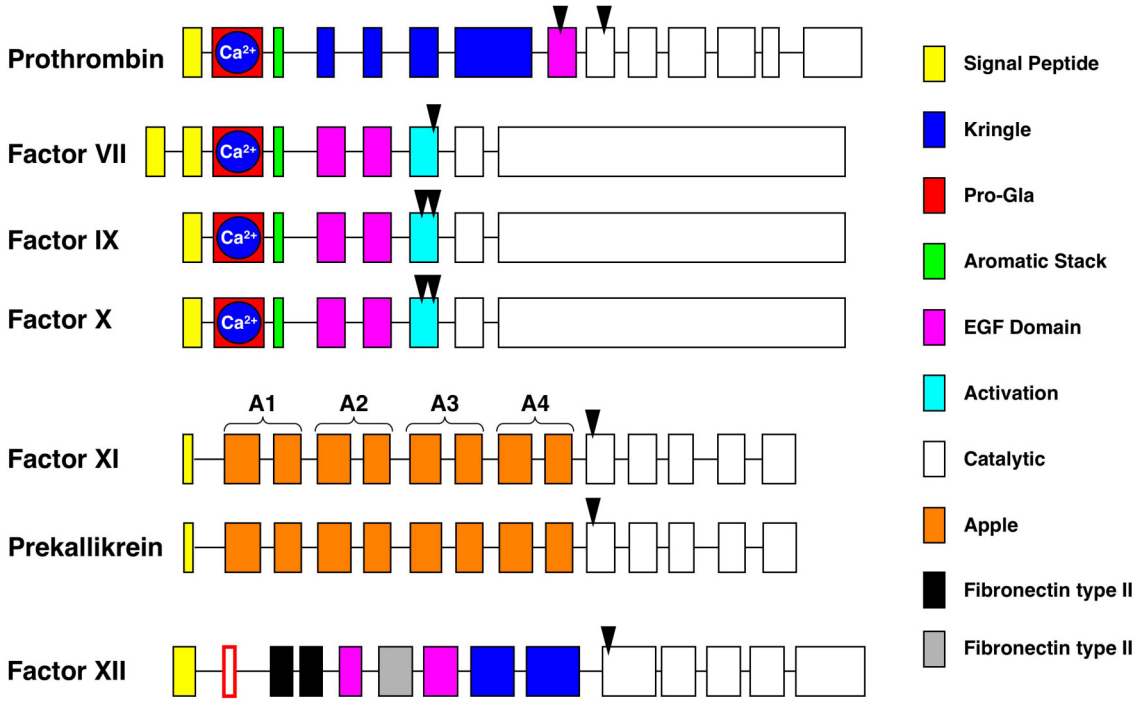

Figure I-2. Schematic diagram of coagulation protease domain structures. Each box represents the amino acids encoded by a single exon in the gene for the protein. Signal peptides (yellow) and the Pro portion of the Pro-Gla domains (red) are removed prior to secretion from hepatoctyes. The Gla domains undergo post-translational modifications that facilitate Ca^{2+} binding. The black arrows indicate the locations where protease zymogens must be cleaved to generate the active protease. Note that the apple domains of factor XI and prekallikrein are each encoded by two exons. Adapted from Furie and Furie, 2009.

surfaces. These auxiliary domains embody the evolutionary pressure that drove the development of specificity of each clotting factor and, thus, its contribution to hemostasis.

The Catalytic Domains of Trypsin and Coagulation Proteases

Vertebrates share a common complement of genes encoding peptidases, indicating that the expansion of the common peptidase families occurred prior to the appearance of the vertebrate lineage (Page and DiCera, 2008). Of these families, the trypsin-like serine peptidases are the largest homologous group. The catalytic domain of

each coagulation protease belongs to the S1A trypsin family of peptidases that cleave on the C-terminal side of amino acids with a positively charged side chain such as Arginine or Lysine. Chymotrypsinogen, which cleaves after large hydrophobic residues (Phe, Trp, and Tyr), was the first member of this family to be crystallized, and residues of the catalytic domains of trypsin-like enzymes are conventionally numbered using the chymotrypsinogen numbering system (Bode *et al.*, 1989). When describing catalytic domain residues in this thesis, we will hereafter use the chymotrypsin numbering system unless otherwise indicated.

Trypsin is a "serine protease" because it contains an unusually reactive, basic serine residue (Ser^{195}) in its active site (Blow, 1990). Trypsin-like serine proteases contain a catalytic triad consisting of Asp^{102} and His^{57}, in addition to Ser^{195} (**Figure I-3**). Binding of a substrate to the enzyme orients the catalytic triad such that Asp^{102} stabilizes an electropositive form of His^{57}, which in turn abstracts a proton from Ser^{195}, rendering that residue nucleophilic. Nucleophilic Ser^{195} attacks the carbonyl carbon of a properly positioned peptide bond, forming an oxyanion tetrahedral intermediate that is stabilized by backbone N atoms of Gly^{193} and Ser^{195}. This interaction takes place in an

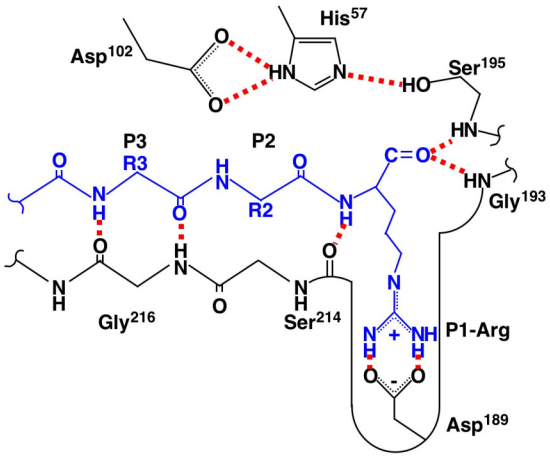

Figure I-3. The active site of a trypsin-like serine protease. The diagram shows the amino acids of the active site (black) interacting with a hypothetical substrate (blue). Hydrogen bonds between substrate and enzyme are in red. The substrate P1 Arg side chain forms hydrogen bonds with Asp^{189} in the S1 specificity pocket. The catalytic triad Asp^{102}, His^{57} and Ser^{195} is shown at the top, with Gly^{193} on the right. The backbone amido groups of Ser^{195} and Gly^{193} form part of the oxyanion hole structure. Chymotrypsinogen numbering used throughout. (After Craik and Perona, 1995)

area of the enzyme active site referred to as the oxyanion hole (**Figure I-3**), and it stabilizes the transition state of the substrate by 1.5 to 3 kcal/mole (Stryer, 1995; Page and DiCera, 2008). The oxyanion hole also stabilizes a second tetrahedral intermediate in which water displaces the polypeptide fragment generated by bond cleavage. Zymogens of most serine proteases related to chymotrypsinogen, including all coagulation proteases, must be cleaved in order to generate an active enzyme. Cleavage occurs between the residues corresponding to Arg^{15} and Ile^{16} in chymotrypsinogen numbering. The cleavage generates a new N-terminus (residue 16) that forms an electrostatic interaction with Asp^{194}, resulting in formation of the oxyanion hole and substrate-binding site (Bode *et al.*, 1976; Fehlhammer *et al.*, 1977). Formation of the salt bridge between residue 16 and Asp^{194} is a defining feature of an active serine protease.

The substrate specificity of trypsin, as well as other degradative enzymes, is primarily driven by interactions involving amino acids flanking the cleavage site on the substrate, and residues surrounding the catalytic triad on the enzyme. In enzymatic nomenclature, substrate amino acids flanking the cleavage site are designated P, and the amino acids in the activating enzyme that interact with them are designated S (Schechter and Berger, 1967). Numbers following P designate the amino acid position on the substrate relative to the scissile bond. By convention, residues on the substrate extending from the scissile bond toward the N-terminus are P_1, P_2, $P_3 \ldots P_n$ (**Figure I-3**). The complementary sites on the activating enzyme that engage these residues are designated S_1, S_2, $S_3 \ldots S_n$. On the C-terminus of the scissile bond, substrate residues are numbered P_1', P_2', P_3' with the complementary protease residues numbered S_1', S_2', and S_3'. Interactions of these residues, particularly the P_1 residue, which is arginine or lysine in

most substrates of trypsin-like enzymes, are important for the docking interaction between the enzyme and substrate (Krishnaswamy, 2004). While trypsin is theoretically capable of cleaving after any accessible lysine and arginine residues in a protein (Roach *et al.*, 1997), the substrate specificity of the coagulation proteases is much more restricted, and the number of cleavages per substrate molecule is typically limited. In contrast to trypsin, coagulation protease specificity for substrates stems from binding interactions at sites remote from the catalytic active site called exosites.

Coagulation Protease Exosites and Auxiliary Domains

Reversible active site inhibitors of coagulation proteases inhibit cleavage of small peptidyl substrates by a classic competitive inhibition (**Figure I-4A**). This is because the substrate is recognized solely by structures surrounding the enzyme active site, and the inhibitor and substrate bind in a mutually exclusive way to the catalytic domain active site. The same inhibitor causes mixed hyperbolic inhibition (**Figure I-4B**) of cleavage of the normal plasma macromolecular substrates of the protease, indicating the binding

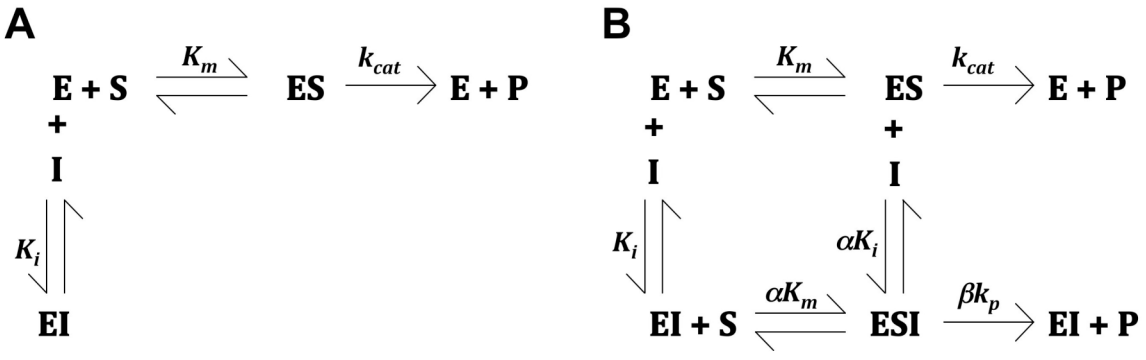

Figure I-4. Models for enzyme inhibition The models illustrate conversion of a substrate (*S*) to product (*P*) by an enzyme (*E*), in the presence or absence of a reversible active site inhibitor (*I*). **(A)** A model for pure competitive inhibition. **(B)** A model for mixed hyperbolic inhibition. Steps that are largely responsible for determining K_m, k_{cat}, and K_i are indicated. α and β represent factors that depends on the nature of the inhibitor.

energy that drives the physiologic enzyme-substrate interaction occurs outside of the areas where the inhibitor binds (i.e. remote from the active site). These extended macromolecular recognition sites are called exosites, and can be located in the catalytic domain, as in the case of α-thrombin (Davie and Kulman, 2006), or in the N-terminal auxiliary domains, as has been demonstrated for factors VIIa, IXa, Xa, and XIa (Baugh *et al.*, 2000; Krishnaswamy, 2004; Bock *et al.*, 2007; Ogawa *et al.*, 2005). For example, during activation of factor IX by factor XIa, factor IX initially binds to one of the auxiliary domains on factor XIa (Ogawa *et al.*, 2005). This exosite interaction presents the cleavage site on factor IX to the factor XIa active site on the catalytic domain for catalysis. The importance of exosite interactions during factor IX activation by factor XIa will be addressed in Chapters V and VI.

In addition to containing exosites, coagulation protease auxiliary domains (**Figure I-2**) serve to localize the enzyme, and thus the clotting process, to areas of vascular injury. A critical auxiliary domain for the coagulation protease zymogens prothrombin and factors VII, IX and X is the N-terminal γ-carboxyglutamic acid (Gla) domain (Furie and Furie, 2009). The Gla domain of factor IX is shown in **Figure I-5**. Gla domains are rich in glutamic acid residues that have been modified by addition of a carboxyl group to the γ-carbon in a process that depends on reduced vitamin K. The γ-carboxyglutamic acid residues in the Gla domains coordinate the binding of calcium and magnesium ions, which are critical for proper folding of the domain and protein function. The divalent cations in the Gla domain interact with negatively charged phospholipids in platelet and cell membranes, with the non-polar side chains of N-terminal residues of the domain inserting into the lipid bilayer. γ-carboxyglutamic acid is widely distributed in the animal

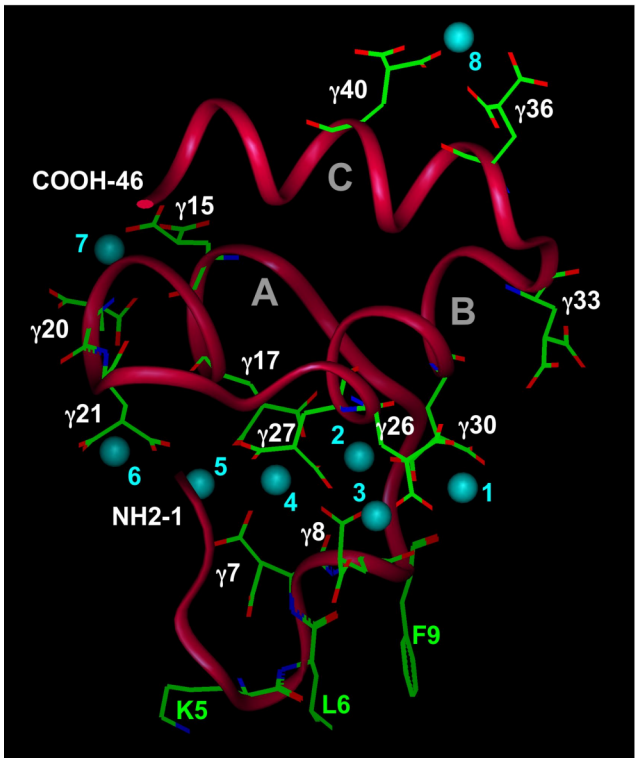

Figure I-5. The Gla domain of factor IX. Shown is a ribbon diagram of the Gla domain from human factor IX, extending from the N-terminal tyrosine residue one to the C-terminal valine residue 46. The peptide backbone is indicated by the red ribbon. Side chains of γ-carboxyglutamic acid residues (γ), and Lys5, Leu6, and Phe9 in the omega loop, are in green. Nitrogen and oxygen atoms on these residues are indicated in blue and red, respectively. Calcium ions are shown as light blue spheres. The three α-helices of the Gla domain are indicates as A, B and C in gray lettering. The omega loop is involved in binding to phospholipid membranes.

Image courtesy of Dr. S. Paul Bajaj (University of California, Los Angeles)

kingdom, having been found in a variety of organisms such as the sea squirt (*Ciona intestinalis*), fruit flies (*Drosophila melanogaster*), and marine snails (*Conus textile*), which do not possess a vertebrate-type coagulation mechanism (Bandyopadhyay, 2008).

Activation of vitamin K-dependent coagulation factors (prothrombin and factors VII, IX and X), for the most part, is characterized by reversible assembly of the activating protease and substrate on a phospholipid surface (Krishnaswamy, 2004). In these reactions, the Gla domain is critical for binding to the phospholipid membrane, and in some cases with other proteins (cofactors and substrate), that are instrumental in proper positioning of substrate and protease relative to each other for efficient catalysis (Waters *et al.*, 2006). An exception to this rule is activation of factor IX by factor XIa, which is not affected by phospholipids (Mannhalter *et al.*, 1984; Gailani and Broze, 2001). This is likely due to the fact that factor XIa lacks a Gla domain and does not bind to

phospholipid membranes. Factor IX does bind phospholipid through its Gla domain, so it is likely that factor XIa interacts with factor IX at a site distinct from the phospholipid binding region. The four auxiliary domains that comprise the N-terminal non-catalytic portion of factor XIa (**Figure I-2**), are distinctly different from the auxiliary domains of vitamin K-dependent proteases. In Chapters V and VI we will show that a protein-protein interaction between auxiliary domains on factor XIa and the factor IX Gla domain is required for normal activation of factor IX by factor XIa.

Factor XI During Vertebrate Evolution

The specific genetic events in which the genes of trypsin-like enzymes were attached to genes for various auxiliary domains to form the coagulation proteases have not been identified; however, evidence from comparative genomics clearly indicates that gene duplication and exon shuffling were involved in placing a diverse array of domains at the N-termini of the genes for these proteases (Jiang and Doolittle, 2003). This process is undoubtedly responsible for the differentiation (specificity) of the enzymes involved in fibrin clot formation. Because blood clotting follows the same basic pattern in all vertebrates, following the development of clotting enzymes through vertebrate evolution allows the identification of an initial and essential set of clotting enzymes for fibrin formation. A comparison of the genomes of the puffer fish (*fugu rubripes*) and the invertebrate *Ciona intestinalis* (the sea squirt) reveal that a functioning coagulation mechanism emerged in the time interval following the last common ancestor of these diverging chordates, a period of less than 100 million years (Jiang and Doolittle, 2003).

Analysis of the genomes of the puffer fish, and more recently the zebra fish (*Danio rerio*) show that jawed fish possess homologs for the coagulation factors shown in **Figure I-6**. Note that while these species have all of the vitamin K-dependent proteases found in mammals, they lack genes for factor XI and its homolog PK, and for factor XII (Ponczek *et al.*, 2008). In factor XI and PK, the trypsin-like protease is linked to four auxiliary domains called apple domains (discussed in Chapter III). Apple

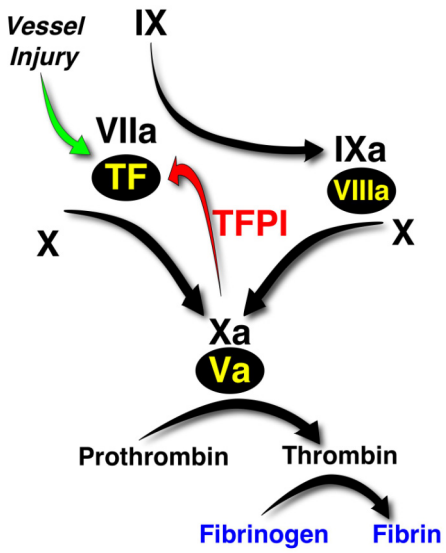

Figure I-6. Schematic diagram of the predicted protease reactions involved in fibrin formation in fish. Jawed fish (teleosts) have orthologs for all of the vitamin K-dependent coagulation proteases found in mammals (prothrombin and factors VII, IX and X); the cofactors tissue factor (TF), factor V and factor VIII, and the regulatory protease inhibitor tissue factor pathway inhibitor (TFPI). Teleosts do not have orthologs for factor XI, prekallikrein or factor XII. However, they do have genes for the likely ancestors of these proteases: hepatoctye growth factor or plasminogen in the cases of factor XI and prekallikrein; and hepatoctye growth factor activator in the case of factor XII. The immediate predecessor of factor XI and prekallikrein first appears in amphibians, as does factor XII.

domains are members of the PAN (Plasminogen/Apple/Nematode) domain family, which are related to the N-terminal regions of plasminogen and hepatocyte growth factor (Tordai, 1999). It is likely that the apple domains of factor XI and PK are derived from a protease such as plasminogen or hepatocyte growth factor, both of which are found in fish (Ponczek *et al.*, 2008). While fish have no homologs for factor XI or PK, a gene for a trypsin-like protease with four apple domains that is clearly the direct ancestor for these proteins first appears in amphibians (frogs - *Xenopus*). A duplication event involving this ancestral gene occurred during mammalian evolution after the divergence of monotremes

(egg-laying mammals such as the platypus) from marsupial and placental mammals, giving rise to factor XI and PK. Similarly, the gene for factor XII first appears in amphibians, as a result of a duplication of the gene for its homolog, hepatocyte growth factor activator. The appearance of factor XII and the predecessor of factor XI/PK in amphibians may indicate that factor XIIa is an activator of the latter, just as it is an activator of factor XI and PK in mammals.

Thus, it is apparent that factor IX appeared much earlier in vertebrate evolution than factor XI, clearly indicating that a mechanism for factor IX activation must have existed that is not depicted in the classic cascade model in **Figure I-1A**. This inconsistency in the old model is not an issue in current models of coagulation (**Figure I-1B** - Discussed in detail below), where factor IX can be activated by both factor VIIa and factor XIa. The selective advantage gained by having factor XIa as a second activator of factor IX is unknown, but it is clear that significant changes have occurred in factor XI since its divergence from the factor XI/PK precursor that facilitate factor IX activation. This issue will be covered in detail in chapters V and VI.

Models of Fibrin Clot Formation

Fibrin clot formation was originally envisioned as a cascade or waterfall-like sequence of proteolytic reactions, with one coagulation protease activating the next zymogen, in a linear sequence (**Figure I-1A**) (Macfarlane, 1964; Davie and Ratnoff, 1964). In this model, coagulation can be triggered by two distinct mechanisms. One mechanism involves activation of factor XII in a process called contact activation (**Figure I-7**), which requires the protease zymogen PK and the cofactor HMWK. Contact

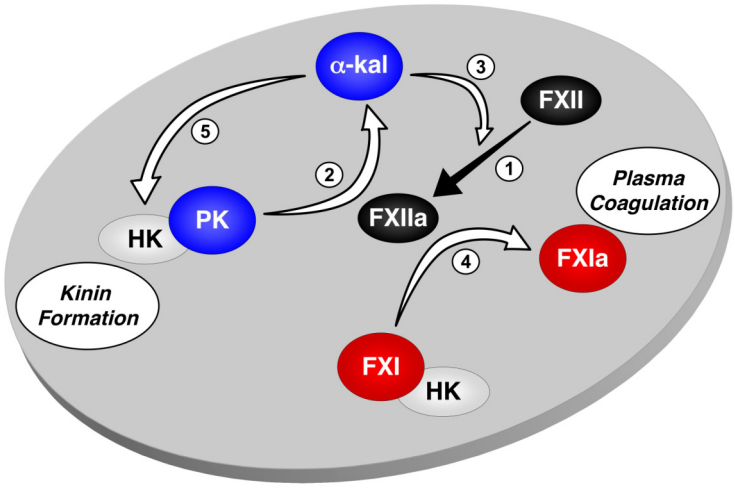

Figure I-7. Plasma contact activation. Contact activation is initiated by the slow activation of factor XII (FXII) to α-factor XIIa (FXIIa), probably by autoactivation, when plasma is exposed to a negatively charged surface (reaction 1). Reciprocal activation of prekallikrein (PK) to α-kallikrein (α-kal) by FXIIa (reaction 2), and FXII by α-kal (reaction 3) enhances FXII activation. FXIIa promotes coagulation through conversion of factor XI (FXI) to factor XIa (FXIa) (reaction 4). α-kal also cleaves high molecular weight kininogen (HK) to liberate bradykinin (reaction 5). Note that HK is required for PK and FXI binding to the contact surface.

activation is triggered *in vitro* by exposure of blood to various surfaces, usually with a negative charge, such as silica or glass (Gailani and Broze, 2001). Possible physiologic activators of contact activation are discussed below. The second initiation mechanism involves the binding of factor VIIa in plasma to its cofactor, tissue factor (TF) (**Figure I-1A**). Note that in the classical model, factor IX is activated only by factor XIa. As discussed in the previous section, this seems unlikely given the relatively recent addition of factor XI to vertebrate coagulation.

Modern coagulation models incorporate a junction that allows for factor IX to be activated by more than one enzyme (**Figure I-1B**), and the resulting mechanism overall is not as much a cascade as a group of proteolytic reactions that may play roles in both initiating and consolidating coagulation (Broze *et al.*, 1990; Davie *et al.*, 1991). Factor IX activation, therefore, lies at a critical junction in the coagulation process because its

activation is accomplished by two distinct reaction pathways, each of which may be triggered by different stimuli related to vascular damage.

Tissue Factor and Initiation of Blood Coagulation

In current models of fibrin formation (**Figure I-1B**), coagulation at a site of vascular damage is initiated by formation of a complex between plasma factor VIIa and the non-enzymatic integral membrane protein TF, which is exposed to blood at a site of injury. TF is a 47,000 Dalton trans-membrane glycoprotein member of the cytokine receptor family that is constitutively expressed on many cells that underlie vascular endothelium, such as fibroblasts, pericytes, astrocytes, and cardiomyocytes (Mackman *et al.*, 2007) (**Figure I-8A**). TF expression can also be induced in platelets and monocytes, establishing an additional source for this cofactor at injury sites. Factors VII and VIIa are the natural ligands of TF. Factor VII must bind to TF in order to be efficiently activated,

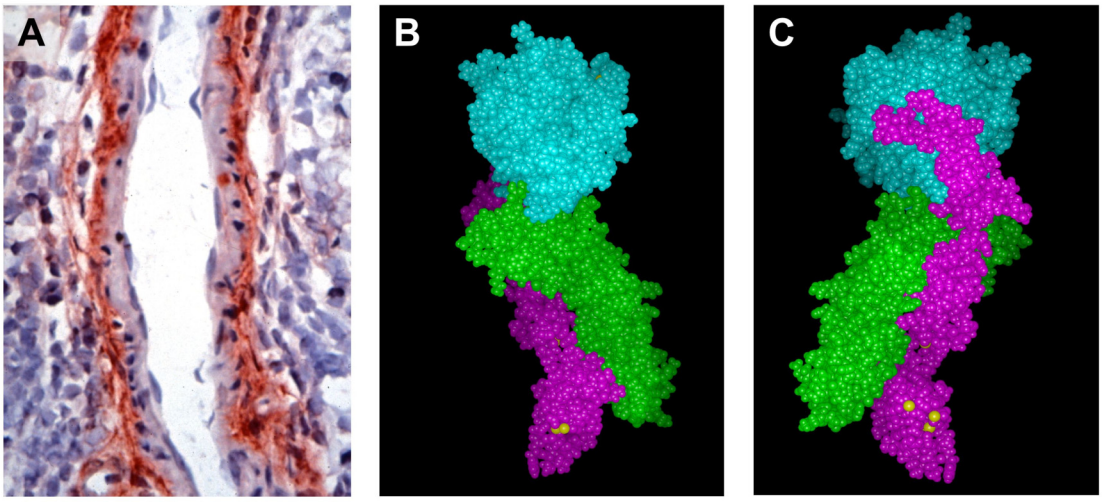

Figure I-8. Tissue factor (TF). (A) Photomicrograph of a human arteriole. Immunohistochemistry was performed with a polyclonal antibody to human TF. Cells expressing TF appear red. Hematoxylin was used as a counterstain. **(B** and **C)** Space-filling models of human factor VIIa in complex with the extra-cellular portion of TF (green). The factor VIIa catalytic domain is blue and the non-catalytic light chain is in purple. Calcium ions in the factor VIIa Gla domain appear as yellow spheres. Model courtesy of Dr. S. Paul Bajaj.

and factor VIIa requires TF to efficiently activate its substrates, factors X and IX. While the majority of coagulation factors circulate as inactive zymogens that require proteolytic activation, approximately 1% of circulating factor VII is in the form of factor VIIa. Factor VIIa and factor VII bind to TF, when TF is exposed to flowing blood (**Figure I-8B** and **8C**). Factor VIIa/TF is able to auto-activate FVII that is in complex with TF. This feedback process amplifies the local concentration of factor VIIa/TF.

Factor VIIa/TF converts some factor X and factor IX to the active proteases factor Xa and factor IX to factor IXaβ, respectively (Broze et al, 1990; Davie *et al.*, 1991; Mackman, 2007). The relatively small amount of factor Xa generated initially converts some prothrombin to α-thrombin (**Figure I-1B**), which cleaves fibrinogen into insoluble fibrin, and activates the cofactors, factor V and factor VIII. Just as TF serves as a cofactor that enhances the efficiency of factor VIIa, factors Va and VIIIa enhance the catalytic efficiencies of factor Xa and factor IXaβ, respectively, resulting in greater than 100,000-fold enhancement of prothrombin activation by the system (Furie and Furie, 2009). However, the activity of factor VIIa/TF at a wound site may be transient, as the complex is a target for inhibition by the Kunitz-type proteinase inhibitor, tissue factor pathway inhibitor (TFPI - **Figure I-1B**). Furthermore, the TF exposed by vascular damage is quickly covered by the growing clot. This has lead to speculation that factor VIIa/TF is critical for the initiation of clot formation, but may not be sufficient to sustain factor X activation over time to consolidate the process in all circumstances. In many instances, sustained factor X activation would require factor IXaβ and factor VIIIa. This hypothesis is consistent with the severe bleeding seen in patients lacking factors IX or VIII, condition known as hemophilia B and A, respectively (Ragni *et al.*, 2009).

The Intrinsic Pathway of Blood Coagulation

As shown in **Figure I-1B**, the factor VIIa/TF complex activates both factor X and factor IX, but it has long been recognized that factor IX can be activated by a factor VIIa/TF-independent process. Indeed, factor IX activation by factor XIa was reported nearly 20 years before it was appreciated that factor IX was activated by factor VIIa/TF. Factor XIa is an enzyme of the traditional intrinsic pathway for initiating coagulation (represented by the white arrow in **Figure I-1A**), which also contains the proteases factor IX, factor XII, and PK, and the cofactor HMWK. Activation of coagulation through the intrinsic pathway requires a charged surface (**Figure I-7**). While the events involved in initiating coagulation through factor VIIa/TF (the classical extrinsic coagulation pathway) are reasonably well understood, the identity of the physiologic counterparts for the surface or cofactor that initiates coagulation through the intrinsic cascade is a matter of debate. Some studies suggest that collagen exposed by vascular damage can facilitate factor XII activation (Renné *et al.*, 2005), while others have identified RNA from damaged cells (Kannemeier *et al.*, 2007) and polyphosphates from platelet α-granules (Smith *et al.*, 2006) as inducers of contact activation. On a surface, factor XII is slowly converted to factor XIIa, which then converts PK to α-kallikrein, which reciprocally activates additional factor XII (**Figure I-7**). Factor XIIa also converts factor XI to factor XIa, which converts factor IX to factor IXaβ. There is no reported functional difference between factor IXaβ that has been produced by factor XIa or by factor VIIa/TF.

The sequence of reactions and molecular interactions in the intrinsic pathway have been studied for many years, but the physiologic significance of the pathway as a whole, as depicted in **Figure I-1A**, is not established and is a topic of debate (Renné *et*

al., 2005; Pedicord *et al.*, 2007; Blat and Seiffert 2008) It has been widely accepted that factor XIIa activation of the intrinsic cascade is not important for normal coagulation (hemostasis) *in vivo* because persons with factor XII deficiency do not have a bleeding disorder (Gailani and Broze, 2001). Factor XI deficiency does result in abnormal bleeding that varies among individuals, typically causing a problem after trauma or surgery (Seligsohn, 2007). The phenotypic difference in the deficiency states of these two proteins suggests that enzymes other than factor XIIa mediate factor XI activation during hemostasis. For example, thrombin is capable of activating factor XI (Naito and Fujikawa, 1991; Gailani and Broze, 1991), and evidence from *ex-vivo* human plasma systems suggest that under some circumstances prothrombin activation is dependent on factor XI in the absence of factor XII (von dem Borne *et al.*, 1995; Kravtsov *et al.*, 2009).

It is now thought that factor XI is not part of a mechanism for initiating fibrin clot formation (at least under normal circumstances), but instead is activated late in the process (possibly by thrombin or factor XIIa), and contributes to hemostasis by sustaining factor IX activation to supplement factor IXaβ produced by the factor VIIa/TF complex. Bleeding in hemophilia B is much more severe than in factor XI deficiency (Seligsohn, 2007), indicating factor IX activation by factor VIIa/TF is likely to be at least as important as activation by factor XIa. Thus, the model in **Figure I-1B** not only explains bleeding disorders associated with various factor deficiencies better than the model in **Figure I-1A**, but is also consistent with genomic analyses showing that factor IX was present in vertebrates before factor XI. The role of factor XIIa in the activation of factor XI during normal coagulation remains a subject of controversy. Experiments in Chapter IV will examine factor XI activation by factor XIIa and α-thrombin.

