STUDIES OF STRUCTURE-FUNCTION RELATIONSHIPS IN TWO HUMAN COAGULATION PROTEINS: FACTOR XII AND PROTHROMBIN

by

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B. Sc (Hon.), Laval University, 1987.

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

Doctor of Philosophy

in

THE FACULTY OF MEDICINE

Department of Biochemistry

We accepted this thesis as conforming

to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

September 1993

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ABSTRACT

The epitope(s) of three anti human factor XII monoclonal antibodies were localized by screening a factor XII cDNA expression library in λgt11. One antibody, B7C9, had been shown previously to inhibit the activation of FXII by negatively charged surfaces. The positive recombinant phage contained inserts that coded for the amino-terminal 31 amino acids of FXII. These results were confirmed by binding of B7C9 to synthetic peptides containing amino acids 1-28 and 1-14 of FXII. Two other mAbs, C6B7 and K0K5 were also mapped. The epitope(s) for C6B7 which had been shown to inhibit factor XII activation was localized to amino acids 336-364, while K0K5 which inhibited the clotting activity of FXII mapped to amino acids 27-73. Four human FXII cDNA constructs were expressed in BHK cells, using the pNUT vector: FXII wild type, FXIIΔ1-20, FXIIΔ5-20 and FXIIΔ28-69. The FXII wild type and FXIIΔ28-69 were secreted into the media at ~5-10 μg/mL while FXIIΔ1-20 and 5-20 were expressed in the cells but not secreted into the culture media. Recombinant human FXII was partially purified, analyzed by N-terminal sequencing, and assayed for amidolytic and clotting activity. These results indicate that the N-terminus of FXII might be involved in the binding to negatively charged surfaces, and that B7C9 blocks that interaction thereby inhibiting activation.

During activation of prothrombin by the prothrombinase complex (FXa, FVa, phospholipids and Ca^{++}), transient activation intermediates are produced. The intermediate meizothrombin has enzymatic activity but very little coagulant activity while intermediate prethrombin-2 has no enzymatic activity. Because meizothrombin is very sensitive to further activation and autolysis (converting it to meizothrombin(desF1) and ultimately thrombin), the isolation of meizothrombin is possible only in the presence of active-site thrombin inhibitors. This complicates studies of the activities and functions

of meizothrombin. As a model, a mutant human prothrombin cDNA (R155A, R271A, R284A) (hMZ) was expressed, with three of the cleavage sites modified so that they are no longer cleaved by factor Xa or thrombin. Other mutants mimicking prethrombin-2 (hPRE2) and "non-activable" prothrombin (hQM) were also expressed using the pNUT expression vector in BHK cells. When cultured in roller bottles, the cell lines secreted between 20 and 400 μg/mL of protein, at various levels of γ-carboxylation. The secreted recombinant hMZ was purified to homogeneity and fractionated by Ca^{++} gradient chromatography to select for Ca^{++}-binding and phospholipid-binding populations. Once activated by the prothrombinase complex or by ecarin, the rhMZ is converted to a meizothrombin-like molecule. Electrophoretic analysis and N-terminal sequence analysis were consistent with cleavage of a single bond between Arg320-Ile321 and proper processing of the prepro-peptide. No other proteolytic cleavage was observed and rhMZa was stable for weeks at 4°C. Compared with human plasma-derived prothrombin, rhMZa demonstrated ~7% clotting activity and 100% TAME esterase activity. The amidolytic activity of rhMZ toward S-2238 was found to be Ca^{++}-dependent, and was identical to that of thrombin in the presence of 2 mM Ca^{++}. Analysis of rhQM under the same conditions showed no cleavage of the molecule and no generation of activity. Furthermore, rhQM inhibited activation of prothrombin by the prothrombinase complex. These results indicate that these prothrombin mutants offer good models for further studies on the activity and physiological function of meizothrombin, and on the kinetics of prothrombin activation and the interactions between the components of the prothrombinase complex.

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LIST OF ABBREVIATIONS

A	absorbance
Amp	ampicillin
aPC	activated protein C
APTT	activated partial thromboplastin time
AT-III	antithrombin III
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BHK	Baby Hamster Kidney
bis	N,N'-methylenebisacrylamide
bp(s)	basepair(s)
BSA	bovine serum albumin
Ca^{++}	calcium ions
cDNA	complementary deoxyribonucleic acid
CHO	Chinese Hamster Ovary
C1INH	C1-inhibitor
CRM^{+}	cross-reactive material
DAPA	dansylarginine N-(3-ethyl-1,5 pentanediyl) amide
DHFR	dihydrofolate reductase
DIC	disseminated intravascular coagulation
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleotide triphosphates
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid

EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbtion assay
ER	endoplasmic reticulum
F1	prothrombin fragment 1
F2	prothrombin fragment 2
F(ab)	the antigen-binding fragment from IgG following papain digestion
FII	prothrombin
FPLC	fast protein liquid chromatography
FV	factor V
FIX	factor IX
FX	factor X
FXI	factor XI
FXII	factor XII
FXIIa or FXIIf	activated factor XII
Gla	γ-carboxyglutamic acid
HAE	hereditary angioedema
HEPES	N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid
HMWK	high molecular weight kininogen
HMWKa	cleaved HMWK
HPLC	high performance liquid chromatography
HUVECs	human umbilical vein endothelial cells
IgG	immunoglobulin G
IL-1	interleukin 1
IPTG	isopropylthiogalactoside
kDa	kilodaltons
Klenow fragment	*E. coli* DNA polymerase (large fragment)

LB	Luria broth
LPS	lipopolysaccharides
mAB	monoclonal antibody
M_r	molecular weight
MT	metallothionein
MTX	methotrexate
NBT	nitro blue tetrazolium
NBS	New-born serum (bovine)
PAGE	polyacrylamide gel electrophoresis
PAI-1	endothelial cell type plasminogen activator-inhibitor
PAI-2	placental type plasminogen activator-inhibitor
PBS	phosphate-buffered saline
PCPS	phosphatidylcholine:phosphatidylserine
PCR	polymerase chain reaction
PGI_2	prostacyclin
pII	plasma-derived human prothrombin
PK	prekallikrein
PPACK	D-Phe-Pro-Arg chloromethylketone
PRE-2	prethrombin-2
PT	prothrombin time
PUK	prourokinase
RT	room temperature
S-2238	D-phenylalanyl-L-pipecolyl-L-arginine-p-nitroanilide-dihydrochloride
SDS	sodium dodecyl sulphate
SRP	signal recognition particle
SV40	Simian Virus 40

TAME	p-toluene-sulfonylarginine methyl ester
TBST	20 mM Tris-HCl pH 7.5, 0.15 M NaCl, 0.5% Tween-20 buffer
TEMED	N,N,N',N'-tetramethylethylenediamine
TM	thrombomodulin
t-PA	tissue-type plasminogen activator
Tris	tris(hydroxymethyl)aminomethane
u-PA	urokinase-type plasminogen activator
X-GAL	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
α_2AP	α_2-antiplasmin
α_2M	α_2-macroglobulin

ACKNOWLEDGEMENTS

I would like to express all my gratitude toward my suprervisor Ross MacGillivray for his unconditional support during all these years, for his science, his friendship, and most of all, for always believing in me, even when I didn't myself. I also want to thank all the members of the lab, past and present, for their help and advice, and for creating a working environment which made the long hours enjoyable. Special thanks to Mike Nesheim and his students for their interest in my research and the sharing of their knowledge with me.

Jamie, I'll take a roller coaster ride with you anytime.

Je voudrais dédier cette thèse à mes parents Maurice et Jocelyne qui n'ont jamais douté...

INTRODUCTION

I. HEMOSTASIS

Vertebrates have a closed circulatory system for transport of blood and nutrients through vessels lined by endothelial cells. Hemostasis is the process by which blood volume and flow are maintained. Four interrelated processes are involved in stopping blood loss and repairing damage in response to injury: vasoconstriction; the formation of a platelet plug; blood coagulation and fibrinolysis. (for review see Colman et al., 1987, Part 1)

A. Vasoconstriction

Endothelial cells line the luminal surface of blood vessels and modulate vascular perfusion, permeability and maintain blood fluidity. When a vessel is injured, it contracts. This intense vasoconstriction temporarily reduces blood loss from the site of injury.

B. The formation of the platelet plug

Platelets do not normally adhere to vascular endothelial cells but at the site of an injured blood vessel, they readily adhere to components of the subendothelial connective tissue. Following adhesion, a number of platelet agonists further activate platelets and cause them to aggregate at the site of injury. Platelet activation causes secretion of granules, exposure of surface receptors for plasma proteins and alterations in lipid structure of the platelets surface membrane, leading to acceleration of plasma coagulation.

C. The contact activation system

The contact-activation system or surface-mediated system comprises four major components: factor XII (FXII) (M_r 80,000) or Hageman factor; prekallikrein (PK) (M_r 88,000) or Fletcher factor; High molecular weight kininogen (HMWK) (M_r 110,000) or Williams-Fitzgerald-Flaujeac factor and factor XI (FXI) (M_r 143,000) or plasma thromboplastin antecedent. These four proteins have been shown to initiate, amplify and propagate surface-mediated defense reactions *in vitro* which participate in coagulation, fibrinolysis and in the inflammatory response. The initiation mechanism of the contact system is unclear, although the most probable cause is the binding of factor XII to a negatively charged surface where autoactivation of the zymogen occurs, converting it to an active serine protease. The presence of a small amount of active FXII (FXIIa) leads to the activation of its substrates prekallikrein, factor XI and HMWK by limited proteolysis. Cleaved HMWK (HMWKa), bound to the surface, acts as a cofactor and enhances greatly the activation of FXII, FXI and PK which are reciprocal.

As shown in Figure 1, the contact activation system interacts with several other pathways. Factor XIIa can initiate the intrinsic coagulation pathway by activating factor XI as well as the extrinsic pathway by activating factor VII (Kisiel et al., 1977). The release of bradykinin, one of the most potent vasodilators, reduces blood pressure and triggers the release of tissue-type plasminogen activator (t-PA) by the endothelial cells, but also has an indirect inhibitory effect on platelet aggregation. T-PA, FXIIa and kallikrein act toward the activation of plasminogen to plasmin, which is responsible for the proteolytic breakdown of the clot.

The physiological requirement for all the components that participate in the contact-activation system *in vitro* is ambiguous because individuals deficient in two of these components (FXII and PK) show prolonged bleeding time *in vitro* but do not exhibit bleeding disorders. On the contrary, these patients seem to demonstrate a lack of protection from thrombotic diseases. In addition, deficiency of HMWK is asymptomatic.

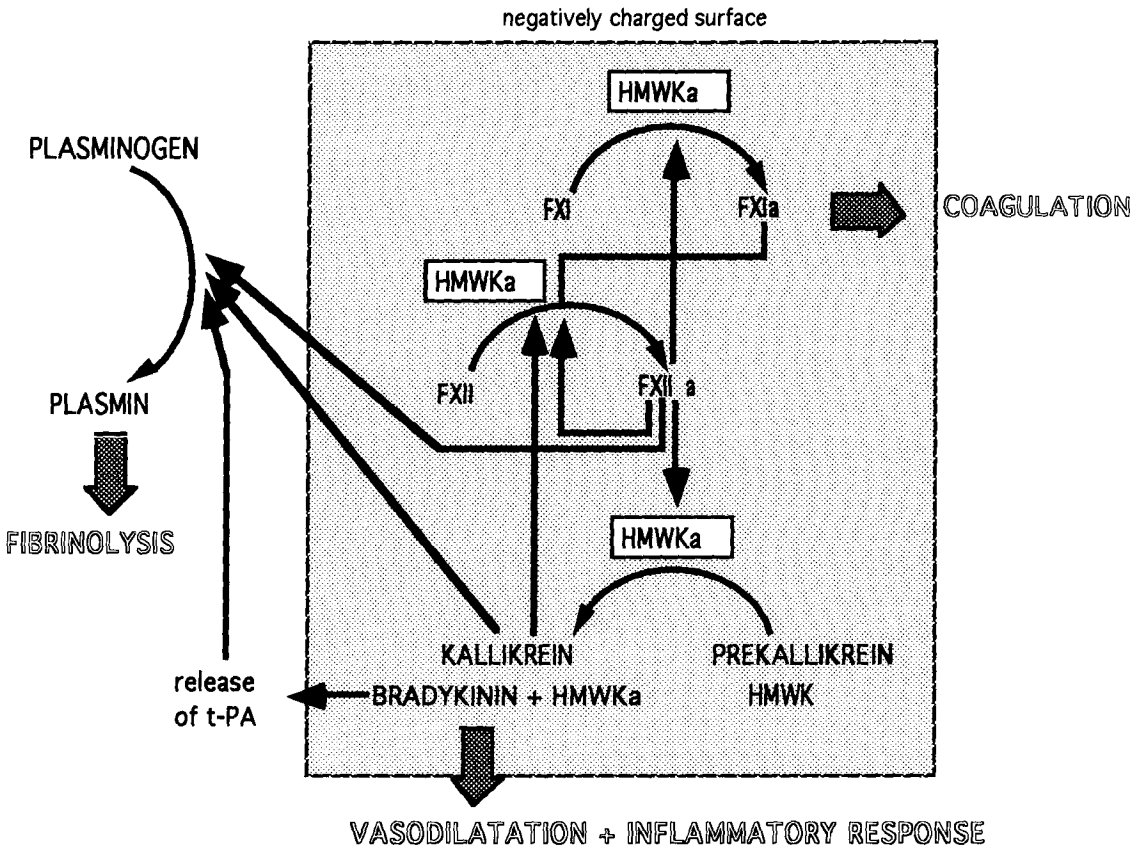


Figure 1. The contact activation system

t-PA, tissue-type plasminogen activator; HMWK, High molecular weight kininogen; HMWKa, cleaved HMWK; FXII, factor XII; FXI, factor XI

This suggests that these proteins, necessary for *in vitro* hemostasis, are not required *in vivo*.

D. Blood coagulation

Blood coagulation involves the sequential enzymatic activation of serine protease zymogens resulting in the formation of an insoluble fibrin clot that strengthens the platelet plug (Jackson and Nemerson, 1980; Davie et al., 1991). Two tentative pathways have been described (MacFarlane, 1964; Davie and Ratnoff, 1964) to explain coagulation: the extrinsic and intrinsic pathways (Figure 2). Both pathways converge in the activation of factor X to FXa which then converts prothrombin to thrombin.

The initiation of the intrinsic pathway (contact activation) has been described in detail above. Recent studies (Gailani and Broze, 1991) proposed a revised model of the intrinsic coagulation pathway in which, in the absence of cofactors, factor XI is activated by thrombin. Once activated, FXIa catalyzes the activation of factor IX to FIXa which then activates factor X to FXa, in the presence of cofactor FVIIIa, calcium ions and a phospholipid surface.

The extrinsic pathway requires tissue factor, a membrane-bound protein, which comes in contact with blood only after vascular injury. When exposed, tissue factor interacts with plasma factor VII to form a calcium-dependent complex that facilitates the conversion of factor VII to a serine protease FVIIa by limited proteolysis. The factor VIIa-tissue factor complex converts factor X to factor Xa (Radcliffe and Nemerson, 1974).

Present evidence suggests that the extrinsic pathway is critical in the initiation of fibrin formation (Davie et al., 1991) while a second overlapping mechanism, the intrinsic pathway, plays an important role in the growth and maintenance of fibrin formation in the coagulation cascade.

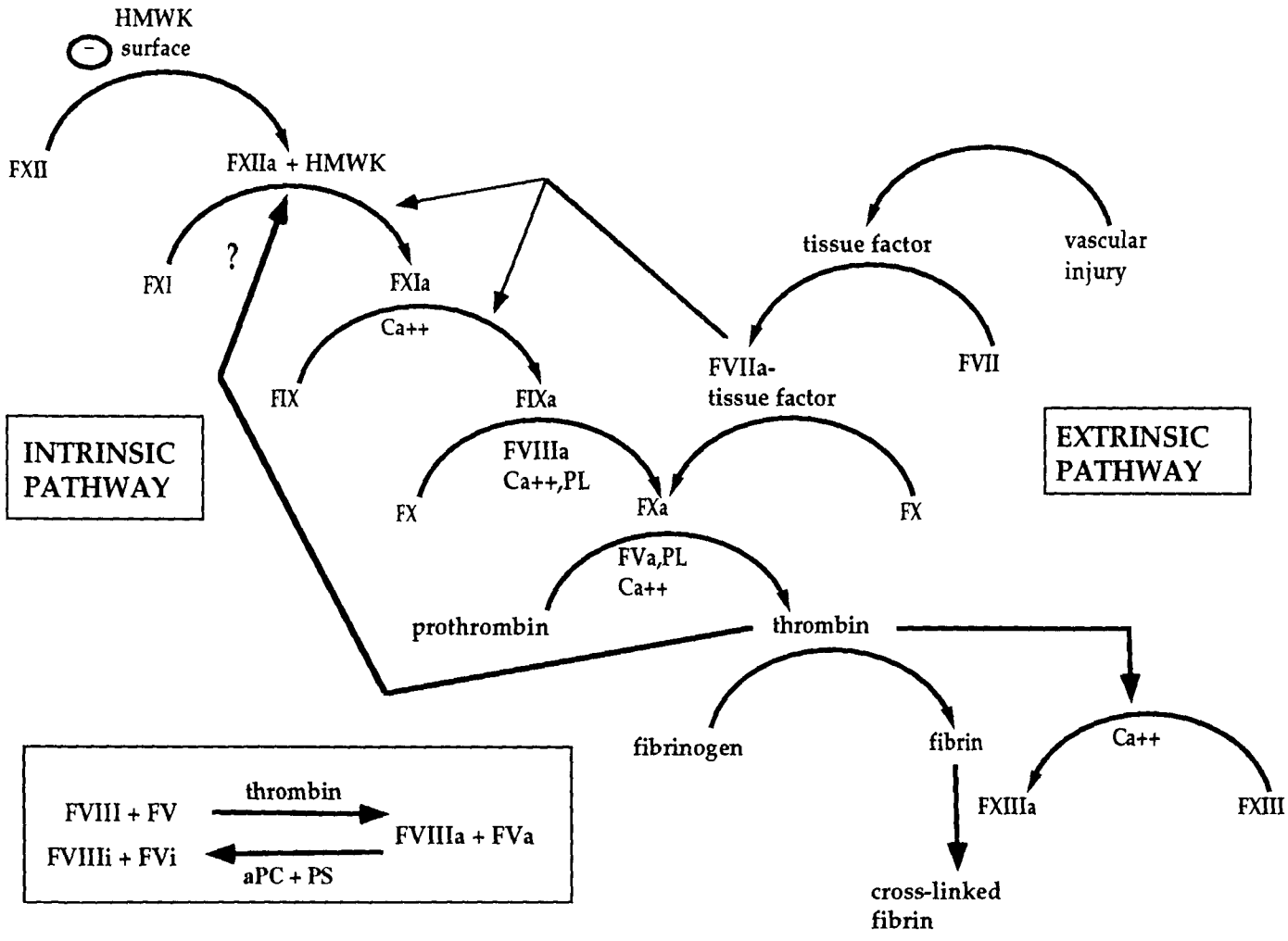


Figure 2. Schematic representation of the coagulation cascade.
Indicated are the two activation pathways, intrinsic and extrinsic, converging at the activation of factor X. PL indicates phospholipids and ⊖ represents a negatively charged surface.

Factor Xa generated by either pathway forms a complex with cofactor FVa in the presence of calcium ions and phospholipid called the prothrombinase complex (Nesheim and Mann, 1983; Krishnaswamy et al., 1987; Krishnaswamy et al., 1988; Mann et al., 1990). This complex binds prothrombin and converts it to the serine protease thrombin by limited proteolysis. Thrombin converts fibrinogen to fibrin by cleavage of a peptide bond in each of the two α and two β chains (Blomback and Blomback, 1972) which promotes the polymerization of fibrin. Thrombin also activates factor XIII, a transglutaminase, which cross-links fibrin monomers leading to the formation of a strong fibrin clot (Davie et al., 1991)

E. Fibrinolysis

The fibrinolytic system is the principal effector of clot removal and controls the enzymatic degradation of fibrin (Colman et al., 1987). Dissolution of the fibrin clot requires binding of the circulating zymogen plasminogen to the fibrin clot, conversion of plasminogen to the active protease plasmin, proteolysis of the clot and inactivation of plasmin by circulating antiplasmin (Colman et al., 1987). Plasminogen activators are released by the liver and vascular endothelium into the circulation. They include urokinase plasminogen activator (u-PA), tissue-type plasminogen activator (t-PA) and products of the contact activation system such as FXIIa and kallikrein. Plasmin, a protease, hydrolyses susceptible arginine and lysine bonds in fibrin and effectively dissolves the thrombus. The activity of the fibrinolytic system is tightly regulated by the abundant plasmin inhibitor α_2-antiplasmin (Sprengers and Kluft, 1987). Also involved in regulating fibrinolysis are the plasminogen activator-inhibitors. These include the endothelial cell type PA-inhibitor (PAI-1), the placental type PA-inhibitor (PAI-2), and the protease nexin-1 (Sprengers and Kluft, 1987).

F. Regulation of hemostasis

The process of hemostasis is highly regulated by activators and inhibitors. The endothelium itself synthesizes prostacyclin (PGI_2) which stimulates adenylate cyclase to convert ATP to cyclic AMP. Cyclic AMP inhibits platelet aggregation, secretion and adhesion to surfaces (Colman et al., 1987) (see Figure 3). The C1 inhibitor regulates the contact activation system by inhibiting the activity of FXIIa and kallikrein. The main inhibitors of the coagulation cascade are α_1-antitrypsin which inhibits FXIa and antithrombin III (AT-III), a potent inhibitor of FXa and thrombin. Concurrently, endothelial cells produce thrombomodulin on their surface. Thrombomodulin binds thrombin and changes its specificity toward activation of Protein C. In the presence of Protein S, activated PC (aPC) destroys FVa and FVIIIa, two cofactors required for coagulation. Fibrinolysis is mainly regulated by α_2-antiplasmin which circulates in plasma. α_2-antiplasmin reacts exceedingly fast with plasmin, irreversibly inhibiting the enzyme by formation of a complex with the active serine in the plasmin catalytic site (Colman et al., 1987)

II. HUMAN FACTOR XII

A. Biosynthesis and post-translational modifications

Factor XII or Hageman factor is a single-chain glycoprotein composed of 596 amino acids (M_r 80,0000) that is present in plasma at a concentration of ~ 30 μg/mL. FXII is synthesized in the liver (Fujikawa and McMullen, 1983; McMullen and Fujikawa, 1985) and following post-translational modifications, is secreted into the circulation. These modifications include the processing of a propeptide region and addition of several N-linked oligosaccharides and at least one O-linked fucose residue (Harris et al., 1992).

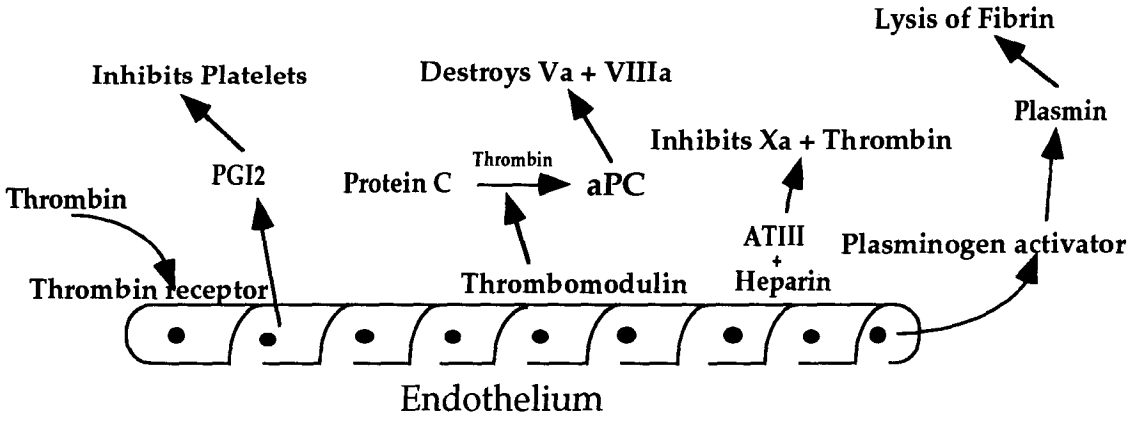


Figure 3. Thromboresistant properties of the endothelium.

Endothelial cells synthesize prostacyclin (PGI2), thrombomodulin, heparin, and plasminogen activators, all of which inhibit hemostasis and contribute to the maintenance of vascular potency. (from Colman et al., 1987)

B. Structure

The known amino acid sequence (Fujikawa and McMullen, 1983; McMullen and Fujikawa, 1985), cDNA sequence (Cool et al., 1985) and gene sequence (Cool and MacGillivray, 1987) of factor XII provide information about the structure of this plasma protein (Figure 4). Based on sequence identities with other proteins, FXII can be divided into several putative structural motifs.

1. Signal peptide

As also found in most secreted proteins (Watson, 1984), FXII includes an amino terminal signal peptide of 19 amino acids responsible for binding to the signal recognition

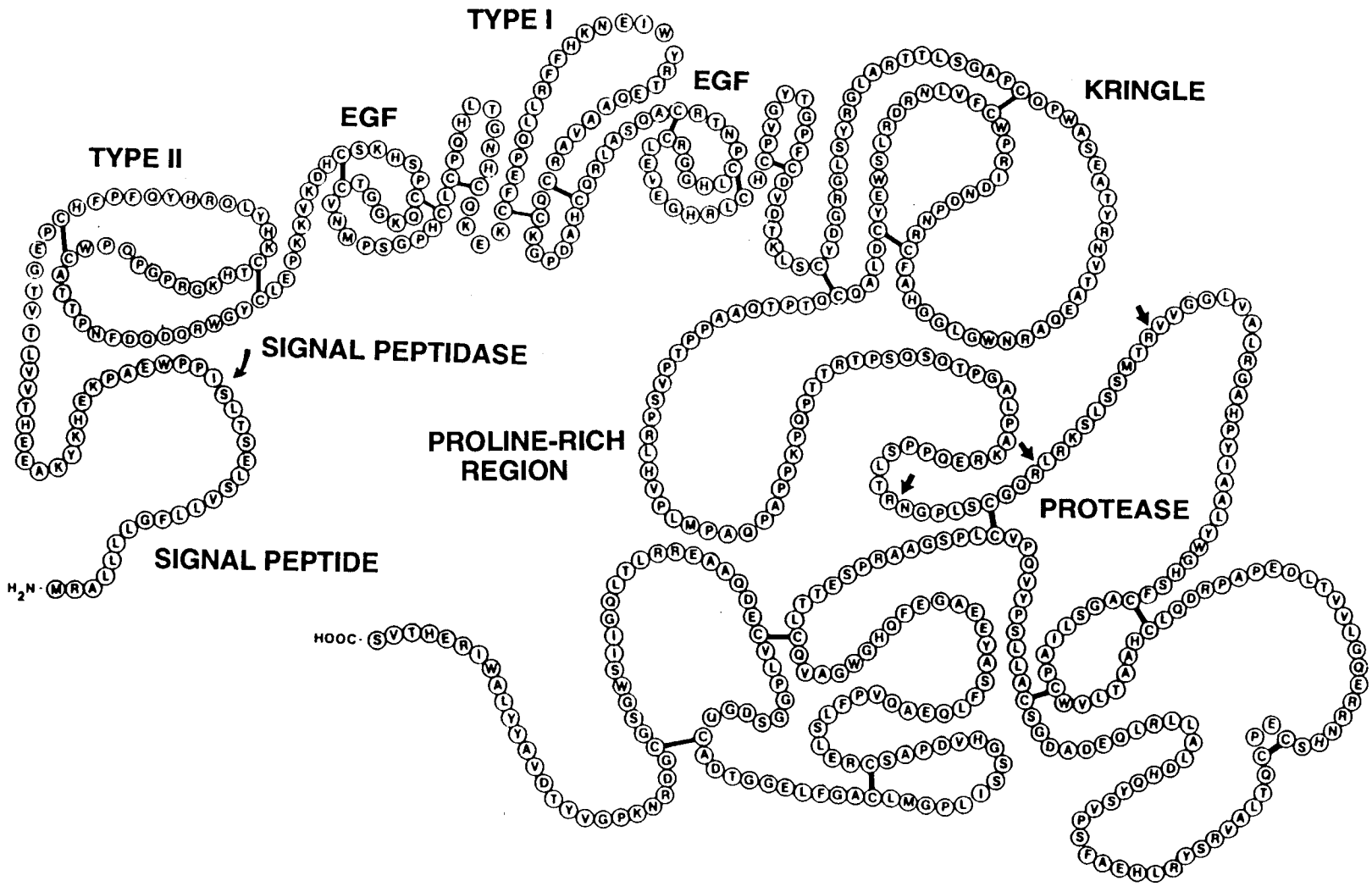


Figure 4. Schematic diagram of human factor XII

The arrows indicate the sites of cleavage during activation of the zymogen (from Cool et al., 1987)

particle, facilitating the co-translational transport of the protein across the endoplasmic reticulum (ER) (Blobel, 1980). As the FXII polypeptide is translocated, the signal peptide is cleaved by a signal peptidase exposing the mature protein N-terminus.

2. N-terminus

The signal peptide is followed by a region that is rich in charged amino acids. This region (1-28) was identified (Clarke et al., 1989) as the putative epitope for anti-factor XII mAb B7C9 (Pixley et al., 1987) and P-5-2-1 (Saito et al., 1985). On binding to factor XII, these antibodies inhibit the ability of FXII to bind to negatively charged surfaces and therefore to undergo autocatalyzed activation (Pixley et al., 1987; Saito et al., 1985). The region contains a hydrophilic core between amino acids 5 and 17, including 9 charged amino acids. Particularly notable is the presence of three positively charged lysine residues that could potentially interact electrostatically with negatively charged surfaces (Clarke et al., 1989).

3. Fibronectin type II homology

The next region of factor XII (Figure 4) shares sequence homology with the type II homology regions of fibronectin (Petersen et al., 1983). These homologies are composed of approximately 60 residues including four half-cysteine residues (Petersen and Skorstengaard, 1985). Residues 13-69 of factor XII share 39% and 40% sequence identity with the two fibronectin type II homologies (Cool et al., 1985). This type II fibronectin-like homology has been found in FXII, and recently, in the human 92-kDa and 72-kDa Type IV collagenases (Collier et al., 1992). Fibronectin is a macromolecular dimer of nearly identical polypeptide chains (Mr 220,000-250,000), synthesized by the liver, megakaryocytes and platelets (Petersen and Skorstengaard, 1985). Interaction with fibrin, collagen and cell surfaces implicates fibronectin in the process of wound repair either as a result of severe injury or in inflammation (Clark and Colvin, 1985). The type

IV collagenases are members of the secreted zinc-metalloprotease family. These enzymes are capable of cleaving macromolecules of the extracellular matrix, thus initiating the process of tissue remodeling. Site-directed mutagenesis of the fibronectin type II-like domain of the 92 kDa type IV collagenase showed its involvement in mediating gelatin binding (Collier et al., 1992).

4. Epidermal growth factor homology (EGF)

Two epidermal growth factor-like regions are also found in factor XII. This 50 amino acid region with nine invariant cysteine and glycine residues is present in a number of proteins, including the 19K protein from Vaccina virus, transforming growth factor type 1, tissue-type plasminogen activator (t-PA), and the proteins from the vitamin K-dependent family such as factor X, factor IX, factor VII, protein S and protein C (Stenflo, 1991). EGF domain-containing proteolytic fragments of factors IX (Handford et al., 1991) and X, and proteins C and S (for review see Stenflo, 1991) have been shown to bind Ca^{++}, thus defining a new class of metal ion-binding sites that is presumably crucial to many biological processes. EGF-like domains in thrombomodulin have been involved in cofactor activity (Tsiang et al., 1992). The function of the EGF homologies in factor XII is unknown. Hydroxylated aspartic acid and asparagine residues are found in the EGF domain of proteins C, S, Z, factors IX and X (Stenflo, 1991). This modification is vitamin K-independent (Sugo et al., 1985) and, in FIX, does not require the presence of the propeptide (Rabiet et al., 1987). The function of hydroxylated aspartic acid and asparagine is unknown.

5. Fibronectin type I homology

Another region of the molecule shares limited sequence identity with the type I region of fibronectin (Petersen et al., 1983). This domain is repeated 12 times in the fibronectin macromolecule (Petersen and Skorstengaard, 1985); within the repeats, two

disulfide bonds and one tyrosine residue are highly conserved. A type I homology is also found in t-PA where it is involved in fibrin binding (Lubin et al., 1993).

6. Kringle

Another type of homology found in FXII is the kringle domain (Magnusson et al., 1975). Kringles are composed of approximately 80 amino acids containing six invariant cysteine residues which form three internal disulfide bridges. Kringle domains are also found in prothrombin (2 kringles) (Magnusson et al., 1975), plasminogen (5 kringles) (Magnusson et al., 1975), tissue-type plasminogen activator (2 kringles) (Pennica et al., 1983; Ny et al., 1984) and in urokinase (1 kringle) (Steffens et al., 1982; Gunzler et al., 1982). The function of the kringle domain in FXII is unknown but the second kringle of prothrombin has been reported to be involved in binding factor Va (Esmon and Jackson, 1974), while the second kringle in t-PA partly mediates interaction with fibrinogen fragments (van Zonneveld et al., 1986; Verheyen et al., 1986). The first kringle present in prothrombin fragment 1 has been crystallized and the three-dimensional structure solved (Park and Tulinsky, 1986; Soriano-Garcia et al., 1989;, 1992). The three dimensional structure of the kringle resembles an eccentric oblate ellipsoid. Its folding is defined by close contact between the sulfur atoms of two of the disulfide bridges, which form a sulfur cluster in the center of the structure (Park and Tulinsky, 1986; Soriano-Garcia et al., 1989).

7. Proline-rich region

Between the kringle and the serine protease domain lies a region (amino acids 279-330) in which 33% of the residues are proline (17 out of 52). This proline-rich region shares sequence identity with residues 29-38 of calf thymus HMG-17 (Walker et al., 1977). The significance and function of this putative homology are unknown.

8. Serine protease domain

Finally, the carboxy-terminus of the protein contains the serine protease domain, the catalytic region of factor XII. The serine protease region of factor XII is part of the trypsin-like family like all the proteases involved in coagulation, believed to originate from a common ancestral gene. The active sites of all the serine proteases employ the same three amino acid unit to catalyze hydrolysis of peptide bonds, namely serine, histidine and aspartic acid. Trypsin-like proteases are specific for the cleavage of a peptide bond following a positively charged amino acid such as arginine or lysine. The diversity between coagulation enzymes results from the way they accommodate their specific substrates in the substrate-binding pocket (Krieger et al., 1974).

Figure 5 shows the structural homologies between FXII, t-PA and fibronectin. It can be seen that FXII shares remarkable homology with t-PA which contains a type I finger, a EGF-like domain, two kringle domains and the catalytic serine protease domain. This organization is very similar to that of factor XII except that the proline-rich region is replaced by a second kringle.

C. Activation

The first step of factor XII activation involves a slow autodigestion and autoactivation of the native surface-bound FXII by active FXII (FXIIa) (Griffin, 1978; Dunn et al., 1982). Uncertainty surrounds the actual initiation of the contact system and activation of FXII. It is postulated that either a trace amount of active factor XII is present in the circulation or that the molecule can undergo a conformational change upon binding to a negatively charged surface and that (in the presence of HMWK) renders it active without necessarily being cleaved (Colman, 1984). Non-physiologic substances with a negative surface that can activate factor XII include glass, kaolin, celite, dextran

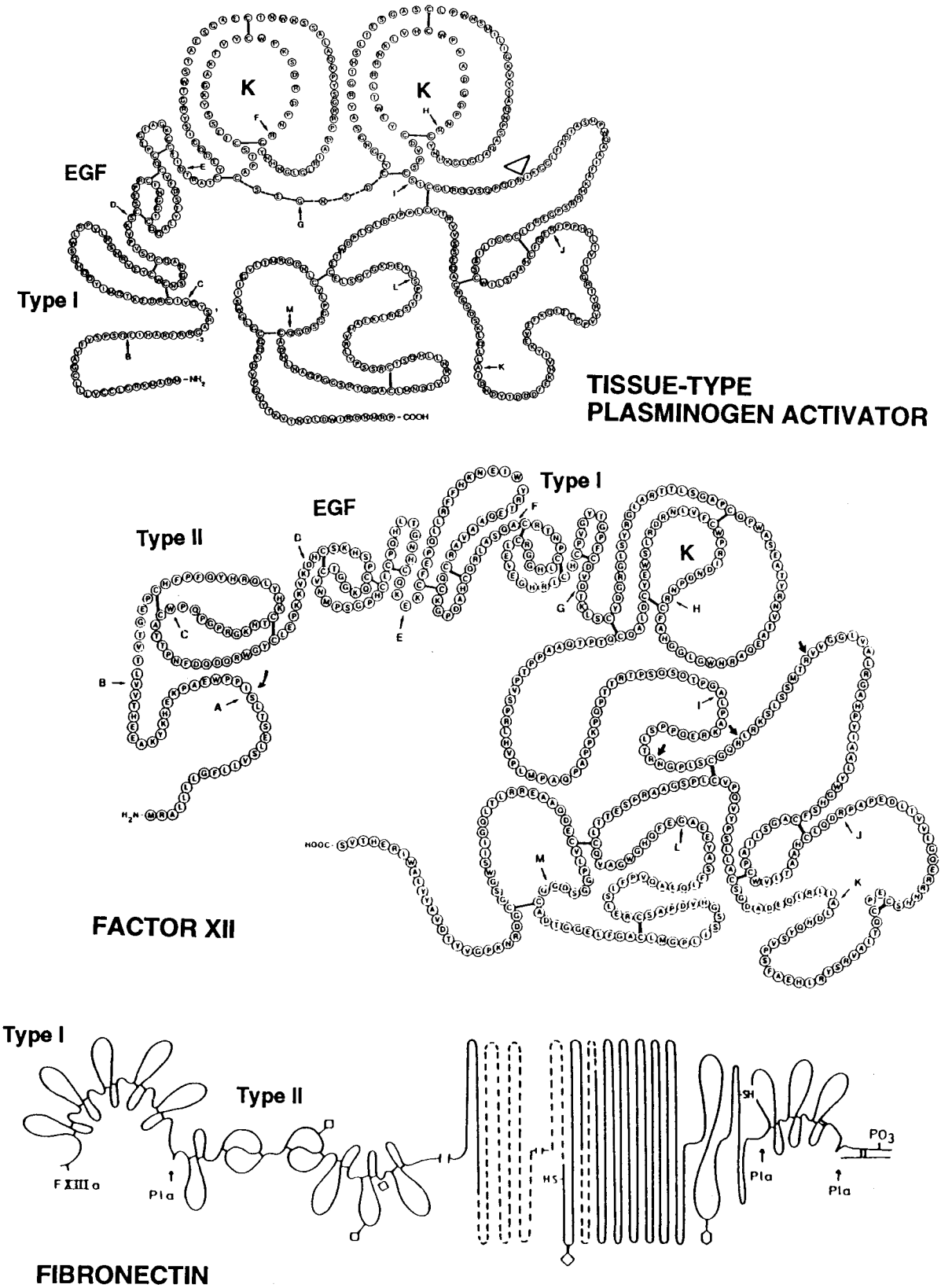


Figure 5. Structural homologies between factor XII, tissue-type plasminogen activator and fibronectin. (factor XII from Cool et al., 1987, t-PA from Ny et al.., 1984, fibronectin from Petersen and Skorstengaard, 1985)

sulfate, and ellagic acid (Colman et al., 1987). Biologic components, which include articular cartilage, skin, fatty acids, endotoxin, sodium urate crystals, calcium pyrophosphate, and L-homocysteine, may act as appropriate activating surfaces (Colman et al., 1987). The physiologic activator is postulated to be subendothelial vascular basement membrane (Colman et al., 1987). The second hypothesis is supported by the fact that FXIIa activity appears slowly, prior to cleavage of the zymogen (Heimark et al., 1980). As soon as some prekallikrein or FXII is cleaved, further activation of FXII by proteolytic cleavage takes place rapidly (see Figure 1). The first peptide bond hydrolyzed is Arg^{353}-Val^{354} (Dunn et al., 1982; Silverberg and Kaplan, 1988) producing FXIIa (Figure 6). FXIIa or α–FXIIa is composed of two peptide chains, a heavy chain containing the amino terminus of the protein (M_r 52,000) and a light chain containing the protease domain (M_r 28,000) linked together by a disulfide bond. Upon longer exposure of FXIIa to its activator, a second peptide bond is cleaved at Arg^{334}-Asn^{335}, yielding FXIIf or β-FXIIa (M_r 30,000). The latter no longer contains the heavy chain and consists of the same light chain linked to a small peptide (M_r 2,000). FXIIf has little coagulant activity but retains the ability to activate prekallikrein (Revak et al., 1978). A third site Arg^{343}-Leu^{344} is also sensitive to proteolytic cleavage which releases a small activation fragment (Asn^{335}-Arg^{343}) and produces FXIIf(2) (Dunn et al., 1982).

D. Functions

As described previously, factor XII is a component of the contact activation system and has multiple activities *in vitro*. Because individuals deficient in factor XII appear to be asymptomatic, the physiological function of FXII remains unclear. FXII is directly or indirectly involved in coagulation, in fibrinolysis and in neutrophil chemotaxis and inflammatory response. The involvement of the contact system has also been implicated in a number of clinical conditions (Saito, 1987).

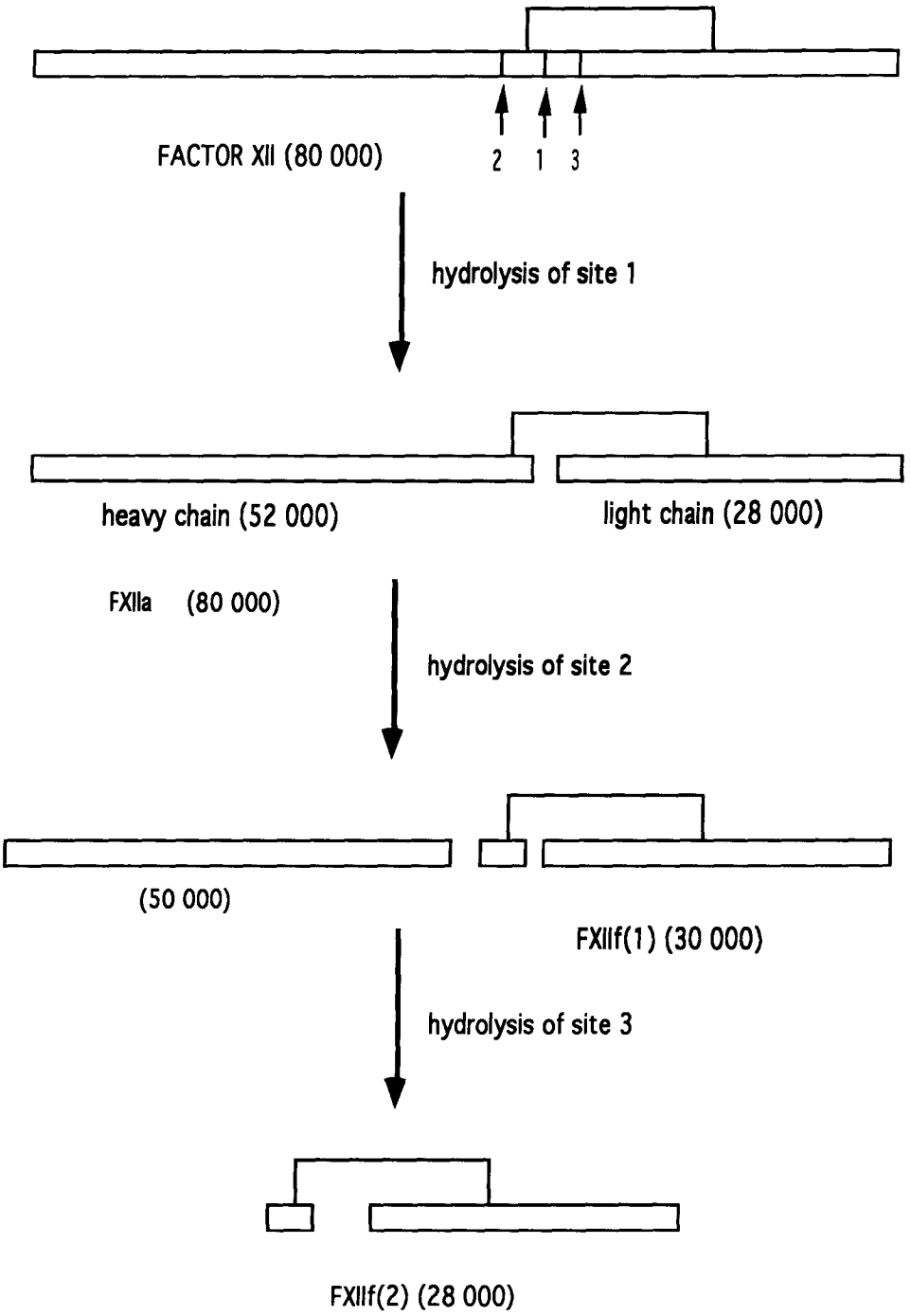


Figure 6. Diagram of human factor XII activation

1. Coagulation

Factor XII was first discovered for its ability to activate factor XI and promote coagulation. FXIIa proteolytically cleaves a single Arg-Ile bond in FXI to produce an amino-terminal 50 kDa heavy chain and a carboxyl-terminal 35 kDa light chain. Activated FXI (FXIa) can then cleave FIX in a reaction that is part of the intrinsic pathway of the coagulation cascade.

Factor XI-deficient patients show bleeding disorders (Colman et al., 1987); therefore, FXI is an essential component of normal hemostasis. This suggests that there must be another activator for FXI *in vivo*, other than FXIIa. Recently, it was demonstrated that thrombin, in the presence of a negatively charged surface, can activate factor XI, independently of the contact activation system and FXIIa (Naito and Fujikawa, 1991; Gailani and Broze Jr., 1991). The physiological relevance of these results is questioned, however, by the finding that addition of α-thrombin to FXII-deficient plasma (with or without a negative surface) leads to instantaneous fibrinogen cleavage, but with no cleavage of factor XI observable (Brunnee et al., 1993). Similarly, addition of tissue factor to plasma does not induce cleavage of factor XI (Brunnee et al., 1993).

FXIIa can also affect FVII activity: prothrombin time (PT) assays performed in a glass tube are shorter than those performed in plastic tubes (Rapaport et al., 1955). Factor XIIa is responsible for an increase in factor VII activity when FVII is cleaved into a two-chain molecule, having a 40-fold enhanced coagulation activity (Radcliffe et al., 1977).

2 Fibrinolysis

Activation of the contact system leads to expression of fibrinolytic activity. Although FXIIa, FXIa and kallikrein are all capable of directly activating plasminogen to plasmin, their contribution to the total plasminogen activator activity in human plasma is small (~15%) (Kluft et al., 1987). However, FXII and kallikrein can have indirect

fibrinolytic effects through the activation of prourokinase (PUK) to urokinase plasminogen activator (u-PA) by kallikrein (Hauert et al., 1989). This fibrinolysis pathway is referred to as the intrinsic pathway. Also, cleavage of HMWK by FXIIa releases bradykinin which stimulates t-PA release by endothelial cells. The fibrinolysis activation pathway involving t-PA is referred to as the extrinsic fibrinolytic pathway (Kluft et al., 1987).

3. Neutrophil chemotaxis and inflammatory response

When endothelial cells are injured, exposure of the subendothelial basement membrane initiates blood coagulation and recruitment of protective cells, such as neutrophils. In humans, kallikrein (Wachtfogel et al., 1983) and FXIIa (Wachtfogel et al., 1986) can aggregate neutrophils and stimulate the release of lactoferrin and elastase. Both plasma enzymes require an active site and an intact heavy chain for this action, as FXIIf and β-kallikrein fail to induce neutrophil degranulation (Wachtfogel et al., 1986).

High molecular weight kininogen (HMWK) circulates as a complex with prekallikrein (Mandle et al., 1976). Exposure of plasma to a negatively charged surface results in interaction and activation of FXII and prekallikrein; HMWK acts as a non-enzymatic cofactor and enhances these reactions (Meier et al., 1977). The resulting kallikrein cleaves HMWK at Arg-Ser and Lys-Arg sites which releases the nonapeptide bradykinin (Kerbiriou and Griffin, 1979). FXIIa, in the presence of a negative surface can also generate bradykinin, although more slowly (Wiggins, 1983). Bradykinin (or kinin) decreases blood pressure, increases vascular permeability, induces smooth muscle contraction and provokes pain (Rocha e Silva et al., 1949: Armstrong et al., 1957) as part of the inflammatory response.

The role of factor XII and the contact activation system in septic shock has also been investigated. Septicemia triggers the activation of the contact system. Patients suffering septicemia or bacteremia develop irreversible hypotension and disseminated intravascular coagulation (DIC). As a consequence of contact activation, bradykinin is

released. Bradykinin is a potent endogenous dilatator and it may contribute to hypotension (Pixley et al., 1993). On the other hand, by generating FXIa, the contact system could theoretically contribute to the DIC associated with septicemia.

In an experimental baboon model which was infused with *E. coli* to produce lethal hypotensive bacteremia, a group of animals was treated with an anti-FXII monoclonal antibody (C6B7). The C6B7 mAb blocks the activation of FXII. The results of the study indicated that the contact system contributed to the observed hypotension but did not affect disseminated intravascular coagulation. Treatment with the mAb significantly extended the survival time of the animals, one of five survived (Pixley et al., 1993).

In a similar baboon model, it has been shown that injection with activated protein C protects against lethal septic shock (Taylor et al., 1987). Activated protein C inhibits coagulation by inactivating factor Va and factor VIIIa. (for reviews see Esmon, 1983; Esmon, 1989) APC is also involved in the inflammation process taking place during septic shock (for review see Esmon et al., 1991).

Activated factor XII (FXIIa/FXIIf) induces production of interleukin-1 (IL-1) by human monocytes (Toossi et al., 1992). This augmentation in production was observed only in the presence of lipopolysaccharide (LPS). These observations provide further evidence for a potential role for FXII in the acute-phase reaction and the cellular immune response.

4. Complement activation

Factor XIIf (but not FXIIa) has been shown to activate enzymatically the first component of complement, C1 (Ghebrehiwet et al., 1981). Such activation however, is significant only in conditions that result in substantial conversion of FXIIa to FXIIf, such as hereditary angioedema (Donaldson, 1967).

Finally, the possible participation of the contact factors has been implicated in a number of clinical conditions including: allergic reactions, arthritis, disseminated intravascular coagulation, carcinoid syndrome, hyperlipoproteinemia, hyperacute renal allograft rejection, liver cirrhosis, nephrotic syndrome, postgastrectomy syndrome, shock and typhoid fever (Saito, 1987)

E. Regulation

C1-inhibitor (C1INH) is the major inhibitor of FXIIa and FXIIf (Schreiber et al., 1973; de Agostini et al., 1984; Pixley et al., 1985). C1INH accounts for ~90% of FXIIa/FXIIf inactivation in normal plasma. Antithrombin III (AT-III), α_2-antiplasmin (α_2AP) and α_2-macroglobulin (α_2M) can also inhibit the enzyme but with much lower effectiveness. C1INH, AT-III and α_2AP exhibit a 1:1 stoichiometry for the reaction with FXIIa/FXIIf (de Agostini et al., 1984; Pixley et al., 1985) while a portion of α_2M seems to covalently bind a subunit of FXIIa (Pixley et al., 1985). Because C1INH inhibits FXIIa and FXIIf equally, it must interact with the light chain of the molecule, containing the serine protease domain.

Deficiency of C1INH gives rise to the disease hereditary angioedema (HAE) which is due to *in vivo* activation of the complement system by FXIIa/FXIIf (Donaldson, 1967).

Novel inhibitors of factor XII activation have recently been reported. Endothelial cells were shown to produce a substance that inhibits contact activation of coagulation by blocking the adsorption of FXII to glass and therefore activation of the zymogen (Kleneiwski and Donaldson, 1993). An inhibitory fraction partially purified from a human umbilical vein endothelial cells (HUVECs) lysate exhibited a single homogeneous band in SDS-PAGE of ~22.5 kDa (Kleniewski and Donaldson, 1993). A similar inhibitor produced by human peripheral blood eosinophils was shown to inhibit contact activation

of factor XII, presumably by neutralizing the negative charge of activators of FXII (Ratnoff et al., 1993).