Summary

Formation of a fibrin clot is mediated by a tightly regulated series of proteolytic reactions involving trypsin-like plasma proteases and their cofactors. Factor IX, which lies at a critical junction in the coagulation process, is activated by two distinct mechanisms mediated by factor VIIa and factor XIa. There has been a recent increase in interest in the biology of factor XIa and factor XIa activation of factor IX, as studies in animal models (Gailani and Renné, 2007a, 2007b) and human epidemiologic data (Meijers *et al.*, 2000; Doggen *et al.*, 2006) suggest that this mechanism may make a significant contribution to pathologic coagulation disorders such as vascular thrombosis (Wang *et al*., 2006; Tucker *et al.*, 2009), reperfusion injury in stroke (Kleinschnitz *et al.*, 2006), and disseminated intravascular coagulation (Tucker *et al.,* 2008). A major goal of the work in this thesis is to investigate the mechanisms by which factor XIa, a protease with a distinctly different structure than other coagulation proteases, activates factor IX. We postulate that the mechanism by which factor IX is activated by factor XIa is markedly different than the mechanism for factor IX activation by factor VIIa/TF, as factor XIa requires neither phospholipid or a cofactor to mediate the process. We have also pursued interesting preliminary data on the mechanism by which zymogen factor XI is converted into an active protease. This work identified a novel form of activated factor XIa that may represent the major physiologic species of this protease.

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CHAPTER II

FACTOR IX STRUCTURE AND ACTIVATION

Introduction

This chapter will cover the structure of factor IX, with a particular emphasis on how the structure relates to factor IX activation. The importance of factor IX in hemostasis is demonstrated by the severity of the bleeding in the disorder hemophilia. Hemophilia B, congenital deficiency of factor IX, is an X-linked disorder that occurs in 1 in 30,000 male births (Ragni *et al.*, 2009). People with severe hemophilia B suffer from spontaneous hemorrhage into soft tissues and joints that can be crippling or life threatening. Factor IX arose from a gene duplication that also produced factor X, with which it shares a similar structure. As discussed in Chapter I, factor IX is classified as an intrinsic pathway protein in the traditional cascade model of coagulation (**Figure I-1A**), and forms a link between contact activation and thrombin generation. Because the duplication of the primitive gene that resulted in formation of the factor IX gene substantially predates the appearance of factor XI in vertebrates (Ponczek et. al., 2008), factor IX must be activated by other mechanisms as well. In current models of coagulation (**Figure I-1B**), the major mechanism for factor IX activation is through the factor VIIa/tissue factor complex, and the most likely contribution of activated factor IX to hemostasis is as a separate activator for factor X that is not inhibited by TFPI. In this scheme, factor IX activation through factor XIa serves to supplement factor IX activation by factor VIIa/TF.

Human factor IX is a 57,000 Dalton single chain glycoprotein composed of 415 amino acids, with approximately 17% carbohydrate (DiScipio *et al.*, 1978). It is synthesized in hepatocytes, and circulates in plasma at a concentration of ~90 nM (Schmidt and Bajaj, 2003). Factor IX is structurally similar to factors VII and X (**Figure I-2**), and the coagulation regulatory protease protein C (Furie and Furie, 2009). Starting from the N-terminus, factor IX is composed of an N-terminal Gla domain, a hydrophobic stack region, two epidermal growth factor (EGF) domains (EGF1 and EGF2) connected by a short linker, an activation peptide region that is removed completely upon protease

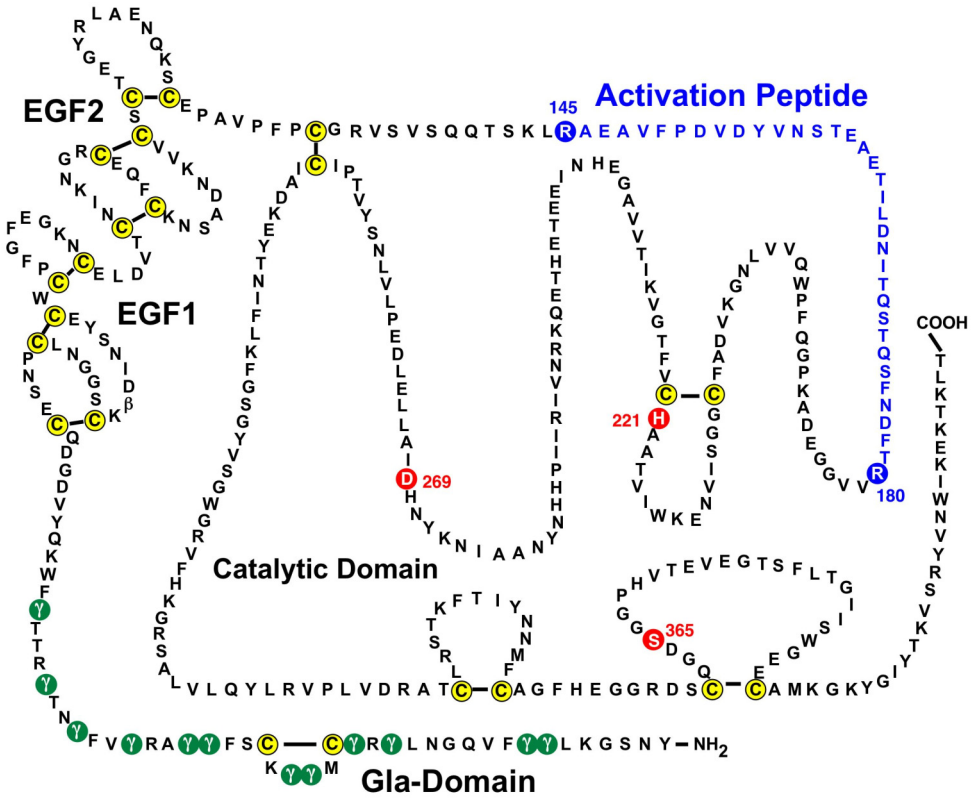

Figure II-1. Primary amino acid sequence and disulfide bond structure of human factor IX. γ-gluamyl-carboxyglutamic acid residues in the Gla domain are shown in green circles, and amino acids of the activation peptide are shown in blue. Cleavage after Arg^{145} and Arg^{180} are required to convert zymogen factor IX to the active protease factor IXaβ. The residues of the protease domain catalytic triad are shown in red circles. The non-catalytic portion of factor IXaβ contains the Gla domain and the two epidermal growth factor domains (EGF1 and EGF2). Cysteine residues are in yellow circles. Not shown are the N-terminal signal peptide and pre-pro-leader sequence that binds to γ-glutamyl-carboxylase.

activation, and a C-terminal trypsin-like catalytic serine protease domain (**Figure II-1**). In **Figure II-2**, a schematic diagram of factor IX is shown along with a space-filling model based on structures of other vitamin-K dependent proteases (there are no published crystal structures for factor IX). Note that the Gla domain and catalytic domains are at the extreme ends of the molecule. When factor IX binds to a phospholipid surface, this elongated arrangement facilitates placement of the catalytic domain at the correct distance above the membrane for optimal activation by factor VIIa (Waters *et al.*, 2006).

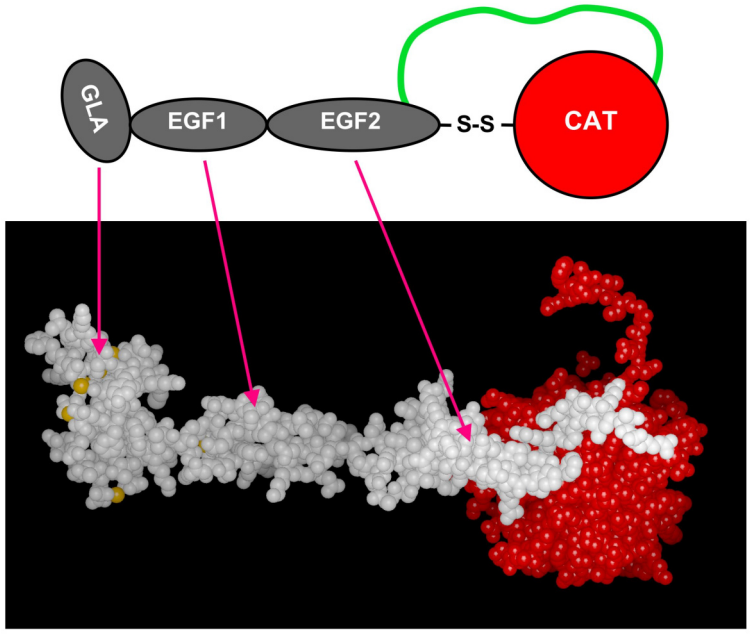

Figure II-2. Domains of factor IX. Shown at the top is a schematic diagram of factor IX with the domains labeled. Abbreviations are: Gla - γ-carboxyglutamic acid domain, EGF - epidermal growth factor domain, CAT - catalytic domain. The activation peptide is shown in green. At the bottom of the figure is a composite space filling model of human factor IX cleaved after Arg^{145} (factor IXα) with the non-catalytic domains indicated by the purple arrows. The catalytic domain is in red. Calcium ions in the Gla domain are represented by yellow spheres. Model courtesy of Dr. S. Paul Bajaj.

Factor IX is converted to the active protease factor IXaβ by proteolytic cleavage after Arg^{145} and Arg^{180}, releasing the internal activation peptide. The resulting polypeptides that comprise factor IXaβ, referred to as the light chain (Gla domain, and

the EGF1 and EGF2 domains) and the heavy chain (catalytic domain), are held together by a disulfide bond between Cys^{132} and Cys^{289}. The Gla, aromatic stack, EGF1, EGF2, and catalytic domains of factor IX are all involved in protein-protein interactions with substrates, phospholipid surfaces and cofactors. In addition, the Gla domain appears to be of primary importance in interacting with factor XIa in solution (Aktimur *et al.*, 2003).

Gla Domains

As briefly discussed in Chapter I, Gla domains are membrane binding motifs that support interactions with phospholipid membranes containing phosphatidylserine, such as those typically found on activated platelets, cells undergoing apoptosis, and damaged cells at a site of vascular injury. The factor IX Gla domain (residues 1-40 - **Figure II-3**) is post-translationally γ-carboxylated on its 12 glutamic acid residues in a reaction catalyzed by the vitamin K-dependent enzyme γ glutamyl-carboxylase (Suttie, 1985; Wu,

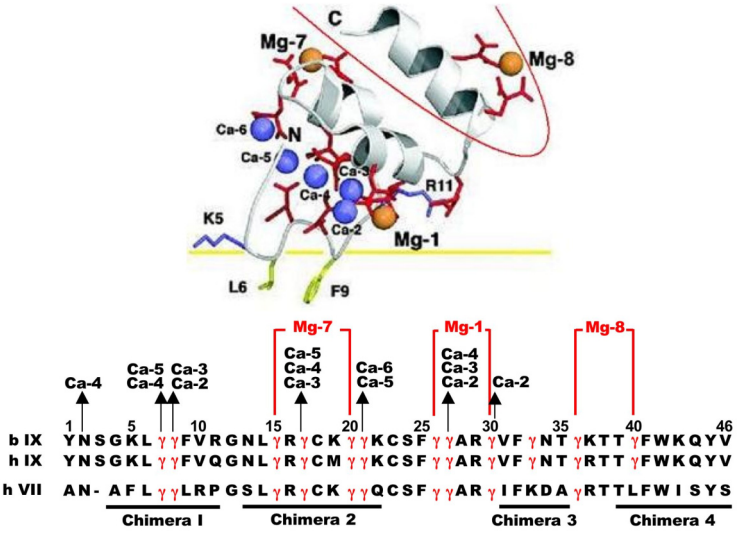

Figure II-3. The factor IX Gla domain. The top image shows the factor IX Gla domain structure, from a crystal structure of amino acids 1-46 in complex with a binding protein from the venom of the snake *Trimeresurus flavoviridis*. γ-carboxyglutamic acids are in red and residues that insert into phospholipid membranes are in yellow. The yellow line indicates the membrane surface. The red arc indicates residues 40-46, which were implicated in initial studies as a factor XIa binding site. The image is adapted from Shikamoto *et al.* (*J. Biol. Chem.* 278;24090-24094, 2003). At the bottom of the figure is a comparison of Gla domain sequences for bovine (b IX) and human (h IX) factor IX, and human factor VII (h VII). Amino acids involved in binding calcium or magnesium ions are indicated at the top. Residues in factor IX replaced by factor VII sequence in the recombinant chimeras discussed in the text are indicated at the bottom.

1991). Once modified, these glutamic acid residues coordinate binding of calcium and magnesium ions, resulting in proper structural rearrangement of the entire Gla domain. The properly folded factor IX Gla domain binds negatively charged phospholipids, such as phosphatidylserine, by calcium and/or magnesium bridging with the negatively charged phospholipid head groups (Swairjo *et al.*, 1995; Nelsestuen and Ostrowski, 1999; Huang *et al.*, 2003), as well as by hydrophobic interactions involving residues Lys^5, Leu^6 and Phe^9 as shown in **Figure II-3** (Huang *et al.*, 2003).

The factor IX Gla domain is essential for normal binding of factor IX to factor XIa, as factor IX with the homologous Gla domain from factor VII (factor IX/VII-Gla) does not bind to factor XIa (Aktimur *et al.*, 2003). Factor IX/VII-Gla is activated poorly by factor XIa, but is activated normally by factor VIIa/TF. Similarly, recombinant factor IX expressed in media containing warfarin, which inhibits vitamin K-dependent γ-carboxylation, is activated poorly by factor XIa (Aktimur *et al.*, 2003). Replacement of the factor IX Gla domain with the Gla domain from protein C also resulted in a factor IX molecule that could be activated normally by factor VIIa/TF, but not by factor XIa (Ndonwi *et al.*, 2007). Because the N-terminal portion of the Gla domain, known as the ω-loop, is involved in lipid interactions, and lipid does not affect factor XIa catalyzed factor IX activation (Mannhalter *et al.*, 1984; Gailani and Broze, 2001), it is reasonable to conclude that the area of the Gla domain that interacts with factor XIa lies in the C-terminus of the Gla domain. These findings suggest different epitopes within the factor IX Gla domain bind factor XIa and the factor VIIa/TF complex, and may explain structural differences between the Gla domain of factor IX and those of other vitamin K dependent proteins, which are not activated by factor XIa.

The coordinated binding of Ca^{2+} ions is required for proper ω-loop conformation and membrane binding during factor IX activation by the factor VIIa/TF complex, and for activation of factor X by factor IXaβ (Huang *et al.*, 2003). The factor IX Gla domain is thought to interact with an area of tissue factor near the phospholipid surface when engaged with the factor VIIa/TF complex. The modeled structure of the factor IX Gla domain proposed by Chen *et al.* (Chen *et al.*, 2002) predicts that residues 18 and 19, which are located in a turn between the first and second α-helices (helices A and B), are involved in an interaction with Arg^{36} on factor VIIa. Recombinant chimeras in which α-helices from the factor IX Gla domain have been exchanged for the equivalent helices from factor VII are activated normally by the factor VIIa/TF complex (Sun and Gailani, unpublished data). However, factor IX activation by factor XIa is abnormal for the chimera with replacement of the C-terminal helix C (Chimera 4 - **Figure II-3**), suggesting a unique interaction between this area and factor XIa. In experiments described in Chapters V and VI, activation of factor IX by factor VIIa/TF will be compared to activation by factor XIa.

Epidermal Growth Factor (EGF) Domains

The factor IX EGF1 domain (residues 47-83) is connected to the Gla domain by a short aromatic amino acid stack region that likely contributes to the curvature of the molecule and relative positions of the EGF1 and Gla domains (Chen *et al.*, 2002). Multiple lines of evidence from mutational and biochemical analyses (Ndonwi et al, 2005), and molecular modeling studies (Chen *et al.*, 2002) suggest a role for the factor IX

EGF1 domain in interactions with TF, but not with factor XIa (Zhong et al, 1994; Ndonwi *et al.*, 2007).

The factor IX EGF2 domain (residues 88-127) is implicated in binding factor VIIIa, and in interactions with platelets in the "tenase" complex involved in the activation of factor X (Wilkinson *et al.*, 2002). Experiments with EGF2 chimeras suggest this domain plays a role in assembling the factor X activating complex, and a minor role in platelet binding (Wilkinson 2002). In factor IX, EGF2 makes extensive contacts with the catalytic domain (Hopfner *et al.*, 1999; Fribourg *et al.*, 2006), and is also required for normal binding to the A2 subunit of factor VIIIa (Celie *et al.*, 2002). There is no evidence that the EGF2 domain of factor IX interacts with factor XIa.

Activation Peptide

The Gla and EGF domains, which make up the factor IX light chain, are connected to the catalytic domain (heavy chain) by a 35 amino acid (11,000 Dalton) activation peptide (DiScipio 1978). Removal of the activation peptide to generate factor IXaβ requires two proteolytic cleavages, and the activation peptide has no known function once removed from factor IX. In the space-filling model in **Figure II-2** factor IX has been cleaved at the N-terminus of the activation peptide (Arg^{145}-Ala^{146} bond) where it attaches to EGF2. Cleavage at the C-terminal end of the activation peptide (Arg^{180}-Val^{181} bond) allows Val^{181} to (Val^{16} in chymotrypsinogen numbering) form a salt bridge with Asp^{364} of the catalytic domain (Asp^{194} in chymotrypsinogen numbering) that allows the protease catalytic site to assume an active conformation. Although cleavage of the C-terminus of the activation peptide without cleavage at the N-terminus is

sufficient to generate an active protease (referred to as factor IXaα), the protease is a poor activator of factor X (Griffith *et al.*, 1985). Interestingly, activation of factor IX by factor VIIa/TF appears to always proceed by cleavage at the N-terminal Arg^{145}-Ala^{146} bond first (**Figure II-4A**), producing an non-active intermediate called factor IXα. Thus, complete removal of the activation peptide is required for normal activation of factor IX by factor VIIa/TF. Determining the order of bond cleavage of factor IX by factor XIa is a major goal of this thesis project.

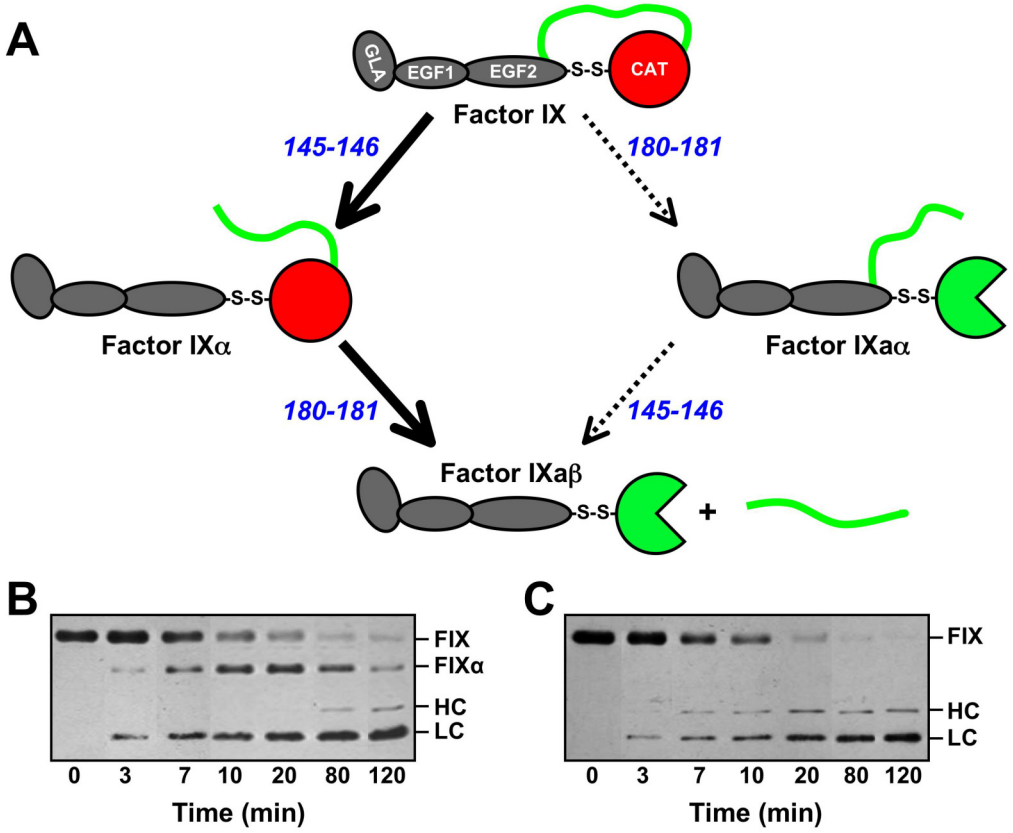

Figure II-4. Factor IX activation. (A) Factor IX is converted to factor IXaβ by cleavage of the Arg^{145}-Ala^{146} and Arg^{180}-Val^{181} bonds, releasing the activation peptide (green ribbon). Conversion of inactive catalytic domain (red circle) to active protease (green three-quarter circle) requires cleavage at Arg^{180}-Val^{181}. **(B)** During activation by factor VIIa/TF, factor IX is cleaved at Arg^{145}-Ala^{146} forming factor IXα. Factor IX cleavage at Arg^{180}-Val^{181} is a minor reaction. **(C)** When factor XIa activates factor IX, intermediate is not observed. Abbreviations: FIX - zymogen factor IX; FIXα - the large fragment of factor IXα containing the activation peptide and protease domain; CD - catalytic domain, the active protease domain of factor IXaβ; and LC - light chain, the Gla domain and EGF domains of factor IXα and factor IXaβ. Bonds cleaved in each step are indicated in blue.

Activation of Factor IX

As discussed above, factor IX must be cleaved after Arg^{145} and Arg^{180} for full conversion to the protease factor IXaβ. The predicted structure of factor IX (**Figure II-2**) does not give an indication of which peptide bond is likely to be cleaved first, as both cleavage sites appear to be accessible at either end of the solvent exposed activation peptide. Reducing western blots of time courses of factor IX activation by the factor VIIa/TF complex typically reveal the accumulation of the activation intermediate factor IXα, cleaved only after Arg^{145} (**Figure II-4B**). Similar time courses in which factor XIa was the activating enzyme revealed only slight accumulation of the same intermediate at most, with most experiments showing no accumulation at all (**Figure II-4C**). Although there is no obvious reason for this difference, the structures and evolutionary lineages of factor VIIa and factor XIa are consistent with different mechanisms for factor IX activation by these proteases.

Exosite interactions align the enzyme and substrate for efficient cleavage, and must account for the geometry of the substrate molecule, as well as changes in conformation that accompany conversion of the zymogen to an activation intermediate. Although both cleavages in factor IX are made by factor VIIa and factor XIa, these enzymes appear to process the individual cleavages differently. It is clear that these proteases have different structures. Factor VIIa, which is structurally similar to factor IX, has the typical elongated profile of a vitamin K-dependent coagulation protease, as shown in the space-filling model in **Figure II-5**. The structure of factor XIa, which is considered in the next chapter, is substantially different, consistent with the structure-function studies discussed above that indicate factor VIIa and factor XIa bind to distinctly

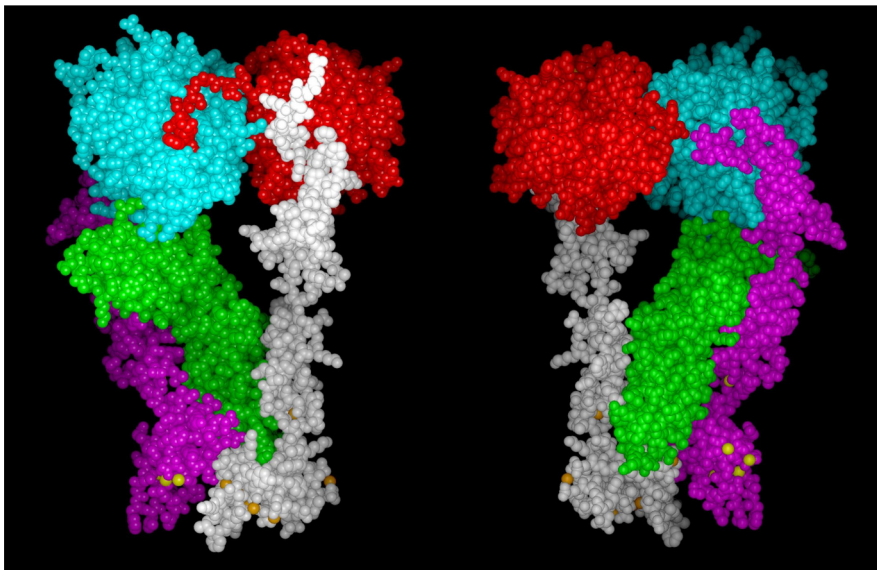

Figure II-5. Factor IX undergoing activation by factor VIIa/TF. Shown are two space-filling models of human factor IX (catalytic domain in red, non-catalytic domains in white) in complex with factor VIIa (catalytic domain in blue, non-catalytic domains in purple) and the extracellular portion of tissue factor (green). Calcium ions in the factor VIIa and factor IX Gla domains appear as yellow spheres. In this image, factor IX has already been cleaved at the Arg^{145}-Ala^{146} bond to form the intermediate factor IXα. Images courtesy of S. Paul Bajaj, University of California Los Angeles.

different domains on factor IX. Furthermore, while factor VIIa/TF activates both factor IX and factor X, factor XIa activates only factor IX. A difference in binding recognition sites for the two proteases is, therefore, expected. If exosite interactions between factor IX and factor VIIa or factor XIa involve the auxiliary domains of these proteases, then presentation of the factor IX scissile bonds to either catalytic domain may be distinctly different. In addition, the manner in which the two proteases interact with a singly cleaved factor IX activation intermediate may also be different.

Factor IX activation by factor XIa is potentially a more complex process than is activation by factor VIIa/TF. Factor XIa appears to generate no intermediate when cleaving factor IX. This could be interpreted as indicating that both cleavages required for factor IX activation are made without formation or release of an intermediate. As

mentioned in Chapter I, and covered in detail in Chapter III, factor XIa is a homodimer, and it has been suggested that the two catalytic domains of factor XIa may cleave both scissile bonds of factor IX simultaneously or in rapid succession without release of an intermediate (Wolberg *et al.*, 1997; Samuel *et al.*, 2007). Alternatively, factor XIa could cleave the scissile bonds of factor IX sequentially, with the second cleavage being as fast or faster than the initial cleavage. While this latter mechanism could require both factor XIa catalytic domains, it is also possible that each subunit of the factor XIa dimer can activate factor IX independently of the other subunit. In this case one factor XIa dimer may bind two factor IX molecules simultaneously. These issues are addressed in detail in the experiments described in Chapter V.

Summary

It seems clear that factor IX is activated by the factor VIIa/TF complex in a process that involves (1) binding of factor IX to the complex, (2) cleavage of the Arg^{145}-Ala^{146} bond to form the intermediate factor IXα, (3) release of the intermediate followed by rebinding to the complex, and (4) cleavage of the Arg^{180}-Val^{181} bond to form the final product, factor IXaβ. Factor IX activation by factor XIa, on the other hand, may involve a distinctly different process. As no intermediate appears to form during factor IX activation by factor XIa, we do not gain significant information on the mechanism involved by studying western blots of time courses such as the one shown in Figure **Figure II-4C**. The main goals of the work described in this thesis are to determine (1) the order of bond cleavage of factor IX by factor XIa, (2) whether factor XIa requires both

catalytic domains to properly process factor IX, and (3) the importance of exosite interactions as they relate to the mechanism of activation of factor IX by factor XIa.

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CHAPTER III

FACTOR XI STRUCTURE

Introduction

Human coagulation factor XI is a 160,000 Dalton homodimer (**Figure III-1A**) composed of two identical 80,000 Dalton subunits (**Figure III-1B**) that are joined by a single disulfide bond. Each subunit is composed of an N-terminal series of four apple domains, numbered A1 through A4, followed by a C-terminal trypsin like serine protease domain (**Figure III-2**) (Fujikawa *et al.*, 1986; McMullen *et al.*, 1991A; Papagrigoriou *et al.*, 2006). Factor XI is synthesized in hepatocytes and circulates in plasma at a concentration of ~30 nM in a complex with the glycoprotein HMWK. Each subunit of factor XI is activated through cleavage by factor XIIa or thrombin. Factor XI is the only coagulation factor, and only plasma serine protease, that is known to be a dimer. The only other homodimeric serine protease that has been reported is granzyme A, which is secreted by cytotoxic T lymphocytes (Bell *et al.*, 2003). Although factor XI and granzyme A both have an S1A trypsin-like serine protease domain, they are not related enzymes. While factor XI has a catalytic domain that is similar to those of other coagulation proteases, it shares the highest homology with the monomeric protease PK (Discussed in Chapter I). The catalytic domains of factor XI and PK are 68% homologous (Fujikawa *et al.*, 1986; Chung *et al.*, 1986), consistent with the fact that the duplication that gave rise to these paralogs was recent in comparison to the appearance of other coagulation proteases. Overall, factor XI and PK are 58% identical at the amino acid level.

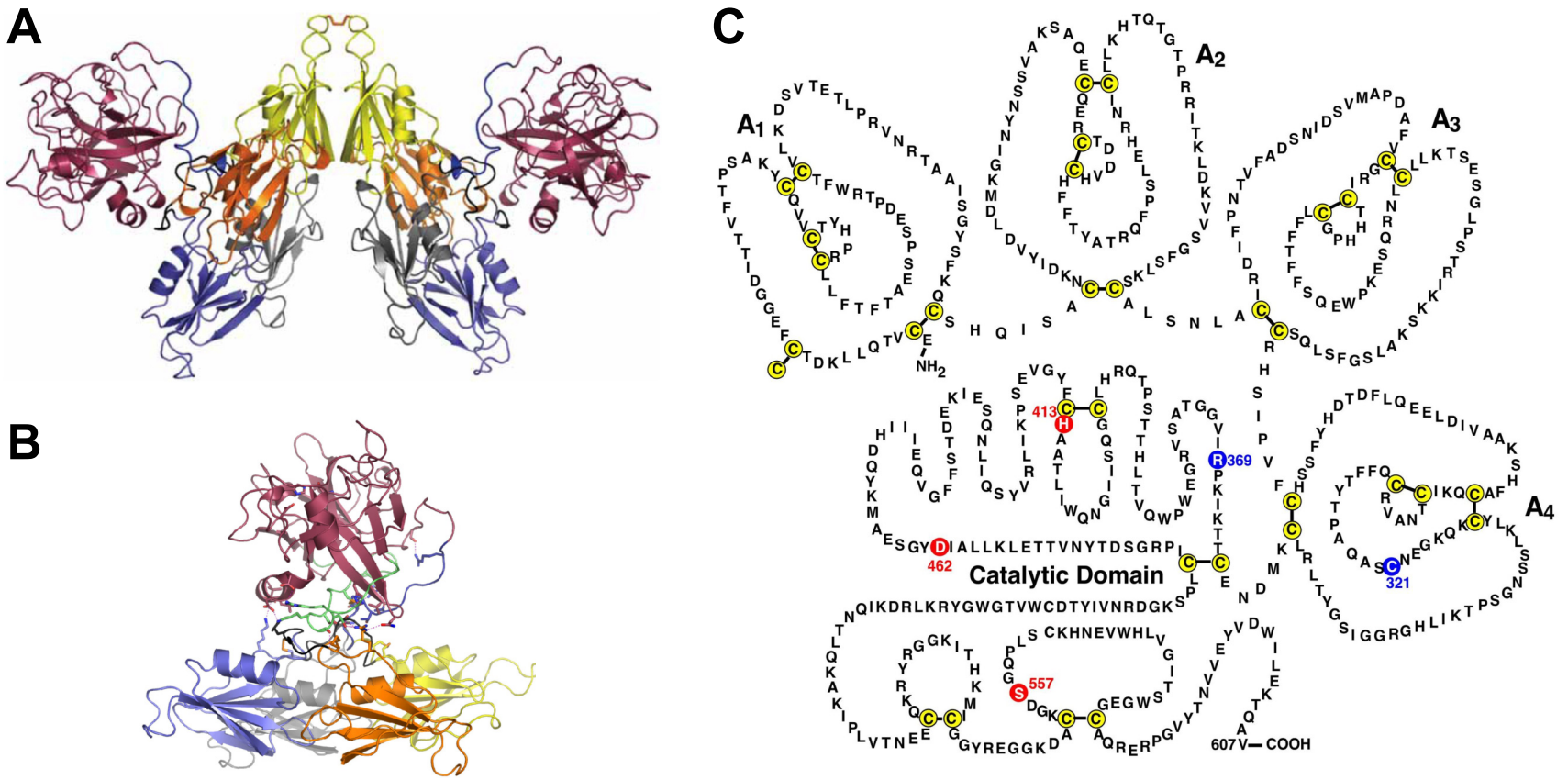

Figure III-1. Structure of human factor XI. (A) Dimer and (B) Monomer. In both images the apple 1 (A1) domains are gray, A2 blue, A3 orange, and A4 yellow. The protease domains are dark red. Note the interchain disulfide bond formed by residue Cys321 in the A4 domains from each subunit at the top of panel **A**. Images courtesy of Jonas Emsley, University of Nottingham (Papagrigoriou *et al.*, 2006). **(C) Amino acid sequence and disulfide bond structure of human factor XI monomer.** The apple domains are designated A1-A4. Cys321 in A4 forms the disulfide bond between the dimer subunits. Factor XI is cleaved after Arg369 to form factor XIa. A disulfide bond between Cys362 and Cys482 connects the catalytic domain and heavy chain in factor XIa. His413, Asp462 and Ser557 (His57, Asp102, Ser195 in chymotrypsin) form the protease catalytic triad, and are shown in red. Diagram modified from McMullen *et al.*, 1991A.