F. Deficiencies

Deficiency of factor XII was first identified in a patient whose surname, Hageman, has become the eponym for this protein (Ratnoff and Colopy, 1955). The autosomal recessive mode of inheritance causes complete deficiency of factor XII only when two abnormal alleles are inherited (Colman et al., 1987). Patients with complete deficiency of factor XII have no clinical bleeding disorder but are not protected from thrombotic disease. At least 16 patients with Hageman factor trait have experienced myocardial infarction and Mr. Hageman himself died of massive pulmonary embolism (Ratnoff et al., 1968). Factor XII deficiency translates in a markedly prolonged activated partial thromboplastin time (APTT), with normal bleeding time and prothrombin time (PT) (Ratnoff and Colopy, 1955).

The majority of the subjects with Hageman trait lack immunologically identifiable factor XII (CRM^-). Rare individuals show normal levels of cross-reactive material (CRM^+) but abnormal activity. Such cases provide an opportunity to study structure-function relationship in factor XII protein. Unfortunately, few abnormal factor XII cases have been analyzed at the molecular level. Here are some of the deficiencies reported in the literature.

Factor XII Washington is characterized by normal antigen level, the same specific antigenicity as purified normal FXII, the same molecular weight as normal FXII, but no clot-promoting activity (Miyata et al., 1989). Limited proteolysis of the abnormal FXII exposed to glass and plasma yields a two-chain FXIIa with normal sized heavy and light chains. These characteristics suggest that the abnormality does not reside in the activation of FXII but may be involved in the catalytic site of the molecule. Amino acid sequence analysis of tryptic peptides isolated from the abnormal factor XII indicated that

Cys^{571} is substituted to serine. It was proposed that the Cys->Ser replacement destroys the formation of the disulfide linkage between Cys^{540} and Cys^{571}. This would give rise to an altered conformation of the active site serine residue or of the secondary substrate binding site and lead to the lack of enzymatic activity (Miyata et al., 1989).

Factor XII Locarno is present in plasma at half the normal antigen level and shows no clotting activity (Wuillemin et al., 1992). FXII Locarno has a normal molecular weight but isoelectric focusing suggests an excess of negative charge when compared to normal FXII. Furthermore, FXII Locarno is not proteolytically cleaved upon prolonged incubation of the patient's plasma with dextran sulfate but is normally adsorbed to kaolin. Following addition of plasma kallikrein, FXII Locarno shows only partial cleavage within the disulfide loop Cys^{340}-Cys^{467}. Partially cleaved factor XII Locarno has no amidolytic or proteolytic activity. It is proposed that an amino acid substitution is affecting the kallikrein cleavage site Arg^{353}-Val^{354} in FXII Locarno (Wuillemin et al., 1992)

Factor XII Bern has a normal molecular weight, low antigen level, no clotting activity and a normal isoelectric point. The abnormal FXII is adsorbed to kaolin but no proteolytic cleavage occurs upon incubation with dextran sulfate. In the presence of plasma kallikrein, normal cleavage occurs but is not accompanied by any proteolytic activity. The molecular defect is unknown but is believed to be located in the light chain region of factor XII, containing the enzymatic active site (Wuillemin et al., 1991b)

Factor XII Toronto, another CRM^+ abnormal FXII has been partially characterized (Takahashi and Saito, 1988). The factor XII Toronto was purified to homogeneity, had a normal apparent molecular weight, an amino acid composition similar to that of normal FXII but no clot-promoting activity. The molecular defect is still unknown.

G. Recombinant FXII

The first expression of recombinant human factor XII was achieved in a human hepatoma cell line (HepG2) by using the vaccinia virus expression system (Citarella et al., 1993). The full-length recombinant FXII was reported to behave like native human factor XII for its activation by kaolin, proteolytic cleavage and substrate recognition. On the other hand, a mutant factor XII containing only exons 1 and 9-14 (resulting in a deletion of 319 amino acids) displayed higher FXII-specific clotting activity than native factor XII. This mutant was also reported to bind to kaolin and to be activated by negatively charged surfaces, even though it does not contain the N-terminus of the native zymogen. The authors therefore conclude that amino acids 319-334 and 344-353 are involved in the negative-charge dependent activation of factor XII (Citarella et al., 1993).

III. HUMAN PROTHROMBIN

A. Biosynthesis and post-translational modifications

During the final stages of blood coagulation, prothrombin (Mr 72 000) is converted from an inactive zymogen to the serine protease thrombin (Mr 37 000) which plays a central role in hemostasis. Prothrombin is synthesized in the liver and undergoes several post-translational modifications prior to secretion. These modifications include glycosylation, cleavage of the pre and pro-peptide and vitamin K-dependent γ-carboxylation of the 10 amino terminal glutamic acid residues (for reviews see Furie and Furie, 1988; Mann et al., 1990; Davie et al., 1991). Human plasma prothrombin consists of 579 amino acids and circulates at levels of 100-200 μg/mL making it one of the most abundant blood coagulation protein.

B. Structure

The amino acid (Butkowski et al., 1977; Hewett-Emmett et al., 1981) and cDNA (Degen et al., 1983) sequences of human prothrombin have been determined. The amino acid sequence gives information about the structure and domain organization of the molecule. A model of the prothrombin molecule is shown in figure 7.

Plasma prothrombin is composed of three structural domains commonly referred to as fragment 1 (F1), fragment 2 (F2) and prethrombin-2 (PRE-2). The fragment 1 region contains the γ-carboxyglutamic acid (Gla) region and the first kringle. Fragment 2 contains the second kringle and prethrombin-2 is the precursor of thrombin (Magnusson et al., 1975).

1. Leader sequence

Human prothrombin is synthesized as a prepro-protein with an amino-terminal leader sequence that contains the signal peptide required for translocation of the nascent polypeptide into the endoplasmic reticulum, followed by a propeptide which directs the vitamin K-dependent γ-carboxylation of prothrombin (Jorgensen et al., 1987b; Suttie et al., 1987). Studies with three naturally occurring factor IX mutants (another vitamin K-dependent coagulation protein) allowed the identification of the signal peptidase cleavage site. Due to point mutations in the propeptide region, these factor IX molecules circulated with the propeptide still attached, thereby defining the length of the FIX propeptide as 18 amino acids (Diuguid et al., 1986; Bentley et al., 1986; Ware et al., 1986). The exact length of the prothrombin propeptide is not known but the whole leader sequence is 43 amino acids long (Degen et al., 1983; Jorgensen et al., 1987a).

The propeptide regions of the vitamin K-dependent proteins (factor IX, prothrombin, factor X, Protein C, factor VII and Protein S) share some sequence identities (Pan and Price, 1985; Bentley et al., 1986; Jorgensen et al., 1987b). Studies on factor IX (Rabiet et al., 1987; Jorgensen et al., 1987b; Ware et al., 1989b; Handford et al.,

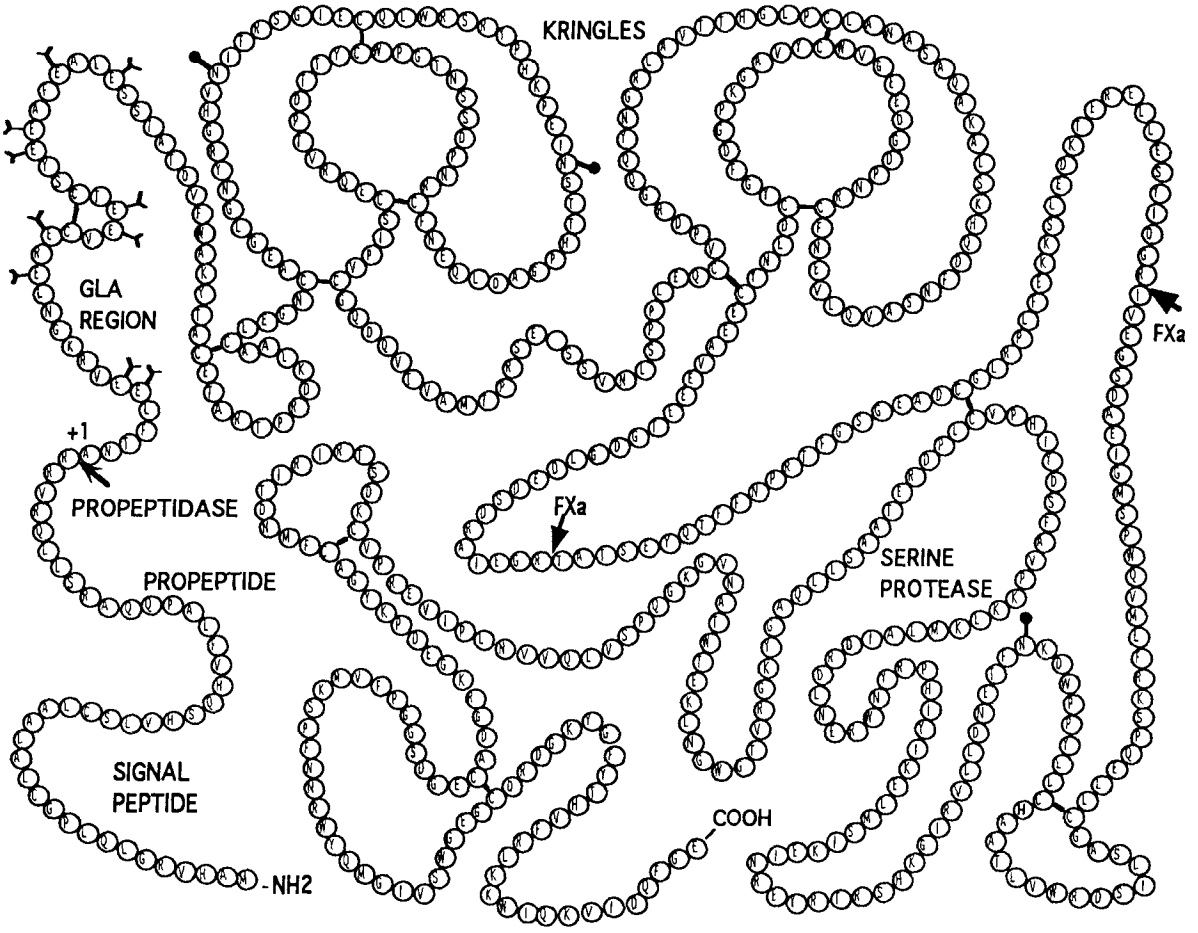


Figure 7. Schematic diagram of human prothrombin

The Gla residues are indicated as ⊁ and the N-linked carbohydrate chains are indicated by ↖. The FXa cleavage sites are indicated by arrows.

1991), Protein C (Foster et al., 1987), and prothrombin (Huber et al., 1990), have demonstrated that if the propeptide region is mutated or deleted, in most cases, γ-carboxylation is impaired or abolished. It is hypothesized that the propeptide contains two recognition elements: one for vitamin K-dependent carboxylase recognition located toward the N-terminus, and one for propeptidase recognition located near the C-terminus. Cleavage of the propeptide at Arg^{-1}-Ala^{+1} by an unidentified propeptidase, prior to secretion, produces plasma prothrombin.

2. γ-carboxyglutamic acid (Gla) domain

The γ-carboxyglutamic acid (Gla) domain is located at the N-terminus of prothrombin, within the fragment 1 (F1) region. The first 10 glutamic acid residues are modified by the vitamin K-dependent carboxylase during post-translational modification of prothrombin. The Gla residues are essential for the formation of calcium binding sites involved in binding of prothrombin to phospholipid surfaces (Nelsestuen and Suttie, 1972; Esmon et al., 1975). Upon exposure of the protein to calcium ions, a first transition occurs rapidly as prothrombin binds Ca^{++} (Nelsestuen, 1976). Two or three Gla residues bind a single calcium ion and form a noncovalent intramolecular bridge between regions of the polypeptide backbone (Furie et al., 1979), stabilizing the tertiary structure of the fragment 1 region. Studies on the X-ray structure of prothrombin F1 show that the region is structurally ordered only in the presence of metal ions (Tulinsky et al., 1988; Soriano-Garcia et al., 1989).

The conformational change resulting from the binding of calcium ions exposes a membrane-binding site (Nelsestuen, 1976; Nelsestuen et al., 1976; Borowski et al., 1986). Such binding is required for proper interaction between the components of the prothrombinase complex (see below) (Mann et al., 1988) and efficient activation of prothrombin (Jackson, 1981).

Further support for the role of the Gla domain in the activation of vitamin K-dependent proteins comes from studies on naturally occurring factor IX mutants. Several factor IX deficiencies involving mutations at specific Gla residues (Chen et al., 1987; Wang et al., 1990; Hamaguchi et al., 1991a) result in severe bleeding tendencies, presumably subsequent to decreased activation potential. Binding studies with chimeric proteins composed of portions of factor VII and factor IX suggest that the high-affinity interaction between FIX and the endothelial cell binding site is mediated by the Gla domain (Toomey et al., 1992).

3. Aromatic amino acid stack

Between the Gla region and the first kringle is a short segment known as the aromatic amino acid stack (Furie and Furie, 1988). In fragment 1, the aromatic amino acid residues Phe-Trp-X-X-Tyr have their side chains stacked into a ring cluster which stabilizes the protein structure (Park and Tulinsky, 1986; Soriano-Garcia et al., 1989). Although hydrophobic, this region is oriented toward the surface where it may play a role in recognition of a receptor (Park and Tulinsky, 1986).

4. Kringle domain

The next domains are two structures known as kringles (Figure 7). The kringle was described earlier (see FXII-kringle).

5. Serine protease domain

The X-ray crystal structures for the D-Phe-Pro-Arg chloromethylketone (PPACK)-inhibited α-thrombin (Bode et al., 1989; Bode et al., 1992a) and for the thrombin/hirudin complex (Grutter et al., 1990; Rydel et al., 1990) have been elucidated. The thrombin molecule can be described as a prolate ellipsoid of approximate dimensions 45 x 45 x 50 Å, and the A and B chains are not organized in separate domains (Bode et

al., 1989). An anion-binding exosite, distant from the catalytic residues is important for fibrinogen cleavage and is involved in the binding of other proteins. Another feature of thrombin is the existence of a unique loop, within the B chain, composed of Tyr^{367}-Phe^{374}. The B loop, together with the loop segment around Trp^{468}, shapes, narrows and deepens the active-site cleft (Bode et al., 1989). Steric hindrance by this segment is one of the presumed reasons for the narrow substrate specificity of thrombin (Bode et al., 1989). The thrombin A chain is arranged in a boomerang-like shape against the B chain globule opposite to the active site, and it is not involved in substrate and inhibitor binding (Bode et al., 1992a,b).

C. Activation

Activation of prothrombin to thrombin results from the proteolytic cleavage of Arg^{271}-Thr^{272} and Arg^{320}-Ile^{321} by factor Xa (Figure 8). Although factor Xa alone will catalyze the activation slowly, the reaction is accelerated greatly in the presence of the prothrombinase complex which consists of factor Xa, cofactor factor Va, calcium ions and a negatively charged phospholipid surface (Nesheim et al., 1979; Krishnaswamy et al., 1987). The formation of the prothrombinase complex results in a 10^5-fold enhancement in the rate of prothrombin activation catalyzed by FXa.

Depending upon the order of peptide bond cleavage, two intermediate products accumulate transiently, meizothrombin and prethrombin-2 (Figure 8). Meizothrombin is produced by proteolytic cleavage of the Arg^{320}-Ile^{321} bond by FXa yielding activation fragment 1.2-thrombin A chain linked to the thrombin B chain by a disulfide bond. Meizothrombin is capable of catalyzing the cleavage of the Arg^{155}-Ser^{156} bond which releases the fragment 1 (F1) domain giving rise to another active species meizothrombin(desF1), no longer containing the Gla region and exhibiting different functional activities from that of meizothrombin (Doyle and Mann, 1990). Prethrombin-2 results from the cleavage of the Arg^{271}-Thr^{272} bond. Although prethrombin-2, with the

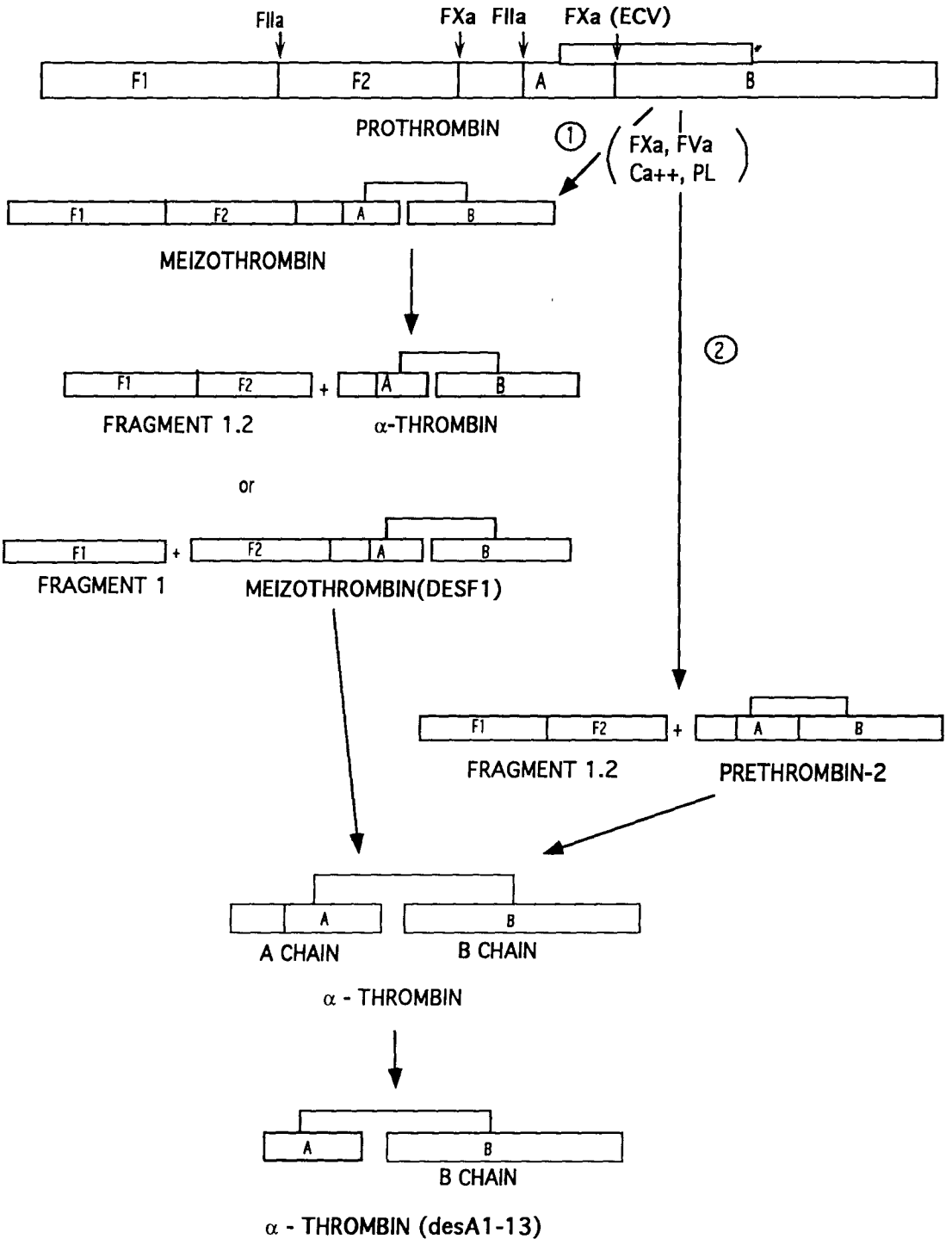


Figure 8. Prothrombin activation pathways.

exception of the bond at Arg^{320}-Ile^{321}, is identical in covalent structure to thrombin, it has no proteolytic activity (Owen et al., 1974).

The existence of meizothrombin as an intermediate in the prothrombinase-catalyzed activation of prothrombin has been described (Rosing et al., 1986), and further studies indicated that it is the main if not sole intermediate of the activation of prothrombin by the fully assembled prothrombinase complex *in vitro* (Krishnaswamy et al., 1986; Boskovic et al., 1990). Human thrombin formed on the surface of endothelial cells influences the formation of meizothrombin via a feedback mechanism, leading to accumulation of meizothrombin(desF1) in the final phase of prothrombin activation (Tijburg et al., 1991). The specific role of factor Va in meizothrombin formation has not been clarified to date. Factor Va, however, interacts with both factor Xa and prothrombin and presents them to one another in the formation of a ternary enzyme-substrate-cofactor complex (Boskovic et al., 1990). In addition, if factor Va is omitted from the reaction, prethrombin-2 is the main intermediate observed (Esmon et al., 1974; Rosing et al., 1980).

D. Function

1. Thrombin

a. Enzymatic

Fibrinogen is a large glycoprotein (M_r 340 000) present at high concentration in plasma and platelets (Colman et al., 1987) and comprises six polypeptide chains: 2 Aα, 2 Bβ, and 2 γ chains (Doolittle, 1984). Thrombin cleaves four peptide bonds in fibrinogen (one in each of the Aα and Bβ chains) releasing fibrinopeptides A and B, and the fibrin monomer. The monomers polymerize to form long fibrin strands or short broad sheets.

Thrombin is sequestered within the matrix of the fibrin gel where it remains active and intact for extended periods of time (Wilner et al., 1981; Mann, 1987).

The fibrin network is further strengthened by the formation of covalent cross links between monomers by activated factor XIII (FXIIIa) (Lorand and Radek, 1992). FXIIIa, a transglutaminase, catalyzes the formation of isopeptide bonds between lysine and glutamine residues, branching the fibrin fibers together. Factor XIII is activated by limited proteolysis by thrombin, in the presence of calcium ions. Polymeric fibrin, the physiological substrate of factor XIIIa, is a potent promoter of the α-thrombin-catalyzed cleavage of the zymogen FXIII (Lorand and Radek, 1992).

Factor Va and factor VIIIa are essential to the blood clotting cascade. Factor V consists of a single polypeptide chain (M_r 330,000) (Nesheim and Mann, 1979). Thrombin converts factor V to factor Va by limited proteolysis of three peptide bonds (Mann et al., 1988). FVa, as a cofactor of the prothrombinase complex, markedly increases the rate of the factor Xa-catalyzed activation of prothrombin (reviewed in Davie and Fujikawa, 1975). In a similar manner, factor VIIIa is required as a non-enzymatic cofactor for the activation of factor X by factor IXa, in the presence of Ca^{++} and phospholipids (Vehar and Davie, 1980). Factor VIII (M_r 280,000) is also activated by limited proteolysis by thrombin (Jackson and Nemerson, 1980; Mann et al., 1988).

The zymogen Protein C (PC) (M_r 62,000) is activated by the membrane-bound Ca^{++}-dependent complex composed of α-thrombin and thrombomodulin (TM). Thrombomodulin, a platelet and endothelial cell receptor for thrombin (M_r 100,000) enhances 1000-fold the activation of PC (Esmon et al., 1982). The association of thrombin with TM, in the presence of Ca^{++}, alters the activity and specificity of thrombin towards fibrinogen and factor V (Mann et al., 1988) and directs it toward protein C activation by limited proteolysis (Figure 3). The binding of thrombin to TM structurally alters the active site of thrombin (Ye et al., 1991). Activated protein C (aPC) degrades cofactors Va and VIIIa (Fay et al., 1991) thereby inhibiting further production of

thrombin (Vehar and Davie, 1980). aPC also facilitates fibrinolysis *in vivo* (Esmon, 1983) and *in vitro* by its ability to inhibit the activation of prothrombin (Bajzar et al., 1990; Bajzar and Nesheim, 1991).

b. Non-enzymatic

At the cellular level, thrombin promotes migration of human peripheral blood monocytes involved in the inflammatory response (Bar-Shavit et al., 1992). α-thrombin is also capable of initiating proliferation in quiescent fibroblast cells, as well as promoting growth in macrophage (Bar-Shavit et al., 1992). Thrombin interaction with endothelial cell surface receptors promotes the production and release of diverse cellular mediators and proteins, such as: prostacyclin (PGI_2), adenine nucleotides, plasminogen activator, plasminogen activator inhibitor, von Willebrand factor (vWF), fibronectin, platelet-activating factor, and platelet-derived growth factor (PDGF) (Bar-Shavit et al., 1992). In addition, thrombin can pass through the endothelial cell layer and reach subendothelial structures. These observations potentially involve thrombin in wound healing as well as in hemostasis.

2. Meizothrombin

Bovine meizothrombin has between 2 and 10 % of the activity of bovine thrombin toward fibrinogen, but similar activity to thrombin toward the chromogenic substrate S-2238 (Doyle and Mann, 1990; Rosing et al., 1986). Bovine meizothrombin has 75 % of the activity of thrombin toward the activation of Protein C and, in the presence of phospholipids, the kinetics for activation of Protein C by the meizothrombin-thrombomodulin (rabbit) complex are identical to those of thrombin-thrombomodulin (Doyle and Mann, 1990). The recombinant human prothrombin active site mutant (Ser^{205}->Ala) which mimics inactive meizothrombin, however, does not bind to human

recombinant thrombomodulin (Wu et al., 1992). The physiological function of this intermediate (enzymatic or not) is still unclear.

Studies of human meizothrombin are hampered by autolysis which results in rapid formation of meizothrombin(desF1), and further activation to α-thrombin (Figure 8). Furthermore, electrophoretic analysis under non-reducing conditions is complicated because meizothrombin and meizothrombin(desF1) have molecular weights that are identical to those of prothrombin and prethrombin-1. Reversible thrombin inhibitors allow the isolation of meizothrombin but make subsequent enzymatic characterization difficult. Even in the presence of the reversible inhibitor dansylarginine N-(3-ethyl-1,5 pentanediyl) amide (DAPA), the meizothrombin generated is only stable for a few hours at 4°C before autolysis occurs and meizothrombin(desF1) appears (Doyle and Mann, 1990).

E. Regulation

Considering the wide range of thrombin functions, it is apparent that a high degree of regulation of thrombin activity is required for the maintenance of normal hemostasis. The activity of thrombin generated during coagulation is primarily regulated by inactivation of the enzyme by plasma proteinase inhibitors. Four such inhibitors, antithrombin III (AT-III), α_2-macroglobulin (α_2M), α_1-proteinase inhibitor (α_1PI) and heparin cofactor II have been shown to inhibit thrombin. α_1-proteinase inhibitor and α_2-macroglobulin are general protease inhibitors that can inactivate several different enzymes while AT-III and heparin cofactor II are more specific for thrombin.