Activation of Factor XI

Conversion of a factor XI subunit to factor XIa involves cleavage of the Arg^{369}-Ile^{370} bond. Cleavage results in a heavy chain (the four apple domains) and catalytic domain (light chain) that remain connected by a disulfide bond between Cys^{362} on the A4 domain and Cys^{482} on the catalytic domain. Ile^{370} (Ile^{16} in chymotrypsinogen numbering) that forms the new N-terminus of the catalytic domain and transits approximately 20 Å to form a salt bridge with Asp^{556} (Asp^{194} in chymotrypsin numbering) that stabilizes the protease oxyanion hole (Navaneetham *et al.*, 2005; Papagrigoriou *et al.*, 2006). A similar cleavage between Arg^{371} and Ile^{372} activates PK, with a predicted shift of 17 Å in the new N-terminus (Hooley *et al.*, 2007). Both PK and factor XI are activated by factor XIIa (Davie *et al.*, 1991). However, because factor XII deficient people do not have a bleeding disorder, it is not clear that factor XII is the most physiologically relevant activator of factor XI. In addition to factor XIIa, factor XI can be activated by α-thrombin (Naito and Fujikawa, 1991; Gailani and Broze, 1991), the prothrombin activation intermediate meizothrombin (von dem Borne *et al.*, 1997), factor XIa (autoactivation) (Naito and Fujikawa, 1991; Gailani and Broze, 1991) and trypsin (Mannhalter *et al.*, 1980). Work in progress in our laboratory indicates that β-thrombin and γ-thrombin, two degradation products of α-thrombin can also activate factor XI.

Activities of Factor XIa

α-Kallikrein, the active form of PK, is a potent activator of factor XII and cleaves HMWK to release bradykinin (Gailani and Broze, 2001). However, in contrast to factor XIa, it is a poor activator of factor IX (Sun and Gailani, 1996). Factor XIa, is able to

cleave factor XII and HK, but with a catalytic efficiency that is far poorer than α-kallikrein. The binding epitopes that confer specificity to the protein-protein interactions of factor XI, PK, and their activated forms are likely to be found to a large extent outside of the catalytic domains of these molecules.

Apple Domains

The structural features setting factor XI and PK apart from other coagulation molecules are the apple domains. As discussed in Chapter I, apple domains are members of the PAN domain family (Tordai *et al.*, 1999). Apple domains are composed of seven β-strands that form a curved anti-parallel sheet, with an α-helix attached to the concave side of the sheet (**Figure III-2**) (Papagrigoriou *et al.*, 2006). Each end of the α-helix is attached to the β-4 and β-5 strands of the β-sheet by a disulfide bond. The four apple domains of each factor XI subunit are arranged into a planar disc, with 60 X 60 X 20 Å dimensions (**Figure III-2**). Two types of interdomain interactions are important for the packing stability of apple domains in the disc. These are referred to as *side* and *edge*

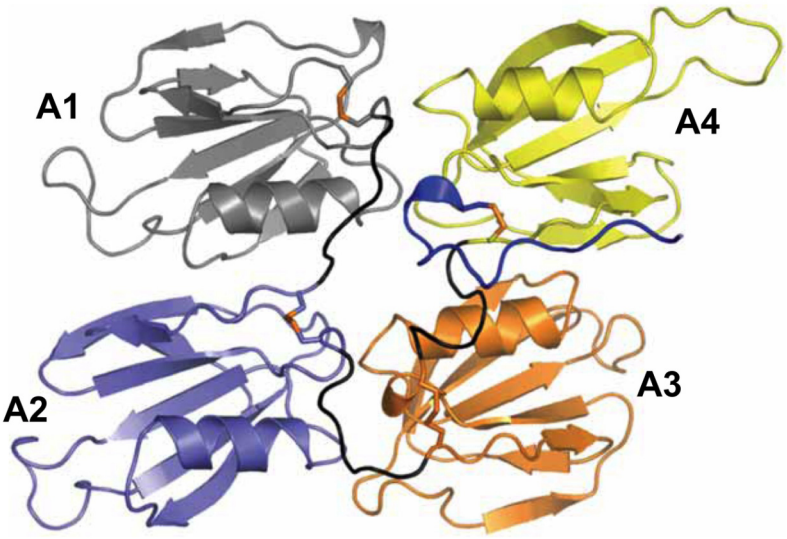

Figure III-2. Factor XI heavy chain. The apple domains of factor XI (A1-A4) form a planar structure measuring 60 by 60 by 20 Å. The hydrophobic residues and Cys^{321}, which form the interface between the dimer subunits, are on the loop extending from the upper right hand corner of A4. Image courtesy of J. Emsley (Papagrigoriou *et al.*, 2006)

interfaces, with reference to the alignment of the β-strands composing each apple domain (**Figure III-2**). A1-A2 and A3-A4 domain interfaces are *side* interfaces, and bury 441 $Å^2$ and 444 $Å^2$, respectively. The interfaces between A1-A4 and A2-A3 are *end* interfaces, and bury 380 $Å^2$ and 284 $Å^2$, respectively (J Emsley, personal communication). The catalytic domain rests on the planar structure formed by the apple domains, like a "cup in a saucer", giving each subunit an overall pyramidal shape (**Figure III-1B**) (Papagrigoriou *et al.*, 2006). It is likely that PK has a similar structure (Hooley *et al.*, 2007).

Factor XI and Dimerization

The major difference between factor XI and PK is that the former is a dimer and the latter a monomer. The factor XI A4 domain mediates dimer formation by a mixture of hydrophobic interactions and a single disulfide bond involving Cys^{321} (Meijers et al, 1992; Papagrigoriou *et al.*, 2006). Cys^{321} is conserved in factor XI in all mammalian species studied with the exception of the rabbit, which has a histidine at this position. Gel filtration chromatography shows that rabbit factor XI is in fact a non-covalently associated dimer, indicating that non-covalent interactions are sufficient to maintain dimeric structure (Sinha *et al.*, 2002). Similarly, human factor XI in which Cys^{321} has been replaced with another amino acid is also a dimer (Meijers et al, 1992; Sinha *et al.*, 2002; Cheng *et al.*, 2003).

The factor XI crystal structure reveals a mixture of electrostatic and hydrophobic interactions in the dimer interface. Recent studies have shown that salt bridges and hydrophobic interactions are responsible for the relatively tight association constant of factor XI subunits in the absence of Cys^{321} (Samuel *et al.*, 2007). The factor XI dimer

subunit interface is 567 Å^2, which is about 2.24% of the overall surface area of the factor XI monomer (25,312 Å^2) (Wu *et al.*, 2008). Most other dimeric proteins have a much larger interface, with areas ranging from 3-44% of the total protein surface area. Crystal structure studies and mutagenesis work show that the A4 residues Leu^{284}, Ile^{290} and Tyr^{329} form the relatively small hydrophobic interface between the two subunits (Papagrigoriou *et al.*, 2006; Wu *et al.*, 2008).

PK cannot form an inter-subunit disulfide bond because Cys^{321} in its A4 domain forms an intrachain disulfide bond with Cys^{326}, which is unique to PK (McMullen *et al.*, 1991B). In factor XI the residue at position 326 is glycine. However, recombinant PK with an unpaired Cys^{321} (Cys^{326} changed to Gly) does not form a dimer, indicating other components of the A4 domain are required for dimer formation in factor XI (Cheng *et al.*, 2003). The PK A4 domain does in fact differ from that of factor XI in key residues thought to be critical for dimer formation, including residues thought to mediate hydrogen bonding (Asn^{322} to Lys and Gln^{318} to Pro), residues involved in interfacial salt bridges, (Arg^{345} to Thr and Asp^{289} to Asn), and residues directly involved in forming the interface (Leu^{284} to Gly and Ser^{295} to Gly) (Hooley *et al.*, 2007).

Binding Sites on Factor XI and Factor XIa Apple Domains

Sites on the apple domains are thought to contribute the majority of binding energy for the interactions of factor XI and factor XIa with various ligands and substrates. Studies using recombinant factor XI in which individual apple domains were replaced by the corresponding domain of PK strongly suggest that factor IX interacts with an exosite on the factor XIa A3 domain. Factor XI with the PK A3 domain (factor

XIa/PKA3) supported factor IX activation poorly, with a substantially increased K_m for the reaction (Sun and Gailani, 1996). Factor IX binds to factor XIa, but does not bind to zymogen factor XI (Aktimur *et al.*, 2003). Thus, when factor XI is activated, a conformational change must occur that exposes the exosite on the A3 domain. In saturation mutagenesis studies, amino acids 183-191 in the factor XIa A3 domain are required for factor IX activation (Sun *et al.*, 1999). As expected, these residues are partly obscured by the catalytic domain in zymogen factor XI (**Figure III-3**). Experiments demonstrating the importance of the factor XIa A3 domain to interactions with factor IX will be shown in Chapters V and VI. Recently, Sinha and colleagues presented evidence for another factor IX-binding exosite within the catalytic domain of factor XIa, distinct from the protease active site (Sinha *et al.*, 2007). The relative importance of the different factor IX-binding exosite on factor XIa is currently under investigation in our laboratory.

The factor XI A3 domain also supports interactions with heparin (Zhao *et al.*, 1998) and the platelet receptor Glycoprotein 1bα (GP1bα) (Baglia *et al.*, 2004). A unique

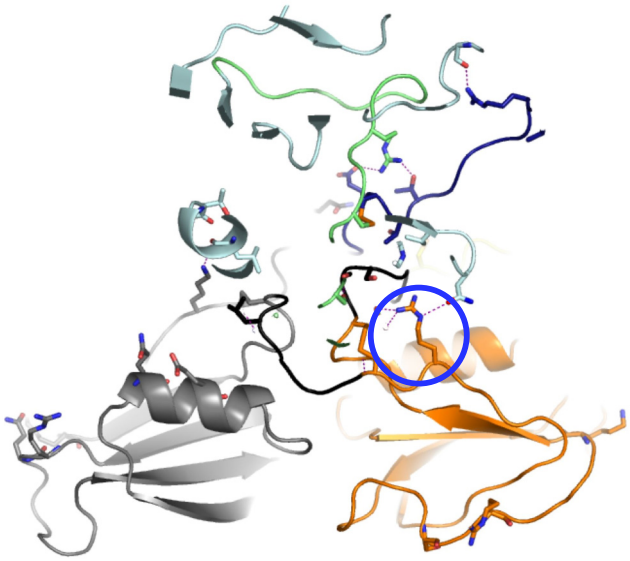

Figure III-3. Putative factor IX binding site on the A3 domain. Shown is an "edge-on" view of zymogen factor XI monomer with the end-to-end interface between the A2 domain (gray) and the A3 domain (orange). The catalytic domain (green and light blue at top) is partially cut away to show an area of A3 that is covered by the catalytic domain in the zymogen. Arg^{184} in A3 (circled) is required for factor IX activation, but is buried in the zymogen structure. A salt bridge between Arg^{184} and the catalytic domain is probably broken upon activation of factor XI, exposing this residue to the solvent phase where it can participate in factor IX binding. Image courtesy of J. Emsley. (Papagrigoriou *et al.*, 2006).

aspect of the apple domain arrangement of factor XI, that probably holds for factor XIa as well, is that it positions the A3 domains of the two subunits on opposite sides of a major plane of the molecule (**Figure III-4**), while the A2 and A4 domains straddle the plane. This arrangement has been postulated to have implications for ligand binding (Papagrigoriou et al, 2006). For example, a Gp1bα platelet receptor could theoretically bind to one A3 domain without causing steric interference with factor IX binding at the other A3 domain. Both factor XI and PK circulate in plasma bound to HMWK. The A2 domain in both factor XI and PK is thought to contain a major binding site for HMWK, with contributions from the A1 and A4 domains (Renné *et al.*, 2002) via a charged "channel" that traverses these domains (Hooley *et al.*, 2007).

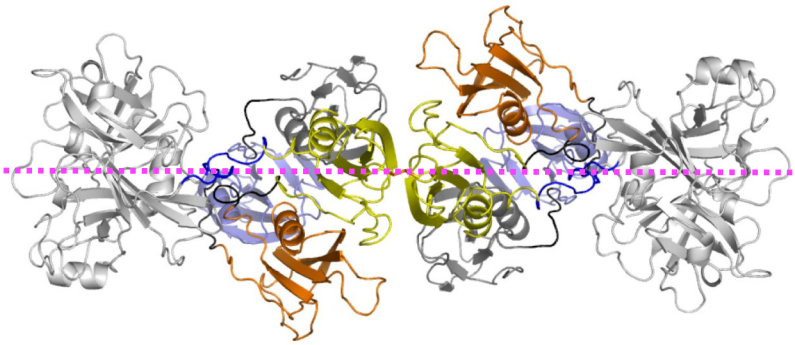

Figure III-4. Factor XI. View from above the dimer interface in the A4 domain (yellow). Note that the A3 domains (orange) are on opposite sides of the long axis of the molecule indicated by the magenta line. Catalytic domains are in gray. Image courtesy of J. Emsley (Papagrigoriou *et al.*, 2006)

The Importance of the Dimeric Conformation to Factor XI/XIa Function

The functional importance of the dimeric structure of factor XI has not been resolved, and there has been limited investigation of this issue because of technical problems involved in isolating factor XI or factor XIa comprised of a single subunit (monomeric factor XI - addressed in Chapter V). Papagrigoriou *et al.*, and later Wu *et al.*, suggested a *trans*-activating mechanism for factor XI activation in which the activating protease binds to the apple domains of one factor XI subunit and activates the opposing

subunit by cleavage of the Arg^{369}-Ile^{370} bond (Papagrigoriou *et al.*, 2006; Wu *et al.*, 2008). The *trans*-activating mechanism could be a natural consequence of factor XI binding to a platelet receptor by one of its subunits, while the other subunit is unbound. As discussed in Chapter II, another recent hypothesis regarding the unique dimeric structure proposes that factor XIa, which has two active catalytic domains, could cleave both scissile bonds on one factor IX molecule simultaneously. This would offer a reasonable explanation for why factor XIa activates factor IX without generation of an intermediate. Alternatively, if only one factor XIa subunit is required for factor IX activation, the other subunit could be involved in tethering the protease to a platelet receptor. Most coagulation protease-mediated reactions take place on the surface of platelets or TF-bearing cells. The dimeric structure of factor XI/XIa could be an adaptation to facilitate this type of binding in a protein that lacks a phospholipid-binding Gla domain.

Summary

As discussed in Chapter II, factor XIa appears to activate factor IX by a mechanism distinct from that used by factor VIIa. It is reasonable to suspect that one or more of the unique aspects of the factor XIa structure are responsible for this difference. Factor XIa differs markedly in structure from the vitamin K-dependent coagulation proteases in several important features including the lack of a Gla domain, the presence of apple domains, and its dimeric structure. Because of these differences, it is difficult to extrapolate from the structure-function relationships established for the vitamin K-dependent protease to factor XIa. The observation that factor XIa can activate factor IX

in the absence of a phospholipid surface or a cofactor emphasizes its differences from other coagulation proteases, and raises questions as to how it would be regulated *in vivo*.

The fact that factor XI is a homodimer in all mammalian species raises an important issue that has only been briefly addressed in the thirty years since the dimeric structure was identified (Bouma and Griffin, 1978); that a form of factor XIa may exist that is only activated on one of its subunits. In Chapter IV, we examine activation of factor XI by factor XIIa and α-thrombin in detail, and demonstrate that conversion of factor XI to factor XIa proceeds through an intermediate with only one activated subunit. This novel species is a valuable reagent for investigating the importance of two activated catalytic domains to normal activation of factor IX in Chapter V.

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CHAPTER IV

DISCOVERY AND PURIFICATION OF A NOVEL FORM OF FACTOR XIa

Introduction

During coagulation, factor XI subunits are converted to factor XIa by cleavage of the Arg^{369}-Ile^{370} bond by factor XIIa and various activated forms of thrombin (Discussed in Chapter III). Since factor XI is a homodimer, consisting of two identical subunits joined by a disulfide bond, it must be cleaved after two spatially separated Arg^{369}-Ile^{370} bonds per molecule for full activation (**Figure IV-1**). The conversion of factor XI to factor XIa has been treated functionally as a simple one-step process, in which only fully activated factor XIa is produced. However, it has been postulated that factor XI activation should proceed through a singly cleaved intermediate in which one subunit is activated, while the other remains uncleaved (Bouma and Griffin, 1978). However, no activation time course experiments have been performed that are capable of detecting the accumulation of a singly activated form of factor XIa, and only factor XIa in which both subunits are cleaved and activated has been described and characterized in

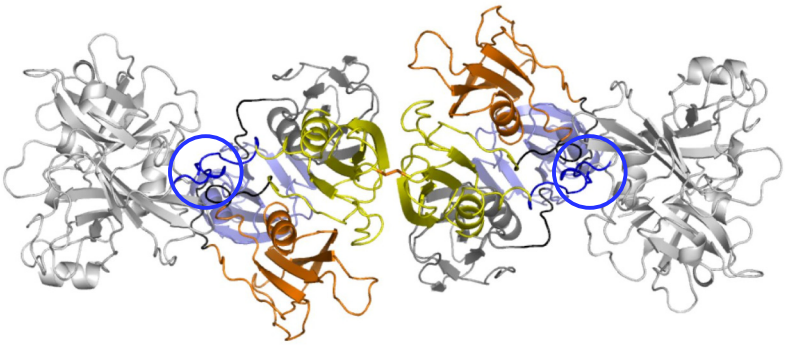

Figure IV-1. Factor XI activation sites. View from above the dimer interface in the A4 domain (yellow). A3 domains are in orange, A2s in light blue, A1s in dark gray. The catalytic domains are at the extremes of the molecule (light gray). The activation cleavage sites are circled in blue. Image courtesy of J. Emsley (Papagrigoriou *et al.*, 2006)

existing literature. During factor XI activation, appearance of factor XIa activity is typically monitored by cleavage of a chromogenic substrate. Alternatively, cleavage of the Arg^{369}-Ile^{370} bonds in factor XI can be followed by SDS-polyacrylamide gel electrophoresis run under reducing conditions. Reducing conditions have traditionally been used to monitor factor XI activation time courses because they show the disappearance of the 80,000 Dalton factor XI zymogen subunits, and the appearance of the 50,000 Dalton heavy chain (apple domains) and the 30,000 Dalton light chains (catalytic domains) (**Figure IV-2**). Note that neither technique can determine if zymogen and activated subunits are on separate molecules (factor XI and factor XIa) or are on the same molecule (a half-activated intermediate). Because factor XI does not lose mass when converted to factor XIa, there is no reason to expect that electrophoretic migration under non-reducing conditions would show a difference between factor XI to factor XIa.

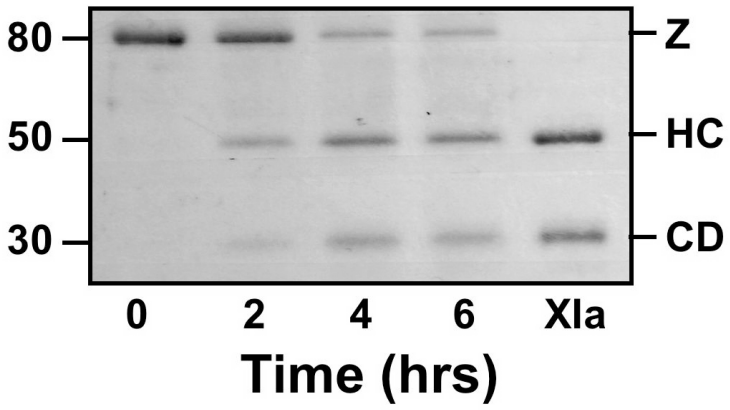

Figure IV-2. Factor XI activation. Shown is a 12% polyacrylamide gel of samples of human factor XI (1.7 μM) incubated with factor XIIa (0.625) μM. Samples were collected into reducing sample buffer at the indicated times. Positions of zymogen factor XI (Z) and the heavy chain (HC) and catalytic domain (CD) of factor XI are shown on the right. Positions of molecular mass standards are on the left.

Our initial efforts to activate plasma-derived human factor XI were intended to produce a molecule with two activated subunits. As factor XI is activated slowly in the absence of a surface, we used high enzyme:substrate (E:S) ratios so that we could follow the progress of activation on conventional stained SDS-polyacrylamide gels. Complete

activation of factor XI by either factor XIIa or α-thrombin required greater than 24 hours of incubation at 37 °C, with E:S ratios as high as 1:1. These prolonged incubation times appeared to result in significant degradation of factor XI. It was our impression that the reaction initially proceeded relatively quickly, but then became substantially slower. While it is possible that product inhibition was occurring, the observation that it was still difficult to drive the reaction to completion at high ES ratios suggested another mechanism might be operating. Negatively charged surfaces are typically used to enhance activation of plasma factor XI by either factor XIIa or thrombin, and activation kinetics are known to be unfavorable in the absence of these surfaces (Gailani and Broze, 1991; Gailani and Broze, 1993). However, the surface must be removed to generate pure factor XIa, and this process was deemed inefficient for use in later experiments.

Interestingly, recombinant human factor XI activated by the same protocol was fully activated in ~12 hours. We hypothesized that the difference in the observed activation rate of recombinant and plasma factor XI could be attributed either to a contaminant in the plasma protein that retarded activation, or differences in post-translational modifications (such as glycosylation) that may render cleavage of the second subunit of plasma factor XI more difficult to achieve than cleavage of the first subunit. We were unable to definitively identify an inhibiting contaminant in our plasma factor XI preparations. We did, however, observe slight differences in migration between standards for factor XI and factor XIa under non-reducing conditions on SDS-polyacrylamide gels, likely due to changes in protein shape that accompany activation. We reasoned that this might provide us with a method for following factor XI activation that could detect an intermediate species with only a single activated subunit.

The experiments and data presented in this chapter summarize the discovery of a novel, single active site form of activated factor XI that is produced during the activation of plasma derived factor XI in solution. This intermediate can be generated by either factor XIIa- or α-thrombin-catalyzed activation of factor XI. The intermediate, which we have designated 1/2-factor XIa, cleaves a chromogenic substrate as efficiently as fully active factor XIa when numbers of active sites are normalized. Importantly, 1/2-factor XIa can be formed in plasma, suggesting a possible physiologic role for this new enzyme.

Activation of Factor XI by Factor XIIa and α-Thrombin

As discussed in the previous section, non-reducing SDS gels have not been used to follow activation of factor XI. It is reasonable to expect that no change in migration would occur upon activation, because no mass is lost from factor XI when it is cleaved by either factor XIIa or α-thrombin. The negatively charged sodium dodecylsulfate (SDS) detergent used in the Laemmli gel electrophoresis system (Laemmli, 1970) coats proteins evenly, solubilizing hydrophobic internal amino acids, eliminating secondary and many tertiary structures, and allowing the protein to adopt an elongated rod like conformation that migrates largely (although not exclusively) based on mass. However, we observed a slight difference in mobility between factor XI and factor XIa standards on 12% SDS-polyacrylamide gels, and suspected that a lower percentage gel would exaggerate this difference. When standards for factor XI and factor XIa were run beside samples from a factor XI activation time course on a 6% SDS-polyacrylamide gel, factor XI migrated noticeably faster than factor XIa (**Figure IV-3**). As the actual masses of factor XI and factor XIa are the same, this supports the notion that there is a significant shape change

upon activation of factor XI. As expected, samples from the activation time-course contained bands that migrated with the standards for factor XI and factor XIa, but also contained a distinct band that migrated between factor XI and factor XIa (**Figure IV-3A**). Note the rapid disappearance of the zymogen band, but relatively slow disappearance of the intermediate band, in keeping with our observation that initial rate of factor XI activation is relatively rapid compared to activation later in the time course.

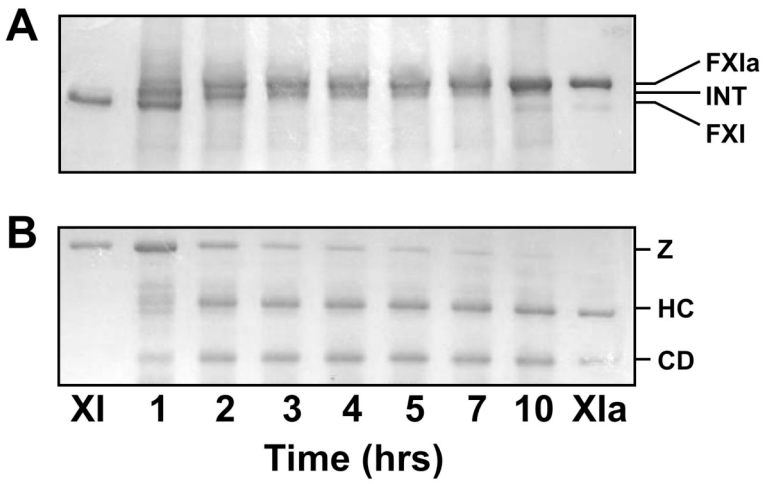

Figure IV-3. Time course of factor XI activation by factor XIIa. Factor XI (12 μM) in Assay Buffer was incubated at 24˚C with factor XIIa (860 nM) as described under Methods. At various times, duplicate aliquots were removed into reducing and non-reducing sample buffer, and fractionated on **(A)** a 12% polyacrylamide-SDS gel (reducing) or **(B)** a 6% polyacrylamide gel run (non-reducing), and stained with GelCode Blue (Pierce, Rockford, IL). Migration of standards for unreduced factor XI (FXI), unreduced factor XIa (FXIa), reduced factor XI (Z), and the heavy chains (HC) and catalytic domains (CD) of reduced factor XIa are shown. The position of migration of the reaction intermediate is indicated by INT.

Suspecting that the intermediate band could be a singly cleaved factor XIa species, we activated factor XI with factor XIIa and collected samples into reducing and non-reducing buffer for gel analysis. We reasoned that if the intermediate band was a singly cleaved factor XIa species, its appearance on the non-reducing gel would coincide with appearance of the heavy and light chains of factor XIa on the reducing gel. In **Figure IV-3A** the appearance of the intermediate on the non-reducing gel is coincident with appearance of the heavy and light chains of factor XIa on a reducing gel (**Figure IV-3B**). These complementary time-course gels demonstrate, as discussed above, that

traditional reducing gels cannot distinguish between a singly cleaved factor XI species (the intermediate) and a mixture of factor XI and factor XIa.

The most logical conclusion from these observations was that factor XIa migrates more slowly than factor XI through polyacrylamide gels because it has been cleaved at its Arg^{369}-Ile^{370} activation sites. Cleavage after Arg^{369} could allow each factor XIa subunit to adopt a more elongated shape in SDS-containing buffer, or interact differently with SDS, than the non-cleaved subunit, increasing the drag that the molecule experiences when passing through the pores of a gel. We further reasoned that cleavage after only one Arg^{369} residue would result in a protein that encountered approximately half as much drag when passing through the same gel. These experiments cannot be expected to detect or predict which factor XI subunit has been cleaved first. Indeed, the crystal structure of factor XI indicates that the protein is symmetric around a central axis, and it would not be possible to assign a designation to a specific subunit.

Factor XI is also activated by α-thrombin (Naito and Fujikawa, 1991; Gailani and Broze, 1991), giving a similar activation pattern on reducing gels as with factor XIIa (**Figure IV-4**). In our hands, thrombin activation of factor XI is slower than activation by factor XIIa, so we used a 1:1 E:S ratio when activating factor XI by α-thrombin to generate a similar degree of activation to factor XIIa. Factor XI activation by α-thrombin proceeded through an intermediate. Although the same intermediate is likely to be generated with factor XIIa and α-thrombin, it is interesting to note that the distribution of the intermediate over time is different for the two proteases. The intermediate accumulates and persists for a longer period of time in reactions with α-thrombin compared to factor XIIa, which appears to process the second cleavage more rapidly.

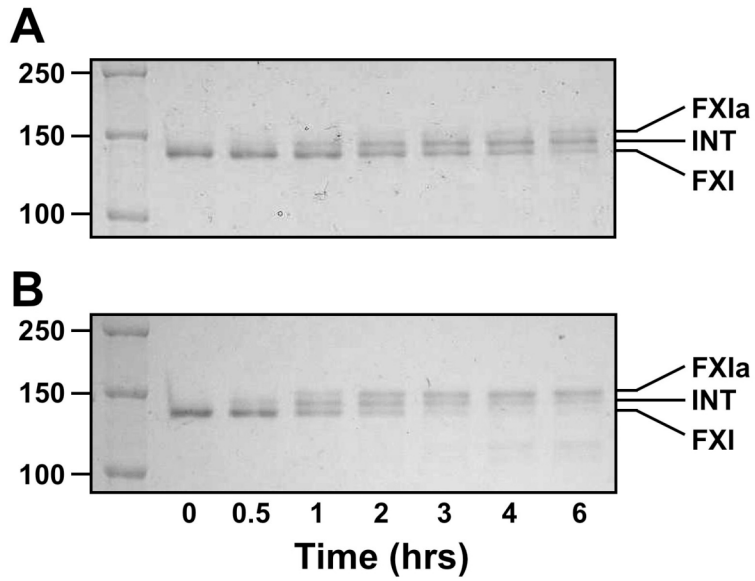

Figure IV-4. A comparison of factor XI activation by α-thrombin and factor XIIa. Plasma factor XI (600nM) was incubated at 37°C with either 600nM α-thrombin or 60 nM factor XIIa. Samples of each reaction were quenched in non-reducing sample buffer at the times indicated and size fractionated on 6% polyacrylamide gels. Positions for markers, and protein standards for factor XI (FXI), factor XIa (FXIa) and the activation intermediate (INT) are noted.

We also examined activation of recombinant factor XI by α-thrombin. As expected, the recombinant material was activated considerably more rapidly than plasma factor XI (**Figure IV-5**), but we still observed formation of the activation intermediate. Here again, we see that the initial cleavage of recombinant factor XI by α-thrombin appears to be substantially more rapid than the second cleavage. Our overall impression is that the catalytic efficiency for processing the intermediate to factor XIa is higher for factor XIIa than for α-thrombin.

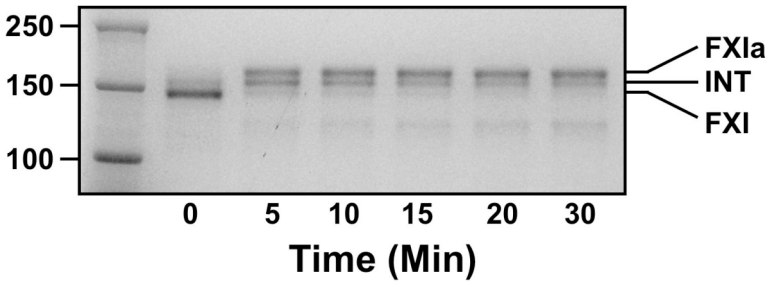

Figure IV-5. Recombinant factor XI activated by α-thrombin. 600 nM factor XI was activated by 600 nM α-thrombin. Samples were collected into non-reducing buffer at the indicated time points. Positions for markers, and protein standards for factor XI (FXI), factor XIa (FXIa) and the activation intermediate (INT) are noted.

Formation of the Factor XI Activation Intermediate in Plasma

We wanted to determine if the factor XI activation intermediate observed in the purified protein systems described in the previous section could be observed in plasma at physiologic protein concentrations. The partial thromboplastin time (PTT) assay is used in hospitals to assess plasma coagulation, and it is affected by the levels of all coagulation factors with the exception of factor VII/VIIa. The PTT is triggered by contact activation, and requires the contact factors factor XII, HMWK, and PK to activate factor XI (**Figure I-6**). The assay uses a negatively charged surface to initiate and propagate the activation of factor XII. In the PTT assay, a substantial portion of the factor XII in plasma is converted to factor XIIa, which rapidly converts factor XI to factor XIa. HMWK serves as a cofactor to anchor factor XI and PK to the contact surface.

We postulated that the factor XI activation intermediate might be formed during contact activation in plasma. To test this, we conducted an experiment in which normal human plasma, factor XII deficient plasma, or HMWK deficient plasma (**Figure IV-6**) was exposed to a silica-based PTT reagent. Western blots of the resulting activation time courses with an anti-human factor XI polyclonal antibody revealed rapid generation of both the activation intermediate and factor XIa in normal plasma (**Figure IV-6A**). As expected, no activation pattern was observed in the absence of factor XIIa or HMWK (**Figure IV-6B** and **C**). The results show that the novel factor XIa activation intermediate is generated *ex-vivo*, albeit in a system that requires an exogenous activator of factor XII. It is interesting to note that the appearance of the intermediate and factor XIa and the disappearance of factor XI follow the pattern seen in factor XIIa-catalyzed factor XI activation reactions in purified systems (**Figure IV-4**).

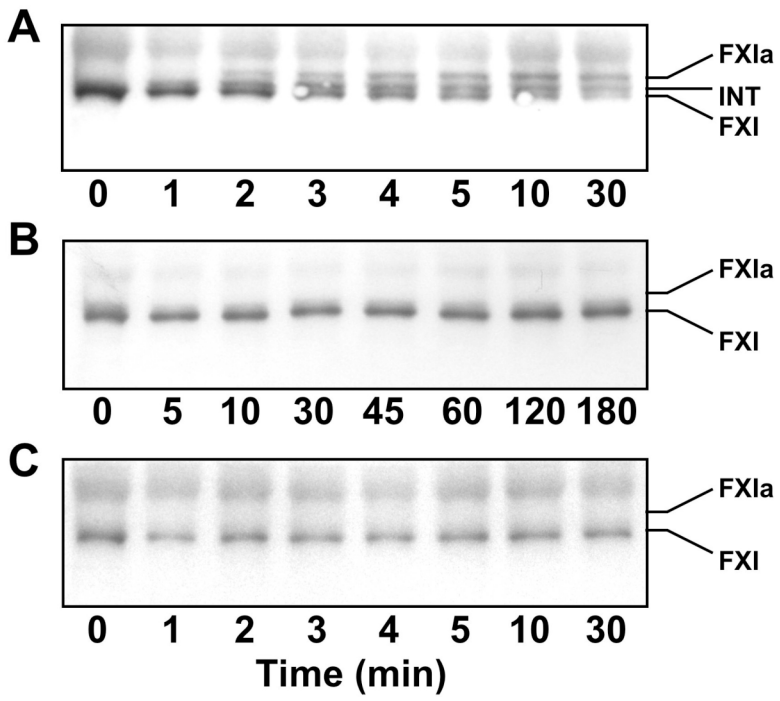

Figure IV-6. Western blots of factor XI activation in plasma. (A) Normal human plasma, and plasmas lacking **(B)** factor XII or **(C)** HMWK were mixed with a PTT reagent at 37˚C. At various times, samples were removed into non-reducing buffer and fractionated on 6% polyacrylamide gels, followed by western blotting as described under Methods. Positions for markers, and protein standards for factor XI (FXI), factor XIa (FXIa) and activation intermediate (INT) are noted. The time course is longer for factor XII deficient plasma than for normal or HMWK deficeint plasma.

The Factor XI Activation Intermediate Can Cleave a Chromogenic Substrate

To confirm the that factor XI activation intermediate described above is an activated form of factor XI, we followed the appearance of factor XIa during α-thrombin-catalyzed activation of factor XI, and compared the changes in activity to the appearance of factor XI activation products on a western blot (**Figure IV-7**). The presence of activated factor XI was detected by cleavage of the tripeptide chromogenic substrate S-2366 (see Methods). Note that the rapid generation of protease activity correlates with the appearance of the activation intermediate. After a lag phase in which only the zymogen and intermediate are seen, there is a further increase in protease activity that corresponds to formation of fully activated factor XIa. Based on the observation that appearance of the activation intermediate is associated factor XIa protease activity we postulated that the intermediate form, indeed, represented a form of activated factor XI in

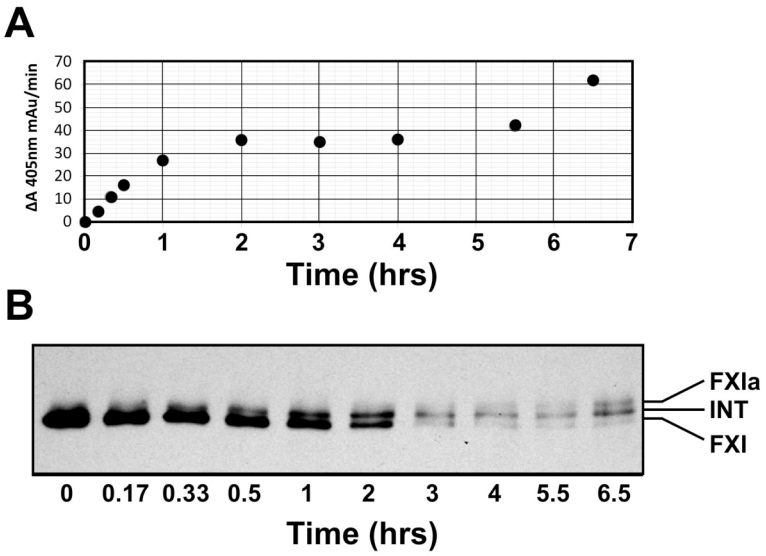

Figure IV-7. Time course of factor XI activation by α-thrombin. Factor XI (625 nM) was activated by 625 nM α-thrombin. At the indicated time points samples were tested for **(A)** factor XIa activity by chromogenic substrate assay as described in methods, or **(B)** western blotting of non-reduced samples on a 6% acrylamide gel. Positions for markers, and protein standards for factor XI (FXI), factor XIa (FXIa) and activation intermediate (INT) are noted.

which only a single subunit was activated (**Figure IV-8**). We will refer to this novel species hereafter as 1/2-factor XIa.

Purification of 1/2-Factor XIa

We developed a method to separate 1/2-factor XIa from factor XI through a purification protocol based on the assumption that 1/2-factor XIa has a functional

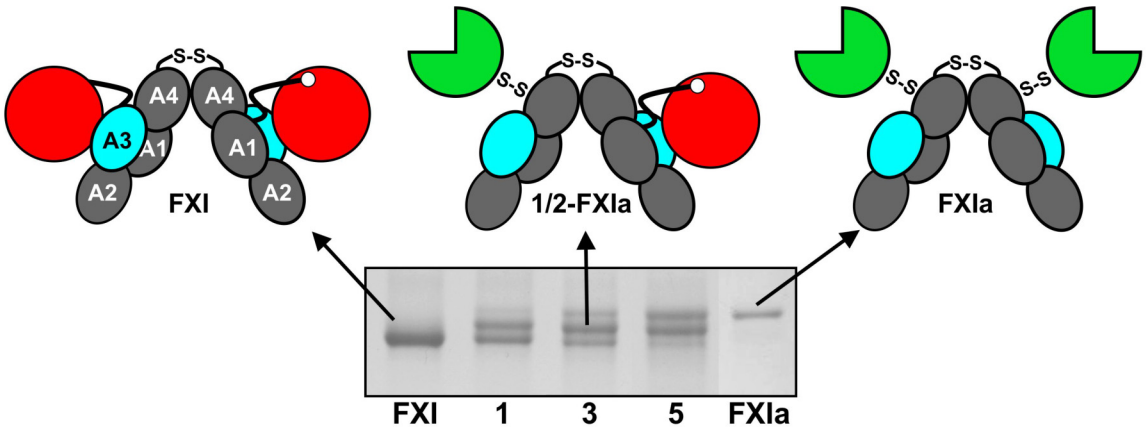

Figure IV-8. Model for factor XI activation. Factor XI migrates faster than factor XIa on non-reducing SDS-PAGE. Factor XI activation by α-thrombin (above) or factor XIIa (shown) goes through an intermediate migrating between factor XI and factor XIa. We predicted that the intermediate was factor XI with one activated subunit (1/2-factor XIa). Numbers below the gel indicate hours of incubation. In the diagrams, red circles are zymogen, and green three-quarter circles activated catalytic domains. A1 - A4 are apple domains.

catalytic domain on one subunit, whereas factor XI does not. This seemed reasonable based on the ability of 1/2-factor XIa to cleave a tripeptide chromogenic substrate. We reasoned that if 1/2-factor XIa was able to cleave S2366, then it could also bind active site inhibitors that block factor XIa cleavage of this substrate. Benzamidine is a small molecule general inhibitor of serine proteases that inhibits factor XIa with an IC_{50} of 100 μM (Jin *et al.*, 2005). Benzamidine binds to the S_1 specificity pocket of factor XIa with its amidine group forming hydrogen bonds with Asp^{189}, the carbonyl oxygen of Gly^{218} (both chymotrypsinogen numbering) and a conserved water molecule. The commercial availability of benzamidine-Sepharose (Sigma, St. Louis) led us to test this inhibitor-resin for its ability to bind 1/2-factor XIa, and separate it from zymogen factor XI (**Figure IV-9**).

We developed a strategy to produce 1/2-factor XIa with minimal generation of factor XIa by using limited digestion of factor XI with α-thrombin. Recall that α-thrombin appears to make the cleavage converting 1/2-factor XIa to factor XIa much more slowly than does factor XIIa. Time course experiments indicated that a 1 hour

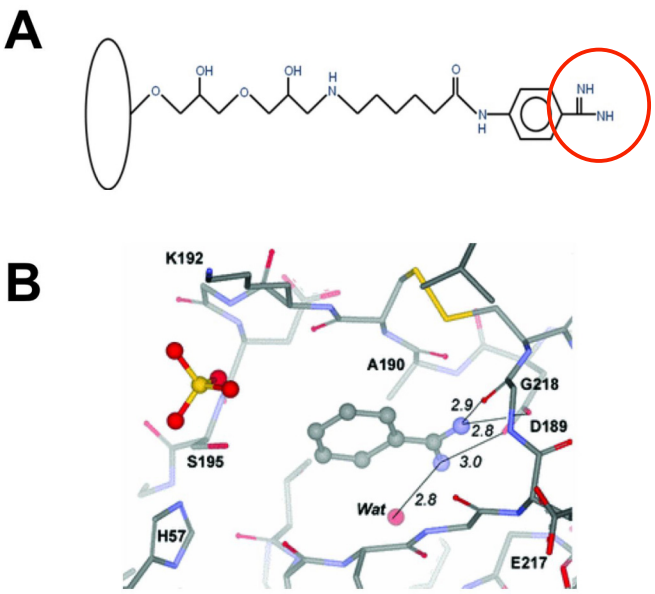

Figure IV-9. Benzamidine-Sepharose resin with details of its interaction with the factor XIa catalytic domain. (A) Schematic representation of benzamidine-Sepharose resin with the amidine group of benzamidine circled in red. **(B)** Crystal structure of recombinant factor XIa catalytic domain showing the interaction of the amidine group of benzamidine (gray ball and stick representation) with residues in the active site of factor XIa. Asp^{189} (D^{189} in the figure) is at the bottom of the protease S1 specificity pocket and forms hydrogen bonds with the amidine group of benzamidine. (After Jin *et al.*, 2005)

incubation of 625 nM factor XI with 625 nM α-thrombin yielded the maximum amount of 1/2-factor XIa that could be attained without noticeable contamination by factor XIa (**Figure IV-10A**). When a mixture of factor XI and 1/2-factor XIa was chromatographed on benzamidine-Sepharose, 1/2-factor XIa was retained (Schematic diagram below the gel in **Figure IV-10A**), whereas FXI passed through the column and appeared in the flow through. 1/2-factor XIa was eluted from the resin by competition with a solution of 100 mM benzamidine, indicating the band corresponding to 1/2-factor XIa was an active protease. Unlike factor XIa, 1/2-factor XIa displayed non-cleaved (zymogen) subunits as well as the heavy and light chains of activated factor XI and is, therefore, a species with one activated, and one non-activated subunit per factor XI dimer (**Figure IV-10B**). Note that a zymogen factor XI subunit cannot bind to benzamidine unless it is attached to an active subunit. The number of moles of active sites per mol of 1/2-factor XIa protein was 0.93, compared with 1.98 for factor XIa, as determined by active site labeling with an excess of the covalent inhibitor fluorescein-Phe-Pro-Arg-CH_2Cl. As expected, 1/2-factor XIa cleaved S-2366 at about half of the rate (58%) of an equimolar concentration of factor XIa (**Figure IV-10C**). While factor XIa is not seen in the sample in **Figure IV-10B**, we did observe traces of factor XIa in some 1/2-factor XIa preparations.

Preparation of Fully Activated Factor XIa with One Inhibited Catalytic Domain

The existence of an activated factor XI species with only one active subunit can be used as a tool to aid in understanding the importance of the unusual dimeric structure of factor XI structure to its function, and in particular as an activator of factor IX. We prepared an additional single active site species in which both catalytic domains have

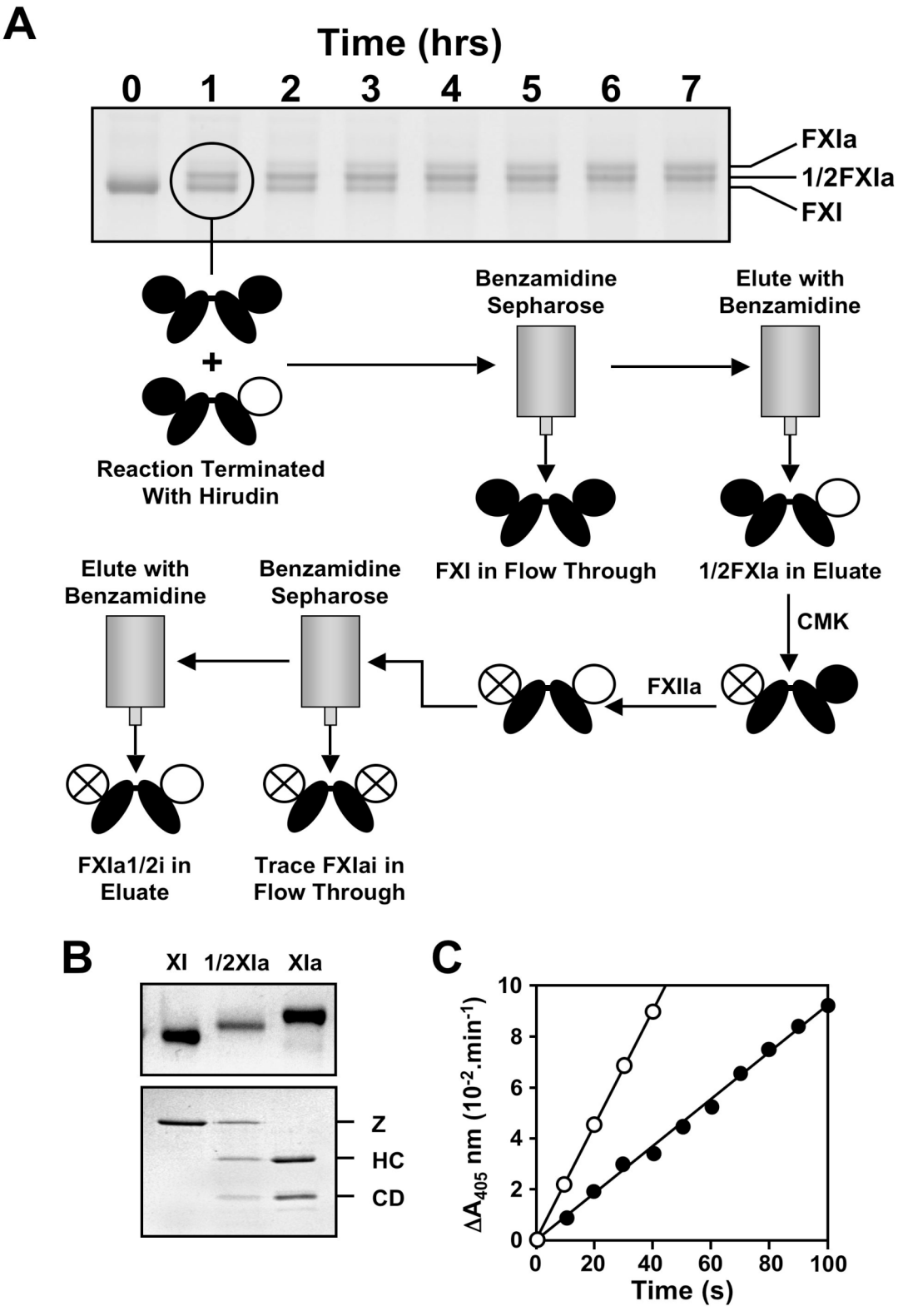
A
Time (hrs)
0 1 2 3 4 5 6 7
FXIa
1/2FXIa
FXI
Reaction Terminated With Hirudin
Benzamidine Sepharose
Elute with Benzamidine
FXI in Flow Through
1/2FXIa in Eluate
CMK
FXIIa
Elute with Benzamidine
Benzamidine Sepharose
FXIa1/2i in Eluate
Trace FXIai in Flow Through
B
XI 1/2XIa XIa
Z
HC
CD
C
ΔA405 nm (10-2.min-1)
Time (s)
0 20 40 60 80 100
0 2 4 6 8 10

Figure IV-10. (A) Time course of factor XI activation by α-thrombin monitored by SDS-polyacrylamide gel electrophoresis, and schematic diagram of the generation and purification of 1/2-factor XIa and factor XIa-1/2i from plasma factor XI. FXI (625 nM) in Assay Buffer (see Methods) was incubated at 37˚C with α-thrombin (625 nM). At various times, aliquots were removed into non-reducing sample buffer and fractionated on 6% polyacrylamide-SDS gels. Migration of protein standards for factor XI (FXI) and factor XIa (FXIa) are shown, and migration of the reaction intermediate is indicated by INT. Below the one hr time point on the gel are schematic diagrams of factor XI and factor XIa dimers, with ellipses representing heavy chains, and circles representing the catalytic domains. A filled circle ● represents an unactivated factor XI subunit, an open circle ○ an activated subunit, and a circle with an "X" (⊗) an activated subunit in which the active site is inhibited with a chloromethyl-ketone (CMK). To prepare 1/2-factor XIa, factor XI undergoes limited activation by thrombin, followed by separation of the active species from residual factor XI by chromatography on benzamidine-Sepharose as described under Methods. Factor XIa with one inhibited active site per dimer (FXIa-1/2i) was prepared by treating 1/2-factor XIa with CMK to inhibit all active sites. After dialysis, the unactivated subunit of inhibited 1/2-factor XIa was activated by incubation with factor XIIa. The final product, factor XIa-1/2i, was separated from traces of inhibited factor XIa by chromatography on benzamidine-Sepharose. **(B) Purified 1/2-factor XIa.** Samples of purified factor XI, 1/2-factor XIa, and factor XIa were fractionated under non-reducing conditions on a 6% polyacrylamide-SDS (top) or reducing conditions on a 12% polyacryalmide-SDS gel (bottom), and stained with GelCode blue. **(C) 1/2-factor XIa activity in a chromogenic substrate assay.** Factor XIa (○) or 1/2-factor XIa (●) (6 nM) in Assay Buffer containing 500 mM S-2366 was incubated at 24˚C and changes in absorbance at 405 nm were followed as described under Methods.

been activated, but in which one of the active catalytic domains has been irreversibly inhibited. We call this protease factor XIa-1/2i. We decided to prepare this protease out of concern that 1/2-factor XIa may be converted to factor XIa during subsequent experiments with factor IX (discussed in Chapter V). Assuming that there is a significant conformational change that accompanies conversion of a factor XI subunit to the active form (Samuel *et al.*, 2007), the conformation of factor XIa-1/2i should be the same as for factor XIa, but with only one active subunit. Factor XIa-1/2i may, in fact, mimic a form of factor XIa found *in vivo*; that is a factor XIa molecule that has one of its active catalytic domains inhibited by a natural protease inhibitor such as antithrombin.

We prepared this half-inhibited FXIa using the procedure shown in **Figure IV-10A**. Tripeptide chloromethyl-ketone inhibitors are commonly used to covalently inhibit serine proteases. We speculated that the activated subunit of 1/2-factor XIa could be inhibited by D-Phe-Pro-Arg-CH_2Cl, which is used to irreversibly inhibit factor XIa. The inhibited 1/2-factor XIa could then be activated on its zymogen subunit, yielding a molecule that is conformationally equivalent to factor XIa, but that has half the active sites per molecule of factor XIa. 1/2-factor XIa was incubated with D-Phe-Pro-Arg-CH_2Cl. After dialysis, the uncleaved subunit was activated with factor XIIa. The only new active sites formed in this step are due to cleavage of the zymogen subunit of /2-factor XIa. The preparative technique employed prevents contamination of factor XIa-1/2i with factor XIa. No fully active factor XIa can be generated because zymogen factor XI was removed during preparation of 1/2-factor XIa, and any factor XIa in the 1/2-factor XIa preparation was inactivated by the chloromethyl ketone. Factor XIa-1/2i has half the chromogenic substrate cleavage activity of factor XIa per mole of protein, and is

indistinguishable from factor XIa by electrophoresis on 6% non-reducing or 12% reducing SDS-polyacrylamide gels. This protease will be compared to factor XIa and 1/2-factor XIa in factor IX activation assays described in Chapter V.

Factor XIa/PKA4

Availability of monomeric factor XIa would offer another approach to address the importance of the dimeric structure of factor XI to factor IX activation. Removing the interchain disulfide bond involving Cys^{321} in the factor XI A4 domain (**Figure IV-1**) does not produce a pure monomeric protein because of the non-covalent interactions between the two factor XI subunits (see Chapter III). As discussed, factor XI has a high degree of structural homology to PK, which is a monomer. Previously, we described recombinant factor XIa in which the A4 domain is replaced with the PK A4 domain (Sun and Gailani, 1996). The chimera, factor XIa/PKA4, is a monomer on size exclusion chromatography (**Figure IV-11**) (Cheng et al, 2003). Factor XI/PKA4 is activated normally by factor XIIa, and factor XIa/PKA4 cleaves S-2366 and factor IX with similar kinetic parameters to factor XIa (Sun and Gailani, 1996). Factor XIa/PKA4 will be used in studies described in Chapter V on factor IX cleavage.

Cleavage of S-2366 by Single Active Site Species of Factor XIa

Cleavage of the tripeptide chromogenic substrate S-2366 by 1/2-factor XIa and factor XIa-1/2i was compared to factor XIa (**Table IV-1**). For this study, each active site of factor XIa was considered an independent enzyme. The K_ms and k_{cat}s for cleavage of S-2366 were similar for the three proteases. Previously, we showed that factor XIa/PKA4 and factor XIa cleaves S-2366 with similar kinetic parameters (Sun and Gailani, 1996).

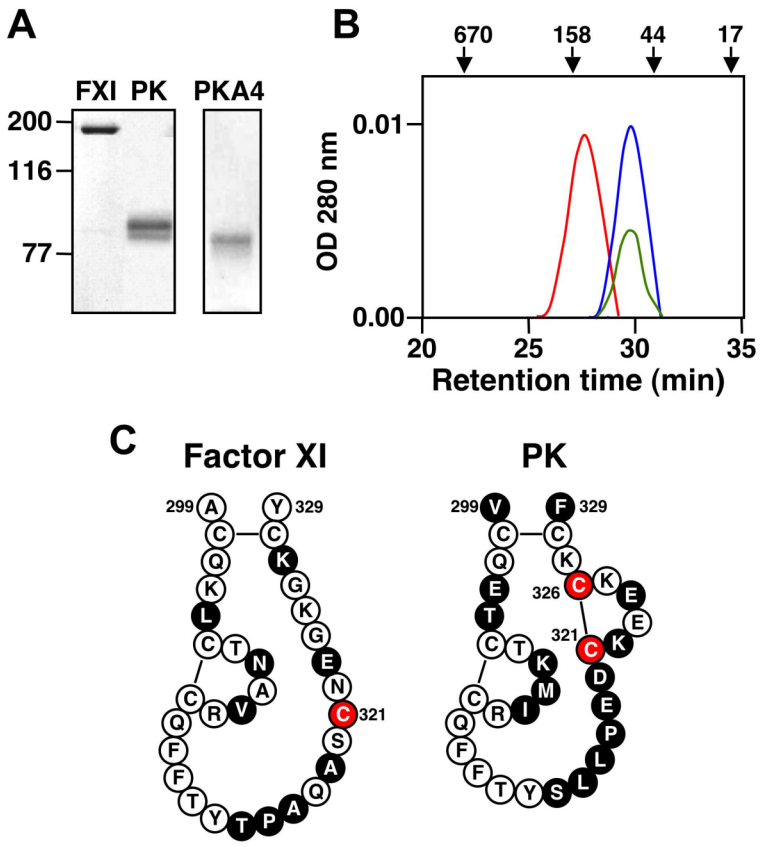

Figure IV-11. Factor XI/PKA4. (A) Non-reducing 12% Polyacrylamide gels of recombinant factor XI/PKA4 (PKA4) in comparison to factor XI (FXI) and prekallikrein (PK). Note that factor XI/PKA4 migrates as a monomeric protein, similar to PK. **(B) Size-exclusion chromatography.** Twenty microgram samples of factor XI (red), PK (blue curve) or factor XI/PKA4 (green) were chromatographed on a Superose-12 size-exclusion column. Retention times are shown at the bottom and the positions of protein standards in kilo-Daltons are at the top. **(C) Amino acids 299-329 in the A4 domains of factor XI and PK.** Note that Cys^{321} is unpaired in factor XI, and is paired with Cys^{326} in PK.

Properties of Factor XIa Immobilized to Acrylamide Beads by One Active Site

Binding interactions of coagulation proteases have been studied by affinity chromatography, in which the enzyme to be studied was immobilized specifically by its active site to an insoluble matrix (Dharmawardana and Bock, 1998). This work demonstrated the utility of immobilized coagulation proteases in studying exosite driven interactions. The ability to selectively inhibit 1/2-factor XIa on its active site raised the

Table IV-1. Kinetic parameters for cleavage of S-2366 by factor XIa, 1/2-factor XIa, and factor XIa1/2i. Values for K_m and k_{cat} for S-2366 cleavage were determined by fitting the Michaelis-Menten equation to substrate dependence curves using eight concentrations of S-2366. Errors in parameters represent 95 % confidence intervals.

Protease	K_m (μM)	k_{cat} (sec^{-1})
Factor XIa	440 ± 20	16.1 ± 0.3
1/2-factor XIa	300 ± 20	12.4 ± 0.3
Factor XIa-1/2i	340 ± 7	21.6 ± 0.2

interesting possibility of using this singly inhibited species, linked to insoluble resin, to directly study its macromolecular binding interactions.

We activated plasma-derived factor XI with α-thrombin to obtain a mixture of factor XI and 1/2-factor XIa as described above. The 1/2-factor XIa in the mixture was then inhibited by ATA-Phe-Pro-Arg-CH_2Cl inhibitor (Bock, 1992A; Bock, 1992B) and reacted with iodoacetyl beads by the general reaction strategy illustrated in **Figure IV-12A**. This strategy resulted in a homogeneous resin in which the inhibited activate subunit of 1/2-factor XIa is linked to the surface of the bead, while the zymogen subunit is oriented away from the surface and exposed to the solution phase. When a slurry of 1/2-factor resin was exposed to reducing SDS-sample buffer and analyzed by reducing SDS-gel electrophoresis, the 80kDa zymogen subunit and 50kDa heavy chain of 1/2-factor XIa were observed on the stained polyacrylamide gels **Figure IV-12B**. However, no light chain was observed, consistent with the catalytic domain of the active subunit being covalently linked to the matrix.

The free unactivated subunit of the immobilized 1/2-factor XI matrix was activated by incubating the matrix with factor XIIa (see Methods). This essentially converts 1/2-factor XI resin to factor XIa-1/2i, with the inhibited catalytic domain forming the link with the beads. This preparative method eliminates the possibility of factor XIa with two active sites being bound to the resin. Factor XIa linked to beads is able to use its free catalytic domain to cleave S-2366 (**Figure IV-12C**). Each microliter of beads contributed the equivalent of 3.65×10^{-13} moles of active catalytic domains to the assays, as determined by comparison with a factor XIa standard. This correlates to 370 nM factor XIa active sites in the packed resin. Assuming each free catalytic domain

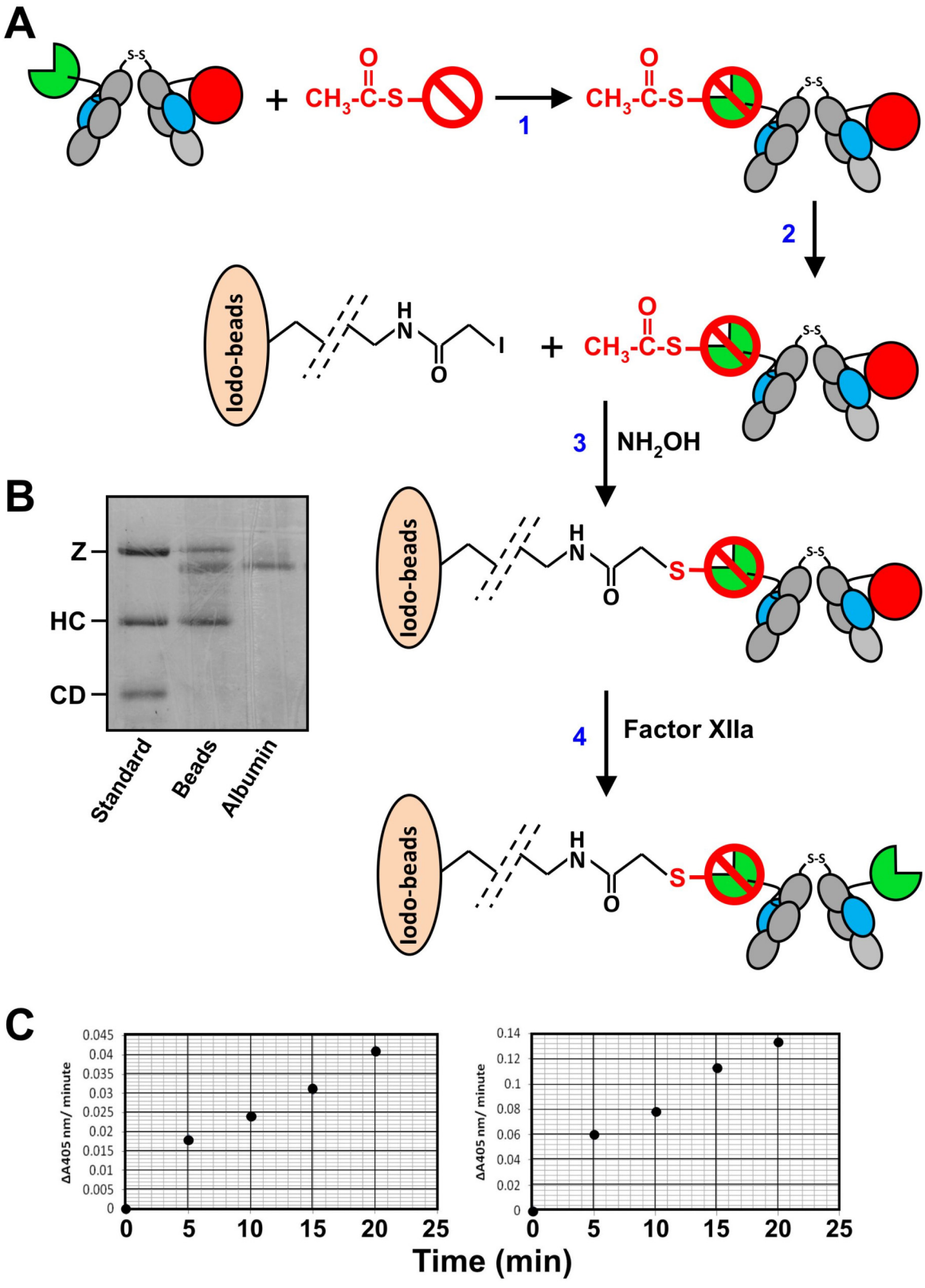
A
S-S
+
O
CH3-C-S
1
2
Iodo-beads
H
N
I
3
NH2OH
B
Z
HC
CD
Standard
Beads
Albumin
4
Factor XIIa
S
C
ΔA405 nm/ minute
0.045
0.04
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
5
10
15
20
25
Time (min)

Figure IV-12. 1/2-factor XIa and factor XIa linked to beads. (A) Protocol for linking 1/2-factor XIa to acryalmide beads. *Step 1:* The single active catalytic domain of 1/2-factor XIa is irreversibly inhibited by incubation with ATA-Phe-Pro-Arg-CH_2Cl (Bock, 1992A). > 99% inhibition was achieved as determined by a chromogenic substrate assay. *Step 2:* Inhibited 1/2-factor XIa is mixed with UltraLink Iodobeads. *Step 3*: NH_2OH is added to initiate the coupling reaction. The product, 1/2-factor XIa covalently linked to the beads, presents the unactivated subunit of 1/2-factor XIa to the solution phase. *Step 4:* 1/2-factor XIa-beads were incubated with factor XIIa to activate the zyomgen subunits of the bound protein. In the diagram of the UltraLink Iodobeads the cross-hatched area represents a 15 carbon spacer arm. In the schematic diagrams of 1/2-factor XIa the red circle indicates the inactive catalytic domain of the zymogen subunit, while the green three-quarter circle indicates the catalytic domain of the activated subunit. The apple domains A1, A2, and A4 are represented by gray ovals, with the A3 domain involved in factor IX binding shown as blue oval. **(B) SDS-polyacryalmide gel of protein removed from 1/2-factor XIa-beads with reducing sample buffer.** Ten μl of acryalmide beads linked to 1/2-factor XIa were mixed with μl of reducing SDS-sample buffer. After removing the beads, the sample buffer was size-fractionated on a 10% polyacryalmide-SDS gel and stained with GelCode blue. Note that in comparison to standards for factor XI and factor XIa (left lane), the protein from the beads (center lane) does not show a band in the position of the factor XIa catalytic domain. This is because the catalytic domain of 1/2-factor XIa is bound to the bead through a covalent linkage that cannot be broken by the reducing-sample buffer. A small amount of bovine serum albumin, which was used to block unreacted sites on the beads can be seen in the sample. Abbreviations to the left of the panel are (Z) zymogen factor XI subunits, (HC) heavy chain of factor XIa subunits, and (CD) catalytic domain of factor XIa subunits. **(C) Hydrolysis of S-2366 by factor XIa-beads compared to standard factor XIa.** In the left-hand panel, factor XIa-beads were assayed at a concentration of 3 μl packed resin per ml assay buffer. Cleavage of S-2366 (500 mM) by this mixture resulted in an increase in absorbance of 0.0047 Au/minute/ul of packed resin. In the right-hand panel factor XIa at 0.01 nM generated an increase in absorbance of 0.022 Au/minute in the same assay.

is not conformationally altered by the inhibited subunit of factor XIa binding to the beads, this activity correlates to each microliter of beads contributing 17 ng of active factor XIa subunit, or 17 ng factor XIa dimer. This value is in good agreement with the quantity of factor XIa observed on reducing gels in which factor XIa removed from resin by a reducing buffer was compared with a factor XIa standard.