Antithrombin III, a member of the protein family called serpins (an acronym for serine protease inhibitor), is the main thrombin inhibitor. AT-III can also inhibit all enzymes of the intrinsic coagulation pathway, as well as plasmin, trypsin and the first component of the complement, C1 (Rosenberg, 1979). AT-III forms a tight complex with thrombin, in which the active site is blocked. The inactivation is promoted greatly

by the presence of the sulfated glycosaminoglycan heparin. A suggested mechanism for this interaction is that thrombin, AT-III and heparin are first assembled into a ternary complex, in which both thrombin and AT-III are bound to heparin. An active-site-dependent interaction between the protease and the inhibitor is then established. Under physiological conditions, the affinity of thrombin for heparin-bound AT-III is nearly 10 000-fold higher than for the free inhibitor. Upon binding of thrombin to AT-III, an interaction between the enzyme and a reactive bond of AT-III (Arg^{393}-Ser^{394}) takes place but subsequent cleavage does not proceed normally and stable complex is formed (Olson and Björk, 1992).

A second heparin-dependent inhibitor of thrombin has been described, heparin cofactor II (Briginshaw and Shanberge, 1974). This protein shows homology to other serpins and like AT-III, it inhibits thrombin by forming a stable equimolar complex with the enzyme, in which the active site is blocked (Tollefsen et al., 1982). Heparin cofactor II differs from AT-III in that its reaction is accelerated by both heparin and the polyanion dermatan sulfate (Tollefsen et al., 1983). The physiological role of heparin cofactor II is unclear. It cannot substitute for AT-III in individuals deficient for the inhibitor. A possible role for the inhibitor has been suggested by regulating thrombin activity in the extravascular space at the site of blood vessel damage (McGuire and Tollefsen, 1987). Heparin cofactor II may also play a role in mediating the inflammatory response to injury (Hoffman et al., 1989).

Antithrombin III inactivates thrombin generated by prothrombinase-activated prothrombin 2 to 4-fold more slowly than purified thrombin (Lindhout et al., 1986; Schoen and Lindhout, 1987). Presumably, this effect results from the noncovalent association of fragment 1.2 or fragment 2 with thrombin. Binding of fragment 2 to thrombin also reduces the rate of AT-III inactivation by 3-fold (Walker and Esmon, 1979). Similarly, although both bovine meizothrombin and human meizothrombin(desF1) are inhibited by bovine and human antithrombin-III (AT-III)

respectively, the reactions are not promoted by heparin (Rosing et al., 1986; Schoen and Lindhout, 1987). This observation is presumably due to the absence of a heparin binding site in this intermediate.

Thrombin binds very tightly to and is selectively inhibited by hirudin, a protein isolated from the European medicinal leech *Hirudo medicinalis* (Markwardt, 1970).

F. Thrombin receptor

Thrombin activates platelets to aggregate through interaction with a receptor which is expressed by both platelets and endothelial cells. A cDNA encoding a functional thrombin receptor was recently obtained by direct expression cloning in Xenopus oocytes (Vu et al., 1991a). The deduced amino acid sequence of the cDNA predicts a novel member of the seven transmembrane domain receptor family. The extracellular amino-terminal extension of this receptor contains a putative thrombin cleavage site (LDPR/S), resembling the one found in the zymogen Protein C (LDPR/I). This apparently highly glycosylated receptor (Brass et al., 1992) also has an acidic region within the extracellular domain, with some similarities to the carboxyl-terminal region of the leech thrombin inhibitor hirudin which is known to interact with the anion exosite of thrombin (Bode et al., 1989; Rydel et al., 1990). A model of the thrombin-receptor interaction suggests that thrombin interacts with its receptor through the primary recognition sequence (LDPR/S) and through the anion-binding exosite binding domain. Thrombin then cleaves its receptor at the Arg-Ser bond. This cleavage exposes a new N-terminus that functions as a ligand and activates the receptor (Vu et al., 1991b; Liu et al., 1991a).

G. Deficiencies

Inherited disorders of prothrombin can be classified in two categories: hypoprothrombinemia results from decreased prothrombin synthesis, while

dysprothrombinemia results from the synthesis of an abnormal prothrombin molecule, with decreased biological activity (Colman et al., 1987). In some cases, patients are described as compound heterozygotes having, for example, one gene for dysprothrombinemia and the other for hypoprothrombinemia.

Although prothrombin deficiencies are rare, at least 19 distinct abnormal prothrombins have been reported (Table 1), and several have been characterized at the molecular level.

Prothrombin Quick I is characterized by a substitution of Arg^{383} by a cysteine. This result identifies residue 382 in human prothrombin as essential for determining the specificity of thrombin toward fibrinogen and also in the cellular responses of platelet aggregation and prostacyclin release (Henricksen and Mann, 1988). Prothrombin Quick II results from the substitution of Gly^{558} to valine. This study establishes that residue 558 is critical for controlling the primary substrate specificity in thrombin and supports the finding that Gly^{558}, which is conserved among serine proteases, plays an essential role in the primary substrate binding pocket (Henricksen and Mann, 1989).

Prothrombin Salakta results from the substitution of Glu^{466} by alanine. It is suggested that this substitution would change the proper conformation around the substrate binding site containing Trp^{468}, which is a unique surface loop on the thrombin molecule (Miyata et al., 1992).

The proband identified as prothrombin Tokushima comprises a compound heterozygote for dys- and hypoprothrombinemia. The mutation for hypoprothrombinemia is a single base pair insertion at position 4177 of the gene. The resulting frameshift causes an altered amino acid sequence from codon 114 and a premature termination codon at amino acid 174 (Iwahana et al., 1992). More interesting is the mutation causing the dysprothrombinemia, the substitution of Arg^{418} by tryptophan. This mutation which is found in the thrombin portion of prothrombin Tokushima seems

Table 1. Characteristics of the hereditary dysprothrombinemias

name	genotype	% activity	% antigen	molecular defect	references
Prothrombin Himi	compound heterozygote	10	88	I: Met^{337}->Thr II: Arg^{388}-> Hi	(Morishita et al., 1991) (Morishita et al., 1992)
Prothrombin Tokushima	compound heterozygote	12	42	I: frameshift -> $stop^{174}$ II: Arg^{418}-> Trp	(Iwahana et al., 1992) (Miyata et al., 1987)
Prothrombin Madrid	heterozygous	3	103	Arg^{271}-> Cys	(Diuguid et al., 1989)
Prothrombin Barcelona	homozygous	12	100	Arg^{271}-> Cys	(Rabiet et al., 1986)
Prothrombin Quick	compound heterozygote	<2	34	I: Arg^{382}-> Cys II: Gly^{558}-> Val	(Henricksen and Mann, 1988) (Henricksen and Mann, 1989)
Prothrombin Salakta	unknown	17	100	Glu^{466}-> Ala	(Miyata et al., 1992)
Prothrombin Habana	heterozygous	<10	50	unknown	(Rubio et al., 1983)
Prothrombin Metz	compound heterozygote	10	50	unknown	(Rabiet et al., 1984) (Josso et al., 1982)
Prothrombin Brussels	heterozygous	46	88	unknown	(Kahn and Govaerts, 1974)
Prothrombin Molise	compound	11	45	unknown	(Girolami et al., 1978)
Prothrombin Mexico City	compound heterozygote	9	3.5	I: fragment 2? II: unknown	(Valls-de-Ruiz et al., 1987)
Prothrombin Gainesville	unknown	34	70	fragment 2?	(Smith et al., 1981)
Prothrombin Perija	homozygous	2	70	unknown	(Ruiz-Saez et al., 1986)
Prothrombin Houston	?compound heterozygote	5-9	51	unknown	(Weinger et al., 1980)
Prothrombin Cardeza	heterozygous	50	100	Pre-2 region?	(Shapiro et al., 1969)
Prothrombin Padua	heterozygous	50	100	unknown	(Girolami et al., 1974)
Prothrombin Denver	homozygous	<1	13	unknown	(Montgomery et al., 1980)
Prothrombin Poissey	homozygous	1.7	50	unknown	(Dumont et al., 1983)

to reduce its interaction with various substrates including fibrinogen and platelet receptor, although the active site appears to be intact (Miyata et al., 1987).

Prothrombin Himi is a compound heterozygote for two dysfunctional prothrombin molecules. One of the mutations, prothrombin Himi I, is the replacement of Met^{337} by Thr . Little is known about the function of Met^{337}. The Met->Thr substitution, although conservative, seems to reduce the interaction of prothrombin Himi I with substrates including fibrinogen (Morishita et al., 1992). The mutation causing prothrombin Himi II is the substitution of Arg^{388} by histidine. Arg^{388} is not conserved in other serine proteases, including the blood clotting factors. Using the model of the spatial structure of α-thrombin (Bode et al., 1989), Arg^{388} would form part of the arginine-rich surface of the 70-80 loop, which probably represents part of the anion-binding exosite. The anion-binding exosite which is found only in thrombin, is located away from the active site and contributes to the remarkable specificity of thrombin interaction with many substrates, cofactors, and inhibitors. Fibrinogen, thrombomodulin and hirudin all seem to bind competitively to this exosite (Tsiang et al., 1990). The Arg->His replacement might impair the site for interaction with fibrinogen, resulting in partial loss of the clotting activity (Morishita et al., 1992).

A very interesting mutation of the prothrombin molecule is the one found in prothrombin Barcelona (Rabiet et al., 1986) and prothrombin Madrid (Diuguid et al., 1989). In both cases, Arg^{271} is substituted for a cysteine. Prothrombin Barcelona and Madrid are cleaved abnormally by factor Xa which only cleaves the Arg^{271}-Thr^{272} bond, between the fragment 2 region and the A chain of thrombin. The mutation causes alteration of the activation of the molecule yielding meizothrombin, which shows little clotting activity but retains proteolytic activity (Doyle and Mann 1990). An analogous defect is found in factor IX Chicago (Duiguid et al., 1989) and factor IX Chapel Hill (Noyes et al., 1983) in which there is substitution of arginine residues preceding peptide bonds normally cleaved during activation of the molecules.

H. Recombinant human prothrombin

Wild type human prothrombin cDNA has been expressed in several different eukaryotic expression systems, including Chinese hamster ovary cells (CHO) (Jorgensen et al., 1987a), baby hamster kidney cells (BHK) (Le Bonniec et al., 1991), and VERA cells using a vaccina virus vector (Falkner et al., 1992). In all systems, the recombinant human prothrombin activity was equivalent to that of plasma-derived prothrombin and the proteins were reported to be fully γ-carboxylated. These results contrast with those of other vitamin K-dependent proteins such as factor IX (Anson et al., 1985; Busby et al., 1985; Kaufman et al., 1986; Lin et al., 1990), protein C (Grinnel et al., 1987; Yan et al., 1990) or factor X (Wolf et al., 1991), where incomplete γ-carboxylation results in partial biological activity of the recombinant proteins.

IV. STRUCTURE-FUNCTION STUDIES

From the information gathered through protein sequencing and molecular biology, the primary structure of many proteins have been elucidated. The next goal is to determine the folding pattern and three-dimensional structure inherent to proteins, and understand how a particular function or binding relates to the polypeptide structure. The numerous serine proteases involved in coagulation and fibrinolysis are a good example of a family of proteins with diverse functional properties but common structural elements. As such, they offer an interesting model for structure-function relationship studies. Those proteins resemble trypsinogen but possess a much larger amino-terminal, non-catalytic segment. They also differ from trypsin in having a very limited protein substrate specificity. In general, the N-terminal region is believed to mediate the interaction of the proteases or their zymogen with other proteins or macromolecules (Patthy, 1985). A variety of approaches are available for structure-function relationship studies.

A. Antibodies

Monoclonal antibodies (mAbs) raised against complex proteins can prove to be useful tools for the study of structure-function relationships within large polypeptides. The use of mAbs has been applied to several coagulation proteins including factor XII (Small et al., 1985; Saito et al., 1985; Pixley et al., 1987; Clarke et al., 1989; Nuijens et al., 1989), prothrombin (Jorgensen et al., 1987a; Church et al., 1991; Noe et al., 1988), factor V (Annamalai et al., 1987), factor X (Church et al., 1988), factor IX (Liebman et al., 1985; Kaufman et al., 1986; Frazier et al., 1989), factor VIII (Ware et al., 1989a), and HMWK (Schmaier et al., 1987) among others. Here are some examples.

Three anti-FXII mAbs have been described: B7C9 (Pixley et al., 1987), P-5-2-1 (Saito et al., 1985), and a murine anti-HF IgG (Small et al., 1985) that inhibited activation of FXII and the surface-mediated coagulant activity of FXII without affecting its amidolytic activity. The epitope for all three antibodies was located to the amino-terminal heavy chain of the molecule, as determined by Western blot analysis. Since these mAbs inhibited the adsorption of FXII to negatively charged surfaces, it was postulated that their epitope would help localize the putative surface-binding domain of the protein within the 50 kDa fragment. Western blot analysis of FXII peptides, limited N-terminal analysis of immunoreactive fragments and synthetic peptide binding studies located the putative epitope for B7C9 to amino acids 134-153 of FXII (Pixley et al., 1987). This region lies within the fibronectin type I homology. This same structure in fibronectin may be involved in fibrin and heparin binding (Yamada, 1983). However, further studies by Clarke et al. indicated another location for the same antibody binding, namely the N-terminal part of the heavy chain. (See later section)

Another anti-FXII mAb, F1, as well as its $F(ab')_2$ and F(ab') fragments were shown to induce activation of the contact system in plasma, as reflected by the generation of FXIIa (Nuijens et al., 1989). Experiments with trypsin-digested ^{125}I-FXII revealed that the epitope for mAb F1 was located in the NH_2-terminal portion of the molecule.

FXII in fresh plasma bound 190 times less to mAb F1 than cleaved FXII. However, no difference in accessibility of the epitope was observed between cleaved and single-chain FXII when bound to glass. mAb F1-induced contact activation required the presence of FXII, prekallikrein, and HMWK and, in contrast to activation by negatively charged surfaces, was not inhibited by the presence of polybrene (polybrene coats surfaces, rendering them neutral). The authors proposed that a conformational change in FXII is a key event in the activation process of the molecule, and that this change can be induced by binding of FXII to a surface, by proteolytic cleavage, or by binding to mAb F1.

The effect of anti-FXII mAb C6B7 was described earlier. C6B7 blocks the activation of FXII and subsequently the activation of the remaining contact system *in vivo* (Pixley et al., 1993). This antibody was raised against purified fragment FXIIf(1) composed of the 28 kDa catalytic unit of activated FXII and a small 2 kDa portion of the heavy chain linked by disulfide bond to the light chain. Incubation of normal human plasma with mAb C6B7 at concentrations greater or equal to 1 μM resulted in 92-95% inhibition of coagulant activity. Monitoring of HMWK cleavage also indicated that HMWK remained ~80% intact, reflecting little activation of prekallikrein to kallikrein by FXIIa. Western blot analysis indicated that mAb C6B7 epitope was localized to the catalytic light chain region of the protein.

Conformation-specific antibodies that bind solely to the metal ion-stabilized factor IX (Liebman et al., 1985) and prothrombin (Jorgensen et al., 1987a) conformer have been developed. Those antibodies have been used for affinity chromatography and characterization of the Gla content in recombinant FIX and prothrombin (Kaufman et al., 1986; Jorgensen et al., 1987a).

Two mAb raised against the second kringle of prothrombin inhibited activation of prothrombin by the prothrombinase complex by 90 and 50% (Church et al., 1991). This inhibition was also present when human platelets provided the reaction surface. This study suggests a role for prothrombin fragment 2 (F2) in activation, possibly by

mediating the interaction of substrate prothrombin with FXa or FVa on the phospholipid surface (Church et al., 1991).

In another example, polyclonal antibodies were raised against a synthetic peptide comprising residues 62-73 of the B chain of human α-thrombin (Noé et al., 1988). These antibodies were found to bind to the peptide as well as to the thrombin molecule. Although they had no effect on the amidolytic activity of thrombin toward a small synthetic substrate and caused minimal decrease (20%) in the rate of inactivation by AT-III, the antibodies competitively inhibited the binding of hirudin. The release of fibrinopeptide A from fibrinogen was also competitively inhibited, as well as the activation of protein C in the presence of thrombomodulin. In contrast, the antibodies had no effect on the activation of PC in the absence of thrombomodulin. Since residues 62-73 are located on a surface loop relatively far from the catalytic center (Bode et al., 1989), it is hypothesized that the positively charged region 62-73, or areas in close proximity in the three-dimensional structure, form a secondary binding site for negatively charged areas on the surface of hirudin and fibrinogen, and that thrombomodulin, directly or indirectly, affects this region (Noé et al., 1988).

B. Naturally occurring mutations

Mutant proteins from patients suffering from a coagulation deficiency can also provide precious information regarding the structure-function relationships within coagulation proteins. The most insight is probably gained by studying the blood proteins of CRM^+ (cross-reactive material) patients. The latter show a normal level of antigen but reduced or non-detectable activity for a particular protein. Such mutant proteins have been studied from hemophiliacs (lacking factor IX or factor VIII activity) and also from deficiencies in factor XII, prekallikrein, HMWK, factor XI, prothrombin, factor X, factor V and factor VII (Colman et al., 1987). By determining the hemostatic function affected in a patient and correlating it to the molecular abnormality causing the genetic defect,

associations can be made relating to the role of certain amino acids or regions within the molecule.

A number of examples of abnormal factor XII and prothrombin have been described earlier. Two congenital dysprothrombinemia cases have been described, prothrombin Barcelona and prothrombin Madrid (Table 1) for which the molecular defect is a substitution of Arg^{271}->Cys (Josso et al., 1971; Rabiet et al., 1986; Diuguid et al., 1989). Exposure of prothrombin Barcelona or Madrid to factor Xa results in cleavage of the Arg^{320}-Ile^{321} bond, yielding meizothrombin. This mutant prothrombin could offer a model to study the activity of meizothrombin although since the thrombin cleavage sites are intact, generation of meizothrombin(desF1) and α-thrombin(des A 1-13) would probably occur and complicate the analysis.

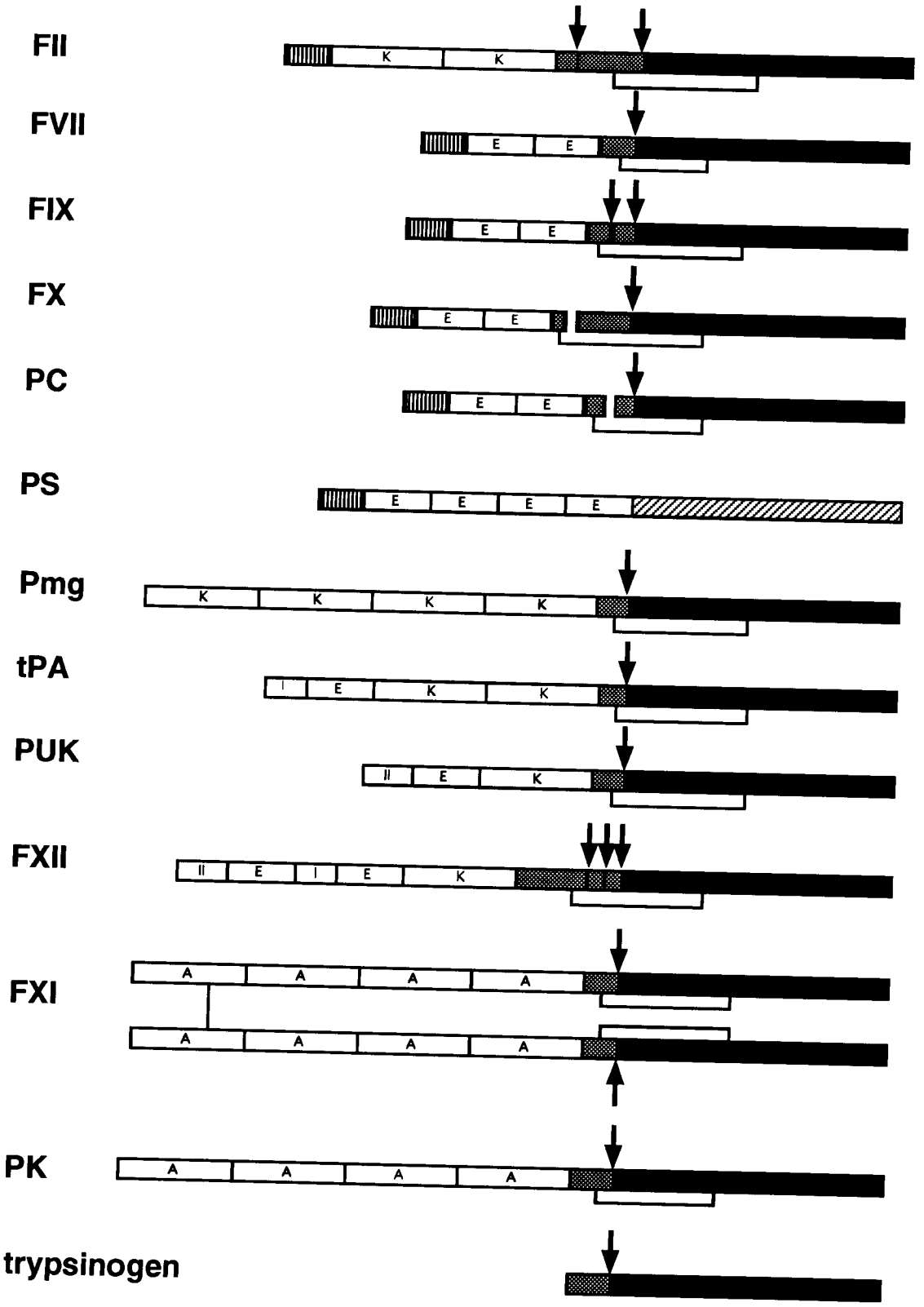
C. Comparison and evolution studies

As mentioned earlier, many proteins involved in hemostasis share homologous domains (Furie and Furie, 1988). Figure 9 illustrates the structural domains and homologies found in the hemostatic serine protease family and trypsinogen. Because the primary mechanism involved in evolution of these proteins is probably exon shuffling, the domain organization of the proteins is often a reflection of their intron-exon organization (Patthy, 1985; Doolittle and Feng, 1987). Some information can be gained by comparing protein-protein or protein-macromolecule interactions between proteins sharing domain structures. For example, the protease function resides in the common serine protease domain, and the Gla domain interact with calcium ions and phospholipids in a similar fashion. The exact roles of other domains such as the kringle or EGF domains are still unclear.

Comparison of amino acid sequences from a number of different species can also help to identify regions or residues required for structural or functional properties such as active-site or substrate specificity, protein-protein interactions and species-specific

Figure 9. Structural domains of the proteins involved in hemostasis and of related proteins

Comparison of the structure of coagulation and fibrinolytic zymogens to trypsinogen. Sites of proteolytic cleavage associated with zymogen activation are indicated by the arrows. The proteins shown from top to bottom are: prothrombin, FII; factor VII, FVII; factor IX, FIX; factor X, FX; protein C, PC; protein S, PS; plasminogen, PMG; tissue type plasminogen activator, tPA; prourokinase, PUK; factor XII, FXII; factor XI, FXI; prekallikrein, PK; and trypsinogen. The solid bar represents the protease domain, the grey region represents the activation peptide and the striped region represents the Gla domain. K represents the kringle domain, E represents the regions homologous to the EGF precursor, A represents the apple domain, and I and II represent the regions homologous to the type I and II homologies of fibronectin. Solid lines represent the disulfide bonds. The lengths of the bars are approximately proportional to the lengths of the polypeptide chains. (from Furie and Furie, 1988)


FII
K
K
FVII
E
E
FIX
E
E
FX
E
E
PC
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PS
E
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E
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Pmg
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A
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A
trypsinogen

differences in biological processes. For example, amino acid comparison of the thrombin B chain from a diverse group of vertebrates indicate that the catalytic triad, the B loop and the chemotactic domain are the most conserved features. The least conserved regions correspond to surface loops (other than the B loop). This variability may explain some of the species-specific interactions observed between thrombin and its numerous substrates (Banfield and MacGillivray, 1992).

D. X-Ray crystallography and NMR spectroscopy

An adequate understanding of the specific and characteristic functions of a protein requires knowledge of the determined three-dimensional structure. The crystal structure of the serine proteases trypsin and chymotrypsin have been resolved below 2 Å, by X-ray crystallography (Bode and Schwager, 1975; Cohen et al., 1981; Tsukada and Blow, 1985). These studies defined the catalytic triad (serine, histidine and aspartic acid) typical of serine proteases as well as subsites surrounding the active site. In contrast to trypsin and chymotrypsin which are relatively non-selective, thrombin is much more selective toward macromolecular substrates in that it cleaves many fewer bonds (Blombäck et al., 1967). The thrombin specificity is not determined by subsites surrounding the active residues alone. A thrombin exosite, quite distant from the catalytic residues is important for efficient cleavage of fibrinogen, as well as binding of fibrin monomers, thrombomodulin and hirudin (Bode et al., 1989; Bode et al., 1992a,b). The existence of an apolar binding site located close to the catalytic center has also been suggested as accounting for thrombin specificity with tripeptide substrates (Sonder and Fenton, 1984).

Three-dimensional models have been proposed for the thrombin B chain, based on sequence homology with bovine chymotrypsin and trypsin; however, these models provide only a general impression of the arrangement of sites involved in the various interactions of thrombin (Bode et al., 1992b).

Although the primary structure of the coagulation factors has been known for a number of years, the three-dimensional structure of most of the clotting factors has not been determined. Neither plasma-derived zymogens nor their activated forms could be crystallized in a form suitable for x-ray diffraction analysis. Crystals were obtained and a three-dimensional structure was elucidated at 2.8Å for the fragment 1 of bovine prothrombin (Park and Tulinsky, 1986). This revealed the folding of the first kringle but the Gla domain was disordered in the absence of calcium ions, as were the two polysaccharide chains of the molecule (Tulinsky et al., 1988). If crystallized in the presence of Ca^{++}, the Gla domain was structurally ordered (Soriano-Garcia et al., 1989). It appears that calcium ions induce the folding and are responsible for the maintenance of the integrity of the Gla domain. All the Gla residues of Ca^{++}-fragment 1 are on the surface of the molecule and most of them line the top edge of the domain, creating a potentially intense electronegative environment in the molecule. This region may bind to phospholipid through bridging calcium ions (Soriano-Garcia et al., 1989).