The Utility of Factor XIa Linked to Beads in Isolating Plasma Binding Partners of Factor XI and Factor XIa

The matrices described in the previous section contain factor XIa derivatives that are spatially oriented to present either one factor XI or factor XIa subunit to the surrounding solution. This could mimic a physiologic situation in which factor XI or XIa is bound to a platelet through one of its subunits, while interacting with macromolecules in plasma with the other (Gailani et al, 2001). Factor IX is known to bind to factor XIa, but not to factor XI (Aktimur *et al.*, 2003). HMWK binds to factor XI (Thompson *et al.*, 1977), and probably also binds to factor XIa (Warn-Cramer and Bajaj, 1985). We used factor XIa-linked resins to perform pull-down assays from human plasma anticoagulated with the thrombin inhibitor hirudin. These experiments were designed to determine whether the factor XI or XIa subunits on the factor XIa-resin were in an arrangement that would support normal physiologic interactions. It should be noted that the factor XIa subunit that is covalently bound to the resin may not be able to bind to other proteins, leaving only the free subunit to engage in binding interactions. Beads presenting either factor XI or factor XIa to the solution phase bound HMWK (**Figure 1V-13A**), whereas control beads reacted with bovine serum albumin (BSA) did not. Interestingly, factor IX did not bind to the free zymogen subunit of 1/2-factor XIa linked to beads (**Figure 1V-**

13B), which indicates that factor IX cannot engage the activated subunit covalently linked to the bead. This result supports the argument that factor IX can only bind a factor XIa subunit that is presented away from a surface to which the protease is bound.

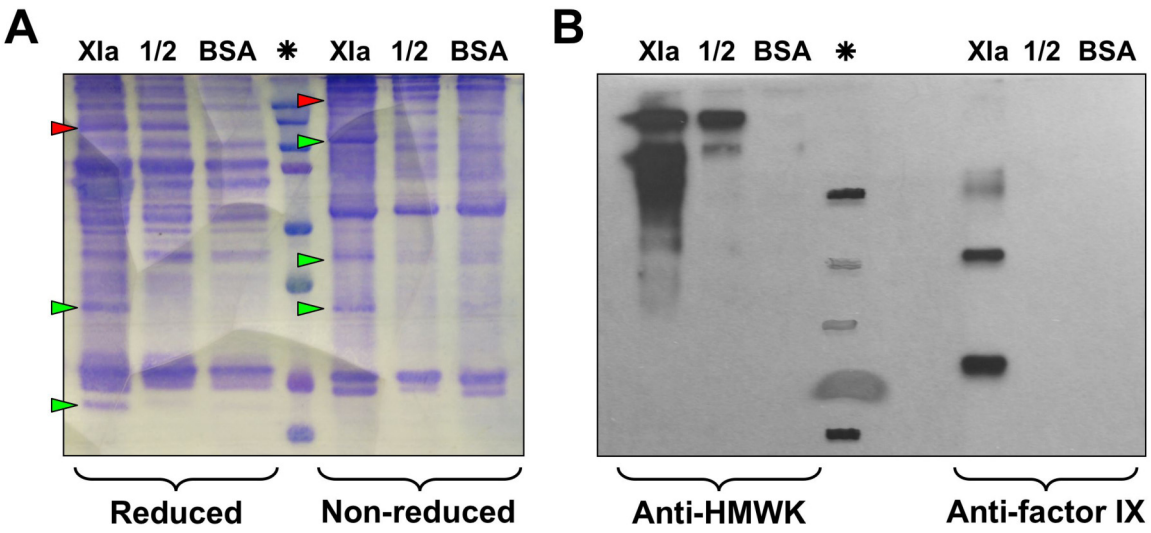

Figure IV-13. Plasma proteins pulled down by factor XIa-beads and 1/2-factor XIa-beads. Protein-linked beads (50 µl) were incubated with one ml of normal human plasma for one hour at 24°C with rocking. Beads were pelleted by brief centrifugation in a microfuge and washed three times with Assay Buffer (see Methods). After the final wash, beads were mixed with 50 µl of reducing or non-reducing buffer. Beads were linked to factor XIa (XIa), 1/2-factor XIa (1/2) or bovine serum albumin (BSA) **(A) GelCode blue stained 12% polyacrylamide gel of reduced and non-reduced samples.** Positions of proteins that bind to factor XIa and 1/2-factor XIa (red arrows) or only factor XIa (green arrows) but not to BSA control are indicated. **(B) Western blot for unreduced samples.** Samples were analyzed by western blotting for HMWK or factor IX as indicated. In both panels BioRad mass standards are indicated by ✱.

Discussion

The data presented in this chapter show for the first time that factor XI is activated by sequential cleavage of its subunits, proceeding through formation of a half-activated intermediate that we have called 1/2-factor XIa. Both of the proteases known to activate factor XI, factor XIIa and α-thrombin, generate 1/2-factor XIa. Both factor XIIa and α-thrombin appear to make the initial cleavage in factor XI relatively quickly, however, α-thrombin appears to be less efficient than factor XIIa at making the second

cleavage that generates fully activated factor XIa. 1/2-factor XIa is generated by factor XIIa in normal plasma during contact activation, showing that this reaction can occur in a complex environment. Recent studies in our laboratory (Kravtsov et al, manuscript in preparation) and work from other laboratories (von dem Borne, 1995) indicate that during TF-initiated coagulation, very small amounts of factor XIa are generated, which have a profound effect on thrombin generation and fibrin clot stability. Given the small amount of factor XIa generated in these assays, and the relatively short time scales of these assays, we postulate that the active factor XI species in these experiments is actually 1/2-factor XIa. 1/2-factor XIa, therefore may represent a physiologic species, or the physiologic species of activated factor XI. In a situation where factor XI is bound to the surface of a cell or platelet, only one of the subunits might be available for activation, and the product would be 1/2-factor XIa.

The availability of several factor XIa molecules with single active sites provides us with the necessary reagents to test a prevailing hypothesis that factor XIa requires both activated catalytic domains to convert a single factor IX molecule to the final product factor IXaβ without formation and/or accumulation of an intermediate (Wolberg *et al.*, 1997; Samuel *et al.*, 2007). If this hypothesis is correct, then activation of factor IX by single active site factor XIa species would be expected to result in factor IX intermediate accumulation, or possibly no cleavage at all. If, on the other hand, each factor XIa subunit acts independently of the other, then these molecules would be expected to yield cleavage patterns similar to factor XIa. An additional possibility is that 1/2-factor XIa could have a unique set of properties related to cooperativity between the subunits. Cooperativity could arise when a structural change in the activated subunit of 1/2-factor

XIa influences the structure of the zymogen subunit. In this situation, we might expect differences in the manners in which 1/2-factor XIa and factor XIa-1/2i interact with factor IX. These major issues are addressed in Chapter V, which covers factor IX activation by factor XIa.

Coagulation proteases in general have domains and/or exosites that facilitate their binding to surfaces to localize the reactions they mediate. Studies done under flow conditions show that factor XI is crucial for promoting fibrin formation, and that platelets are absolutely required to facilitate this process (Tucker *et al.*, 2009). This implies that factor XI and/or factor XIa may need to tether to a cell or platelet in order to remain properly positioned to contribute to coagulation in flowing blood. Binding sites for factor XI (Walsh 2004) and factor XIa (Sinha *et al.*, 1984) have been identified on platelets. The preparations of 1/2-factor XIa and factor XIa bound to beads, therefore, may mimic physiologic situations in which factor XI or an activated species are bound to a physiologic surface. The experiments using these reagents demonstrate that they will be useful tools for isolating plasma or cellular binding partners of factor XI and factor XIa. The image in **Figure 1V-13A** show that several proteins seem to be interacting with factor XI or factor XIa that are not seen in the control BSA beads.

Our results so far point to some interesting conclusions. First, the pull-down experiment examining factor IX binding to factor XIa immobilized on beads suggest that factor IX may be able to interact with a single factor XIa subunit (addressed in the next chapter). In addition, the western blot in **Figure 1V-13B** indicates that the bound factor IX has been converted to factor IXaβ, strongly suggesting that factor XIa only requires a single active subunit to generate the final product. Whether or not this reaction involves

formation and accumulation of an intermediate will be addressed in the next chapter. Previous studies of the interaction of HK with factor XI and factor XIa were done in buffers that did not have physiologic concentrations of relevant plasma cations (Warn-Cramer and Bajaj, 1985) or were done in plasma in which divalent cations such as Ca^{2+} were chelated with sodium citrate. Our pull-down assays were done in plasma anticoagulated with the thrombin inhibitor hirudin, and thus were done in the presence of normal concentrations of all plasma components. This confirmed the interaction of HMWK with factor XI and factor XIa under physiologic conditions.

Methods

Materials—Factor XIIa, HMWK and corn trypsin inhibitor were purchased from Enzyme Research Labs (South Bend, IN). Human α-thrombin, factor XIa, and fluorescein-Phe-Pro-Arg-CH_2Cl were from Hematologic Technologies (Essex Junction, VT). Hirudin (Lepirudin) was from Berlex (Wayne, NJ). Benzamidine-Sepharose was from Amersham Biosciences (Piscataway, NJ). L-Pyroglutamyl-L-prolyl-L-arginine-*p*-nitroanilide (S-2366) was from DiaPharma (West Chester, OH). PTT-A reagent was from Diagnostic Stago (Asnières sur Seine, France). Soybean trypsin inhibitor-agarose was from Sigma (St. Louis, MO).

Expression and purification of recombinant factor XIa—Recombinant factor XIa was prepared as follows. The cDNA for human wild type factor XI (Fujikawa *et al.*, 1986) or the chimera factor XI/PKA4 [Sun, 1996] was introduced into a mammalian expression vector containing the cytomegalovirus promoter (pJVCMV). 5 x 10^6 293 fibroblasts were

transfected with 40 μg of factor XI cDNA/pJVCMV and 2 μg of pRSVneo which contains a gene conferring neomycin resistance. Transfection was by electroporation using an Electrocell Manipulator 600 (BTX, San Diego). Transfected cells were grown in DMEM with 5% fetal bovine serum for 24 hours, then switched to the same medium containing the neomycin analog G418 (500 μg/ml). Media was exchanged every 48 hours for 14 days. G418 resistant clones were tested for protein expression by ELISA. Clones expressing high levels of protein were expanded in 175 cm^2 culture flasks. When cells reached >50% confluence, media was replaced with 75 ml of Cellgro complete media containing SBTI (10 μg/ml) to prevent activation of factor XI. Media was exchanged every 48 hrs. After collection, conditioned media was supplemented with 5 mM benzamidine and stored at -20 ^{o}C pending purification.

Conditioned media (500-2000 ml) was applied to a 5 ml murine anti-human factor XI monoclonal antibody 1G5.12 column (Sun *et al.*, 1999). After loading, columns were washed with 10 volumes of 25 mM Tris-HCl pH 7.4, 100 mM NaCl (TBS) and 5 mM benzamidine, followed by elution with 2 M sodium thiocyanate in 25 mM Tris-HCl pH 7.4, 5 mM benzamidine. Protein containing fractions were pooled and concentrated in an ultra-filtration concentrator (Amicon, Inc., Beverly, MA), dialyzed against TBS, and stored at -80 ^{o}C. Proteins were analyzed by SDS-polyacrylamide gel electrophoresis for purity, and protein concentration was measured by dye binding assay (BioRad, Richmond, CA). Factor XIa was prepared by diluting the zymogen to 100-300 μg/ml in TBS containing 5 μg/ml human factor XIIa and incubating at 37 ^{o}C. Progress of conversion of the 80,000 Dalton zymogen to the 50,000 Dalton heavy chain and 30,000

Dalton light chain of factor XIa was followed by SDS-polyacrylamide gel electrophoresis. Factor XIa was stored at -80 ^{o}C.

Purification of plasma factor XI—Frozen plasma (2 liters) collected in acid-citrate-dextrose was thawed at 4 °C to induce cryoprecipitation of large proteins, and supplemented with benzamidine (20 mM). Factor XI was purified from the cryosupernatant by affinity chromatography using the anti-human factor XI antibody 1G5.12 (Sun *et al.*, 1999). After loading, the column was washed with 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 20 mM benzamidine and eluted with 2 M NaSCN in the same buffer. The eluate was concentrated by ultrafiltration and dialyzed against 50 mM Hepes, pH 7.4, 125 mM NaCl, 20 mM benzamidine. Purity was assessed by SDS-polyacrylamide gel electrophoresis and concentration by colorimetric assay (Bio-Rad, Richmond, CA).

Hydrolysis of S-2366 by factor XIa— Factor XIa (6 nM active sites, 3 nM protein), 1/2-factor XIa (6 nM active sites, 6 nM protein), or factor XIa-1/2i (6 nM active sites 6 nM protein) were diluted in assay buffer (50 mM Hepes, 125 mM NaCl, 5 mM $CaCl_2$, 0.1 mg/ml bovine serum albumin) containing 15.6–2000 μM S-2366. Initial rates of generation of free *p*-nitroaniline in 100-μl reaction volumes were measured by continuous monitoring of absorbance at 405 nm (3 mm path length) in a SpectraMax 340 microtiter plate reader (Molecular Devices Corp., Sunnyvale, CA).

Western blots of factor XI activation in plasma—Human plasmas anticoagulated with 0.38% sodium citrate (George King, Overland Park, KS) were mixed with equal volumes of PTT A reagent at 37 °C. At various times, 9 μl of reactions were mixed with 6 μl of non-reducing sample buffer (233 mM Tris-Cl, pH 6.8, 138 mM SDS, 19% glycerol, 0.01% bromphenol blue), fractionated on 6% SDS-polyacrylamide gels, and transferred to nitrocellulose. The primary antibody was goat anti-human factor XI IgG (Enzyme Research Laboratories, South Bend, IN) and the secondary antibody was horseradish peroxidase-conjugated anti-goat IgG. Detection was by chemiluminescence.

Preparation of factor XI with a single catalytic active site (1/2-factor XIa)— Plasma factor XI (0.6–12 μM) in 50 mM Hepes, pH 7.4, 125 mM NaCl, was subjected to limited digestion by incubating with factor XIIa (625 nM) or α-thrombin (860 nM) for 1 h at 24 °C. Reactions were terminated by addition of corn trypsin inhibitor (8.6 μM) or hirudin (20μM), respectively. The mixture was applied to a 1 ml benzamidine-Sepharose column. After washing with 50 mM Hepes, pH 7.4, 125 mM NaCl, the column was eluted with 50 mM Hepes, pH 7.4, 125 mM NaCl, 50 mM benzamidine. Factor XI was found in the flow through and 1/2-factor XIa in the eluate. 1/2-factor XIa was also prepared by chromatography on soybean trypsin inhibitor-agarose. Elution was with 50 mM Hepes, pH 7.4, 1 M NaCl, 1 M benzamidine, 1 mM EDTA. The active site concentration for 1/2-factor XIa was determined by complete inhibition with fluorescein-Phe-Pro-Arg-CH_2Cl, followed by dialysis to remove free inhibitor. The protein concentration was determined by absorbance at 280 nm (corrected for absorbance of the fluorophore) with ε of 214,400

M^{-1} cm^{-1}, and the fluorescein concentration was determined by absorbance at 491 nm with ε of 79,000 M^{-1} cm^{-1}.

Preparation of factor XIa with one inhibited active site (factor XIa-1/2i)— 1/2-factor XIa (2 μM) in 50 mM Hepes, 125 mM NaCl, 1mg/ml polyethylene glycol 8000, pH 7.4, was incubated with 20 μM D-Phe-Pro-Arg-CH_2Cl at 23°C to irreversibly inhibit active sites. Residual inhibitor was removed by dialysis against the same buffer. The unactivated subunit of inhibited 1/2-factor XIa was activated by incubation with factor XIIa (500 nM) for 7 h at 23 °C. Complete conversion to factor XIa was demonstrated by SDS-polyacrylamide gel electrophoresis. Factor XIa-1/2i was separated from residual inhibited factor XIa by chromatography on benzamidine-Sepharose. This also removed factor XIIa.

Preparation of factor XIa bound to polyacrylamide beads—Factor XIa bound to UltraLink Iodoacetyl Resin (Pierce, Rockford, IL) was prepared by a modification of a published method (Dharmawardana and Bock, 1998). 1/2-factor XIa (1 μM) in 50 mM Hepes, 125 mM NaCl, 1 mM EDTA, 1 mg/ml polyethylene glycol 8000, pH 7.4 (coupling buffer), was incubated with 20 μM ATA-Phe-Pro-Arg-CH_2Cl (Bock, 1992; Dharmawardana and Bock, 1998) to inhibit active sites. After dialysis to remove free inhibitor, the dialysate (500 μl) was mixed with 100 μl of packed UltraLink Resin, previously washed with coupling buffer. Protein coupling was initiated by addition of NH_2OH to 0.1 M, and incubation at 24 °C for 4 h with mixing on a rocker. Beads with bound protein showed no activity in a chromogenic assay using S-2366, indicating the

active sites of bound 1/2-factor XIa were blocked. To generate an active site on the uncleaved 1/2-factor XIa subunit, the beads were incubated with factor XIIa (600 nM) at 37 °C for 6 h with frequent mixing. Unreacted sites were blocked by mixing on a rocker with monomeric bovine serum albumin (Dharmawardana and Bock, 1998) (20 mg/ml) for 12 h at 24 °C. Specific activity of the reacted beads was determined by cleavage of S-2366 using factor XIa as a standard. The activity of the packed resin was 370 nM factor XIa active sites.

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CHAPTER V

FACTOR IX ACTIVATION BY FACTOR XIa

Introduction

Previous work on factor IX activation by factor XIa has not dealt directly with the mechanistic role that the factor XIa dimer structure plays in the activation of factor IX. Wolberg et al (Wolberg *et al.*, 1997) proposed a processive mechanism to explain the lack of factor IX intermediate generation by factor XIa. In a processive mechanism, the cleavages could be made simultaneously or in succession, but no intermediate would be released from the enzyme. In the case of factor XIa it was proposed that the two catalytic domains of the dimer could each cleave one of the scissile bonds at either end of the factor IX activation peptide, releasing it without generation of an intermediate. As discussed, this would be distinctly different from factor IX activation by factor VIIa, which has a single catalytic domain. The mechanism used by factor VIIa/TF to activate factor IX is best explained by a sequential mechanism, where cleavage at Arg^{145}-Ala^{146} to generate factor IXα occurs prior to cleavage at Arg^{180}-Val^{181} to form factor IXaβ (Osterud and Rapaport, 1977; Bajaj *et al.*, 1983; Lawson and Mann, 1991) (**Figure V-1**).

An argument against a processive mechanism for factor IX activation by factor XIa can be made based on the relative positions of the factor XIa two catalytic domains in the crystal structure of zymogen factor XI (Papagrigoriou *et al.*, 2006), which are too far apart to simultaneously engage a single factor IX molecule. Recent, nuclear magnetic resonance work with isolated factor XI A4 domains suggested that cleavage at the

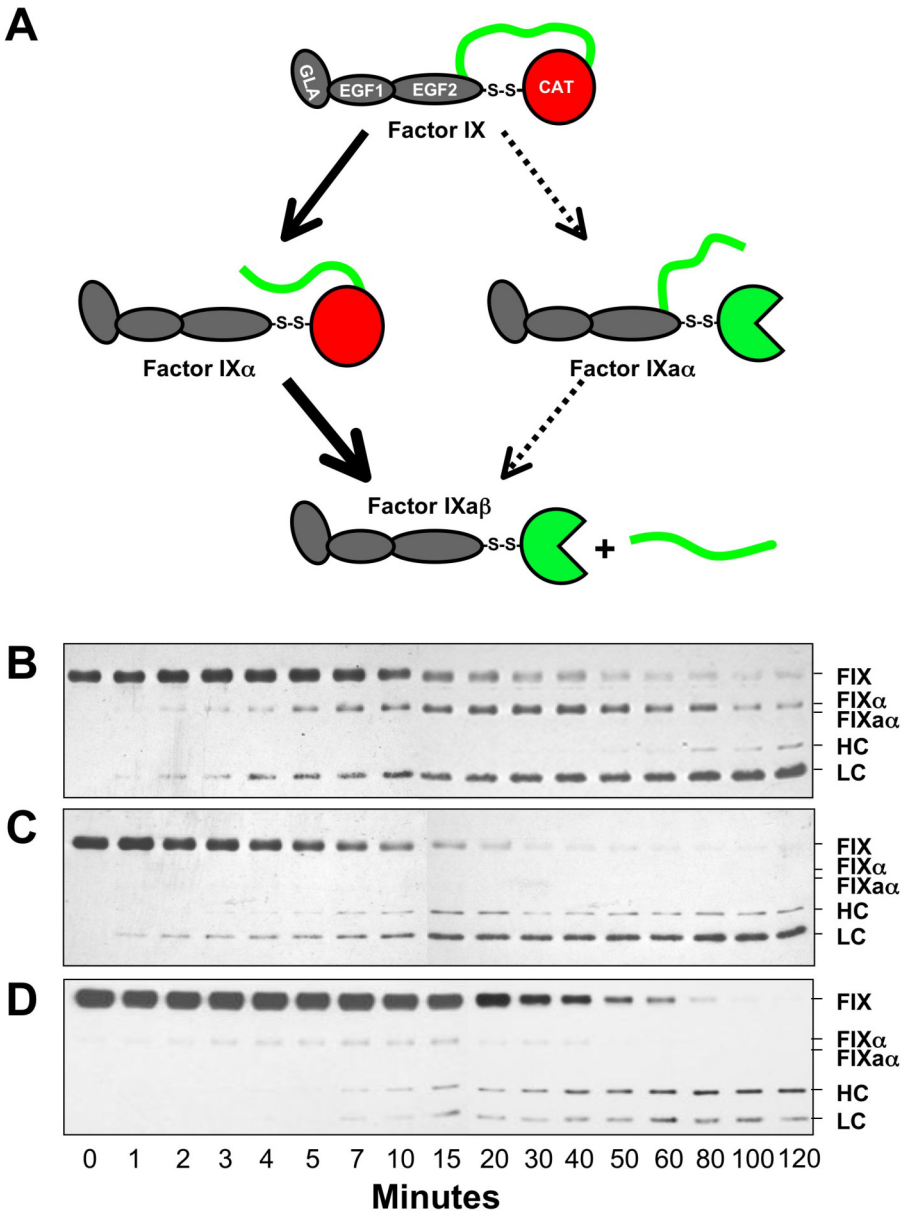

Figure V-1. Time courses of factor IX activation by factor VIIa and factor XIa monitored by western blotting. (A) Factor IX is cleaved after Arg^{145} and Arg^{180} to release the activation peptide (green ribbon) and produce the active protease factor IXaβ. Initial cleavage after Arg^{145} separates the light chain (Gla and EGF domains) and the N-terminus of the AP, producing the intermediate factor IXα. This is the preferred pathway for factor IX activation by factor VIIa/TF (bold solid arrows). Cleavage initially after Arg^{180} results in separation of the factor catalytic domain (CAT) from the C-terminus of the AP, generating a partially active intermediate, (factor IXaα. **(B-D)** Factor IX (100 nM) in Assay Buffer (see Methods) was incubated at 24 ˚C with 1 nM factor VIIa in the presence of saturating TF **(B)** or 1 nM active sites of two different preparations of factor XIa (**C** and **D)**. Aliquots were removed at indicated reaction times into denaturing reducing buffer, fractionated on 12% polyacrylamide-SDS gels, and analyzed by western blotting as described under Methods. Migration of protein standards for zymogen factor IX (FIX), the large fragment of factor IXα (FIXα), the large fragment of factor IXaα, the heavy chain of factor IXaβ or factor IXaα (HC), and the light chain of factor IXaβ or factor IXα (LC).

Arg^{369}-Ile^{370} bond converting factor XI to factor XIa results in a structural change that probably alters the relationship of the two catalytic domains to each other (Samuel *et al.*,2007). Electron microscopy studies using rotary shadowing techniques do indicate a significant change in overall protein conformation when factor XI is converted to factor XIa (Samuel *et al.*, 2007), possibly moving the catalytic domains sufficiently close to each other so that they would be able to engage each end of the activation peptide of a single factor IX molecule.

Previously, we showed that the monomeric variant factor XIa/PKA4 (see Chapter IV) can activate factor IX with similar kinetic parameters to activation by factor XIa (Sun and Gailani, 1996). However, it is not known if an intermediate is generated in this reaction. If a processive mechanism involving both factor XIa catalytic domains is required for activation of factor IX without accumulation of an intermediate, then molecules such as factor XIa/PKA4, 1/2-factor XIa, and factor XIa-1/2i should presumably cleave factor IX in a manner similar to factor VIIa; that is by cleaving the protein at one bond to generate an intermediate. The experiments in this chapter were designed to test the hypothesis that factor XIa needs two activated catalytic domains per molecule to activate factor IX normally, without releasing one of the two possible intermediates factor IXα or factor IXaα (**Figure V-1**).

We show here that factor XIa with one active catalytic domain per molecule activates factor IX without accumulation of either possible singly cleaved factor IX intermediate, and that the mechanism involved cannot be explained by cooperativity between factor XIa subunits within a molecule or between two factor XIa molecules. We also demonstrate that factor XIa with both subunits activated binds two factor IX or

factor IXaβ molecules, implying that each factor XIa subunit is capable of acting as an individual enzyme toward its substrate that can be inhibited by its product.

Kinetic Parameters of Factor IX Activation by Factor XIa

As shown in Chapter IV, the kinetic parameters for cleavage of tripeptide substrate S-2366 by factor XIa and single active site species of factor XIa are similar on an active basis. This experiment established that the catalytic domains are in the expected active conformation in these proteases, and that there are no significant alterations in the conformations of the active sites. Next, we studied the kinetics of factor IX activation by factor XIa using a chromogenic substrate assay (Ogawa *et al.*, 2005). Initial rates of activation were determined from analysis of full progress curves and from the factor IX dependence of the initial rate of factor IXaβ formation (**Figure V-2**). The results, summarized in **Table V-1**, indicate that factor XIa and its singly active site derivatives

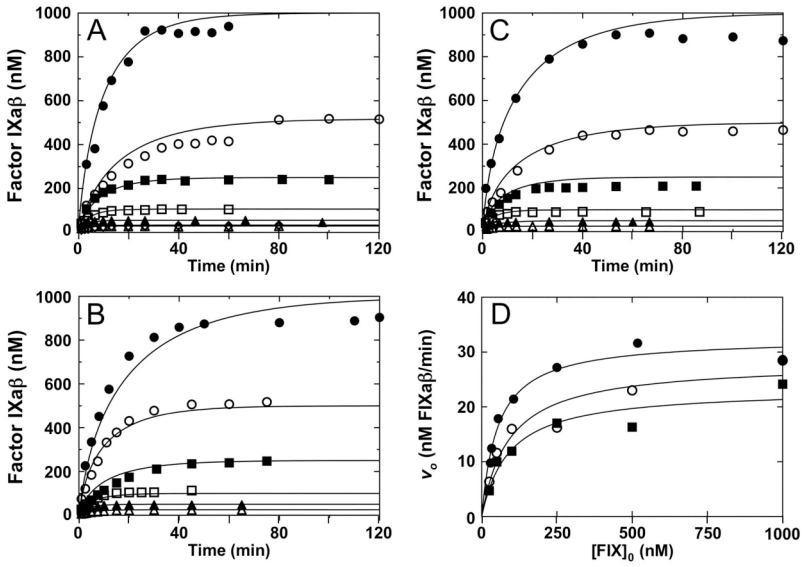

Figure V-2. Factor IX activation by factor XIa, 1/2-factor XIa and factor XIa-1/2i followed by chromogenic assay. Factor IX at 25 nM (△), 50 nM (▲), 100 nM (□), 250 nM (■), 500 nM (○), or 1000 nM (●) in Assay Buffer was incubated at 24˚C with 1-10 nM of active site of **(A)** Factor XIa, **(B)** 1/2-factor XIa, or **(C)** factor XIa1/2i. At appropriate time points aliquots were assayed for factor IXaβ activity as described under Methods. (D) The initial rates for activation of factor IX (25-1000 nM) by factor XIa (●), 1/2-factor XIa (○), and factor XIa1/2i (■) for factor IX were determined from the progress curves in **A-C** as described under Methods, and are plotted as a function of initial factor IX concentration in each reaction.

Table V-1. Kinetic parameters for activation of factor IX by factor XIa, 1/2-factor XIa, and factor XIa-1/2i. K_m and k_{cat} for factor IX activation were based on initial rates for factor IX activation from full progress curves (**Fig. V-2**) as described under Methods. The resulting v_o values were analyzed by fitting the Michaelis-Menten equation, and values for K_m and k_{cat} were obtained from direct non-linear least squares analysis. Values for K_m and k_{cat} were used to analyze the complete progress curves by the integrated Michaelis-Menten equation. With values for K_m and k_{cat} fixed, full progress curves were fitted simultaneously by the integrated rate equation with product inhibition to obtain estimates of K_i for factor IXaβ. Errors in parameters represent 95 % confidence intervals.