Recently, the crystal structures of PPACK-human thrombin (Bode et al., 1989; Bode et al., 1992) and hirudin-thrombin (Rydel et al., 1990; Rydel et al., 1991) complexes have been described. The NH_2-terminal domain of hirudin binds at the active site region and the long COOH-terminal tail occupies a narrow canyon, the exosite (Rydel et al., 1990).

NMR spectroscopy can also be a useful tool for the study of protein-peptide interactions in solution. Although the technique can not yet be applied to large proteins, it is relatively easy to assign all the proton resonances of short peptides (up to 20 amino acid residues) by using two-dimensional NMR spectroscopy (Ni et al., 1988). Upon interaction between a peptide and a protein, the peptide adopts a conformation whose structure can be determined. Such techniques have been applied to the interaction of thrombin with fibrinopeptide A (Ni et al., 1989a,b) and hirudin (Ni et al., 1990). The

secondary structure of the NH_2-terminal EGF module of factor X has also been determined by 2D-NMR spectroscopy (Selander et al., 1990).

E. Protein expression and engineering

The field of protein engineering offers a valuable tool toward the study of structure-function relationships in proteins in general. This allows the expression of wild type proteins as well as mutant variants. The main advantage of recombinant protein expression over purified proteins from natural sources such as blood is the possibility of isolating large quantity of mutant protein, which would be difficult if the protein in question had to be purified from a patient's plasma. It also allows for the design of mutants or chimeras that do not exist in nature. There is also a vast interest in expressing hemostatic proteins as therapeutic pharmaceuticals for the treatment of various deficiencies, and for pro- and anti-coagulant therapy.

One of the first recombinant human proteins involved in hemostasis was t-PA in *E. coli* (Pennica et al., 1983). Because polypeptides synthesized in *E. coli* are not glycosylated while most hemostatic proteins normally undergo this post-translational modification, subsequent expression systems were mostly developed in mammalian cells. A great number of coagulation and fibrinolysis proteins have now been successfully expressed. These include human factor IX (Busby et al., 1985; de la Salle et al., 1985; Kaufman et al., 1986; Rabiet et al., 1987; Jorgensen et al., 1987b; Lin et al., 1990; Hamaguchi et al., 1991b; Yao et al., 1991), human prothrombin (Jorgensen et al., 1987a; Le Bonniec et al., 1991; Wu et al., 1991; Falkner et al., 1992), human protein C (Grinnel et al., 1987; Yan et al., 1990), human plasminogen (Busby et al., 1991), human factor VIII (Toole et al., 1984; Wood et al., 1984; Hironaka et al., 1992), human von Willebrand factor (Bonthron et al., 1986; Verweij et al., 1987; Randi et al., 1992), human factor X (Wolf et al., 1991), human factor VII (Bjoern et al., 1991), human pro-urokinase (Lenich et al., 1992), and human factor XII (Citarella et al., 1993).

The expression of mutant recombinant proteins has given insight into the specificity of proteases toward their substrates, the function of various domains, the regions of molecules involved in binding and recognition, the importance and role of post-translational modifications such as γ-carboxylation, glycosylation and β-hydroxylation, the explanation for decreased activity of naturally occurring mutated proteins, etc...

V. GOALS OF THIS STUDY

The primary structure of the polypeptide chain of many proteins involved in hemostasis reveals a complex domain organization, as illustrated in Figure 9. To study the relationships between protein structure(s) and function(s), two human coagulation proteins, factor XII and prothrombin were investigated. Antibodies were used to determine regions or structures of factor XII involved in negatively charged surface binding or clotting activity. In an effort to relate a particular structure with a known function or characteristic of the protein, deletion mutants of the protein were expressed in mammalian cells. Similarly, prothrombin activation intermediates, including meizothrombin, were produced by protein engineering and characterized. The results of these experiments are discussed in the following sections.

MATERIALS AND METHODS

I. MATERIALS

Restriction endonucleases, T4 DNA ligase, T7 polymerase and *E. coli* DNA polymerase fragment 1 (Klenow) were purchased either from Bethesda Research Laboratories or Pharmacia. Sequenase version 2.0 was from USB and Recombinant *Taq* polymerase was from Perkin-Elmer-Cetus (Rexdale, Ontario). Bacteriophage arms (λgt11), λ phage packaging extracts (Gigapack Gold) as well as the *E. coli* strains used to propagate λ phage were purchased from Stratagene (La Jolla, Ca.). The Geneclean kit was from BIO 101(La Jolla, Ca.). Oligonucleotides were synthesized either on an Applied Biosystems 380A or 391A DNA Synthesizer. The anti-human factor XII mAbs B7C9 and C6B7 were provided by Drs. Robin Pixley and Robert Colman (Thrombosis Research Center and the Department of Medicine, Temple University, Philadelphia), K0K5 and F1 were from Dr. Erik Hack (Central Laboratory of the Red Cross Blood Transfusion Service, Amsterdam) and P-5-2-1 was from Dr. H. Saito (Saga Medical School, Nabeshima, Saga, Japan). Rabbit anti-mouse IgG alkaline phosphatase was from Promega Biotech. Sheep anti-human prothrombin was from Affinity Biologicals (Yarder, Ontario) and anti-sheep IgG alkaline phosphatase was from Chemicon (Temecula, Ca). The eukaryotic expression vector pNUT and the Baby Hamster Kidney (BHK) tk^- cell line were kindly provided by Dr. R. Palmiter (Howard Hughes Medical Institute, University of Washington). Dulbecco's modified Eagle Medium: Nutrient Mixture F-12 (ham) (1:1) powder, trypsin-EDTA (0.25% w/v) and new-born calf serum were purchased from Gibco (Grand Island, N.Y.). Protein molecular weight standards were from Gibco-BRL (Burlington, Ontario) or Pharmacia . Low protein serum replacement (LPSR-1), ecarin and the benzamidine-Sepharose were from Sigma

Chemical Co. (St-Louis, Mo.). Methotrexate sodium injection U.S.P. from Bull Laboratories (Mulgrave, Victoria, Australia) and vitamin K_1 from Sabex (Boucherville, Quebec) were purchased at the local hospital pharmacy. Human factor XII was purchased from Enzyme Research Laboratories Inc. (erl) (South Bend, In). The biotin-conjugated goat anti-mouse IgG antibody was from Jackson Laboratories (West Grove, Pa). The biotin/avidin/peroxidase complex (Vectastain ABC kit) was purchased from Vector Laboratories (Burligame, Ca). The Affi-Gel Hz Immunoaffinity Kit and the Bradford protein assay were purchased from Bio-Rad. The microscope used for immunocytochemistry studies was a Zeiss Axiophot. All fluorescence studies were performed with a Perkin-Elmer MPf-66 fluorescence spectrophotometer equipped with a model 7500 minicomputer, and the amidolytic assays were performed with a Titre-Tek Twin reader (Flow Laboratories) in the Department of Biochemistry at Queen's University, Kingston, Ontario. S-2238 was from Helena Laboratories (Mississauga, Ontario). S-2222 and S-2302 were from Kabi Vitrum (Stockholm, Sweden). Human coagulation factor II and XII deficient substrate plasma, as well as Actin FSL activated PTT reagent were from Baxter (Miami, Fl). Phospholipid vesicles (75% PC/25% PS) were prepared as described by Bloom et al., 1979. Factor X (Krishnaswamy et al., 1987) and factor V (Nesheim et al., 1981) were isolated as described previously. Human prothrombin was provided by Dr. M. E. Nesheim. The prothrombin activator of *E. carinatus* venum (Sigma) was isolated by anion-exchange chromatography and preparative electrophoresis in polyacrylamide as described previously (Boskovic et al., 1990).

II. STRAINS, VECTORS, AND MEDIA

A. Bacterial strains

E. coli strain DH5α (Hanahan, 1983) was the host for transformation and DNA isolation of clones in pUC 18, pUC 19 , Bluescript KS and SK and pNUT. *E. coli* strain JM 101 was the host for plasmids which had to undergo BclI restriction digest, the enzyme being sensitive to methylation. *E. coli* Y1088 and Y1090 (Young and Davis, 1983) were the host for screening and isolation of DNA from λgt11.

B. Vectors

Plasmids pUC 18, pUC 19 (Messing, 1983), Bluescript KS and SK (Yanisch-Perron et al., 1985) were used for cloning and isolation of DNA fragments. For expression in mammalian cells, the pNUT vector (Palmiter et al., 1987) was used.

C. Media

The medium for growth and screening of λ clones and bacterial hosts was NZCYM (Maniatis et al., 1982). The phage library was screened by plating the phage on NZCYM agar (1.5% w/v) plates with overlay of NZCYM agarose (0.8% w/v). The medium for growth of plasmid-containing bacteria was Luria Broth (LB) (Maniatis et al., 1982) supplemented with 100 μg/mL of ampicilin (AMP). For selection of plasmid-containing bacteria, the cells were plated on LB-agar (1.5% w/v) plates supplemented with 100 μg/mL AMP, 25 μg/mL IPTG and 50 μg/mL X-GAL. M13 phage were cultured in YT medium (Maniatis et al., 1982), or YT plates with an overlay of YT agarose (0.8%) supplemented with 25 μg/mL IPTG and 50 μg/mL X-GAL.

For transfection and selection, Baby Hamster Kidney (BHK) cells were cultured in DMEM-F12 supplemented with 5% new-born calf serum. During selection, the medium contained 0.44 mM methotrexate (MTX). For large scale expression, the cells

were cultured in DMEM-F12, 1% LPSR, with 10 μg/mL vitamin K_1 in the case of the prothrombin variants.

III. OLIGONUCLEOTIDES

Synthetic oligonucleotides were designed for use in DNA amplification (polymerase chain reaction), site-directed mutagenesis and DNA sequence analysis. The oligonucleotides used in the FXII studies are shown in Table 2 and those used in the prothrombin studies are listed in Table 3.

IV. EPITOPE MAPPING

A. Preparation of the λgt11 hFXII cDNA expression library

A factor XII cDNA expression library in λgt11 was prepared by Dr. Bryan J. Clarke (Clarke et al., 1989). Briefly, 100 μg of the plasmid pcHXII501 (Cool et al., 1985) was sheared by sonication, the resulting fragments were rendered blunt and were fractionated by electrophoresis in a polyacrylamide gel. Fragments in the 200-300 bp range were electroeluted from the gel, rendered blunt again and ligated to EcoR1 linkers. The library was constructed by ligation of the EcoR1-cut fragments with EcoR1-cut dephosphorylated λgt11 arms. The DNA was packaged into phage particles and the phage titer was estimated by plaque assay using the *E. coli* strain Y1088r$^-$.

B. Screening of the λgt11 expression library

Screening of the library was performed as previously described (Clarke et al., 1989). For mAb KOK5, the λgt11 library was plated and screened following which, the library was amplified by adding ~2 mL of phage diluent to each plate, to rescue the phage. The amplified library was subsequently stored at 4°C and used for screening the other antibodies. Recombinant phage were plated on *E. coli* Y1090r- and phage plaques

TABLE 2. Oligonucleotides used in the FXII studies

oligonuclotide	sequence	use
λgt11 F	5' ATCGCGGCCGCGACTCCTGGAGCCCG 3'	amplification of phage insert
λgt11 R	5' ATCGCGGCCGCGGTAGCGACCGGCGC 3'	amplification of phage insert
M13-20	5' GTAAAACGACGGCCAGT 3'	sequencing primer
Reverse	5' AACAGCTATGACCATG 3'	sequencing primer
FXII 5'	5' ACATCTAGAGTCGACGCGGCCGCCATGAGGGCTC TGCTGC 3'	5' end modification of hFXII cDNA
FXII BclI	5' CTGGTCCTGATCAAAGTTGGGG 3'	5' end modification of hFXII cDNA
FXII 3'	5' ACATCTAGAGTCGACTCAATCAATCAGCGGCCGCT CAGGAAACGGTGTGCTCCC 3'	3' end modification of hFXII cDNA
FXII XhoI	5' TCTGCGCAGGGTTCCTCGAG 3'	3' end modification of hFXII cDNA
FXII B7C9	5' ACATCTAGAGTCGACGCCATGAGGGCTCTGCTGCT CCTGGGGTTCCTGCTGGTGAGCTTGGAGTCAACACTT TCGCTCACTGTCACCGGGGAGCCCT 3'	deletion of amino acids 1-20 of hFXII cDNA
FXII Δ5-20	5' TTGGAGTCAACACTTTCGATTCCACCTTGGCTCACT GTCACCGGGGAG 3'	deletion of amino acids 5-20 of hFXII cDNA
FXII K0K5	5' CTGTCACCGGGGAGCCCTTGGAGCCCAAGAAAGTG AA 3'	deletion of amino acids 28-69 of hFXII cDNA

TABLE 3. Oligonucleotides used in the FII studies

oligonucleotide	sequence	use
FII 5'	5' ACACCCGGGCAGGAGCTGACACACTATGG 3'	5' end modification of hFII cDNA
FII HindIII	5' GCAGCAAGCTTATCTCGAGG 3'	5' end modification of hFII cDNA
FII 3'	5' ACATCTAGACGCTGAGAGTCACTTTTATT 3'	3' end modification of hFII cDNA
FII PstI	5' AGTGTCCTGCAGGTGGTGAA 3'	3' end modification of hFII cDNA
FII R155A	5' GATGACTCCAGCCTCCGAAGGC 3'	Arg155->Ala in hFII cDNA
FII R271A	5' CATCGAAGGGGCTACCGCCACA 3'	Arg271->Ala in hFII cDNA
FII R284A	5' CAATCCGGCGACCTTTGGCTCG 3'	Arg284->Ala in hFII cDNA
FII R320A	5' TCGACGGGGCCATTGTGGAG 3'	Arg320->Ala in hFII cDNA
FII SP1	5' CCTGGTGCTACACTACAGAC 3'	sequencing primer for hFII
FII SP2	5' TGACCACACATGGGCTCCCC 3'	sequencing primer for hFII
FII SP3	5' ACCTCAACTATTGTGAGGAG 3'	sequencing primer for hFII
MTMIN30	5' ACTATAAAGAGGGCAGGCTG 3'	sequencing primer for pNUT
pNUT 3'	5' CCCCAGTGCCTCTCCTGGCCCT 3'	sequencing primer for pNUT
primer B	5' GGAGTACTAGTAACCCTGGCCCCAGTCACG ACGTTGTAAA 3'	PCR mutagenesis oligonucleotide
primer C	5' CAGGAAACAGCTATGACCAT 3'	PCR mutagenesis oligonucleotide
primer D	5' GGAGTACTAGTAACCCTGGC 3'	PCR mutagenesis oligonucleotide

were lifted onto nitrocellulose filters. The filters were blocked in TBS buffer (20 mM Tris-HCl, 150 mM NaCl, pH 8.0) containing 0.05% Tween-20 and 0.25% gelatin for one hour at room temperature. The nitrocellulose filters were then incubated with ~20 μg/mL anti-factor XII monoclonal antibody in TBST, for two hours. The filters were rinsed with TBST and reaction of the antibody with the plaques was visualized by using alkaline phosphatase-conjugated goat anti-mouse IgG and the enzyme substrate BCIP and NBT. Positive phage were purified by two further cycles of immunological screening and isolated phage were obtained.

C. Epitope determination

For the mapping of mAb K0K5, phage lysates (50 mL) of each of the recombinant phage were prepared and phage DNA was isolated as described (Maniatis et al., 1982). Purified phage DNA was digested with EcoR1 and randomly ligated into EcoR1-cut M13mp18. Preparation of M13 single-stranded template DNA and subsequent sequence analysis by the chain termination method were as described (Messing, 1983, Sanger et al., 1977). For the mapping of mAbs C6B7 and F1, the pure recombinant phage were resuspended in phage diluent (20 mM Tris, 0.15 M NaCl, 10 mM $MgSO_4$, 2% gelatin (w/v)) and the phage DNA was amplified by subjecting 5 μL of the diluent buffer to the polymerase chain reaction (PCR) using oligonucleotides λgt11 F and λgt11 R (see Table 2). The PCR conditions were 94°C/20 sec, 55°C/20 sec, 72°C/20sec. The PCR product was then digested with EcoR1, separated on agarose gel, purified by using the Geneclean glass beads and ligated into EcoR1-digested Bluescript. Plasmid DNA was isolated by using the rapid plasmid procedure (Birnboim and Doly, 1979) and the sequence analyzed using the chain termination method (Sanger et al., 1977).

D. Competitive ELISA with short peptides

To define the immunoreactive epitope(s) further, short peptides were synthesized on *p*-methylbenzyl hydrylamine resin (Clark-Lewis et al., 1986). The resulting peptides were purified by reverse-phase high performance liquid chromatography (HPLC) by using a Vydac C4 (25 cm x 10 mm) column developed using a water/acetonitrile gradient in 0.1% trifluoroacetic acid. The composition of each peptide was verified by amino acid analysis. The synthetic peptides were dissolved at a concentration of 10 mg/mL in 0.1M acetic acid. Competitive ELISA of factor XII peptides was performed by incubating 10 ng of B7C9 antibody in 200 µL of TBS containing 0.05% Tween 20 and 0.25% gelatin with 0.01-1000 nmol of peptide in an equal volume of the above buffer for one hour at room temperature. The antigen/antibody mixtures (100µL) were then added in triplicate to Immulon-2 (Dynatech, Fisher) microtiter wells onto which 100 ng of factor XII 1-28 peptide diluted in 0.1 M sodium carbonate/sodium bicarbonate pH 9.6 had been allowed to bind by incubation in the microtiter well overnight at 4°C. B7C9 antibody binding to factor XII peptide 1-28 was detected by colorimetric assay using alkaline phosphatase-conjugated goat anti-mouse IgG and the enzyme substrate NBT and BCIP.

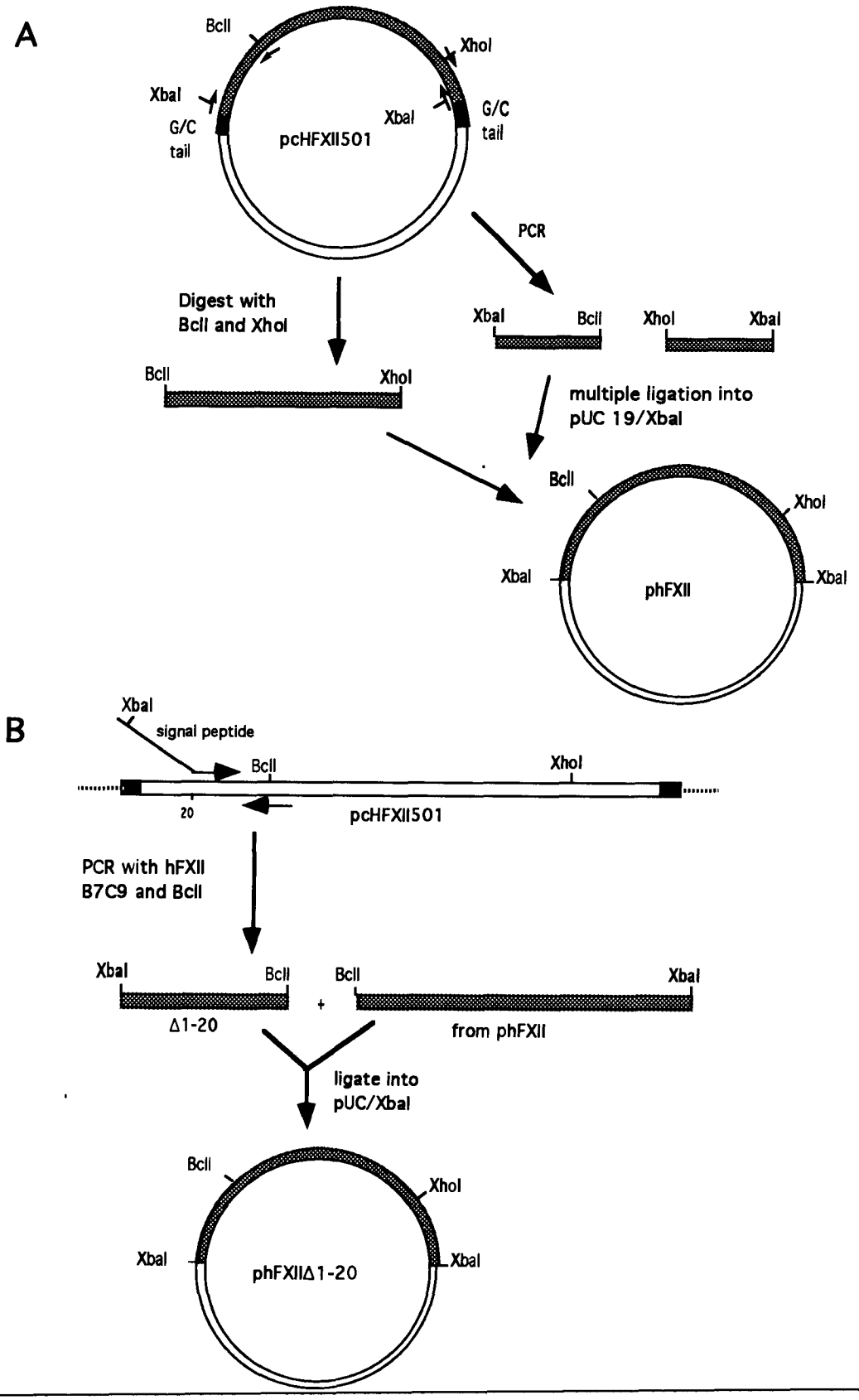
V. CONSTRUCTION OF EXPRESSION VECTORS

A. Assembly of the human FXII cDNA

The 5' and 3' ends of the human cDNA encoded by pcHXII501 were modified using the polymerase chain reaction (PCR) to eliminate G/C tails and unnecessary long untranslated regions (Figure 10A). The 5' and 3' ends of the cDNA were subjected to PCR using oligonucleotides FXII 5'-FXII BclI and FXII 3'-FXII XhoI respectively (see Table 2). The two fragments were rendered blunt-ended, cloned into the HincII site of pUC 19 and the DNA sequences were determined to ensure the absence of PCR errors. The plasmid containing the 5' end fragment was transformed into JM 101 to allow

Figure 10. Construction of the human FXII cDNAs

A, Strategy used for the assembly of the human wild type factor XII cDNA using PCR; B, Strategy used for the assembly of the human FXIID 1-20 cDNA using PCR; C, Strategy used for the assembly of the human FXIID 5-20 cDNA using PCR mutagenesis; D, Strategy used for the assembly of the human FXIID 28-69 cDNA using PCR mutagenesis.


A
BclI
XhoI
XbaI
G/C tail
XbaI
G/C tail
pcHFXII501
PCR
Digest with BclI and XhoI
XbaI
BclI
XhoI
XbaI
BclI
XhoI
multiple ligation into pUC 19/XbaI
BclI
XhoI
XbaI
phFXII
XbaI
B
XbaI
signal peptide
BclI
XhoI
20
pcHFXII501
PCR with hFXII B7C9 and BclI
XbaI
BclI
BclI
XbaI
Δ1-20
+
from phFXII
ligate into pUC/XbaI
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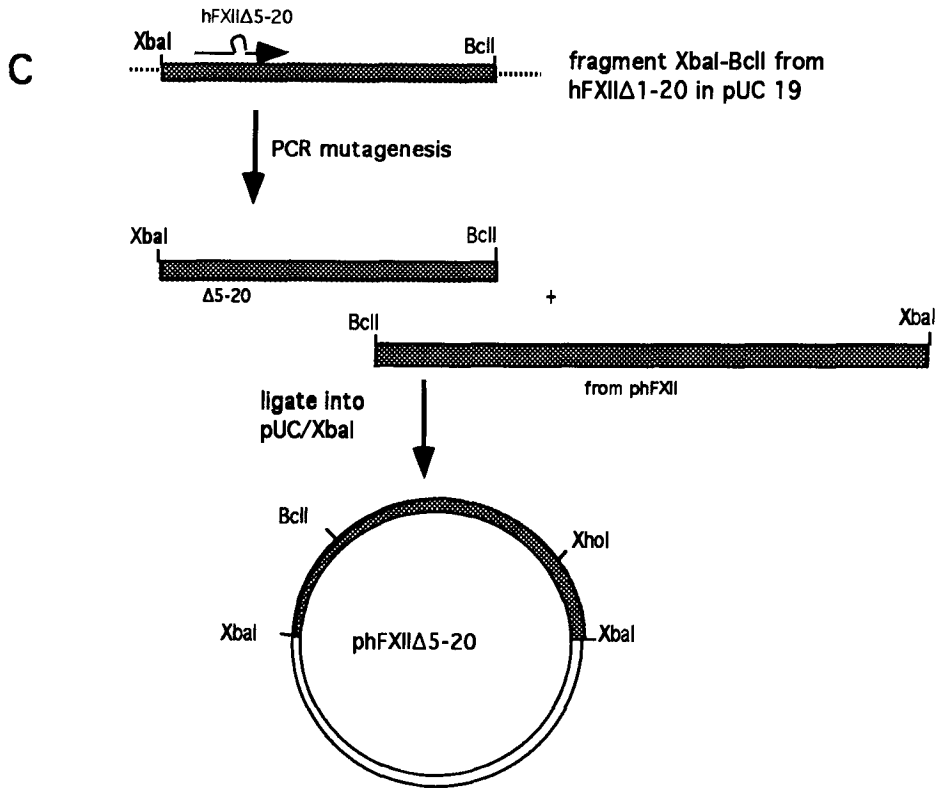
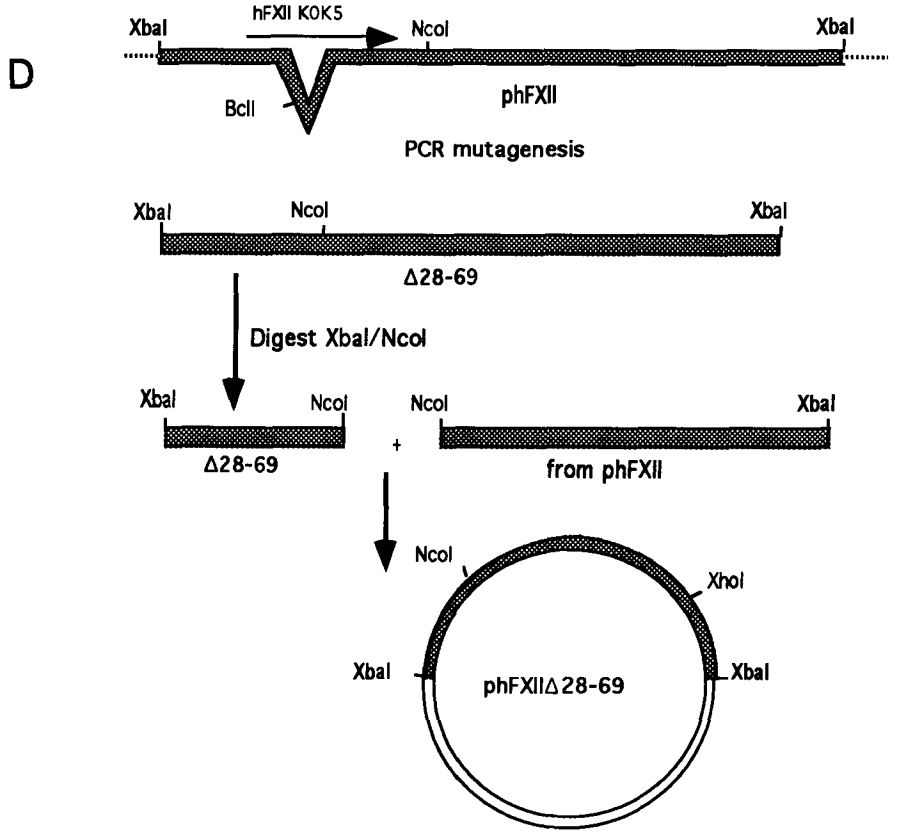
XbaI
phFXIIΔ1-20
XbaI


C
hFXIIΔ5-20
XbaI
BclI
fragment XbaI-BclI from hFXIIΔ1-20 in pUC 19
PCR mutagenesis
XbaI
BclI
Δ5-20
+
BclI
XbaI
from phFXII
ligate into pUC/XbaI
BclI
XhoI
XbaI
phFXIIΔ5-20
XbaI


D
XbaI
hFXII K0K5
NcoI
XbaI
BclI
phFXII
PCR mutagenesis
XbaI
NcoI
XbaI
Δ28-69
Digest XbaI/NcoI
XbaI
NcoI
+
NcoI
XbaI
Δ28-69
from phFXII
NcoI
XhoI
XbaI
phFXIIΔ28-69
XbaI

restriction digest with BclI which is methylation sensitive. The 5' and 3' fragments were then digested with XbaI/BclI and XbaI/XhoI respectively, isolated and ligated with the central BclI-XhoI fragment from the original plasmid, into the XbaI site of pUC 19. The new plasmid (phFXII) was analyzed by DNA sequencing to verify the integrity of the original restriction enzyme sites.

B. Preparation of the human factor XII cDNA mutants

To delete the region of the cDNA coding for amino acids 1 to 20, a 97 mer oligonucleotide was synthesized (FXII B7C9) (see Table 2). The 5' end of the wild type cDNA from phFXII was subjected to PCR using FXII B7C9 and FXII BclI (Figure 10B). The conditions for the reaction were the same as those used to modify the 5' end of the cDNA. The resulting PCR product was rendered blunt-end, ligated into pUC 19/HincII and transformed into JM 101. Following DNA sequence analysis, the plasmid was digested with XbaI and BclI and ligated with the BclI/XbaI 3' fragment of hFXII into pUC/XbaI to create phFXIIΔ1-20.

Oligonucleotide hFXII Δ5-20 (see Table 2) was used for mutagenesis on the XbaI-BclI fragment of hFXIIΔ1-20 to reintroduce the four first amino acids encoded by the hFXII cDNA. The mutagenesis was accomplished by using the PCR mutagenesis procedure described by Nelson and Long (1989) (Figure 11). The mutated XbaI-BclI fragment was then ligated with the 3' end of the hFXII cDNA originating from phFXII to create phFXIIΔ5-20 (Figure 10C).

The DNA encoding for amino acids 28 to 69 was deleted by using the PCR mutagenesis procedure using oligonucleotide hFXII K0K5 (see Table 2). The resulting PCR product sequence was analyzed, digested with restriction enzymes XbaI and NcoI and ligated with the 3' NcoI-XbaI fragment from phFXII to create phFXIIΔ28-69 (Figure 10D).

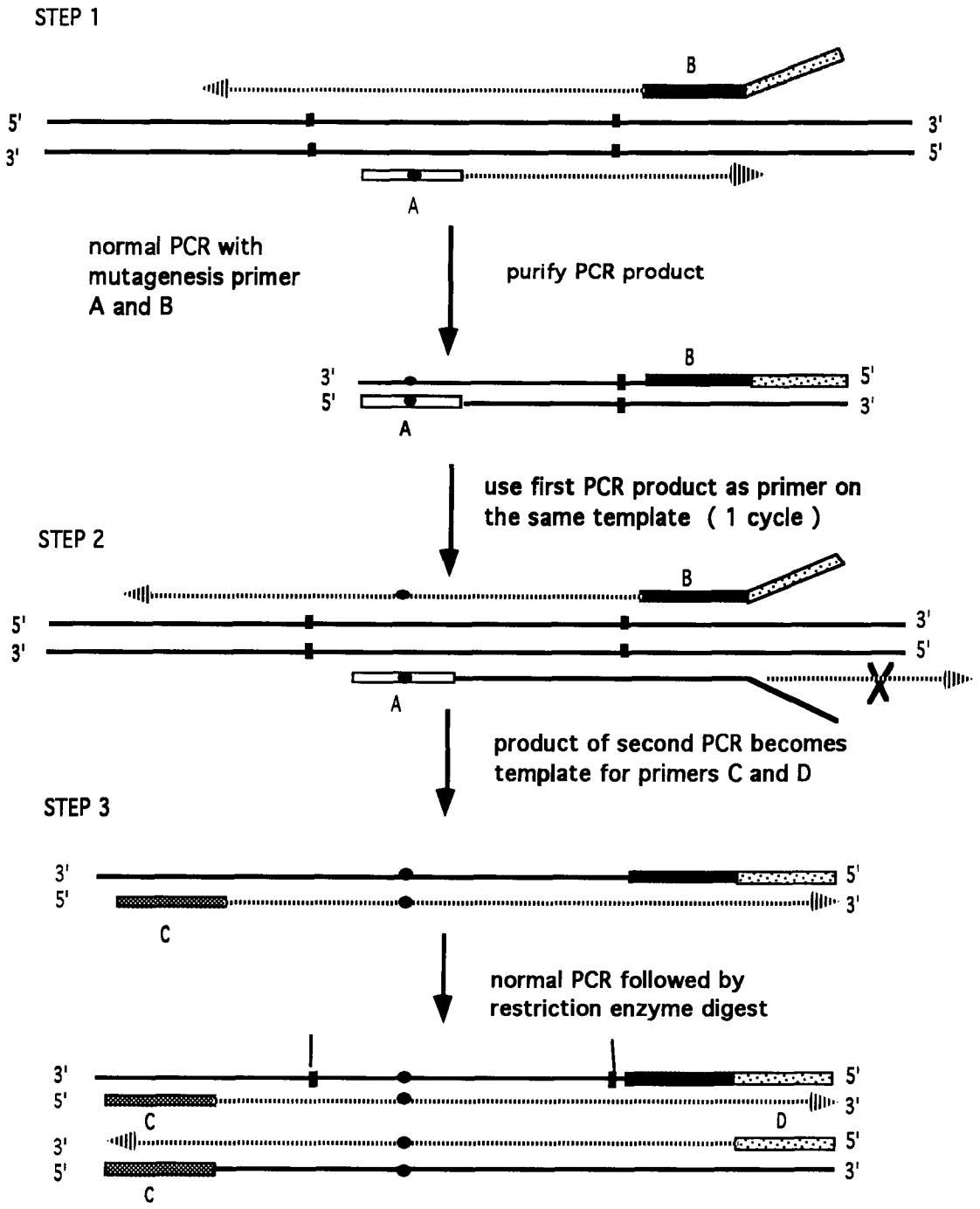


Figure 11. Polymerase chain reaction mutagenesis

(from Nelson and Long, 1989)

C. Assembly of the human prothrombin cDNA

The plasmid pIIH13 containing the full-length human prothrombin cDNA (MacGillivray et al., 1986) was modified using PCR to eliminate G/C tails (Figure 12). The 5' and 3' ends were subjected to PCR using oligonucleotides FII 5'-FII HindIII and FII 3'-FII-PstI respectively (see Table 2). The two fragments were cloned into Bluescript, analyzed by DNA sequencing to ensure the absence of PCR errors, redigested, isolated and ligated with the central HindIII-PstI fragment from the original plasmid, into Bluescript. The new plasmid (phFII) was analyzed by sequencing beyond the ligation sites to verify the integrity of the restriction enzyme sites.

D. Preparation of human prothrombin cDNA mutants

The HindIII-SstI fragment from phFII was subcloned into Bluescript and the three mutations R155A, R271A, R284A were introduced by using the dut^- ung^- mutagenesis technique (Kunkel, 1985) with oligonucleotides FII R155A, FII R271A and FII R284A respectively (this mutagenesis was done by David Banfield in this laboratory). The triple mutant HindIII-SstI fragment was then ligated back into phFII to create phMZ (Figure 12). The intermediate mutagenesis product containing the mutations R155A and R284A was ligated into phFII to create phDM. The SstI-PstI fragment from phFII was subcloned into Bluescript and the substitution R320A was accomplished by using the PCR mutagenesis procedure with oligonucleotide FII R320A. The resulting fragment was then ligated into phMZ to create phQM. The phPRE-2 variant was obtained by ligating the HindIII-SstI fragment from phDM and the SstI-PstI fragment from phQM with the rest of the phFII cDNA.

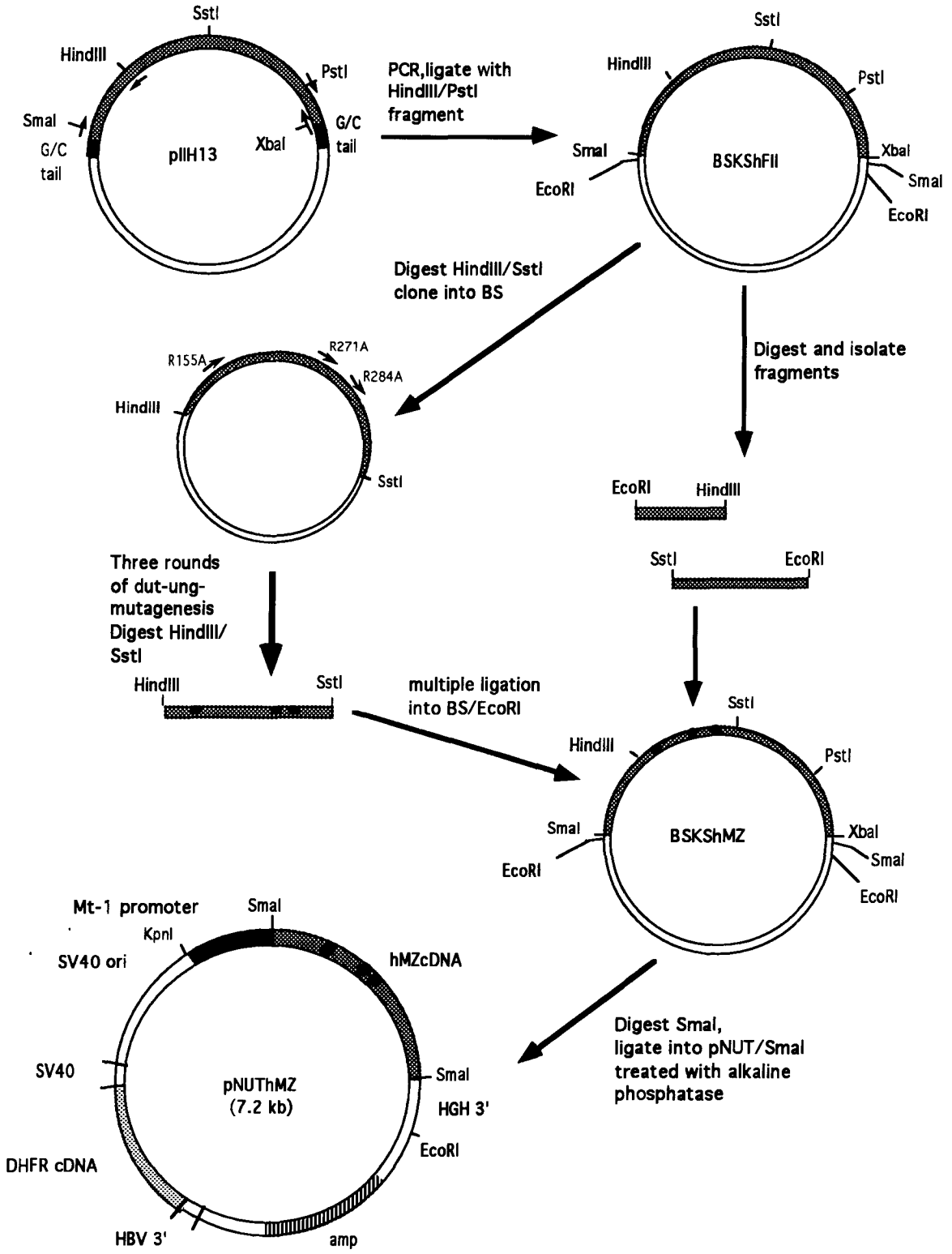


Figure 12. Cloning strategy for the human prothrombin and hMZ cDNA.
Construction of the hMZ expression vector in pNUT

E. Assembly of the cDNAs into the pNUT vector

The human factor XII cDNA was released from phFXII by digesting the plasmid with XbaI. The insert was separated on agarose gel, purified by Geneclean method and blunt-ended by using Klenow polymerase. The blunt-ended fragment was then ligated into the SmaI-cut pNUT vector that had been treated with alkaline phosphatase. Because pNUT does not allow for color selection and because the efficiency of such blunt-end ligation is low, a large number of bacterial colonies were analyzed. PCR was used to facilitate the analysis. Typically, 48 ampicilin resistant colonies were inoculated in 0.5 mL of LB broth containing 100 μg/mL Amp. The cultures were shaken at 37°C for approximately 2 hours. Once growth was visible, 5 μL of the culture were subjected to PCR using oligonucleotides FXII XhoI and PNUT 3'. The reaction mixture was preheated at 94°C for 3 minutes and the enzyme *Taq* polymerase was added. The amplification conditions were: 94°C/20 sec, 55°C/20 sec, 72°C/30 sec. The reaction product was then analyzed on agarose gel. Any PCR product of the expected size was indicative of a successful ligation-transformation but also of the proper orientation of the cDNA within the construct. Construction of the factor XII mutant cDNA expression vectors was performed in a similar fashion.

For the prothrombin expression vector, the plasmid phFII was digested with SmaI to release the cDNA insert which was then ligated into pNUT (Figure 12). Colony selection was achieved by using PCR with oligonucleotides FII PstI and pNUT 3'. Construction of all other variants of prothrombin was performed in a similar fashion.

VI. MAMMALIAN CELL CULTURE,TRANSFECTION AND SELECTION

Baby Hamster Kidney cells (BHK) were cultured in Dulbecco's modified Eagle's Medium: nutrient mixture F-12 (1:1) (DMEM/F-12) supplemented with 5% new-born

calf serum during transfection and selection. The cells were transfected by the calcium-phosphate coprecipitation technique (Searle et al., 1985). The DNA (20 μg) was ethanol-precipitated and the dried pellet was resuspended completely in 450 μL of sterile dH_20. The calcium-phosphate precipitate was formed by addition of 50 μL 2.5 M $CaCl_2$ and 500 μL of the 2X HBS solution (42 mM HEPES, 0.27 M NaCl, 10 mM KCl, 1.5 mM Na_2HPO_4, 8.4 mM sucrose, ph 6.95) and added to the cells (~50% confluence for the hFXII constructs and ~20% confluence for the hFII constructs) for a period of 5 to 7 hours. The cells were then rinsed to remove the DNA precipitate and after approximately 12 hours (for the hFXII constructs) or immediately (for the hFII constructs), the media was changed to DMEM/F-12/5% new-born serum containing 0.44 mM methotrexate (MTX). After approximately 12 days of selection, MTX-resistant colonies were isolated by trypsin treatment at the tip of a transfer pipette, grown to confluence in low-protein-serum-replacement (LPSR) medium and levels of expression were evaluated by Western blot analysis with anti-FXII mAb C6B7 or a sheep anti-human prothrombin antibody.

For factor XII, attempts were made to develop a sandwich ELISA using the various anti-FXII antibodies available in the laboratory. Unfortunately, even when a combination of murine mAb and rabbit polyclonal antibody was used, the first antibody was partly recognized by the secondary antibody and high background resulted. To avoid background, the medium being assayed was diluted in DMEM-F12/1% LPSR previously conditioned by culturing pNUT-transfected cells in roller bottles under identical conditions as recombinant cells. Following appropriate dilution, the medium was adsorbed to the microtiter wells by drying completely at 37°C overnight. The plasma-derived FXII standard curve and the negative control (conditioned media) were prepared the same way. The microtiter plate was then rinsed three times with TBST and blocked with a 2% BSA solution in TBST. The C6B7 mAb was chosen to detect the presence of FXII antigen because all the mutants retained the putative epitope. After rinsing three times again with TBST, a rabbit anti-mouse alkaline phosphatase conjugated antibody

was applied and the color developed by using NBT and BCIP. The reaction was followed by monitoring the change in absorbance at 405 nm.

For prothrombin, an ELISA using a sheep anti-human prothrombin polyclonal antibody was developed. Because the antigen levels were much higher in the case of prothrombin and related mutants, the media was diluted in carbonate antigen coating buffer (0.1 M sodium carbonate/sodium bicarbonate pH 9.6) and allowed to adsorb to the microtiter well at 4°C overnight. The standard curve and the negative control (conditioned media) were treated alike. Pure rhFII of determined concentration was used for the standard curve.

Constructs which did not demonstrate any secretion were further analyzed to detect expression. Briefly, the cells (~10^6) were treated with trypsin, resuspended in media, and recovered by centrifugation. The cells were resuspended in 2 mL of lysis buffer (50 mM Tris-HCl pH 8.0, 0.25 M sucrose, 5 mM DTT, 0.5 mM EDTA), with or without 0.1% Triton, and homogenized with a pestle. The cell lysate was then subjected to centrifugation and the two fractions (pellet and supernatant) were analyzed by SDS-PAGE and immunoblot.

The expressed but not secreted recombinant proteins were also visualized by immunocytochemistry. Recombinant-, pNUT-transfected- and wild type-BHK cells were cultured in small petri dishes, at low confluence. The cells were fixed in Bouin's solution (75% picric acid (v/v) (from a saturated solution), 25% formaldehyde (v/v) (from 37% stock solution), 3% acetic acid (v/v)) for 5-10 minutes at room temperature, and washed in PBS. The cells were incubated with anti-FXII mAb C6B7 at a concentration of ~20 µg/mL in TBST, at 4°C, overnight. Bound antibodies were localized using a biotin-conjugated anti-mouse IgG antibody at a dilution of 1:4000, for one hour at room temperature. The cells were then washed with PBS and incubated with avidin/biotin/peroxidase complex at a dilution of 1:1000 in PBS. The peroxidase reaction was developed with 0.25 mg/mL diaminobenzidine in PBS with 0.025% H_2O_2 for 10

minutes at room temperature following which the cells were washed in PBS. Coverslips were applied with PBS:glycerol (1:9) and the cells were screened using a Zeiss Axiophot microscope under oil immersion.

VII. EXPRESSION OF RECOMBINANT PROTEINS

The highest expressing-secreting clones were seeded into roller bottles (~4 x 10^6 cells). For large scale expression, the cells were cultured in DMEM/F-12, 1% LPSR and 10 μg/mL vitamin K_1. The media (250 mL) was collected every two to three days during the first two weeks and every day subsequently. A 2 mL aliquot of media was withdrawn and stored each time media was collected and expression levels were monitored by ELISA.

VIII. RECOMBINANT PROTEIN PURIFICATION

A. Purification of human factor XII

1. From human plasma

Fresh frozen human plasma (440 mL) was provided by Dr. Dana V. Devine (Canadian Red Cross, B.C. and Yukon Division). Soybean trypsin inhibitor was added for a final concentration of 100 μg/mL. The plasma was precipitated with 25% ammonium sulfate and stirred at 4°C for one hour. The suspension was subjected to centrifugation at 10 000 rpm for 10 minutes, the supernatant brought to 50% ammonium sulfate, stirred, and spun down as before. The pellet was dissolved in 250 mL of 50 mM Tris-HCl, 0.15 M NaCl, pH 8.0. The solution was then dialyzed against four times 4 L of the same buffer.

Several affinity columns were prepared by coupling monoclonal antibodies to the agarose support matrix Affi-Gel Hydrazide. Coupling of the antibody through the

carbohydrate group to form stable covalent hydrazone bonds was performed according to the manufacturer's protocol.

The ammonium-cut solution was loaded onto a anti-FXII mAb C6B7-affinity column, under gravity, at room temperature (flow rate ~1 mL/min). The column was washed extensively with 20 mM Tris-HCl, 0.15 M NaCl, pH 8.0, followed by another wash at higher salt concentration (20 mM Tris-HCl, 0.5 M NaCl, pH 8.0) until no A_{280} reading was detectable in the flow-through. The protein was eluted (2 mL fractions) with 0.05 M sodium citrate pH 2.2 and immediately neutralized to pH ~7 by addition of 1.0 M Tris pH 10.6. The elution of the protein was followed by absorbance at 280 nm ($E^{1\%}_{280}$ = 14.2). The peak fraction were pooled and concentrated on a centricon with a PM30 membrane.

2. From culture medium

The collected medium was passed directly through the C6B7 column. The column was washed and the protein eluted as described for plasma FXII.

B. Purification of rhFII

Recombinant prothrombin and other variants were adsorbed by the slow addition of 1/25 volume of 0.4 M sodium citrate and 1/10 volume of 1.0 M $BaCl_2$. The resulting suspension was stirred for 45 minutes at 22°C. The precipitate was collected by centrifugation (approximately 5 000 x g, 4°C, 10 minutes) and the barium-citrate pellet was washed four times with successive volumes of 0.1 M $BaCl_2$ comprising 1/2, 1/4, 1/8 and 1/8 of the original medium volume. The adsorbed protein was eluted by dissolving the pellet in 0.2 M EDTA pH 8.0 (1/6 of the original volume). The solution was clarified by centrifugation and was subsequently dialyzed and concentrated against 20 mM Tris-HCl pH 7.4, 1 mM benzamidine using an Amicon ultrafiltration stirred cell with a PM10 membrane at 4°C. The protein was then subjected to anion exchange chromatography on

a Pharmacia FPLC Mono Q HR 5/5 column. The column was rinsed with 5.0 mL of starting buffer and the protein was eluted with a 0 to 1.0 M NaCl linear gradient in 20 mM Tris-HCl pH 7.4 at room temperature (26.0 mL total volume, flow rate 1.0 mL/min). rhMZ eluted at approximately 0.45 M NaCl. The peak fractions, identified by absorbance at 280 nm, were pooled and dialyzed against 20 mM Tris-HCl, 0.15 M NaCl pH 7.4 or diluted 1/10 with starting buffer. The sample was loaded onto the same FPLC column and eluted with a 0 to 30.0 mM $CaCl_2$ linear gradient in the same buffer (15.0 mL total volume, flow rate 1.0 mL/min). This step was performed to resolve fully γ-carboxylated from partially γ-carboxylated species of rhMZ (Yan et al., 1990). The first and main peak eluted at 15.0 mM $CaCl_2$ while the second peak extended between 18.0 and 25.0 mM $CaCl_2$. Fractions from each peak were pooled and precipitated against 75% ammonium sulfate. All protein concentrations were determined by absorbance readings at 280 nm ($E^{1\%}_{280}$=13.8) (Mann et al., 1981). The material eluted in the first and second peak will be referred to as rhMZ(I) and rhMZ(II). Purification of the other prothrombin mutants was performed similarly although they were not all separated on the $CaCl_2$ gradient.

IX. AMINO-TERMINAL SEQUENCE ANALYSIS

For rhFXII, rhMZ and rhII, partially purified protein (~90% pure as estimated on SDS-PAGE) was separated on a 10% SDS-PAGE. The protein (~80 μg) was then transferred onto an immobilon membrane. The membrane was stained using Coomassie Brilliant Blue with 20% methanol and destained by diffusion. The clearly visible protein bands were then excised and sent to the University of Victoria Microsequencing Facility for sequence analysis. The amino-terminal sequences of rhFXII, rhMZ, and rhII were determined on an Applied Biosystems 473 pulse liquid protein sequencer or an Applied Biosystems 470 gas phase protein sequencer, according to the manufacturer's guidelines.

X. RECOMBINANT MUTANT PROTHROMBIN STUDIES

A. Ca^{++} binding properties

The Ca^{++} binding properties of populations of the recombinant prothrombin variants were inferred by the decrement in intrinsic tryptophan fluorescence as described (Nelsestuen, 1976; Prendergast and Mann, 1977). The sample of protein (1.6 mL, 30.0 μg/ml) in 0.02 M HEPES, 0.15 M NaCl pH 7.4, was placed in a quartz cuvette equipped with a microstirrer in the temperature regulated (22°C) sample holder of a Perkin Elmer model MPF-66 fluorescence spectrophotometer. Intrinsic fluorescence was continuously monitored with λ_{ex}= 280 nm, λ_{em}= 340 nm, with respective excitation and emission band passes of 4 nm and 20 nm, and with a 290 nm cut-off filter in the emission beam. An aliquot (8.0 μL) of $CaCl_2$ was added to give a final concentration of 5.0 mM and the time course (approximately 5 minutes) of the fluorescence decrease was monitored. The progress curves were typified by an initial rapid exponential decrease followed by a modest linear decrease thereafter. The magnitude of the initial decrement was determined by extrapolation of the linear portion to the time of addition of $CaCl_2$. The increments in fluorescence were compared to that obtained with plasma prothrombin under identical conditions.