Protease	K_m (nM)	k_{cat} (min^{-1})	K_i (nM)
Factor XIa	53 ± 8	33 ± 1	23 ± 46
1/2-factor XIa	90 ± 40	28 ± 3	23 ± 26
Factor XIa-1/2i	90 ± 30	23 ± 2	18 ± 23

have similar apparent affinities (K_m) and turnover numbers (k_{cat}) for factor IX. The results for 1/2-Factor XIa are unlikely to be due to contaminating factor XIa. If 1/2-factor XIa (the vast majority of protease in the preparation) did not cleave factor IX, and all factor IX activation was due to traces of factor XIa, the turnover number (k_{cat}) would be very low relative to the factor XIa control. Previously, our laboratory demonstrated that factor IX and factor IXaβ bind to factor XIa in a mutually exclusive manner, and that the K_i for product inhibition and the K_m for factor IX activation are similar (Ogawa *et al.*, 2005). In the present studies, the K_i values for product inhibition were similar for factor XIa, 1/2-factor XIa, and factor XIa-1/2i (**Table V-1**), and were reasonably similar to K_m values, given the techniques used. The results indicate that factor XIa and its single active site derivatives bind to and activate factor IX similarly. The results are consistent with the observation that monomeric factor XIa/PKA4 activates factor IX with similar kinetic parameters to factor XIa (Sun and Gailani, 1996)

Single Active Site Factor XIa Species Have Activity in Plasma Clotting Assays

As described in Chapter IV, 1/2-factor XIa is formed in plasma induced to undergo contact activation, suggesting it may also be formed *in vivo* as part of normal physiology. Since factor XIa contributes to fibrin clot formation and maintenance by activating factor IX, and half active forms of factor XIa appear to activate factor IX similarly to factor XIa, we predicted that these species would have procoagulant activity in plasma. To determine if 1/2-factor XIa and factor XIa-1/2i can induce coagulation, the enzymes were compared with factor XIa in a clot formation assay. In this assay, preparations of the proteases and phospholipid were added to factor XI deficient plasma anticoagulated with the calcium chelator sodium citrate, which was then triggered to form a clot by addition of calcium to reverse the anticoagulation. Formation of a clot after addition of calcium is monitored by changes in viscosity of the plasma over time using a fibrometer. The relative activities of 1/2-factor XIa (102%) and factor XIa-1/2i (130%) were comparable to fully active factor XIa (activity arbitrarily set at 100%) when corrected for the number of active sites per molecule. Again, these results are consistent with those previously generated with the factor XIa/PKA4 (Sun and Gailani, 1996). These results demonstrate that activated factor XI with a single active site is capable of inducing plasma to form a clot, almost certainly through activation of factor IX.

Single Active Site Factor XIa Cleaves Factor IX Without Intermediate Generation

The time courses of factor IX activation by factor VIIa/TF and factor XIa shown in **Figure V-1** clearly demonstrate the different patterns of cleavage produced by the two proteases. Time courses of factor IX activation by the single active site factor XIa species

1/2-factor IXa, factor XIa-1/2i and factor XIa/PKA4 were analyzed by the same western blotting technique (**Figure V-3**). The data convincingly show that factor XIa with only one active catalytic domain per molecule is capable of making both activation cleavages in factor IX without significant accumulation of activation intermediates, in contrast to reactions with factor VIIa/TF as the activating enzyme. While a slight accumulation of factor IXα was seen in the activation time courses catalyzed by 1/2-factor IXa and factor XIa-1/2i, it did not accumulate, and was also seen in the starting material. This is occasionally seen in activation time courses with fully active factor XIa (**Figure V-1D**). In **Figure V-3C**, the activating enzyme is the monomer factor XIa/PKA4. There is slight accumulation of factor IXα with this protease, but note that it appears simultaneously with the formation of factor IXaβ, and not prior to it as is seen with factor VIIa/TF.

Again, the results with 1/2-factor XIa cannot be explained by traces of contaminating factor XIa in the preparation, as demonstrated by consideration of the

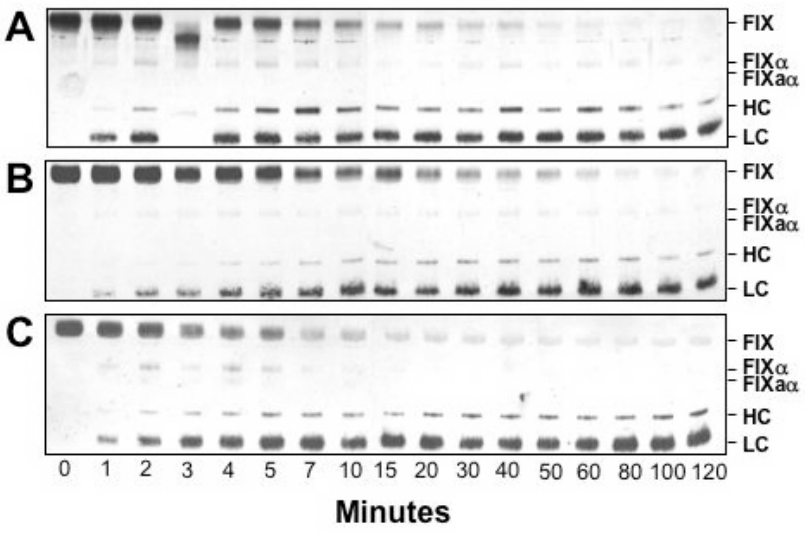

Figure V-3. Time courses of factor IX activation by single active site factor XIa species monitored by western blotting. Factor IX (100 nM) in Assay Buffer was incubated at 24˚C with 2 nM (active sites) **(A)** 1/2-factor XIa, **(B)** factor XIa1/2i, or **(C)** factor XIa/PKA4. Aliquots were removed at indicated times into reducing sample buffer, fractionated on 12% polyacrylamide-SDS gels, and analyzed by western blotting as shown in the Methods section. Migration of protein standards is shown for zymogen factor IX (FIX), the large fragment of factor IXα (FIXα), the large fragment of factor IXaα, the heavy chain of factor IXaβ or factor IXaα (HC), and the light chain of factor IXaβ or factor IXα (LC). There was a defect in the well for the three minute sample for 1/2-factor XIa **(A)**, which resulted in an abnormal pattern of electrophoresis.

following scenarios. If two factor XIa active sites are required for normal factor IX cleavage, intermediate will accumulate when 1/2-Factor XIa cleaves factor IX and should be seen on the western blots, even if a trace of factor XIa activates some factor IX. This is because the vast majority of protease is 1/2-factor XIa. Alternatively, 1/2-Factor XIa may not cleave factor IX at all, and all factor IX activation could be through factor XIa. We would not expect the intermediate to accumulate in this situation; however, the rate of factor IX activation would be significantly reduced relative to the factor XIa control. This was not evident in **Figure V-3A**. In the case of factor XIa-1/2i, which is a dimer with one functional and one blocked active site, the method used to prepare the enzyme makes contamination with either factor XI or factor XIa highly unlikely, and factor IX activation must be through the single active site species. These results are not consistent with a model in which both catalytic domains of factor XIa are required for normal activation of factor IX, and suggest that each factor XIa molecule is comprised of two complete enzymes that likely function independently of each other.

Activation of Factor IX by Factor XIa Linked to a Surface by One of Its Active Sites

The results in the previous section rule out a processive mechanism for factor IX activation that require two active sites per factor XIa molecule, and strongly favors a mechanism in which each factor XIa subunit is independently capable of activating factor IX. An alternative possibility that could be consistent with a processive mechanism requiring two factor XIa catalytic domains would involve cooperativity between separate factor XIa molecules. This mechanism would involve factor IX engaging a factor XIa

subunit, which would cleave one scissile bond in factor IX, with the second bond cleaved by an active subunit of another factor XIa molecule.

Factor XIa linked to acrylamide beads, as described in Chapter IV, cannot freely diffuse across the surface of the bead and, therefore, may be in a situation somewhat analogous to the single catalytic domain of factor VIIa in complex with tissue factor at a site of vascular injury. We activated 100 nM factor IX with factor XIa linked to beads with 1 nM equivalent factor XIa catalytic activity, and the time course was followed by western blotting (**Figure V-4**). No factor IX intermediate accumulated in this reaction, which appeared to progress at roughly the same rate as with factor XIa in solution. These results show that factor XIa can cleave factor IX normally in a surface bound orientation with only one active subunit available. The results are also consistent with those with single active site species of factor XIa in solution, but suggest that cooperativity between two separate factor XIa molecules is unlikely. The results still leave open the possibility that each free subunit of factor XIa makes both cleavages in factor IX without

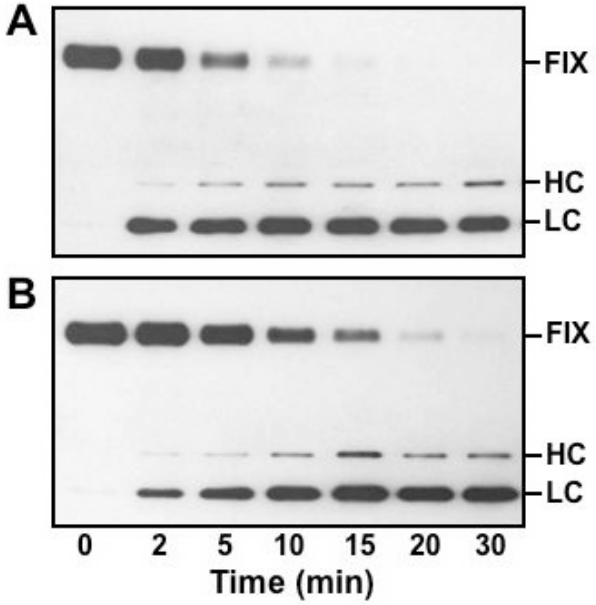

Figure V-4. Time course of factor IX activation by factor XIa immobilized on polyacrylamide beads monitored by western blotting. Factor IX (100 nM) in Assay Buffer was incubated at 24˚C with 2 nM active sites of (A) factor XIa or (B) factor XIa linked to polyacrylamide beads through a chloromethyl ketone inhibitor occupying one of the active sites. Aliquots were removed at indicated times into denaturing reducing buffer, fractionated on 12% polyacrylamide-SDS gels, and analyzed by western blotting as described under Methods. Migration of protein standards is shown for zymogen factor IX (FIX), the heavy chain of factor IXaβ (HC), and the light chain of factor IXaβ (LC).

intermediate release (a processive mechanism). This will be addressed in the next experiment.

The Effect of Active Site Inhibited Factor XIa (Factor XIai) on Factor IX Activation

Data from the preceding experiments supports a model in which each subunit of factor XIa behaves as an independent enzyme. The mechanism for factor IX activation, therefore, likely involves an interaction between the substrate and only one factor XIa subunit. It seems unlikely that a molecule with a single active site, such as a subunit of factor XIa, would operate by a true processive mechanism. Processive mechanisms are typically employed by larger enzymes that partially or fully enclose the substrate molecule to be cleaved, and that often have multiple active sites (Breyer and Matthews, 2001). The crystal structures of factor XI, and the structural predictions pertaining to its activation, do not indicate factor XIa would operate by a true processive mechanism.

The initial interaction of factor IX with factor XIa involves one or more exosites on the factor XIa heavy chain that should also be available on factor XIa with an inhibitor in its active sites (factor XIai). Factor XIai should, therefore, compete with factor XIa for binding to factor IX, behaving as a competitive inhibitor of factor IX activation. We reasoned that factor XIai would also compete with factor XIa binding to factor IXα, if this intermediate was released, and would trap that intermediate in a non-productive complex. This should result in accumulation of factor IXα during an activation time course.

When factor IX was activated by factor XIa in the presence of 1000-fold molar excess of factor XIai, we observed a reduction in the rate of zymogen factor IX cleavage,

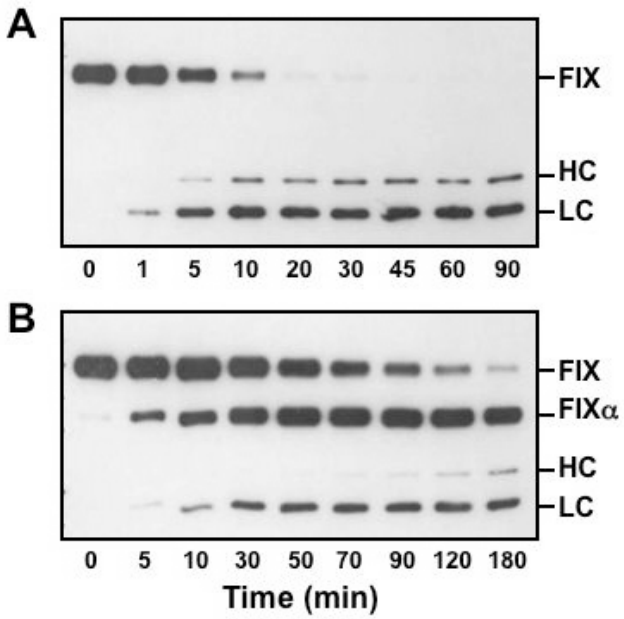

Figure V-5. Time course of factor IX activation by factor XIa in the presence of factor XIai monitored by western blotting. Factor IX (100 nM) in Assay Buffer was incubated at 24˚C with 2 nM active sites of factor XIa in the **(A)** absence or **(B)** presence of 1000 nM factor XIai. Aliquots were removed at indicated reaction times into reducing buffer, fractionated on 12% polyacrylamide-SDS gels, and analyzed by western blotting as described Methods. Note that sampling times differ for the two reactions. Migration of protein standards is shown for zymogen factor IX (FIX), the large fragment of factor IXα (FIXα), the heavy chain of factor IXaβ (HC), and the light chain of factor IXaβ (LC).

as expected in the presence of an inhibitor (**Figure V-5**). In addition, there was a significant accumulation of factor IXα in a pattern similar to that observed with factor VIIa catalyzed activations. This result strongly suggests that factor XIa initially cleaves the factor IX Arg^{145}-Ala^{146} bond to form factor IXα, and that factor IXα is released from factor XIa and is available to bind to factor XIai. To complete conversion to factor IXaβ, factor IXα must rebind to factor XIa, followed by cleavage of the Arg^{180}-Val^{181} bond. Therefore, in addition to competing with factor XIa for binding to factor IX, factor XIai also appears to compete for binding to factor IXα. The result argues against a mechanism for factor IX activation where both activation sites on factor IX are cleaved without release of an intermediate (the processive mechanism), and indicates that the Arg^{145}-Ala^{146} and Arg^{180}-Val^{181} bonds in factor IX are cleaved sequentially by each factor XIa subunit. It is important to note that while these results demonstrate the likely order of bond cleavage by factor XIa, they do not predict which bond represents the rate limiting cleavage. However, given the lack of factor IXα accumulation during activation by factor

XIa in the absence of an inhibitor, one could predict that the second cleavage at the Arg^{180}-Val^{181} must be at least as rapid as the initial cleavage. This issue will be addressed in Chapter VI.

Activation of Factor IX by the Isolated Catalytic Domain of Factor XIa (Factor XIa-CD)

To determine the importance of the heavy chain exosites on factor XIa in factor IX activation, we prepared recombinant isolated factor XIa catalytic domain (factor XIa-CD). This was achieved using recombinant factor XI in which Cys^{362} and Cys^{482} are replaced by serine residues (factor XI-$Ser^{362,482}$). These residues form the disulfide bond that connects the catalytic domain to the heavy chain in factor XIa (see **Figure III-1**). When factor XI-$Ser^{362,482}$ is converted to the active protease by incubation with factor XIIa, the catalytic domain dissociates from the heavy chains, and can be purified. The resulting factor XIa-CD cleaves a chromogenic substrate similarly to factor XIa, but is a poor activator of factor IX, likely due to the loss of substrate binding exosites on the heavy chain (Ogawa *et al.*, 2005). Recently, Sinha *et al.* showed that factor XIa-CD cleaved factor IX with accumulation of factor IXα (Sinha *et al.*, 2007). In these studies, which used stained gels and high substrate concentrations, factor IXα was also evident during factor IX activation by wild type full-length factor XIa.

We activated a physiologic concentration of factor IX (100 nM) with a high concentration of Factor XIa-CD (50 nM) and also observed significant accumulation of factor IXα (**Figure V-6A**). The result is distinctly different from those for the other single active site factor XIa species described above, and indicates that the mechanism by which factor XIa subunits activate factor IX with limited intermediate accumulation requires the

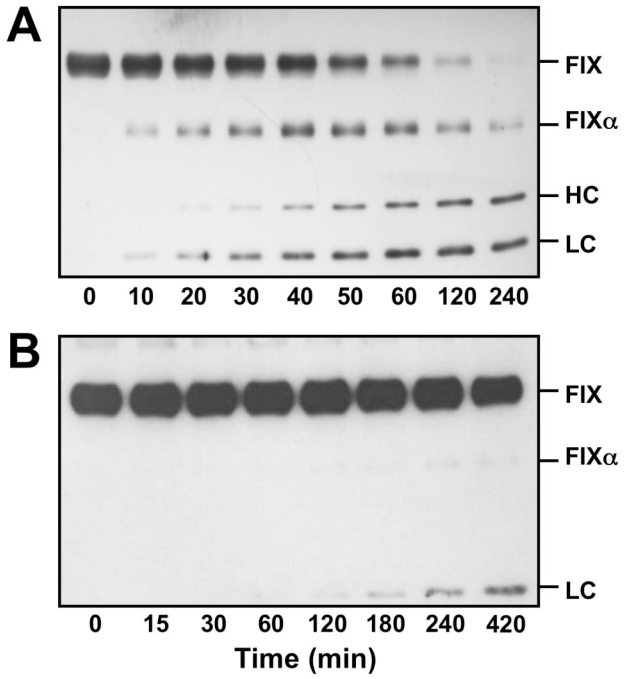

Figure V-6. Time courses of factor IX activation by factor XIa-CD monitored by western blotting. Factor IX (100 nM) in Assay Buffer was incubated at 24˚C with factor XIa-CD **(A)** 50 nM active sites or **(B)**, or 2 nM active sites. Aliquots were removed at indicated reaction times into denaturing reducing buffer, fractionated on 12% polyacrylamide-SDS gels, and analyzed by western blotting as described under Methods. Note that the sampling times differ for the two reactions. Migration of protein standards is shown for zymogen factor IX (FIX), the large fragment of factor IXα (FIXα), the heavy chain of factor IXaβ (HC), and the light chain of factor IXaβ or factor IXα (LC).

factor XIa heavy chain. Activation of factor IX at the typical 100 nM physiologic concentration with 2 nM equivalent factor XIa-CD active sites (an enzyme concentration comparable to that used in the time courses in **Figure V-3**) resulted in extremely slow conversion of factor IX to factor IXα (**Figure V-6B**), likely caused by an increased K_m due to lack of sufficient binding energy at physiologic concentrations. These results indicate that, at concentrations relevant to physiologic conditions, the binding energy needed to tether factor IX to factor XIa is provided by exosites in the heavy chain of each factor XIa subunit.

These results clearly show that factor XIa-CD cleaves the factor IX Arg^{145}-Ala^{146} bond before it cleaves the Arg^{180}-Val^{181} bond. This suggests that the factor XIa heavy chain would normally orient factor IXα in a position that facilitates cleavage after Arg^{180}. Taken together with the data from the factor XIai inhibition study (above), it appears that factor XIa subunits cleave the factor IX Arg^{145}-Ala^{146} and Arg^{180}-Val^{181}

bonds sequentially, with the heavy chain tethering factor IXα in position to cleave after Arg^{180} efficiently. The heavy chain also appears to be critical for the initial binding of factor IX to the protease as the cleavage of the Arg^{145}-Ala^{146} bond is significantly slower with factor XIa-CD compared to factor XIa.

Titration of Active Site-Labeled Factor XIa by Factor IX and Factor IXaβ

The data presented so far strongly indicate that each factor XIa subunit behaves as a complete enzyme toward factor IX. Each factor XIa dimer, therefore, should bind two factor IX molecules. Factor XIa was inhibited with a tripeptide chloromethyl-ketone linked to a fluorescent dansyl group that functions as a reporter of change in the microenvironment around the factor XIa active site (Warn-Cramer and Bajaj, 1985). The fluorescence probe is covalently linked via the chloromethyl ketone to the active site catalytic histidine and serine residues (**Figure V-7A**). Because initial binding of factor IX to factor XIa involves interactions with exosites remote from the active site (Ogawa *et al.*, 2005), blocking the active site should not significantly affect factor IX binding to the active site inhibited factor XIa. Titration of active site-labeled factor XIa with factor IX resulted in increased fluorescence (**Figure V-7B**), with a maximum enhancement at saturation of 43 ± 3%, and a stoichiometry of 1.9 ± 0.4 mol of factor IX per mol of factor XIa. The K_d for the interaction (70 nM ± 40 nM) was consistent with published results (Aktimur *et al.*, 2003; Ogawa *et al.*, 2005). The observed smooth increase in fluorescence with addition of factor IX suggested there was no intra-subunit cooperativity, where binding to one subunit confers a change in binding affinity to the other subunit. Earlier work showed that factor IX and factor IXaβ have similar affinities

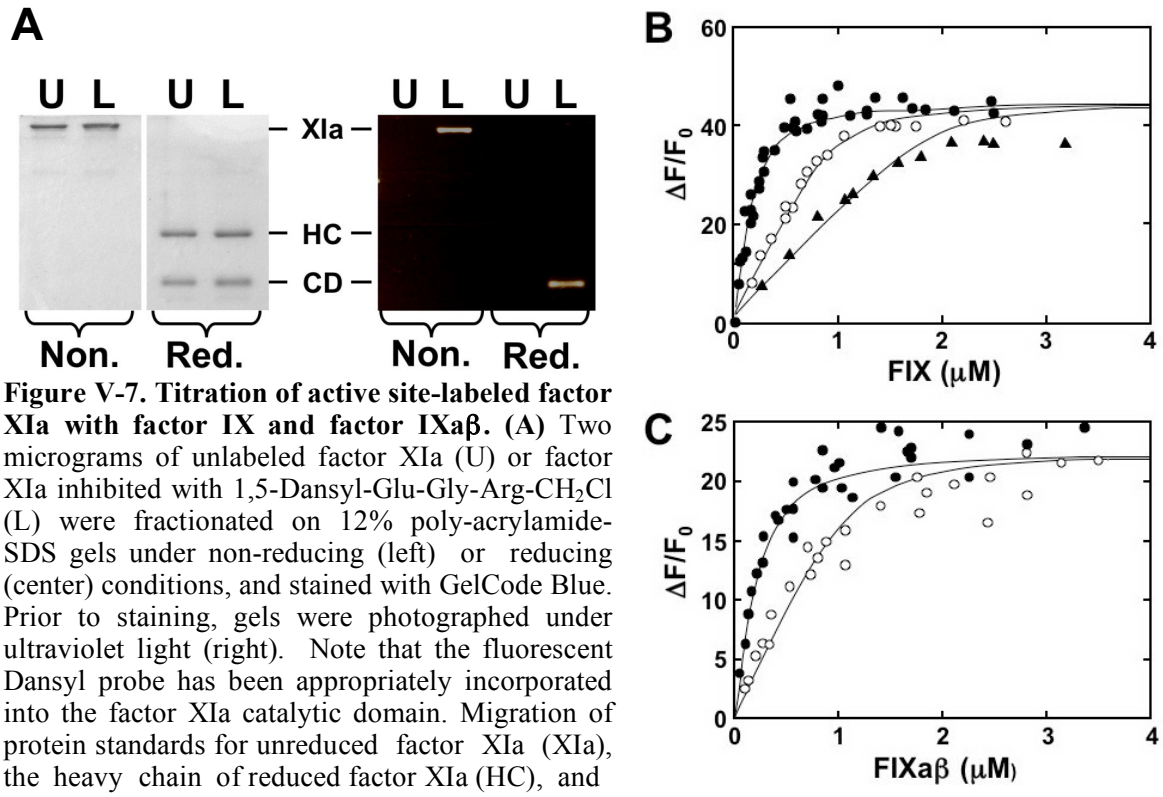

Figure V-7. Titration of active site-labeled factor XIa with factor IX and factor IXaβ. (A) Two micrograms of unlabeled factor XIa (U) or factor XIa inhibited with 1,5-Dansyl-Glu-Gly-Arg-CH_2Cl (L) were fractionated on 12% poly-acrylamide-SDS gels under non-reducing (left) or reducing (center) conditions, and stained with GelCode Blue. Prior to staining, gels were photographed under ultraviolet light (right). Note that the fluorescent Dansyl probe has been appropriately incorporated into the factor XIa catalytic domain. Migration of protein standards for unreduced factor XIa (XIa), the heavy chain of reduced factor XIa (HC), and the catalytic domain of reduced factor XIa (CD) are shown. **(B** and **C)** Fluorescence titrations of Dansyl-labeled factor XIa (100 nM [●], 500 nM [○] or 1000 nM [▲]) with factor IX (A) or factor IXaβ (B) were performed as described under Methods. The solid lines represent the nonlinear least-squares fits by the quadratic binding equation with the parameters given in the text.

for factor XIa (Ogawa *et al.*, 2005). Binding of factor IXaβ to labeled factor XIa increased the fluorescence to a maximum of 23 ± 1% (**Figure V-7C**), again with no evidence of cooperativity. The stoichiometry of factor IXaβ binding to factor XIa was approximately 2:1 (2.2 ± 0.4), with a K_d of 100 ± 50 nM. The results support the conclusion that each half of the factor XIa dimer can function as a complete enzyme toward factor IX in solution.

Discussion

The data presented in this chapter show that each subunit of factor XIa behaves as an independent enzyme toward its physiologic substrate, factor IX. Previously, it has been assumed that factor XIa formed during coagulation has both subunits activated, and this is the only form that has been studied in detail. Furthermore, it was postulated that the mechanism by which factor XIa activates factor IX is dependent on factor XIa having two active subunits, allowing processive cleavage of the two scissile bonds in factor IX. Our discovery of 1/2-factor XIa that is cleaved at the Arg^{369}-Ile^{370} activation site on only one subunit allowed us to investigate the importance of the unique dimeric structure to protease function. The observation that 1/2-factor XIa can be generated in plasma, and the ability of this protease to initiate clot formation in plasma indicates that it contributes to factor IX activation under physiologic conditions. Our data directly address two hypotheses put forward in the literature; that factor XIa uses a processive mechanism to activate factor IX without intermediate generation, and that factor VIIa and factor XIa activate factor IX by different mechanisms.

The assumption that all factor XIa formed in plasma has two active catalytic domains per molecule allowed the construction of a reasonable hypothesis to explain the mechanism by which factor IX is cleaved at two distinct sites without accumulation of an intermediate. Our studies with single active site factor XIa proteases refute this attractive hypothesis. Our kinetic data confirm that factor XIa with a single active site, or with both subunits activated, converts factor IX to factor XIaβ at comparable rates, indicating each subunit is sufficient for normal activation of factor IX. We constructed factor XIa linked to acrylamide beads to reduce the likelihood that two factor XIa molecules could interact

with one factor IX molecule to cleave the Arg^{145}-Ala^{146} and Arg^{180}-Val^{181} cooperatively. This is an important consideration since factor XIa, unlike other coagulation proteases, is not known to associate with a phospholipid surface or surface-bound co-factor and is, therefore, presumably free to activate factor IX in solution. These results indicated that, if factor XIa uses a true processive mechanism to convert factor IX to factor IXaβ, that mechanism is contained within one subunit of the dimer.

Since factor XIa requires only one catalytic subunit to make both cleavages in factor IX, we thought it likely that one cleavage would be made initially, as with factor VIIa/tissue factor, and that a processive mechanism was unlikely. We postulated that the reason factor IXα accumulates during activation by factor VIIa/tissue factor is because cleavage after Arg^{180} is rate-limiting. In contrast, if a sequential cleavage mechanism was operating, factor XIa would need to make its rate limiting cleavage first to explain the lack of intermediate accumulation (Osterud *et al.*, 1978). Factor XIai was used as a "trap" to bind and retain intermediates released during factor IX activation. When factor IX was activated by factor XIa in the presence of a 1000-fold molar excess of factor XIai, a large amount of factor IXα accumulated, indicating factor XIa makes its initial cleavage after Arg^{145}. If factor IXα is released from factor XIa, one might expect that it should accumulate early during time course reactions if it binds to a similar exosite on factor XIa as zymogen factor IX. This is because, early in the reaction, factor IX is in vast excess of factor IXα, as factor XIai was in our experiment. That this is not observed raises the possibility that factor IX and factor IXα may bind to factor XIa at different sites (not in a mutually exclusive manner as one would expect for a competitive inhibitor). Indeed, we

noted that a fairly high concentration of factor XIai is required to observe factor IXα accumulation in the time course experiments.

Many symmetrical enzymes with multiple identical catalytic sites display cooperativity. Binding curves did not show evidence of cooperativity between factor XIa subunits. Cooperativity could involve binding of factor IX to one subunit enhancing binding of another factor IX to the opposite subunit. A titration of this type of enzyme would be sigmoidal, reflecting the lower affinity valence being occupied first. The opposite form of cooperativity would involve factor IX binding to one subunit lowering the affinity for binding to the opposite subunit, either by allosteric structural rearrangement or by steric hindrance by the initial binding factor IX. Our data indicates each factor XIa subunit binds factor IX or factor IXaβ independently of the other.

Exosite interactions are important for initial recognition of a substrate by a coagulation protease (Krishnaswamy, 2004; Bock *et al.*, 2007). The factor XIa heavy chain appears to be responsible for most if not all exosite binding energy in the factor IX/factor XIa complex (Sun and Gailani, 1996; Ogawa *et al.*, 2005). We activated factor IX with factor XIa-CD, which has no heavy chain, to determine the role of the heavy chain exosite interaction in cleavage of the two factor IX activation bonds. Factor XIa-CD was severely impaired in its activation of factor IX, demonstrating the importance of the heavy chain for both factor IX cleavages. However, unlike other single active site species of factor XIa, activation of factor IX by factor XIa-CD resulted in significant accumulation of factor IXα. This retention of the initial preference for cleavage at the Arg^{145}-Ala^{146} site suggests that Arg^{180}-Val^{181} may not be readily available for cleavage in the zymogen. This issue will be addressed in Chapter VI. In addition, the fact that

there is a significant accumulation of factor IXα during activation by factor XIa-CD indicates there is a relatively greater impairment of cleavage of the Arg^{180}-Val^{181} bond compared to the initial cleavage at Arg^{145}-Ala^{146}, indicating that loss of the factor XIa heavy chain is more detrimental to cleavage of factor IXα. It has recently been reported that factor IX activation by factor XIa-CD is non-competitively inhibited by a small molecule inhibitor, *p*-aminobenzamidine (Sinha *et al.*, 2007), suggesting that an exosite on the factor XIa catalytic domain may be required for normal recognition of factor IX that is less important for recognition of factor IXα. If confirmed, this would offer an explanation for the cleavage pattern of factor IX by factor XIa-CD.

Materials and Methods

Materials— Factor IX, factor IXaβ, factor XI, and factor XIa were purchased from Hematologic Technologies (Essex Junction, VT). Factor IXaα was purchased from Enzyme Research (South Bend, IN). In addition, plasma factor XI was prepared as described in the Methods section of Chapter IV. Aprotinin was from Sigma (St. Louis, MO). Factor XI deficient plasma was from George King (Overland Park, KS). Methyl-sulfonyl-D-cyclo-hexyl-glycyl-glycyl-arginine-*p*-nitroanilide (S-299) from American Diagnostics (Greenwich, CT). 1,5-Dansyl-Glu-Gly-Arg-CH_2Cl was from Calbiochem (La Jolla).

Factor IX activation by factor XIa followed by chromogenic substrate cleavage— Factor IX (25–2000 nM) in assay buffer (50 mM hepes pH 7.4, 125 mM NaCl, 5 mM $CaCl_2$, 1 mg/ml polyethylene glycol 8000) was activated by factor XIa (1–6 nM active

sites), 1/2-factor XIa (1–4 nM active sites), or factor XIa-1/2i (2.5–10 nM active sites) at 24°C. At various time points between 0 and 120 min, 60-μl aliquots were removed and mixed with 6 μl of assay buffer containing 150 μM aprotinin. Aprotinin completely inhibited factor XIa without affecting factor IXa activity. Sixty-six microliters of 1 mM S299 in assay buffer with 66% ethylene glycol was added to the quenched sample, and substrate hydrolysis was followed by measuring the change in absorbance at 405 nm. Generation of factor IXaβ as a function of time was determined by interpolation of the linear dependence of the initial rate of S299 hydrolysis on known concentrations of factor IXaβ. Initial rates for progress curves of factor IXaβ generation were obtained by analyzing the first 5 min of each curve with a second order polynomial equation. Resulting *vo* values were fit by the Michaelis-Menten equation, and values for K_m and k_{cat} were obtained from direct non-linear least squares analysis. The initial 5 min of factor IX activation are minimally influenced by product inhibition, and defined the K_m and k_{cat} values adequately. The values for K_m and k_{cat} were used to analyze complete progress curves by the integrated Michaelis-Menten equation. With values for K_m and k_{cat} fixed, full progress curves were fitted simultaneously by the integrated rate equation with product inhibition to obtain estimates of K_i for factor IXaβ. SCIENTIST Software was used to analyze the data (MicoMath Scientific Software. Salt Lake City, UT). Parameter errors represent 95% confidence intervals.