B Phospholipid binding properties

The phospholipid binding properties of the populations of rhMZ were inferred by right angle light scattering as described before (Nelsestuen and Lim, 1977; Bloom et al., 1979). Aliquots of the proteins were dialyzed at 4°C overnight against 0.1 M HEPES, 0.075 M NaCl, 5.0 mM EDTA, pH 7.4. The samples were then centrifuged at 12,000 x g and 22°C for 10 minutes to remove any particulate material. Samples of protein were then diluted into the same buffer (filtered, 2.2 μm) to a final concentration of 15.0 μg/mL in a thermally regulated (22°C) quartz cuvette equipped with a microstirrer in the sample

holder of the fluorescence spectrophotometer. The right angle scattering intensity at 320 nm then was monitored continuously over time by setting the excitation and emission monochromators to 320 nm, both with 10 nm slit widths. An aliquot of concentrated PCPS vesicles was then added to yield a final PCPS concentration of 33.0 μg phospholipid/mL. The addition of vesicles produced an immediate and stable increment in scattering. An aliquot (16.0 μL) of 1.0 M $CaCl_2$ then was added to induce the Ca^{++}-dependent protein-phospholipid interaction. The time course of the change in scattering was monitored until a stable increment was obtained (<5minutes). The fluorescence intensities before and after Ca^{++} addition were averaged for two minutes each, with data collected at 0.1 second intervals. The increments in light scattering were compared to those obtained with plasma prothrombin under identical conditions.

C. Activation of human pII and recombinant prothrombin variants

Reaction mixtures (2.4 mL) containing the protein sample (1.4 μM), 5.0 mM $CaCl_2$, and 10.0 μM PCPS with or without 3.0 μM DAPA were prepared in 20.0 mM Tris-HCl, 0.15 M NaCl pH 7.4, at 22°C and were placed in a quartz cuvette equipped with a microstirrer in the sample holder of the fluorescence spectrophotometer. The reactions were initiated by the addition of either factor Xa (0.62 nM) or ecarin (1.3 μg/mL) and were monitored continuously by recording fluorescence intensity. When factor Xa was used, factor Va (2.0 nM) was included also. In the presence of DAPA, measurements of extrinsic fluorescence of the DAPA-product complex were made with excitation and emission wavelengths of 335 and 545 nm, respectively, with respective slit widths of 10 and 20 nm, and a 430 nm cut-off filter in the emission beam. In the absence of DAPA, intrinsic fluorescence was monitored with excitation and emission wavelengths of 280 and 340 nm, respectively, with respective slit widths of 3 and 10 nm, and a 290 nm cut-off filter in the emission beam. The reactions were followed until a stable reading was reached, typically 10 to 25 minutes.

D. Analysis of activation by SDS-PAGE

From the above reactions, aliquots of 100.0 µL were withdrawn at intervals and added to 200.0 µL of 0.2 M acetic acid. These solutions were then lyophilized and the residues dissolved in 50.0 µL of 14.0 mM HEPES, 105.0 mM NaCl pH 7.4, 4 % (w/v) Bromophenol Blue, 10 % (v/v) glycerol and 1 % (w/v) SDS. The reconstituted samples were divided in two equal aliquots and 2-mercaptoethanol (1.0 µL) was added to one of the aliquots. Both were heated at 90°C for 2 minutes and subjected to electrophoresis in 5 - 15 % polyacrylamide gradient gels using the buffers and conditions described by Neville, 1971. The gels were stained with Coomassie Brilliant Blue and destained by diffusion. In some instances gels were scanned with an LKB model 2202 laser densitometer.

E. Functional studies

a. Fibrinogen clotting assays

To a 10 x 75 mm glass tube were added 100.0 µL of human fibrinogen (2.0 mg/mL) dissolved in 20.0 mM Tris-HCl, 0.15 M NaCl, pH 7.4 and 100.0 µL of 20.0 mM Tris-HCl, 0.15M NaCl, 5.0 mM $CaCl_2$, pH 7.4. The contents were equilibrated at 37°C (approximately 30 seconds) and the reaction was initiated by the addition of 20.0 µL of sample (fully activated plasma prothrombin or rhMZ(I)a) at various dilutions in assay buffer containing 0.01% tween 80. The time required for clot formation was determined manually at 37°C.

b. Esterase assays

A cuvette containing 0.87 mL of 50 mM Tris-HCl, pH 8.1 and 30.0 µL of sample (fully activated plasma prothrombin or rhMZ(I)a) at a final concentration of 0.14 µM was maintained at 22°C in a quartz cuvette in a the sample compartment of a Perkin-Elmer

λ4B spectrophotometer. The reaction was initiated by addition of 0.1 mL of 0.01 M TAME (in H_20) and followed at 247 nm at 30 second intervals for 10 minutes. An extinction coefficient of 409 M^{-1} cm^{-1} for the TAME hydrolysis product was used for calculations.

c. Amidolytic assays

Samples (fully activated plasma prothrombin or rhMZ(I)a) were diluted in 20.0 mM HEPES, 0.15 M NaCl, 0.01% tween-80 pH 7.4 and 150.0 µL aliquots were pipetted into the wells of a microtitre plate. The solutions were warmed to 37°C and the assays were initiated by the addition of 150.0 µL of 0.4 mM S-2238 dissolved in assay buffer. The conversion of S-2238 was followed by monitoring the absorbance at 405 nm at 30 second intervals for 10 minutes at 37°C.

d. Coagulation assays

Coagulation assays were performed with the various mutants as well as plasma-derived prothrombin using human prothrombin-deficient plasma. The concentration of each protein was determined by absorbance at 280 nm. The human plasma prothrombin was diluted in series from 20 µg/mL to 0 in 20 mM HEPES, 0.15 M NaCl, pH 7.4, for the standard curve. The prothrombin-deficient plasma was resuspended in dH_20. For each assay, 50 µL of pII-deficient plasma and 50 µL of the diluted sample (20 µg/mL) (rhFII, rhMZ(I), rhMZ(II), rhDM, rhPRE2 and rhQM) were mixed in a plastic tube and prewarmed at 37°C for 2 minutes. The assay was initiated by addition of 100 µL of thromboplastin reagent diluted in 20 mM $CaCl_2$, according to the manufacturer. The tube was tilted at 37°C and the time required for clot formation was recorded manually.

F. Preparation of rMZ(I)a with ecarin

rMZ(I) isolated from the Ca^{++} gradient was dialyzed against 75% ammonium sulfate and the precipitate resuspended in 50% glycerol. For the isolation of active rMZ(I), the protein (~2 mg) was resuspended in 10 mL of 20 mM Tris-HCl, 0.15 M NaCl, 5 mM $CaCl_2$, pH 7.4. Approximately 17 µg of ecarin was added and the suspension was shaken gently for 25 minutes at 22°C. The activation was followed either by fluorescence or by assaying amidolytic activity with S-2238 as described previously. rhMZ(I)a was recovered by chromatography on benzamidine-sepharose. The column was equilibrated and washed with 15 mL of 20 mM Tris-HCl, 0.15 M NaCl, pH 7.4, and eluted with 7.5 mM benzamidine in the same buffer (flow rate ~ 2 mL/min). Fractions of 1 mL were collected and the protein was detected using BioRad Bradford microassay procedure. The fractions containing the active protein were pooled and dialyzed against 20 mM Tris-HCl, 0.15 M NaCl, pH 7.4 prior to precipitation against 75% ammonium sulfate. The precipitate was then resuspended in 50% glycerol and kept at -20°C until used.

G. Stability of rhMZ(I)a

A sample of rhMZ(I) was activated with prothrombinase as indicated above. The reaction solution was then stored at 4°C and at intervals up to 28 days, aliquots (100 µL) were removed. Assays of activity against TAME and S-2238 were performed, and samples were subjected to electrophoretic analysis by SDS-PAGE under reducing and non-reducing conditions.

H. Ca^{++} titration of amidolytic activity

To a microtiter plate were added 150 µL of $CaCl_2$ dilutions (from 25.6 to 0 mM), and 50 µL of plasma thrombin or rhMZ(I)a for a final concentration of 1 nM in the assay. The plate was incubated at 37°C for 5 minutes before the addition of 100 µL of S-2238

(0.25 mM final concentration). The conversion of S-2238 was followed as described before.

I. Inhibition studies with rhQM and F1

Recombinant hQM was purified as described for rhMZ and the fully γ-carboxylated species rhQM(I) was isolated on the $CaCl_2$ gradient. Human plasma prothrombin fragment 1 was isolated as described previously (Stevens and Nesheim, 1993). To a microcuvette were added factor Va (2 nM), PCPS vesicles (10 μM), $CaCl_2$ (5 mM), DAPA (1.5 μM), human thrombin (0.5 μM) (final concentrations in TBS pH 7.4, final volume: 200 μL) and various molar ratios of rhQM(I) or human prothrombin fragment 1 (F1) in 50% glycerol. Because the stock solution of rhQM(I) and F1 were in 50% glycerol, the negative control and the assays were all performed in the presence of an equal volume of 50% glycerol. The reactions were initiated by the addition of factor Xa (1nM) and were monitored continuously by recording fluorescence intensity. The excitation and emission wavelengths were 280 and 545 nm respectively.

RESULTS

1. ANTI-FXII MONOCLONAL ANTIBODY EPITOPE MAPPING

This laboratory was provided with a number of murine monoclonal antibodies raised against human factor XII (Table 4). Preliminary work with these mAb (by the investigators who raised them) indicated that they affected some functions of FXII.

mAb B7C9 raised in Dr. Colman's laboratory is described in the introduction, and shows the same properties as mAb P-5-2-1, raised in Dr. Saito's laboratory. Both antibodies have been shown to inhibit the activation of factor XII zymogen by negatively charged surfaces (Pixley et al., 1987; Saito et al., 1985). B7C9 had also been shown to react with reduced FXIIa by ELISA and Western blot analysis indicating that the epitope may be primarily determined by a linear sequence of the protein.

A. Screening of λgt11 expression library

In previous studies, Dr. Bryan Clarke prepared fragments of the FXII cDNA which were then expressed as fusion proteins in a λgt11 expression library. The library was screened using mAbs B7C9 and P-5-2-1 as described in the Materials and Methods section. Strongly positive phage (20) were plaque purified and the cDNA insert was isolated from each phage. The cDNA sequences of 16 of the inserts were then analyzed in order to determine which region of the protein was encoded by the reacting fusion protein. Two factor XII inserts, contained in clones 9 (amino acids -6 to +31) and 16 (amino acids +1 to +47) limit the B7C9 epitope to the amino-terminal 31 amino acids of factor XII (Figure 13). Each of the other 14 insert DNAs also encoded for this region but were longer. To determine if mAb P-5-2-1 recognized the same region in FXII, 14 of the 16 phage clones for which the insert had been analyzed were immunoscreened with P-5-2-1 under identical conditions as those used with B7C9.

Table 4. Characteristics of anti-FXII monoclonal antibodies

anti-FXII mAb	epitope	characteristics
B7C9	heavy chain	- inhibits surface-mediated contact activation - reacts under reduced conditions
F1	heavy chain	- slightly inhibits clotting (~15%) - no effect on amidolytic activity - epitope accessible in cleaved FXII (FXIIa) or upon binding to a negative surface - induces activation of the contact system in plasma - does not react under reduced conditions
KOK5	heavy chain	- inhibits clotting - binds to the zymogen FXII - does not react under reduced conditions
B6C7	FXIIf	- inhibits activation of FXII - does not react under reduced conditions

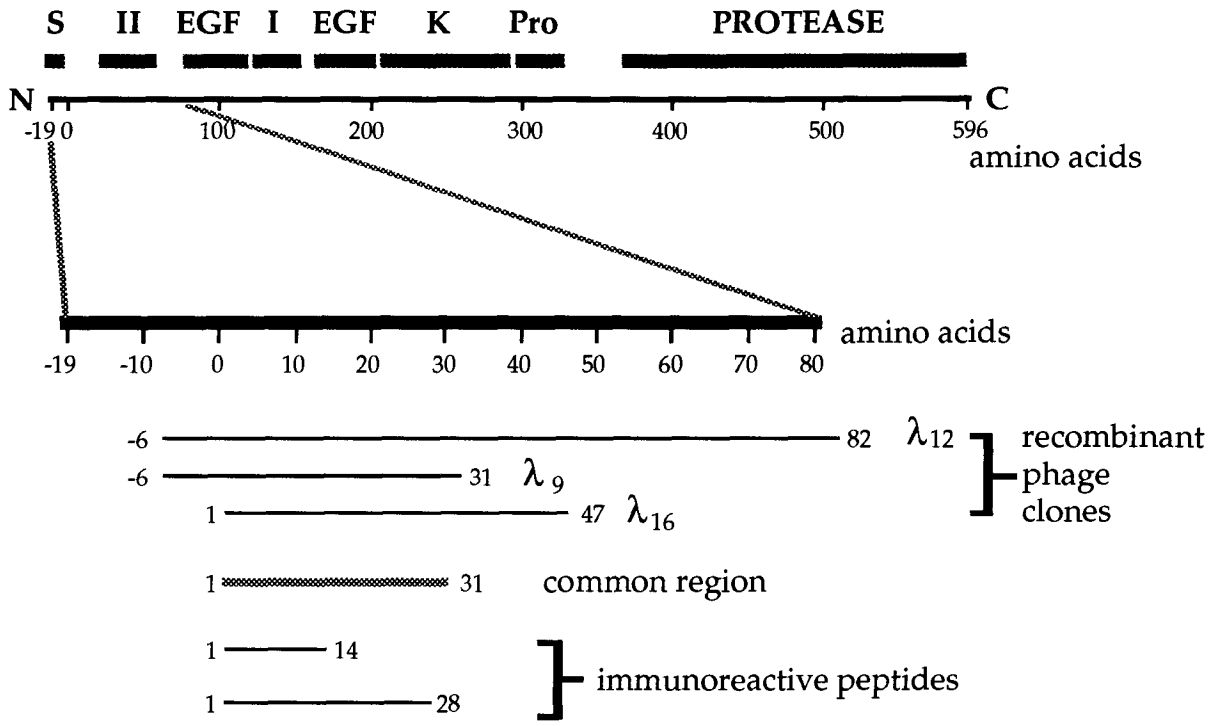


Figure 13. Molecular mapping of the putative surface-binding epitope of human factor XII using recombinant techniques.

The polypeptide chain of prefactor XII (amino acids -19 to +596) is indicated by the line from the amino terminus (N) to the carboxyl terminus (C). Regions of homology are indicated above the polypeptide chain and include a signal peptide (S), a fibronectin type II homology (II), two epidermal growth factor-like regions (EGF), a fibronectin type I homology (I), a kringle (K), a proline-rich region (Pro), and the serine protease domain (PROTEASE). The region from amino acids -19 to +80 is expanded below the polypeptide chain. The region of factor XII encoded by the three recombinant phage clones λ12, λ16 and λ9 are indicated together with the common region encoded by all 16 immunoreactive phage. Also shown are the positions of the two immunoreactive synthetic peptides.

For every phage tested, the plaques were strongly reactive to P-5-2-1 (Clarke et al., 1989).

B. Binding of mAb B7C9 to synthetic peptides

To establish the reactivity of the B7C9 monoclonal antibody with the amino-terminal region of factor XII and to define the immunoreactive epitope(s) implicated in the surface-mediated binding of FXII, a set of four peptides was synthesized. Those included amino acids 1-28, 5-28, 9-28 and 14-28 of FXII. A control peptide containing residues 1-17 of interleukin-3 (Clark-Lewis et al., 1986) was also synthesized. The binding of the B7C9 antibody was tested by slot blot analysis using the alkaline phosphatase-conjugated antibody as described for the phage screen. The B7C9 mAb reacted only with the peptide containing amino acids 1-28 of FXII. To test the specificity of this binding, a duplicate blot of peptides was incubated with anti-FXII mAb KOK5 (Table 4) which does not inhibit the surface-mediated activation of FXII. This antibody bound to none of the peptides (data not shown).

These results confirmed the previous data that the B7C9 epitope resides in the first 31 amino acids of FXII but suggested that amino acids 1-4 are critical for binding of the antibody. Because different peptides may not bind quantitatively to nitrocellulose, an ELISA assay was established to test the ability of B7C9 to react with peptides bound to microtiter dish wells. Again, only peptide 1-28 reacted with the mAb, confirming the blot results. At that time, Dr. Bryan J. Clarke left the laboratory and returned to McMaster University.

To map the epitope more precisely, a competitive ELISA was established in order to test the ability of various peptides to block the binding of B7C9 to 33 nmol of peptide 1-28 that had been immobilized on the microtiter dish well. When peptides 1-28, 5-28, 9-28 and 14-28 were tested, only 1-28 competed for the antibody binding (Figure 14). In addition, peptide 1-14 competed with peptide 1-28, although a 10-fold higher

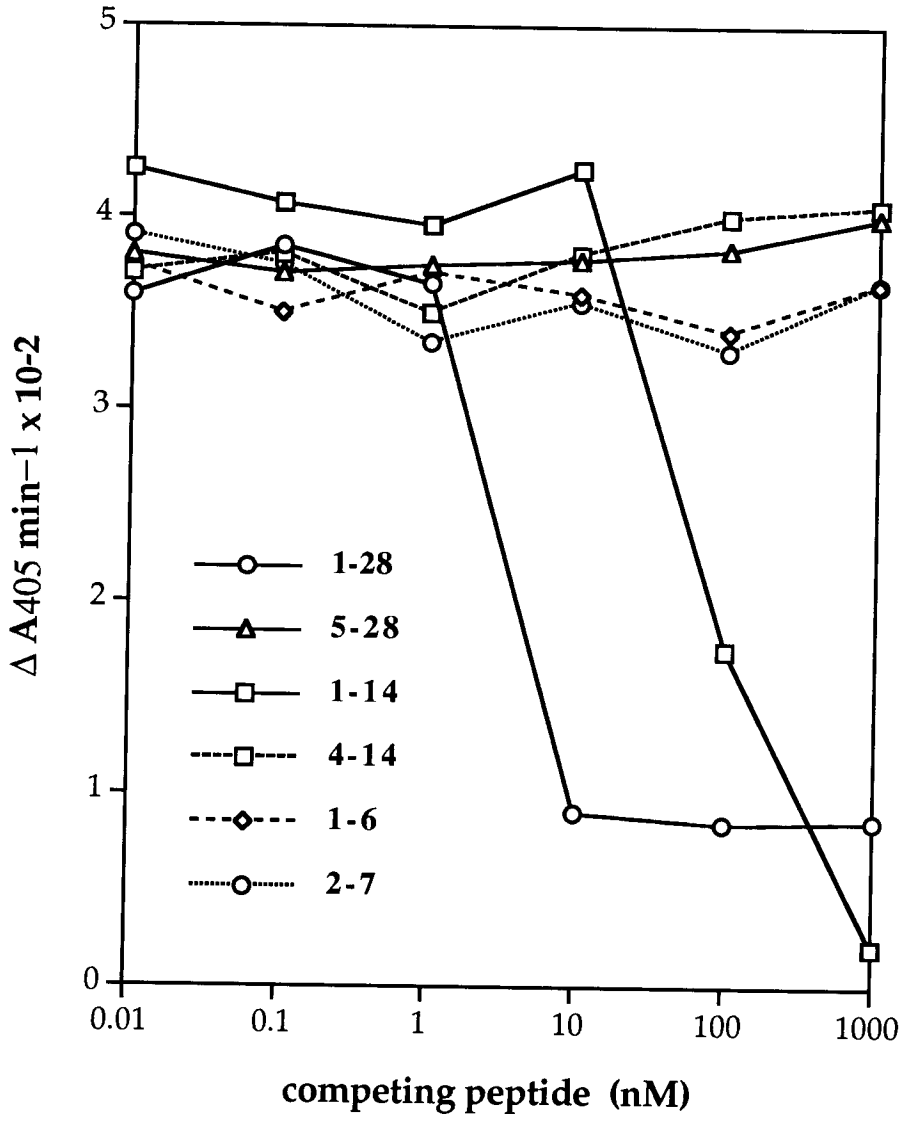


Figure 14. Competitive ELISA of anti-FXII mAb B7C9 binding to synthetic peptide 1-28 in the presence of other peptides

Synthetic factor XII peptides were preincubated with 10 ng of purified B7C9 antibody followed by ELISA. The various peptides are indicated in the legend.

concentration of peptide 1-14 was required to obtain the same degree of competition as with peptide 1-28. Interestingly, peptide 4-14 did not compete at all (Figure 14).

Because these results suggested that the region from 1 to 4 was important for binding of B7C9, a series of short peptides (1-4, 1-5, 1-6, 2-7, 3-8, and 4-9) were synthesized and tested in the competitive ELISA. None of them affected binding at the concentrations used (up to 1 μM) (Figure 14).

Taken together, these results suggest that residues 1-4 are essential but not sufficient for binding of mAb B7C9. Moreover, although peptide 1-14 was sufficient to compete for binding, it was not as effective as peptide 1-28, suggesting that the epitope may also involve some as yet undefined secondary structure.

C. Screening of other mAbs

The results obtained for the epitope mapping of mAb B7C9 encouraged the application of the same technique to other anti-FXII mAbs known to affect the function of FXII. Monoclonal antibodies F1 and B6C7 have been described previously (see Introduction section). Additionally, mAb KOK5, which inhibits clotting, was provided by Dr. Hack (Amsterdam). These antibodies had been partially characterized. They were also tested on Western blot for their ability to bind native human plasma FXII under reducing and non-reducing conditions. Table 4 offers a summary of their characteristics. Unlike B7C9, mAbs B6C7, F1 and K0K5 did not react with native FXII under reduced conditions. This is an indication that the epitope might be dependent on a particular conformation of the molecule rather than a linear sequence.

All three monoclonal antibodies were used to screen the λgt11 fusion expression library by using the same procedure applied to mAb B7C9. The library screened with K0K5 was the same one used for B7C9 while C6B7 and F1 were studied after the library had been amplified. For mAb K0K5, 35 positive phages were identified, of which 29 were cloned and analyzed. All clones shared a common region for which the shortest

overlap, defined by clones 8AK and 21AK, spanned amino acids Pro^{27}-Lys^{73} (Table 5). The region of FXII identified as the putative epitope(s) of KOK5 corresponds to the fibronectin type II homology. The finger-like domain is bordered by two cysteine residues (Cys^{28} and Cys^{69}) which form a disulfide bond. The presence of the disulfide bond appears necessary for binding to the antibody as all the positive clones encoded both cysteines. It is unclear whether *E. coli* is capable of folding properly the short fusion proteins and of forming disulfide bonds, but this result suggests that it probably does.

Antibodies B6C7 and F1 did not react as strongly with phage plaques in general, and as a result, fewer strong positives were identified. Because the library had been amplified, several positives encoded the same region of the protein. Table 6 and 7 describe the clones reacting with each antibody and the amino acids they encoded. The shortest overlapping region encoded by clones reacting with mAb C6B7 comprised amino acids Gly^{336}-Ala^{364}, including clone SC15 (Gly^{336}-Leu^{423}) which reacted very strongly with the antibody. Monoclonal antibody C6B7 was known to bind to FXIIf comprising the light chain of FXII linked to a small 2 kDa peptide after activation. The epitope identified here spans the three sites cleaved by kallikrein during activation of FXII. The binding of a large antibody molecule in the vicinity of the activation region of FXII would explain the lack of cleavage by kallikrein, by rendering Factor XII unavailable for the proteolytic action of its activator.

mAb F1 reacted with clones encoding amino acids Lys^{115}-Leu^{122}. However, one clone encoded a different region (Leu^{154}-Val^{231}) (Table 7). The small number of positive and the discrepancy between them led to the conclusion that monoclonal antibody F1 is not suitable for the application of this technique or that further screening would be necessary to identify the epitope reliably.

Table 5. Characterization of factor XII recombinant bacteriophage reacting with anti-human factor XII monoclonal antibody K0K5

phage clone	Factor XII amino acids encoded	phage clone	Factor XII amino acids encoded
3AK	His17 to His82	18AK	Gly25 to Lys87
3BK	Lys13 to Asp77	18BK	Glu5 to Ser83
4AK	Glu5 to Lys74	18CK	Lys11 to Ser96
5AK	Leu2 to Ser83	20AK	Glu9 to His99
6BK	Trp4 to Lys76	20BK	Glu9 to Gly88
7AK	Glu5 to Cys79	21AK	Pro27 to His78*
8AK	Leu2 to Lys73*	21BK	Thr22 to Gln104
11AK	Glu9 to His99	23AK	Glu9 to His99
14AK	His10 to Val75	24AK	Thr18 to Ser80
14BK	Ala6 to Lys 73	24CK	Thr24 to Cys85
14CK	Leu6 to Lys76	25AK	His10 to Lys81
15CK	Val20 to Asn93	26AK	Val20 to Ser80
16AK	Thr24 to Gly88	27BK	Glu9 to Lys81
16EK	Ala14 to Lys76	28CK	Val19 to Asp77
17AK	Val20 to Ser96		

* Define limits of epitope

TABLE 6. Characterization of factor XII recombinant bacteriophage reacting with anti-human factor XII monoclonal antibody C6B7

phage clone	Factor XII amino acids encoded	phage clone	Factor XII amino acids encoded
SC15	Gly 336 to Leu 423	C15	Gly 336 to Leu 404
15	Pro 285 to Ala 364		

TABLE 7. Characterization of factor XII recombinant bacteriophage reacting with anti-human factor XII monoclonal antibody F1

phage clone	Factor XII amino acids encoded	phage clone	Factor XII amino acids encoded
8A	His 110 to Cys 190	10B	Leu 154 to Val 231
11D	Trp 66 to Gly 146	16A	Pro 58 to Leu 122

II. EXPRESSION OF RECOMBINANT HUMAN FACTOR XII

When this study was undertaken, expression of recombinant human factor XII had never been reported. Because FXII has a complex domain organization and is post-translationally modified, a mammalian cell expression system was desirable. In our laboratory, the amino-terminal half-molecule of human serum transferrin was successfully expressed by Walter Funk using the pNUT expression vector in BHK cells (Funk et al., 1990; Woodworth et al., 1991), and high levels of expression (up to 34 μg/mL) were obtained in roller bottles.

In the pNUT vector, transcription of the cDNA is under the control of the metallothionein (MT-1) promoter and the human growth hormone termination signals (HGH 3'). pNUT also contains the early SV40 promoter driving expression of an altered dihydrofolate reductase (DHFR) cDNA (Simonsen and Levinson, 1983) using transcription termination signals from human hepatitis B virus (HBV). The DHFR cDNA which contains a single nucleotide change produces an abnormal dihydrofolate reductase exhibiting a 270-fold reduction in binding affinity for methotrexate (Haber et al., 1981). This allows for the selection of high copy numbers of pNUT in transfected cells. Since the transfected cells are resistant to high levels of methotrexate (0.5 mM), $DHFR^+$ cell lines can be used for transfection.