Activity of factor XIa in plasma clotting assays— Factor XIa proteases were diluted to 5 μg/ml in 20 mM Tris-Cl, 100 mM NaCl, 1 mg/ml BSA, pH 7.4 and serial 1:2 dilutions were prepared in the same buffer. Sixty μl of each dilution was mixed with an equal

volume of factor XI deficient plasma, and rabbit brain cephalin, followed by incubation for 30 s at 37 ^{o}C. Sixty μl of 25 mM $CaCl_2$ was added and the time to clot formation was determined on a Dataclot 2 fibrometer (Helena Laboratories, Beaumont, TX). Clotting times were plotted against enzyme concentration on a log-log plot, and factor XIa activity was determined as a percent of control by comparison to a control curve constructed with plasma factor XIa.

Factor IX activation followed by western blotting— Plasma factor IX (100 nM) was incubated in assay buffer at 24 °C with various factor XIa species, or with factor VIIa in the presence of saturating human tissue factor (Innovin, Dade-Behring, Miami, FL). In some reactions, factor XIa inhibited by a tripeptide chloromethyl-ketone (factor XIai) was included. At various times, 7 μl samples were mixed with 7 μl of reducing sample buffer (233 mM Tris-Cl, 138 mM SDS, 19% glycerol, 10% 2-mercaptoethanol, 0.01% bromphenol blue, pH 6.8), fractionated on 12% SDS-polyacrylamide gels, and then transferred to nitrocellulose. The primary antibody was goat anti-human factor IX polyclonal IgG (Enzyme Research Laboratories, South Bend, IN), and the secondary antibody was horseradish peroxidase-conjugated anti-goat IgG. Detection was by chemiluminescence. The relative positions of bands representing factor IX, factor IXα, factor IXaα, and factor IXaβ were determined by western blots of standards for each protein.

Titrations of factor XIa by factor IX and factor IXaβ— Factor XIa (6.25 μM) was diluted in titration buffer (50 mM Hepes, 125 mM $CaCl_2$, 1 mg/ml polyethylene glycol

8000, pH 7.4) and inhibited with a 10-fold molar excess of 1,5-dansyl-Glu-Gly-Arg-CH_2Cl at 24 °C. Residual factor XIa activity was determined by diluting aliquots to 20 nM factor XIa in 120 μl of titration buffer with 500 μM S-2366, and monitoring changes in absorbance at 405 nm until factor XIa activity was reduced >99.9%. The concentration of inhibited active sites per mol of factor XIa was determined by measuring dansyl concentration by absorbance at 335 nm with ε of 8,000 M^{-1} cm^{-1}, and factor XIa concentration by absorbance at 280 nm (corrected for absorbance of the fluorophore) with ε of 214,400 M^{-1} cm^{-1}. Probe incorporation was 1.98 moles per mole of factor XIa. Fluorescence titrations were performed with an SLM 8100 fluorometer, using acrylic cuvettes coated with polyethylene glycol 20,000. Fluorescence intensity titrations of dansyl labeled factor XIa were performed with 335 nm excitation (16 nm band pass) and 552 nm emission (16 nm band pass) in titration buffer supplemented with 2 μM D-Phe-Pro-Arg-CH_2Cl at 24 °C. The quadratic binding equation was fit to fluorescence changes ($(F_{obs} - F_o)/F_o = \Delta F/F_o$) as a function of total factor IX concentration, to determine the maximum change in fluorescence ($\Delta F_{max}/F_o$), dissociation constant (K_d), and stoichiometry (n) using SCIENTIST Software. Parameter errors represent 95% confidence intervals.

Preparation of recombinant factor XIa catalytic domain (factor XIa-CD)— Recombinant factor XI with Cys^{362} and Cys^{482} changed to Ser (factor XI-$Ser^{362,482}$) (Ogawa *et al.*, 2005; Smith *et al.*, 2008), was expressed in HEK293 cells as described as described in Chapter IV. Protein from stably transfected clones was purified from conditioned media (Cellgro Complete, Mediatech, Herndon, VA) by chromatography

using anti-human factor XI-IgG 1G5.12 (Sun *et al.*, 1999). The column was eluted with 2 M sodium thiocyanate in 25 mM Tris-HCl, pH 7.5, 100 mM NaCl (Tris/NaCl). Protein-containing fractions were pooled and concentrated by ultrafiltration, dialyzed against Tris/NaCl, and stored at -80 °C. Factor XI-Ser^{362,482} (100–300 μg/ml) were activated by incubation with 2 μg/ml factor XIIa at 37 °C. Complete activation was confirmed by reducing SDS-polyacrylamide gel electrophoresis. Factor XIa-Ser^{362,482}, which lacks the disulfide bond that connects the factor XIa heavy chain to the catalytic domain, was reapplied to the 1G5.12 column. The catalytic domain (factor XIa-CD) binds to the column, whereas the heavy chain is found in the flow through. Factor XIa-CD was eluted as described above and dialyzed against Tris/NaCl.

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CHAPTER VI

FACTOR XIa EXOSITES AND CLEAVAGE OF FACTOR IX

Introduction

Most of the work described in this chapter is unpublished and will serve as the basis of a manuscript that is currently in preparation. The studies follow-up on the observations discussed in Chapter V that the mechanism of factor IX activation by factor XIa involves sequential cleavage of factor IX at the Arg^{145}-Ala^{146} and Arg^{180}-Val^{181} bonds with formation of the intermediate factor IXα. Published results indicate that an exosite interaction involving the factor XIa non-catalytic heavy chain is required for this process (Ogawa *et al.*, 2005; Smith *et al.*, 2008). Work with recombinant factor XI chimeras (Sun and Gailani, 1996) and site-directed mutants (Sun *et al.*, 1999) indicate that the factor XIa A3 domain is required for normal factor IX activation (**Figure VI-1**),

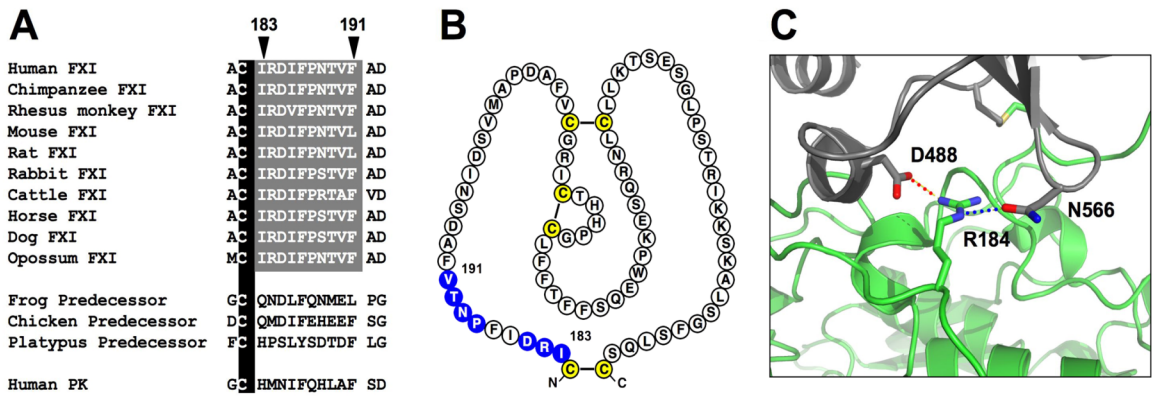

Figure VI-1. Putative factor IX binding site on the factor XIa A3 domain. (A) Residues 183-191 are conserved in the A3 domain of factor XI in mammals, but not in PK or the factor XI/PK precursor found in other vertebrates. **(B)** Diagram of human factor XI A3. Residues identified as critical for the interaction with factor IX are in blue. **(C)** In the crystal structure of factor XI, the putative factor IX binding site on the A3 domain (green) is buried in an interface with the catalytic domain (gray). Arg^{184} on A3 forms a salt bridge with Asp^{488} and a hydrogen bond with Asn^{566} on the catalytic domain. In the crystal structure of the isolated factor XIa catalytic domain, D^{488} and N^{566} interact with other catalytic domain residues, indicating conformational changes that occur with protease activation may free residues 183-191, allowing factor IX to bind. The factor XI numbering system is used in this diagram.

and may contain the proposed exosite. It also appears likely that the C-terminal portion of the factor IX Gla domain is required, as discussed in Chapter II (Aktimur, 2003). In this chapter, we will present results from experiments designed to examine the importance of the factor XIa A3 domain and the factor IX Gla domain to normal activation of factor IX; activation without accumulation of an activation intermediate. The results indicate that both the factor XIa A3 domain and the factor IX Gla domain are necessary for the observed pattern of factor IX activation.

Figure VI-2 shows a schematic diagram of the possible reactions involved in converting factor IX to factor IXaβ. As factor XIa appears to cleave factor IX initially at the Arg^{145}-Ala^{146} bond (*Reaction 1*) to form factor IXα, the lack of accumulation of this intermediate strongly suggests that the second cleavage at Arg^{180}-Val^{181} (*Reaction 2*) must be at least as fast, or perhaps substantially faster than *Reaction 1*. Here we describe experiments comparing the relative rates of cleavage of Arg^{145}-Ala^{146} (*Reaction 1*) and Arg^{180}-Val^{181} (*Reaction 3*) in factor IX with cleavage of the same bonds in the activation intermediates factor IXα (*Reaction 2*) and factor IXaα (*Reaction 4*). The results indicate that cleavage of Arg^{180}-Val^{181} (*Reaction 3*) is substantially slower in factor IX compared to factor IXα (*Reaction 2*), implying that cleavage at Arg^{145}-Ala^{146} (*Reaction 1*) facilitates cleavage at Arg^{180}-Val^{181} (*Reaction 2*).

The Importance of the Factor XIa A3 Domain to Factor IX Activation

We determined the importance of the factor XIa A3 domain to factor IX intermediate formation by replacing the factor XI A3 domain with the equivalent domain from PK. Recall that PK activates factor IX poorly (Sun and Gailani, 1996). Previously

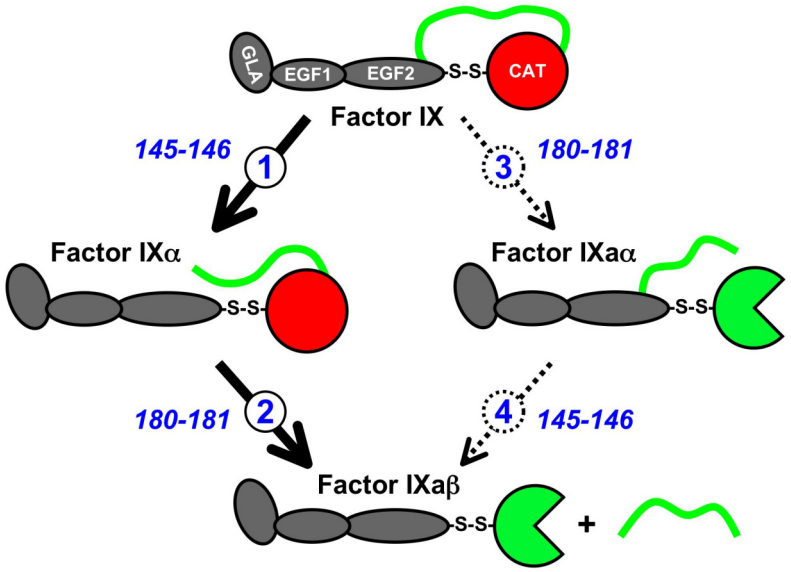

Figure VI-2. Pathways involved in factor IX activation to factor IXaβ. Factor IX is converted to factor IXaβ by cleavage of the Arg145-Ala146 and Arg180-Val181 bonds, releasing the activation peptide (green ribbon). Conversion of inactive catalytic domain (red circle) to active protease (green three-quarter circle) requires cleavage at Arg180-Val181. During activation by factor VIIa/TF, factor IX is cleaved at Arg145-Ala146 forming factor IXα (*Reaction 1*). Factor IXα is subsequently cleaved at the Arg180-Val181 bond (*Reaction 2*) to form factor IXaβ. Initial cleavage of factor IX at Arg180-Val181 (*Reaction 3*) is a minor reaction. The product of *Reaction 3*, factor IXaα is converted to factor IXaβ by cleavage of the Arg145-Ala146 bond (*Reaction 4*). Activation of factor IX by factor XIa, appears to follow the same pathways as for activation by factor VIIa/TF (i.e. through *Reactions 1* and *2*).

we showed that this chimera, factor XIa/PKA3 cleaves the chromogenic substrate S-2366 in a similar manner to factor XIa, but is a poor activator of factor IX. When reducing western blots of factor IX activated by recombinant wild type factor XIa (factor XIa^{WT}) or factor XIa/PKA3 are compared (**Figure VI-3A** and **3B**) two important differences are apparent. First, the rate of cleavage of zymogen factor IX is slower with factor XIa/PKA3, consistent with our published kinetics data (Sun and Gailani, 1996). Second, there is a pronounced accumulation of factor IXα during the reaction with the chimera. Little if any factor IXaβ is observed (very little heavy chain noted on the blot). In fact, the results are similar to those shown in Chapter V for factor IX activation by factor XIai in the presence of factor XIai (**Figure V-5**) and for the isolated factor XIa catalytic

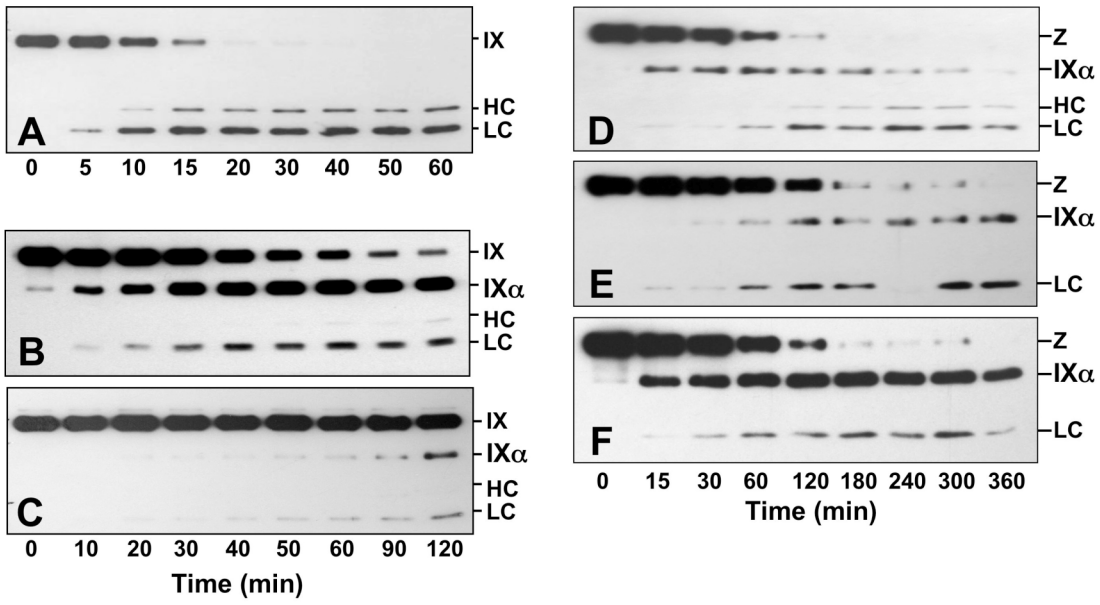

Figure VI-3. Factor IX activation by factor XIa - Importance of the A3 domain. Shown are reducing western blots of time courses of factor IX (100 nM) activation by **(A)** factor XIa - 2 nM, **(B)** factor XIa/PKA3 - 5 nM, **(C)** factor XIa 2 nM in the presence of 50 nM antibody O1A6, **(D)** factor XIa-CD 5 nM **(E)** factor XIa/PKA3 - 5 nM, and **(F)** a chimera consisting of the prekallikrein heavy chain and the factor XIa catalytic domain (PK-HC/FXIa-CD) - 5 nM. All values are active site concentrations. Note that the time courses differ between panels. Migration of protein standards is shown for zymogen factor IX (FIX), the large fragment of factor IXα (FIXα), the heavy chain of factor IXaβ (HC), and the light chain of factor IXaβ or factor IXα (LC).

domain (**Figure V-6**). The result, in conjunction with our previous observation that factor IX activation by factor XIa/PKA3 is characterized by a >30-fold increase in K_m (Sun and Gailani, 1996), are consistent with the factor XIa A3 domain containing an important exosite for factor IX activation. Similarly, factor IXα was seen to accumulate slowly when factor IX was activated by factor XIa in the presence of a monoclonal anti-factor XI IgG (O1A6 - **Figure VI-4**) (Tucker *et al.*, 2009). O1A6 is an antibody that binds to the factor XIa A3 domain (**Figure VI-4A**) at residues that overlap or are in close proximity to the factor IX binding site (compare **Figure VI-4B** and **C**). In this reaction, activation is considerably slower than with factor XIa/PKA3, probably because of steric interference caused by the antibody (**Figure VI-3C**).

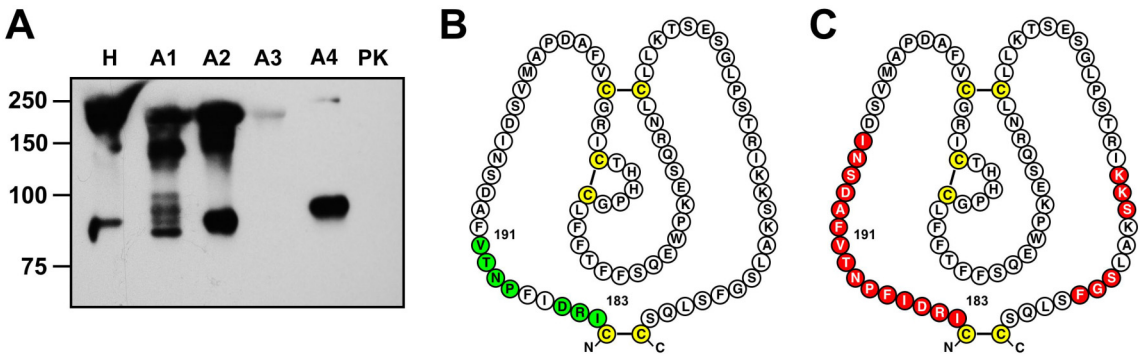

Figure VI-4. Monoclonal anti-human factor XI IgG O1A6. (A) Chemiluminescent western blot of non-reducing SDS-PAGE using O1A6 as the detection antibody. The proteins on the blot are: H - human factor XI; A1, A2, A3, or A4 - human factor XI with the indicated prekallikrein apple domains; PK - human PK. Note that factor XI/PKa4 is a monomer. **(B** and **C)** Schematic diagrams of the A3 domain of human factor XI showing the positions of **(B)** amino acids that are required for factor IX binding (in green) and **(C)** amino acids that may be part of the binding site for the O1A6 antibody (in red). Data in panels **B** and **C** were obtained using a panel of alanine mutants of factor XI (Sun *et al.*, 1991).

We also compared activation of factor IX by factor XIa/PKA3 directly to factor XIa-CD and a chimera consisting of the entire PK heavy chain (all four apple domains) and the factor XIa catalytic domain (PK-HC/FXIa-CD) (**Figure VI-3D-F**) (Cheng *et al.*, 2003). For these reactions, which involved substantially longer time courses than those in **Figure VI-3A-C**, the rate of disappearance of zymogen factor IX is similar, although considerably slower than with factor XIa-WT (**Figure VI-3A**). However, factor IXaβ is observed in the reaction with factor XIa-CD, but not in reactions with factor XIa/PKA3 or PK-HC/FXIa-CD. First, the similarity in reactions with factor XIa/PKA3 and PK-HC/FXIa-CD support the conclusion that most of the binding energy for the factor XIa-factor IX interaction is within the A3 domain, and other apple domains probably do not directly contribute. Once the A3 domain is replaced with a domain that does not bind factor IX, it may actually cause steric interference that prevents the catalytic domain from making the second cleavage at Arg^{180}-Val^{181}, as factor XIa-CD is able to do.

The Importance of the Factor IX Gla Domain to Activation by Factor XIa

The factor IX Gla domain may serve as a recognition site for factor XIa, as replacement of the Gla with factor VIIa Gla results in a molecule (factor IX/VII-Gla) that binds poorly to factor XIa and, probably as a consequence, is activated poorly by factor XIa (Aktimur *et al.*, 2003). The importance of the Gla domain interaction may be why factor IX activation by factor XIa is Ca^{2+} dependent, as other reactions involving factor XI/XIa do not require Ca^{2+}. It is important to note that factor IX/VII-Gla is activated normally by factor VIIa/TF (Aktimur *et al.*, 2003), indicating that the Gla substitution does not interfere with phospholipid binding or protein-protein interactions with factor VIIa/TF. This implies that factor VIIa and factor XIa bind to distinct sites on factor IX.

We conducted experiments to determine if conditions that affect the Gla domain influence the cleavage pattern generated when factor IX is activated by factor XIa. First, we examined activation in the presence of the Ca^{2+} chelator EDTA (**Figure VI-5A**). As with reactions using factor XIa/PKA3, the reaction in the absence of Ca^{2+} is characterized by significant accumulation of factor IXα and a slow generation of factor IXaβ. Factor IXα also accumulated when factor IX was activated by factor XIa in the presence of a monoclonal antibody (SB 249417, John Toomey, GlaxoSmithKline), which is a humanized murine antibody against the human factor IX-Gla domain (**Figure VI-5B**) (Aktimur *et al.*, 2003). The perturbation in activation is similar to the one caused by interfering with the factor XIa A3 domain, raising the possibility that the factor IX Gla domain is interacting with the factor XIa A3 domain. This interaction appears to be necessary for both factor IX cleavages, but disruption of the interaction causes a disproportionate effect on the second cleavage at Arg^{180}-Val^{181}.

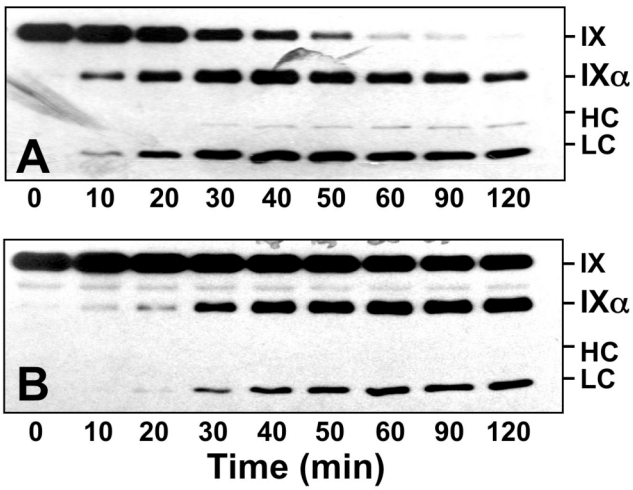

Figure VI-5. Factor IX activation by factor XIa - Importance of the Gla domain. Shown are reducing western blots of time courses of factor IX (100 nM) activation by **(A)** factor XIa - 2 nM in the presence of 25 mM EDTA or **(B)** factor XIa - 2 nM in the presence of the anti-human factor IX Gla domain monoclonal antibody SB 249417. Migration of protein standards is shown for zymogen factor IX (FIX), the large chain of factor IXα (FIXα), the heavy chain of factor IXaβ (HC), and the light chain of factor IXaβ or factor IXα (LC).

Rates of Cleavage of the Arg^{145}-Ala^{146} and Arg^{180}-Val^{181} Bonds in Factor IX and Its Activation Intermediates

As discussed, factor IX must be cleaved at two internal bonds for full activation, and modeling and crystallographic studies do not provide an obvious reason that initial cleavage at either bond would be preferred. If we assume that initial cleavage could occur at either the Arg^{145}-Ala^{146} or the Arg^{180}-Val^{181} bond in factor IX, then there are four half-reactions possible for the conversion of factor IX to factor IXaβ, as shown in **Figure VI-2**. Factor VIIa/TF and factor XIa both make their initial cleavage in factor IX after Arg^{145}, but the mechanistic basis for this is unclear. Conversion of prothrombin to α-thrombin can also proceed through more than one intermediate, but one pathway is favored (Krishnaswamy, 2004; Bock *et al.*, 2007). The mechanistic basis for this involves significantly different k_{cat}s for cleavage at different sites in the zymogen. Cleavage at the preferred site facilitates cleavage at subsequent sites by allowing the substrate to achieve a different conformation that presents the cleavage site more efficiently to the active site of the enzyme. Here we present results from experiments designed to compare the rates of cleavage of the individual scissile bonds.

In experiments in Chapter V examining factor IX activation, we generated full progress curves for factor IX activation using a chromogenic substrate assay, and determined initial rates from these curves to establish K_m and k_{cat} for activation, and K_i for product inhibition (Smith *et al.*, 2008). This technique will not be useful for studying factor IX conversion to factor IXα, which is not an active protease. Furthermore, some of our studies will require recombinant variants of factor IX lacking an activation cleavage site. Species of recombinant factor IX with an activated catalytic domain (factor IXaα and factor IXaβ) do not cleave the chromogenic substrate S299 very well. For these reasons, we opted to follow factor IX activation by densitometry of GelCode blue stained SDS-polyacryalmide gels imaged at infrared wavelengths. The low signal-to-noise ratio with this technique allows us to examine factor IX activation at concentrations as low as 10 nM (**Figure VI-6**).

Initial experiments examined conversion of factor IXα and factor IXaα (*Reactions 2* and *4* in **Figure VI-2**) to factor IXaβ. The intermediates were prepared from plasma factor IX (**Figure VI-7A**, see Methods). The Arg^{180}-Val^{181} bond in factor IXα

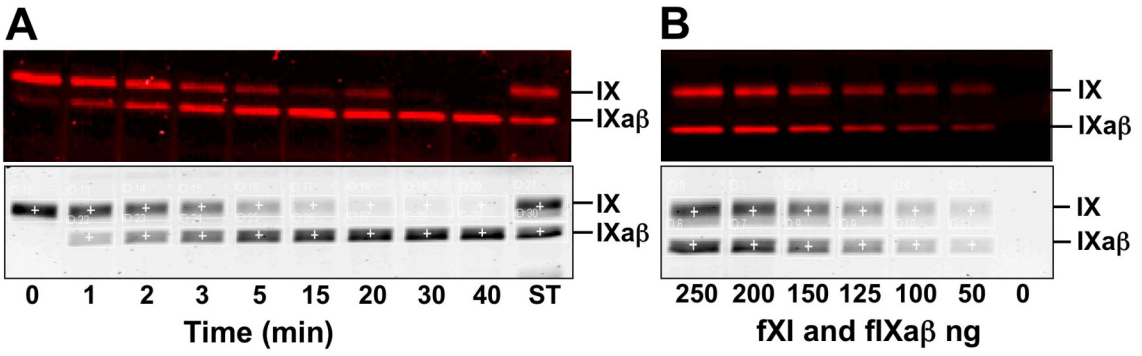

Figure VI-6. Assessing factor IX activation by infra-red densitometry. The top images are derived from infrared images of **(A)** a time course of factor IX (250 nM) activated by 2 nM factor XIa, and **(B)** a set of protein standards used to determine amounts of factor IX and factor IXaβ on the time course. A standard (ST) is also included in the right hand lane of the time course. At the bottom are gray scale images used for densitometry.

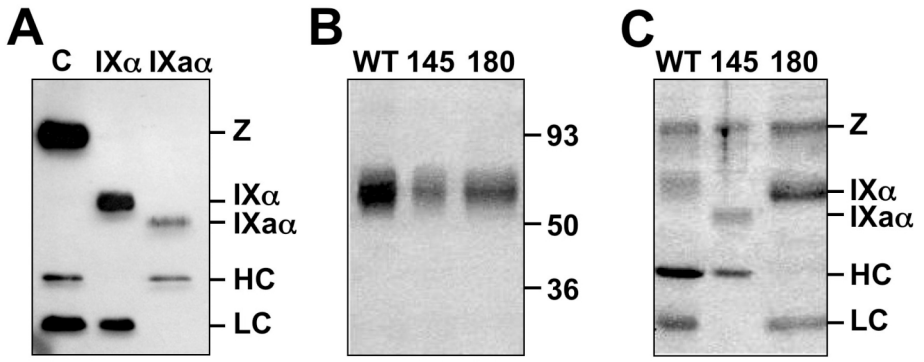

Figure VI-7. Factor IX activation intermediates prepared from plasma factor IX, and recombinant factor IX activation cleavage site mutants. (A) Control (C) mixture of factor IX and factor IXaβ, and purified plasma factor IXα and factor IXaα. **(B)** Recombinant factor IX (WT), factor XI^{R145A}, and factor XIa^{R180A}. Mass standards (kDa) are at right. **(C)** Reduced partially activated proteins from panel **B**. Factor IX (WT) is partially activated by factor VIIa/TF to product and intermediate. Abbreviations for panels **A** and **C**: Z - zymogen factor IX, IXα - large fragment of factor IXα, IXaα - large fragment of factor IXaα, HC - heavy chain (catalytic domain) of factor IXa and factor IXaβ, LC - light chain of factor IXα and factor IXaβ.

and the Arg^{145}-Ala^{146} bond in factor IXaα were cleaved by factor XIa with similar kinetic parameters (**Table VI-1**), and the values agree with those for overall conversion of factor IX to factor IXaβ determined by the same technique. Note that the values agree with those for factor IX activation by chromogenic substrate assay (**Table V-1**). The fact that the parameters for *Reaction 2* (**Figure VI-2**) are similar to those for the overall

Table VI-1. Kinetic parameters for activation of factor IX, factor IXα and factor IXaα by factor XIa. K_m and k_{cat} for activation were based on initial rates for factor IX activation from full progress curves as described under Methods. The resulting v_o values were analyzed by fitting the Michaelis-Menten equation, and values for K_m and k_{cat} were obtained from direct non-linear least squares analysis. Values for K_m and k_{cat} were used to analyze the complete progress curves by the integrated Michaelis-Menten equation. With values for K_m and k_{cat} fixed, full progress curves were fitted simultaneously by the integrated rate equation with product inhibition to obtain estimates of K_i for factor IXaβ. Errors in parameters represent 95 % confidence intervals.

Substrate	K_m (nM)	k_{cat} (min^{-1})	K_i (nM)
Factor IX	82 ± 50	30 ± 6	24 ± 28
Factor IXα	57 ± 48	19 ± 4	20 ± 21
Factor IXaα	108 ± 80	30 (fixed)	22 ± 2

activation of factor IX to factor IXaβ (which proceeds through *Reactions 1* and *2*) suggest that the rates for *Reactions 1* and *2* are similar. This interpretation is consistent with the fact that *Reaction 4* (cleavage at Arg^{145}-Ala^{146} in factor IXaα) has similar kinetic parameters to *Reaction 2*.

To definitively demonstrate that conversion of factor IX to factor IXα has similar kinetic parameters to those for *Reactions 2* and *4*, we are in the process of examining cleavage of recombinant factor IX that can only be cleaved at the Arg^{145}-Ala^{146} bond (factor XIa^{R180A}, Arg^{180} replaced with alanine - **Figure VI-7B** and **Figure 8**). The final product of factor XIa of factor IX-Ala^{180} is factor IXα (**Figure VI-7C**). We also have generated recombinant factor IX that can only be cleaved at the Arg^{180}-Val^{181} bond (factor XI^{R145A}), Arg^{145} replaced with alanine - **Figure VI-7B**). The final product of factor XIa of factor IX-^{R145A} is factor IXaα (**Figure VI-7C**). Comparing the kinetics of cleavage of these mutants to their respective products will allow us to test the hypothesis that cleavage of the Arg^{180}-Val^{181} bond in zymogen factor IX (*Reaction 3* in **Figure VI-2**) is much slower than at Arg^{145}-Ala^{146} (*Reaction 1*). These studies are in progress. In preliminary work, we have indeed observed that factor XIa^{R180A} is converted to its final product at a rate that appears to be much faster than for factor XI^{R145A} (**Figure VI-8**).

Discussion

In this chapter we have presented evidence that factor IX activation by factor XIa is influenced by binding interactions with exosites on the heavy chain of factor XIa (Ogawa *et al.*, 2005). Specifically, one or more sites on the factor XIa A3 domain appear to be involved. Furthermore, the cognate factor IX binding site for this factor XIa exosite

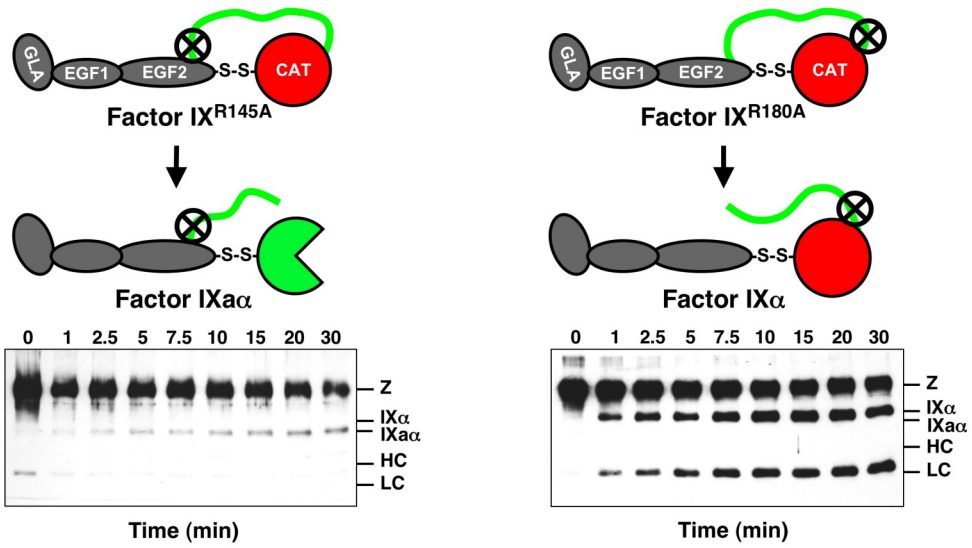

Figure VI-8. Conversion of factor XI^{R145A} to factor IXaα and factor XIa^{R180A} to factor IXα by factor XIa. (Top). The alanine replacement that abolishes each factor IX cleavage site is represented by the symbol ⊗, at either end of the activation peptide (green ribbon). Cleavage of factor IX^{R145A} (left) can only be made after Arg^{180}, producing factor IXaα. Cleavage of factor IX^{R180A} (right) can only produce factor IXα. (**Bottom**) Recombinant factor XI^{R145A} or factor XIa^{R180A} (100 nM) in Assay Buffer were incubated at 24 ˚C with factor XIa (0.2 nM active sites). Aliquots were removed at indicated reaction times into denaturing reducing buffer, fractionated on 12% polyacrylamide-SDS gels, and analyzed by western blotting as described under Methods. Migration of protein standards is shown for zymogen factor IX (Z), the large fragment (activation peptide + heavy chain) of factor IXα (IXα), the large fragment (activation peptide + light chain) of factor IXaα (IXaα), the heavy chain of factor IXaβ or factor IXaα (HC), and the light chain of factor IXaβ or factor IXα (LC).

appears to be the factor IX Gla domain. Since perturbation of either of these sites results in markedly slower factor IX cleavage by factor XIa, the conclusion that these sites are required for initial substrate recognition seems reasonable. Interestingly, binding interactions mediated by these sites also appear to influence the release of the intermediate factor IXα from the enzyme/substrate complex. This observation implies that the A3-Gla domain interaction is more important for cleavage after Arg^{180} than for cleavage after Arg^{145}, but does not explain why we do not normally observe the accumulation of factor IXα.

The implication of the study with factor XIai in Chapter V (**Figure V-5**) is that factor IXα is released from factor XIa. Given the lack of intermediate accumulation on western blots, we would reason that the initial cleavage of factor IX by factor XIa after Arg^{145} is rate limiting and that the cleavage of factor IXα to form factor IXaβ should be significantly faster. However, Wolberg *et al.* estimated that factor IX and its two possible activation intermediates are cleaved by factor XIa at roughly similar rates (Wolberg *et al.*, 1997). The preliminary results presented in this chapter concur with this finding. Given this, it is somewhat surprising that we do not see some degree of intermediate accumulation early in time courses, if factor IXα is released from factor XIa and must be recaptured. At early points in the time course, factor IX zymogen is present in vast excess over factor IXα and should compete with the intermediate for binding to factor XIa, as factor XIai appears to have done in the experiment in **Figure V-5**. This assumes, of course, that factor IX and factor IXα bind with similar affinities and to a similar site (or sites) on factor XIa. We showed that factor IX and factor IXaβ have similar affinities for factor XIa (**Figure V-7**). The comparable K_ms for cleavage of factor IX and factor IXα suggest similar affinities for factor XIa for these two proteins as well.

The issue of whether or not there are distinct binding sites for factor IX and factor IXα is an interesting one. Recently, Sinha *et al.* raised the possibility of a factor IX binding site on the factor XIa catalytic domain (outside of the active site) that was primarily involved with the initial interaction with factor IX (Sinha *et al.*, 2007), and suggested that the interaction with the factor XIa heavy chain was more important for the interaction with factor IXα. This is consistent with our observation that interfering with the factor IX interaction with the heavy chain results in factor IXα accumulation (**Figure**

VI-3 and **4**). Work done previously in our laboratory showed that factor IX and factor IXaβ binding to factor XIa is mutually exclusive (Ogawa *et al.*, 2005), indicating that zymogen and product bind to the same sites on factor XIa. Work is currently underway to determine whether factor IX and factor IXα compete for binding sites on factor XIa.

None of the preceding observations provide a mechanistic explanation for the preferential cleavage of factor IX at the Arg^{145} bond by both factor XIa and factor VIIa/TF. Experiments in which the purified intermediates factor IXα and factor IXaα were activated by factor XIa only address questions of how factor XIa processes these proteins, which do not normally accumulate. The cleavage site mutants factor IX^{R145A} and factor IX^{R180A} were constructed to address the issue of how factor XIa processes zymogen factor IX. The comparatively slow activation of factor IX^{R145A} by factor XIa (**Figure VI-8**) suggests that the factor XIa active site cannot properly access the Arg^{180}-Val^{181} bond in that molecule. It is reasonable to conclude that a decrease in k_{cat}, rather than an increase in K_m, is responsible for this observation, since both cleavage site mutants are zymogens and should bind equally well to factor XIa. We are currently using the densitometry techniques described in this chapter to determine the rate constants for conversion of the cleavage site mutants to their respective products.

The conversion of prothrombin to α-thrombin has been extensively studied (Krishnaswamy, 2004; Bock *et al.*, 2007), and is the most appropriate model from which to consider exosite-mediated mechanisms involved in activation of factor IX. As with factor IX activation, prothrombin must be cleaved at two bonds (Arg^{271}-Thr^{272} and Arg^{320}-Ile^{321}) to generate the final product. Cleavage at the Arg^{320}-Ile^{321} bond is favored because of a 30-fold faster kcat, resulting in formation of the intermediate

meizothrombin. Subsequent cleavage at Arg^{271}-Thr^{272} in meizothrombin is kinetically favorable relative to cleavage of the same bond in zymogen prothrombin. Despite these similarities with factor IX activation by factor XIa, there appear to be two notable differences between the processes. First, meizothrombin is observed to accumulate to various degrees (depending on the experiment) during prothrombin activation, consistent with the intermediate being released from the prothrombinase complex. Second, in the absence of the cofactor factor Va, prothrombin is preferentially cleaved not at the Arg^{320}-Ile^{321} bond, but at the Arg^{271}-Thr^{272} bond to form the intermediate prethrombin 2 - F1.2 complex. This suggests that exosites not only determine order of cleavage of the bonds in prothrombin and, but also which bonds are accessible to the protease. In contrast, factor IX activation by factor XIa or factor VIIa/TF appears to always proceed through factor IXα, suggesting that the second bond is not normally accessible. Thus, it may be difficult to extrapolate from the lessons learned from prothrombin activation to completely explain activation of factor IX by factor XIa.

At this point, we should mention that we have observed cleavage of the Arg^{180}-Val^{181} bond by factor XIa in zymogen factor IX, but only in two situations, and under highly unusual conditions. When recombinant factor IX is expressed in the presence of the vitamin K antagonist warfarin to block γ-carboxylation, the resulting protein is cleaved poorly by factor XIa, and cleavage is preferentially at the Arg^{180}-Val^{181} bond (data not shown). This is difficult to explain purely by a poor interaction between Gla domain and factor XIa A3, as it differs from results for reactions run in the presence of EDTA or the anti-Gla domain antibody SB 249417 (**Figures VI-5**), and may indicate significant distortion of factor IX resulting in an unusual interaction with the protease. A

naturally occurring factor XI mutation in the catalytic domain associated with a dysfunctional circulating factor XI variant (factor XIa with Thr 575 replaced with Met - T213M in chymotrypsinogen) was recently reported (Mitchell *et al.*, 2007). We expressed this protein and noted that it cleaved factor IX relatively slowly, with simultaneous generation of both intermediates (**Figure VI-9**). The methionine substitution in the mutant almost certainly causes significant perturbations in the enzyme active site, changing the nature of the protease. These peculiar findings indicate that the Arg^{180}-Val^{181} is exposed to some extent on the surface of factor IX, but is not readily accessible to factor XIa in the normal zymogen.

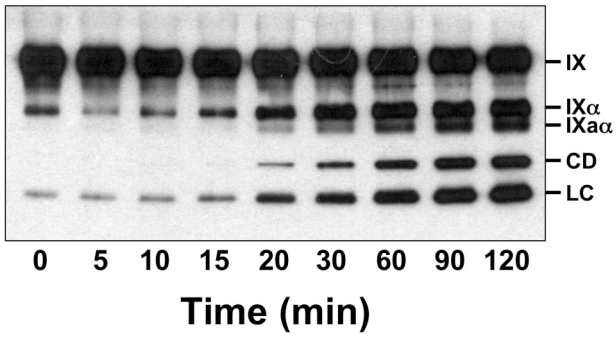

Figure VI-9. Activation of factor IX by factor XIa^{T213M}. Western blot (12% polyacrylamide reducing gel) of factor IX (100 nM) incubated with factor XIa^{T213M} (5 nM). Note that bands corresponding to both possible activation intermediates are present. Migration of protein standards is shown for zymogen factor IX (IX), the large fragment of factor IXα (IXα), the large fragment of factor IXaα (IXaα), the catalytic domain of factor IXaβ or factor IXaα (CD), and the light chain of factor IXaβ or factor IXα (LC).

Methods

Infrared imaging-densitometry of protein on polyacrylamide gels. SDS-polyacrylamide gels are stained with GelCode blue (Pierce). Conversion of factor IX or factor IXα to factor IXaβ is studied with non-reducing gels (factor IXaβ is ~10 kDa smaller than factor IX and factor IXα), while factor IX conversion to factor IXα and factor IXaα is studied with reducing gels. Gels are destained in water for 24 hours, and particulate matter is removed by gently wiping the surface with dishwashing liquid

(Palmolive). Infrared imaging (excitation λ 685 nm, emission λ 720 nm) is done on an Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE). The instrument software converts infrared data to grayscale images for densitometry. To determine amounts of protein per lane, standards are run on a separate gel, with one standard on the time course gel. Full progress curves are plotted from the data.

Recombinant factor IX. cDNAs for factor IX, factor IX-Ala^{145} and factor IX-Ala^{180} are expressed in HEK293 cells (ATCC CRL1573) in Cellgro Complete serum free medium (Mediatech, Herdon, VA) supplemented with 10 μg/ml vitamin K and 10 μg/ml soy bean trypsin inhibitor (Aktimur *et al.*, 2003). A high percentage of vitamin-K dependent protein produced in this line is properly γ-carboxylated (Yan *et al.*, 1990; Wong *et al.*, 1999; Yan 1996) Factor IX is purified from media by affinity chromatography with an antibody to the γ-carboxylated Gla domain (SB 249417).

Preparation of factor IXα and factor IXaα from plasma factor IX. To prepared factor IXα, factor IX (3-4 μM) is incubated with 150 nM factor XIa-Pro^{192} in TBS, pH 7.4, containing 0.1% PEG, 5 mM $CaCl_2$ at 37 ^{o}C for 24 h. Traces of factor IXaβ are inhibited by incubation at 4 ^{o}C with a 20-fold molar excess of biotinylated EGR-CMK, followed by 1 h at RT with streptavidin immobilized on agarose. The supernatant contains pure factor IXα. To prepare, factor IXaα, factor IX (3-4 μM) is incubated with 150 nM Russell Viper Venom protease in TBS, 0.1% PEG, 5 mM $CaCl_2$ pH 7.40 at 37 ^{o}C for 30 min. The mixture is passed over a 5 mL HiTrap Heparin FPLC column in 20 mM citric acid pH 7.40. Protein is eluted with a 0 to 1 M NaCl gradient (Bajaj *et al.*, 1983). Fractions containing pure factor IXaα by SDS-PAGE are dialyzed against TBS, pH 7.4.

Preparation of recombinant factor XI and factor XI/PK chimeras. Factor XI and factor XI/PK chimeras are expressed in HEK293 cells under the same culture conditions for factor IX expression, but without vitamin K (Sun *et al.*, 1999). Protein is purified from conditioned media by chromatography on anti-human factor XI-IgG 1G5.12. Factor XI (300 μg/ml) is converted to factor XIa by incubation in TBS with factor XIIa (2 μg/ml) at 37 °C. Complete conversion to factor XIa is confirmed by reducing SDS-PAGE. Factor XIIa is removed by reapplication to the 1G5.12 antibody column.

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CHAPTER VII

MODELS FOR FACTOR IX ACTIVATION BY FACTOR XIa

Exosites on coagulation proteases are critical for determining substrate specificity and order of bond cleavage. We have shown that this principle applies to activation of factor IX by factor XIa. Specifically, factor XIa (1) requires only a single active subunit to activate factor IX, (2) cleaves factor IX initially at the Arg145-Ala146 bond, and (3) has at least one exosite (A3) required for factor IX activation. The simplest way to treat the reaction, involves postulating a single factor XIa exosite on the A3 domain that would bind factor IX and its activation intermediates (**Figure VII-1**). Here, factor IX engages

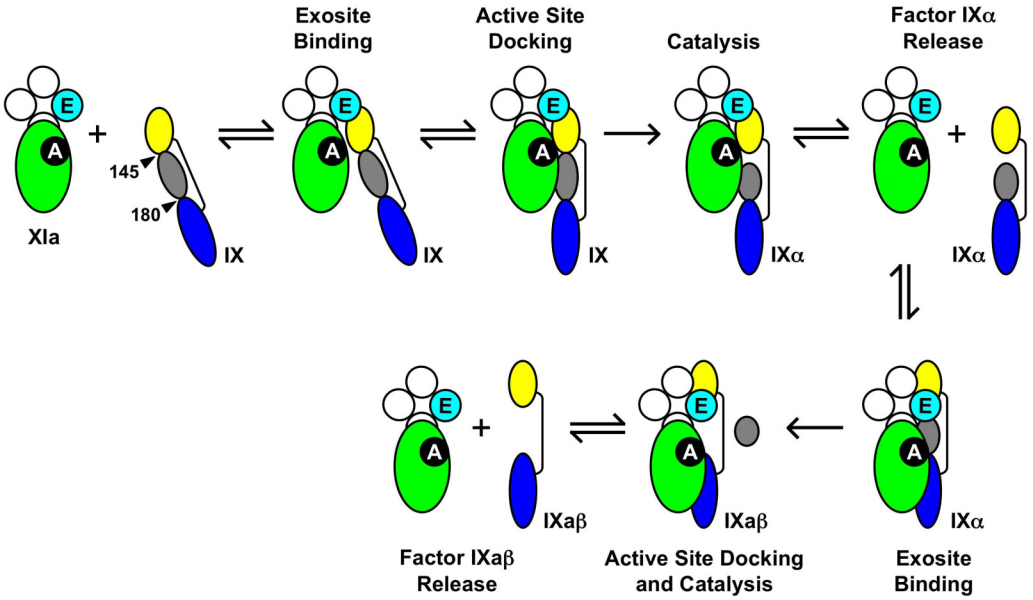

Figure VII-1. Factor IX activation by factor XIa involving a single exosite and release of intermediate. The factor XI apple domains are shown as four circles. A1, A2, and A4 are in white, and A3 with the factor IX binding exosite is in blue (E). The factor XIa catalytic domain is a green ellipse with the active site indicated by (A). The factor IX catalytic heavy chain (dark blue ellipse) and light chain (yellow ellipse) are connected by a line representing a disulfide bond. The activation peptide is the gray oval. Bi-directional arrows represent reversible binding, and directional arrows are activation cleavages. Factor IX is activated by a single factor XIa subunit by sequential cleavage after Arg^{145} and Arg^{180}, with the intermediate factor IXα released from the protease. Factor IX interacts with a single exosite on the A3 domain of factor XIa.

the exosite, followed by a docking interaction at the active site and catalysis of the Arg^{145}-Ala^{146} to form factor IXα. The intermediate is released from the protease, and must rebind to be cleaved at the Arg^{180}-Val^{181} bond to form factor IXaβ. A potential problem with this model is that it seems to require the second cleavage at Arg^{180}-Val^{181} to be more rapid than the initial cleavage; something we have not been able to demonstrate. Also, the more deleterious effect of disrupting the A3 exosite interaction on the Arg^{180}-Val^{181} cleavage suggests the process may be more complex.

The model in **Figure VII-2** is similar to the one in **Figure VII-1**, except that two factor IX binding exosites are proposed, one on the catalytic domain (Sinha *et al.*, 2007) and one on A3. We hypothesize that both exosites are required for the initial cleavage at Arg^{145}-Ala^{146}, but only A3 is required for cleavage of factor IXα at the Arg^{180}-Val^{181}

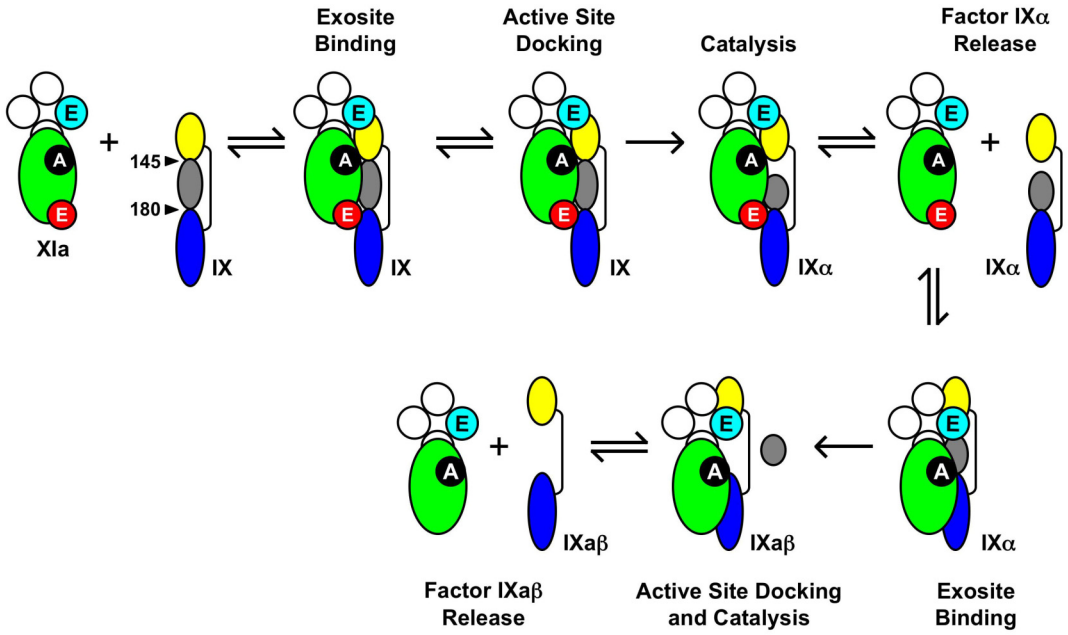

Figure VII-2. Factor IX activation by factor XIa involving two exosites and release of intermediate. See the Figure VII-1 figure legend for details regarding this schematic diagram. In the reaction shown here, a second factor IX-binding exosite on the catalytic domain E is involved. Factor IX is activated by ordered sequential cleavage of its two bonds by a factor XIa subunit, with involvement of two exosites in initial factor IX binding. This also involves release of factor IXα, which rebinds to the protease A3 domain prior to conversion to factor IXaβ.

bond. The second model may offer a better explanation as to why factor IXα accumulates when the interaction with the A3 exosite is disturbed. While much of our data are consistent with either models, both of which involve release of an intermediate, the similar rates of cleavage of the two bonds suggests that we should see some accumulation of factor IXα, at least early in time courses. Another factor to consider is that while including factor XIai in factor IX activation reactions results in factor IXα accumulation, a large excess of factor XIai (1000-fold) is required to see any intermediate accumulation.

The model in **Figure VII-3** is most compatible with all available information. It shows a type of processive mechanism (Breyer and Matthews, 2001). That is, it involves

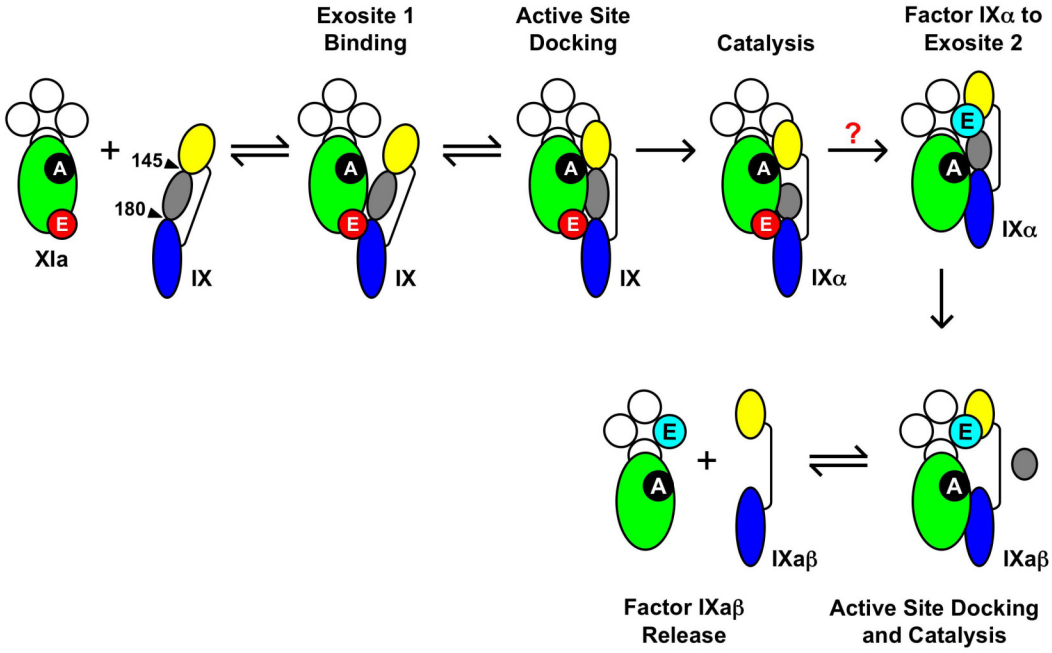

Figure VII-3. A model for factor IX activation by factor XIa that does not involve release of an intermediate (processive mechanism). See the Figure VII-1 figure legend for details regarding this schematic diagram. In the reaction shown here, a second factor IX-binding exosite on the catalytic domain (E) is involved. Factor IX is activated by sequential cleavage of its two bonds by a single factor XIa subunit. Note that factor IXα does not dissociate from factor XIa after cleavage at the Arg^{145}-Ala^{146} bond, but is transferred from the exosite on the catalytic domain to the exosite on A3, which mediates the second cleavage. The unidirectional arrow with the red question mark indicates that we are uncertain if this step is reversible. We postulate that such a mechanism could explain why very high concentrations of factor XIai (**Figure V-5**) are required to induce factor IXα accumulation during activation.

sequential cleavage of the two factor IX bonds but does not involve release of an intermediate. An example of an enzyme relevant to blood coagulation that operates through a processive mechanisms is the vitamin K-dependent γ-glutamyl carboxylase that modifies the Gla domains of prothrombin and factors VII, IX and X (Berkner, 2008). Each protein requires the modification of 10-13 glutamic acid residues, and the enzyme accomplishes this prior to releasing the final product. In the case of factor IX activation by factor XIa, we propose that an exosite on the catalytic domain is necessary for initial interaction with the substrate. After cleavage of Arg^{145}-Ala^{146} to form factor IXα, a conformational change in the substrate allows interaction with the exosite on the A3 domain, which facilitates cleavage at Arg^{180}-Val^{181} to form factor IXaβ.

This scheme offers an explanation for the lack of intermediate accumulation during time courses of factor IX activation that is not dependent on rapid cleavage of the Arg^{180}-Val^{181} bond, as in the models in **Figures VII-1** and **VII-2**. At first glance, this model may not seem to be compatible with the observation that factor IXα accumulates in the presence of factor XIai (**Figure V-5**), which suggests that factor IXα has been released from the protease. We propose an alternative interpretation. Factor XIai, in sufficient concentration, may interfere with the transfer between exosites, giving the impression that factor IXα has been released from the protease. We did not see intermediate accumulation at 500-fold excess factor XIai over factor XIa, even though the rate of the overall reaction was decreased. Intermediate accumulation required at least a 1000-fold molar excess of factor XIai. If transfer of factor IXα from one exosite to the other on factor XIa does not normally result in a free intermediate, then it may require very high concentrations of factor XIai A3 domains to "capture" the intermediate as it

transits from one exosite to the other. Experiments to clearly establish the presence of a factor IX binding exosite on the factor XIa catalytic domain are currently underway in our laboratory. We are also working to determine if factor IX and factor IXα bind to similar sites on factor XIa. This information will facilitate work to establish the validity of the models proposed here.

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CHAPTER VIII

SUMMARY OF THE THESIS AND FUTURE DIRECTIONS

After its initial discovery in the mid-1950's, there was considerable interest in factor XIa because of its interaction with the important coagulation protein, factor IX. Indeed, congenital factor XI deficiency was known at one time as hemophilia C (Gailani and Neff, 2009a). Enthusiasm waned as it became clear that the bleeding disorder associated with factor XI deficiency was far less severe than in hemophilia B (factor IX deficiency). It now appears that factor XIa plays a relatively minor role in hemostasis (normal clot formation at a site of vascular injury) in most situations. By extrapolation, it was assumed that this protease did not contribute significantly to thromboembolic disease. However, recent work with vascular injury models in animals suggests that factor XIa plays a disproportionately greater role in thrombosis (Gailani and Renné, 2007a, 2007b), the production of a pathologic clot that occludes a blood vessel, than it does in hemostasis. These results are consistent with epidemiologic data from human populations showing that elevated factor XI levels are a risk factor for both arterial (Doggen *et al.*, 2006) and venous (Meijers *et al.*, 2000) thrombosis. These observations have stimulated interest in designing novel pharmaceutical agents that target factor XIa, or some aspect of factor XIa-mediated factor IX activation (Gailani *et al.*, 2009b).

Activation of factor IX by factor XIa has features that distinguish it from the biochemical reactions involving the vitamin K-dependent coagulation proteases. For example, factor XIa does not require phospholipid or cofactors for maximum catalytic

efficiency. These features, combined with the highly unusual structure of factor XI, make it difficult to predict the mechanism for this reaction based on the body of knowledge available for other coagulation protease reactions. It was our goal to study the mechanism by which factor IX is activated by factor XIa, to facilitate efforts to develop therapeutics that target this reaction. The work has lead to several novel observations concerning factor XIa and its mechanism of action.

First, we identified a form of factor XIa with only a single activated subunit. While the existence of this species, which we named 1/2-factor XIa, was predicted over thirty years ago (Bouma and Griffin, 1977), we are the first to identify, purify and characterize it. Furthermore, we observed 1/2-factor XIa formation in plasma, raising the distinct likelihood that this protease is a physiologically, or pathologically, relevant molecule. Given the difficulty in producing fully activated factor XIa *in vitro*, it is reasonable to conclude that 1/2-factor XIa may be the predominant species formed in plasma during coagulation. If subsequent work bears this out, it is likely that the entire body of work related to factor XIa may need to be reconsidered, as it is based exclusively on studies with fully activated protease.

The availability of 1/2-factor XIa and related single active site species provided us with unique tools to address another conundrum regarding factor XIa biology - its capacity to activate factor IX without intermediate accumulation. The prevailing opinion was that the dimeric structure of factor XIa was key to its ability to do this, but we showed that the capacity to activate factor IX is contained within a single factor XIa subunit. This, of necessity, implies sequential cleavage of the two scissile bonds in factor IX. We are the first to clearly show that initial cleavage is at the Arg^{145}-Ala^{146} bonds.

The combination of sequential cleavage of the factor IX scissile bonds and the absence of intermediate accumulation could be explained by either of two mechanisms. Initially we thought that the most likely scenario was that the intermediate, factor IXα, would be released form factor XIa, and would need to re-bind to factor XIa to complete activation. In this case, the second cleavage at Arg^{180}-Val^{181} would have to be considerably more rapid than the cleavage at Arg^{145}-Ala^{146} to account for the lack of intermediate accumulation. To our surprise, the rates of cleavage of the two bonds were similar. This, along with the data indicating that factor XIa may have more than one exosite for substrate binding (Sinha *et al.*, 2007) led us to postulate a processive mechanism where factor IX is processed to the final product, factor IXaβ, without intermediate release.

Most processive enzymes are large multi-subunit proteins, with substrates passed from one subunit to another (Breyer and Matthews, 2001). The substrates of processive enzymes also often have repetitive structures. DNA polymerase is a classic example of a multi-unit processive enzyme that catalyzes multiple reactions without dissociating from its substrate. In this regard, factor XIa is considerably smaller and simpler than most processive enzymes. A substantial amount of work remains to be done to definitively establish the mechanism by which factor XIa cleaves factor IX without intermediate accumulation. This continues to be a major area of emphasis in our laboratory.

Having demonstrated that factor XIa does not need to be a dimer in order to activated its substrate, the question of why the protease is dimeric remains open. Our working hypothesis is that 1/2-factor XIa is a hybrid molecule with different properties than either factor XI or factor XIa. For example, factor XI can bind to the platelet

receptor GP1bα (Walsh, 2004) in an interaction involving the A3 domain, while factor XIa does not bind to this receptor (Sinha *et al.*, 1984; Walsh, 2004). As discussed, factor IX binds to factor XIa, but not factor XI. Hypothetically, 1/2-factor XIa could bind to GP1bα through its inactive subunit, while catalyzing conversion of factor IX to factor IXaβ - something that neither factor XI nor factor XIa would be able to do. We think that this chimeric ability is important for 1/2-factor XIa activity *in vivo*, where the protease would need to anchor to platelets at a wound site so as not to be swept away by the flowing blood. Indeed, recent work (Tucker *et al.*, 2009) indicates that platelets are required for factor XIa to contribute to fibrin formation under flow conditions *in vitro*. The dimeric structure of factor XI/XIa, therefore, may be an alternative strategy to having a Gla domain to allow a protein to bind to surfaces during hemostasis. Work in our laboratory is currently addressing the importance of the dimeric structure of factor XI to thrombus formation in animal models.

Current drug design efforts for anti-thrombotic agents that target factor XIa are focused on developing small molecule inhibitors that block the protease active site. This is a standard approach when trying to therapeutically inhibit an enzyme. Because the catalytic active sites of trypsin-like protease share features in common, inhibitors designed to target one protease will often cross-react with other members of the trypsin family, producing undesirable effects. Targeting protease exosites, which hypothetically would be a way to specifically limit a single protease-substrate interaction. has received comparatively less attention, but is gaining in popularity. The drug *Xigris* (recombinant activated protein C) has beneficial anti-inflammatory properties when used as a treatment during sepsis in humans, but has the undesirable side effect of causing excessive bleeding

because of its ability to degrade factors VIIIa and Va (Levi, 2008). Work is underway to modify exosites on this molecule so that it no longer engages these key coagulation factors (Griffin *et al.* 2007). Similarly, α-thrombin has been converted to an anti-coagulant molecule by modifications that cause it to lose its procoagulant properties, but retain its ability to activate the anticoagulant protease protein C (Gruber *et al.*, 2007). Our work suggests that the principle of exosite blockade/modification could apply to factor XI/XIa. The antibody O1A6 (discussed in chapter VI) appears to specifically interfere with factor IXα binding to factor XIa. This antibody was recently shown to be an effective anti-coagulant in a baboon arterio-venous shunt thrombosis model (Tucker *et al.* 2009). We are currently evaluating other antibodies targeting various parts of factor XIa outside the catalytic domain for their potential role as selective anti-thrombotic agents that would have limited impact on normal hemostasis.

Factor XI is probably the least well understood of all of the plasma proteases required for normal blood coagulation. The work in this thesis provides a foundation for future studies to delineate the role of this enzyme in vascular biology, and for efforts to develop therapeutic agents to treat or prevent human disease.

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