A. Recombinant human FXII wild type

The modified human FXII cDNA was introduced in the eukaryotic expression vector pNUT as described in Materials and Methods. pNUT-hFXII was then transfected into BHK cells by using the calcium phosphate co-precipitation technique. After approximately 10-14 days of MTX selection, resistant colonies (~200 per dish) became visible. Isolated colonies were chosen and released with trypsin to clone various hFXII cell lines. In order to determine the highest expressing-secreting cell line, the culture

medium from each clone was analyzed by Western blotting. Figure 15 illustrates the variation in levels of rFXII detected in the media of various clones. The lack of reaction with the antibody in the control lanes confirms that the BHK cells do not secrete any detectable immunoreactive endogenous FXII, and that the serum replacement is also FXII-free. Recombinant human FXII appears as a doublet on Western blot, with the highest band showing an apparent molecular weight slightly lower than 80 kDa. This is also observed with plasma-purified factor XII and pooled human plasma FXII. The two bands probably correspond to different glycosylation forms of the molecule. (The prestained bovine serum albumin contained in the high molecular weight protein standard mixture shows an abnormally slow electrophoretic mobility compared to the non-stained molecule (see Figure 16)).

The highest producing cell line (rhFXII-10) was chosen for large scale culture in roller bottles. Aliquots withdrawn from the roller bottle media were analyzed by ELISA to determine the level of secreted rhFXII achieved in this system. For rhFXII-10, levels of approximately 5 μg/mL were detected. This is consistent with levels of recombinant protein obtained in similar expression system for other coagulation proteins such as prothrombin, factor IX and factor X (Jorgensen et al., 1987a; Le Bonniec, 1991; Falkner et al., 1992; Jorgensen et al., 1987b; Wolf et al., 1991).

An affinity column was prepared using monoclonal antibody C6B7, and both human plasma FXII and recombinant hFXII were partially purified in a single chromatographic step. The rhFXII-10 medium was loaded directly onto the column. Following extensive washing, the protein was eluted from the column, separated on SDS-PAGE, transferred onto an Immobilon membrane and subjected to N-terminal sequence analysis. The result of the analysis indicated the presence of amino acids Ile-Pro-Pro-Trp-Glu at the N-terminus of rhFXII, in agreement with the amino terminus of plasma-derived human factor XII (McMullen and Fujikawa, 1985). This result reflects the proper processing of the signal peptide prior to secretion in BHK cells. rhFXII appeared partly

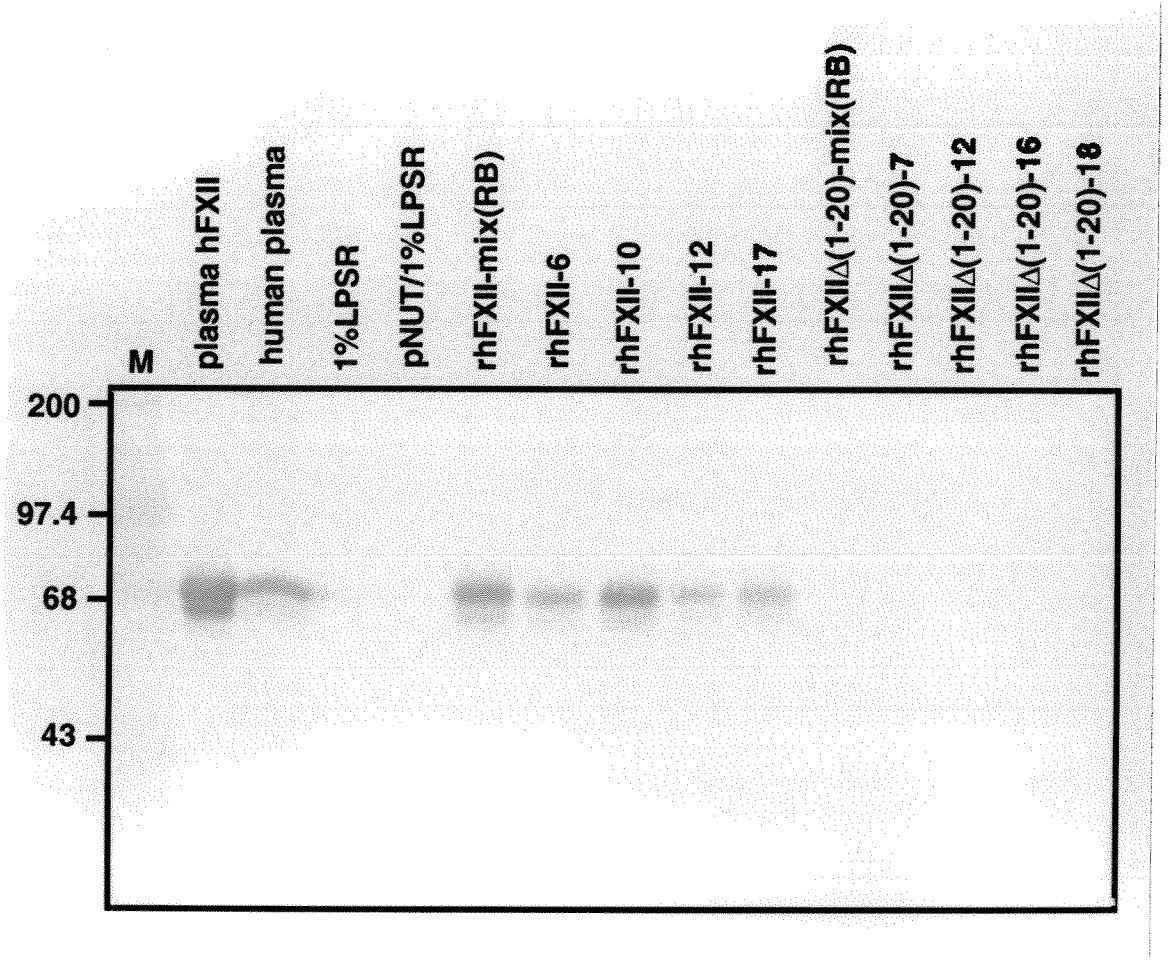


Figure 15. Selection of rhFXII and rhFXIIΔ(1-20) clones.

Following SDS-PAGE (10% acrylamide) under non-reducing conditions, the proteins were subjected to Western blot analysis using mAb C6B7. Lane 1, High molecular weight protein standard; lane 2, purified human plasma FXII (1 μg); lane 3, human plasma (2 μL); lane 4, medium (30 μL); lane 5, medium from pNUT-transfected cells cultured in roller bottle (30 μL); lane 6, medium from non-cloned phFXII-transfected cells cultured in roller bottle (30 μL); lanes 7-10, medium from cloned phFXII-transfected cells cultured in flask (30 μL); lane 11, medium from non-cloned phFXIIΔ(1-20)-transfected cells cultured in roller bottle (30 μL); lanes 12-15, medium from cloned phFXIIΔ(1-20)-transfected cells cultured in flask (30 μL). In all cases, the medium was DMEM-F12/1% LPSR.

activated (Figure 16A,B) after the affinity chromatography step. Although no glassware was used, FXII is extremely sensitive to autoactivation which complicated the purification greatly.

B. Recombinant human FXIIΔ 1-20 and Δ 5-20

In order to investigate whether amino acids 1-28 of the zymogen are involved in binding to negatively-charged surfaces, a mutant cDNA was constructed in which amino acids 1 to 20 were deleted. Amino acid 20 was chosen because it is the last amino acid encoded by exon 2 within the FXII human gene (Cool and MacGillivray, 1987). Leucine 21 seemed like a good candidate to be the first amino acid on the C side of the signal peptidase cleavage site, in place of isoleucine normally found at position +1 in the human FXII protein. This mutant cDNA was introduced into pNUT and transfected into BHK as described for wild type hFXII. Many methotrexate-resistant colonies were obtained following transfection but upon analysis by Western blot and ELISA, no FXII antigen was detected in the culture medium of the cells (see Figure 15).

It was hypothesized that the deletion of the amino acids at the N-terminus of the mature protein (1-20) might affect the recognition and/or cleavage of the signal peptide by the signal peptidase. To verify whether the polypeptide was expressed at all, two hFXIIΔ1-20 cell lines (12 and 16) were lysed, with and without detergent, and the content of the lysis pellet and supernatant were analyzed by SDS-PAGE under non-reducing conditions, followed by Western blot with mAb C6B7. The Western blot revealed the presence of a band of approximately 55 kDa (Figure 17) in the pellet fraction (containing the nuclei and the ER presumably attached to it). It therefore appears that FXIIΔ 1-20 is properly expressed by the BHK cells, as it is recognized by anti-FXII mAb, but improperly processed. The protein was not analyzed by N-terminal sequencing but it is likely that the signal peptide is still attached to the polypeptide thereby blocking secretion

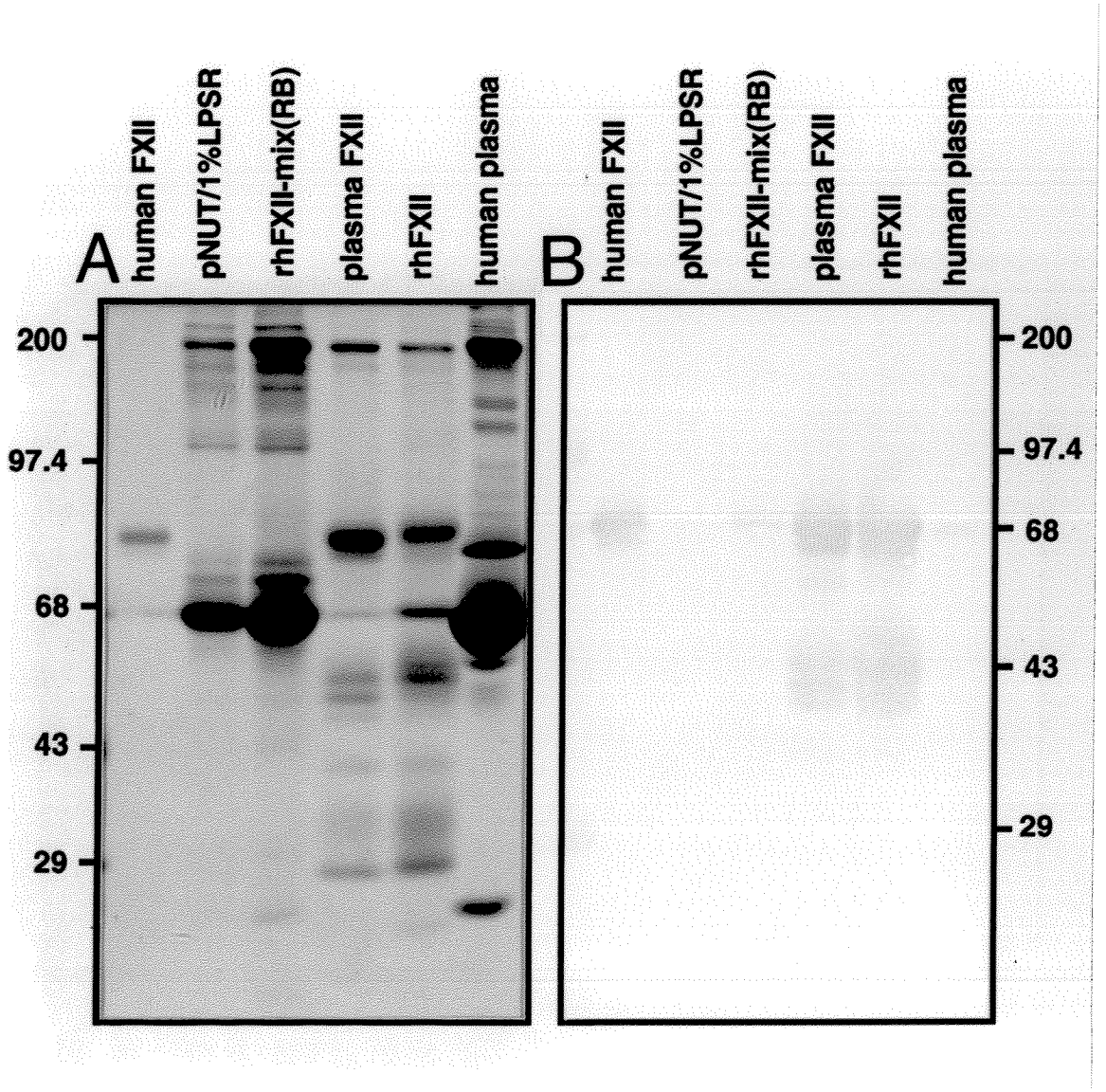


Figure 16. Purification of human plasma FXII and rhFXII.

A. SDS-PAGE (10% acrylamide) under non-reducing conditions. Lane 1, Purified human plasma FXII (1 μg); lane 2, medium (DMEM-F12/1% LPSR) from pNUT-transfected cells cultured in roller bottle (30 μL); lane 3, same medium from non-cloned phFXII-transfected cells cultured in roller bottle (30 μL); lane 4, human plasma FXII after one-step chromatography on C6B7 affinity column; lane 5, rhFXII after one-step chromatography on C6B7 affinity column; lane 6, human plasma (1 μL). B. Western blot analysis of a duplicate of the gel presented in A, using mAb C6B7. The prestained BSA of the high molecular weight standard shows aberrant electrophoretic mobility.

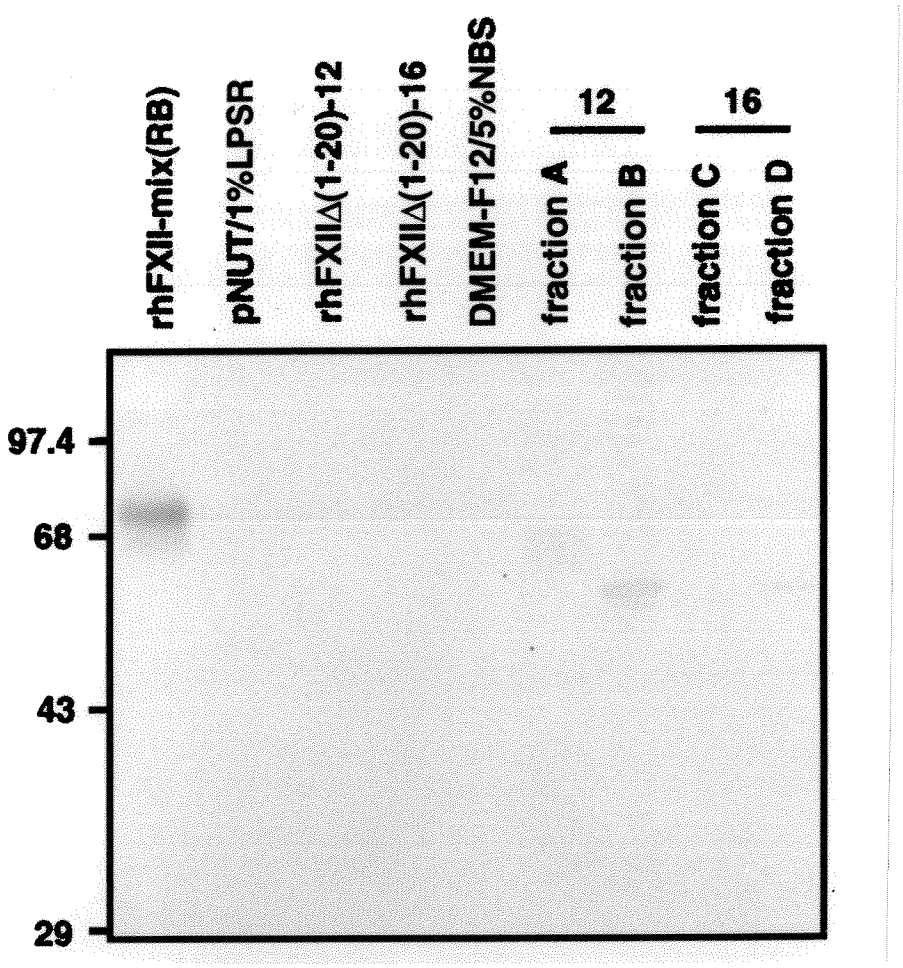


Figure 17. Analysis of non-secreted rhFXIIΔ(1-20) clones.

Western blot analysis following SDS-PAGE (10% acrylamide), using mAb C6B7. Lane 1, medium from non-cloned phFXII-transfected cells cultured in roller bottle (30 µL); lane 2, medium from pNUT-transfected cells cultured in roller bottle (30 µL); lane 3, medium from rhFXIIΔ(1-20)-12 cultured in flask (30 µL); lane 4, medium from rhFXIIΔ(1-20)-16 cultured in flask (30 µL); lane 5, DMEM-F12/5% NBS (30 µL); lane 6, supernatant from the lysis of rhFXIIΔ(1-20)-12 cells, with detergent (30 µL)(fraction A); lane 7, pellet from the lysis of rhFXIIΔ(1-20)-12 cells, with detergent (15 µL)(fraction B); lane 8, supernatant from lysis of rhFXIIΔ(1-20)-16 cells, without detergent (30 µL)(fraction C); lane 9, pellet from lysis of rhFXIIΔ(1-20)-16 cells, without detergent (15 µL)(fraction D). See material and methods for details. In all cases except when indicated, the medium was DMEM-F12/1% LPSR.

by the cell. The low apparent molecular weight might reflect a lack of glycosylation of the mutant protein.

Since the signal peptide sequence is unchanged, it is believed that the new cleavage site sequence is responsible for the lack of processing. The signal peptidase sequence in FXII Δ1-20 is Thr-Leu-Ser/ Leu-Thr-Val-Thr instead of Thr-Leu-Ser/ Ile-Pro-Pro-Trp encoded by the wild type construct.

To overcome this secretion problem, a second construct was made in which amino acids 1 to 4 were restored, to create hFXIIΔ 5-20. Because the first four amino acids of the mature protein are intact, it was hoped that the signal peptidase in BHK would recognize the junction and cleave the expressed mutant. Furthermore, it has been shown that amino acids 1 to 4 were essential but not sufficient for binding to mAb B7C9 (Clarke et al., 1989). Once again, many methotrexate-resistant colonies were isolated but Western blot analysis of the culture media failed to detect secreted rFXII (data not shown).

C. Recombinant human FXIIΔ 28-69

Anti-FXII monoclonal antibody KOK5 inhibits FXII clotting activity without affecting its amidolytic activity. The putative epitope for this antibody is believed to reside within the fibronectin type II homology found in factor XII. A mutant FXII cDNA was constructed in which amino acids 28 to 69 were deleted. Positions 28 and 69 are cysteine residues which border the finger-like domain. Since the disulfide bond they form appears necessary for KOK5 binding, the whole region was deleted, including both cysteine residues to limit the risk of an abnormal disulfide bond formation within the mutant molecule. Western blot analysis on the culture medium revealed the presence of a band with an apparent molecular weight slightly smaller then that of plasma FXII, in agreement with a 41 amino acid deletion (data not shown). Several clones were

compared for their level of expression/secretion and clone hFXIIΔ 28-69-6 was chosen for large scale culture in roller bottle.

D. Immunocytochemistry

BHK cells transfected with pNUT, phFXII, phFXIIΔ 1-20, phFXIIΔ 5-20, phFXIIΔ 28-69 together with untransfected BHK were analyzed by immunocytochemistry using mAb C6B7. As expected, the BHK cells (Figure 18A) and the pNUT-transfected cells (Figure 18B) do not show any binding of the antibody. However, the hFXII (Figure 18C) and the three mutant FXII cell lines (Figure 18D,E and F) are visibly stained. The non-secreted mutants hFXIIΔ 1-20 (Figure 18D) and hFXIIΔ 5-20 (Figure 18E) show a staining pattern similar to that of the secreting cell lines, localized predominantly in the endoplasmic reticulum surrounding the nuclei of the cells. This experiment confirmed the previous result indicating that those two mutant cDNA are expressed by the BHK cells but that the immature polypeptide is retained in the ER. The proteins do not appear to be degraded rapidly as the staining of the mutant cell lines is very similar to that observed for the wild type protein. No obvious lysozomes are visible within the non-secreting cells, which appear as healthy as their secreting counterpart despite obvious protein buildup. The fact that the staining of the secreting cells is as pronounced as that of the non-secreting ones suggests that even when some protein is properly processed and secreted out of the cell, a proportion might never reach maturity and therefore remain in the ER before being targeted for degradation. Because it would be very complicated and time consuming to purify the two mutant FXII from inside the mammalian cells without experiencing proteolytic degradation, these mutant proteins were not purified.

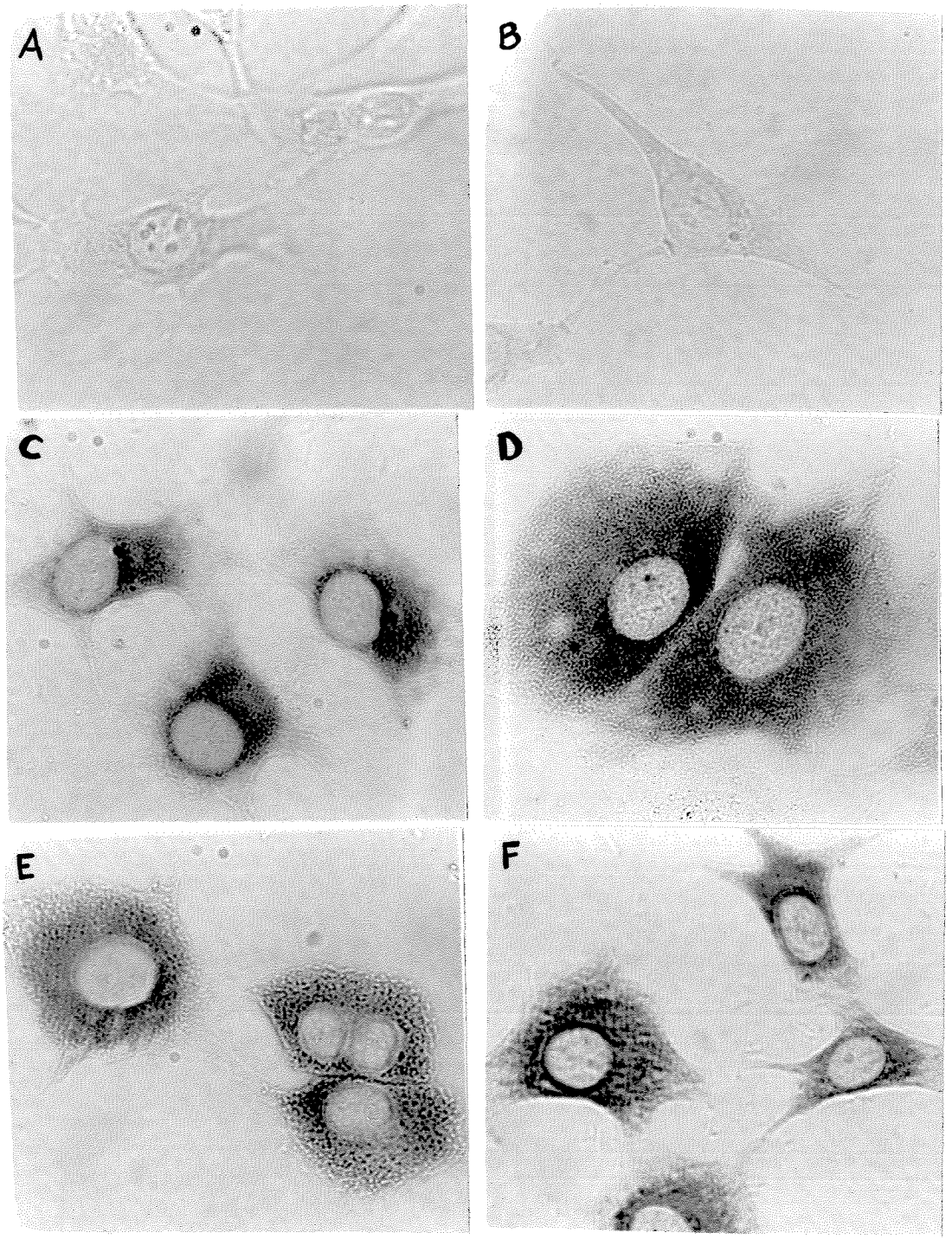


Figure 18. Immunocytochemistry analysis of rhFXII cell lines.

Wild type and transfected BHK cells were cultured at low density and subjected to immunocytochemistry using mAb C6B7. A, wild type BHK; B, pNUT-transfected BHK; C, phFXII-transfected BHK (clone 10); D, phFXIIΔ(1-20)-transfected BHK (clone 16); E, phFXIIΔ(5-20)-transfected BHK (non-cloned); F, phFXIIΔ(28-69)-transfected cells (clone 6).

E. Functional properties

Preliminary activity assays were performed on the rhFXII-10 culture media and with the partially purified rhFXII. These included coagulation assays using FXII-deficient human plasma and amidolytic assays using the chromogenic substrates S-2302 and S-2222. The goal of these experiment was to determine whether rhFXII had any activity. Because the rhFXII samples were not pure, the results were solely qualitative, and will not be presented in detail here. Recombinant hFXII did show clotting activity and amidolytic activity, but the specific activity was not determined.

Because of problems encountered with the non-secreting FXII mutants and with the purification of rhFXII zymogen, and because of the encouraging results obtained with the prothrombin expression system, the FXII project has not been pursued further.

III. EXPRESSION OF RECOMBINANT HUMAN PROTHROMBIN

During activation of prothrombin by the prothrombinase complex, meizothrombin is the main intermediate observed (Krishnaswamy, 1986; Rosing and Tans, 1988; Boskovic et al., 1990). Meizothrombin possesses amidolytic activity but little fibrinogen clotting activity. We chose to express mutant forms of the human prothrombin cDNA in BHK cells using the pNUT expression system. These mutant prothrombin molecules mimic some of the intermediates generated during activation of prothrombin.

A. Construction of vectors and transfection

Prothrombin contains two factor Xa cleavage sites and one site susceptible to thrombin. On longer exposure to thrombin, a second site within the A chain of thrombin is also cleaved. Five different cDNAs (Figure 19) were constructed by using the PCR mutagenesis technique: hFII which encodes the wild type human prothrombin cDNA,

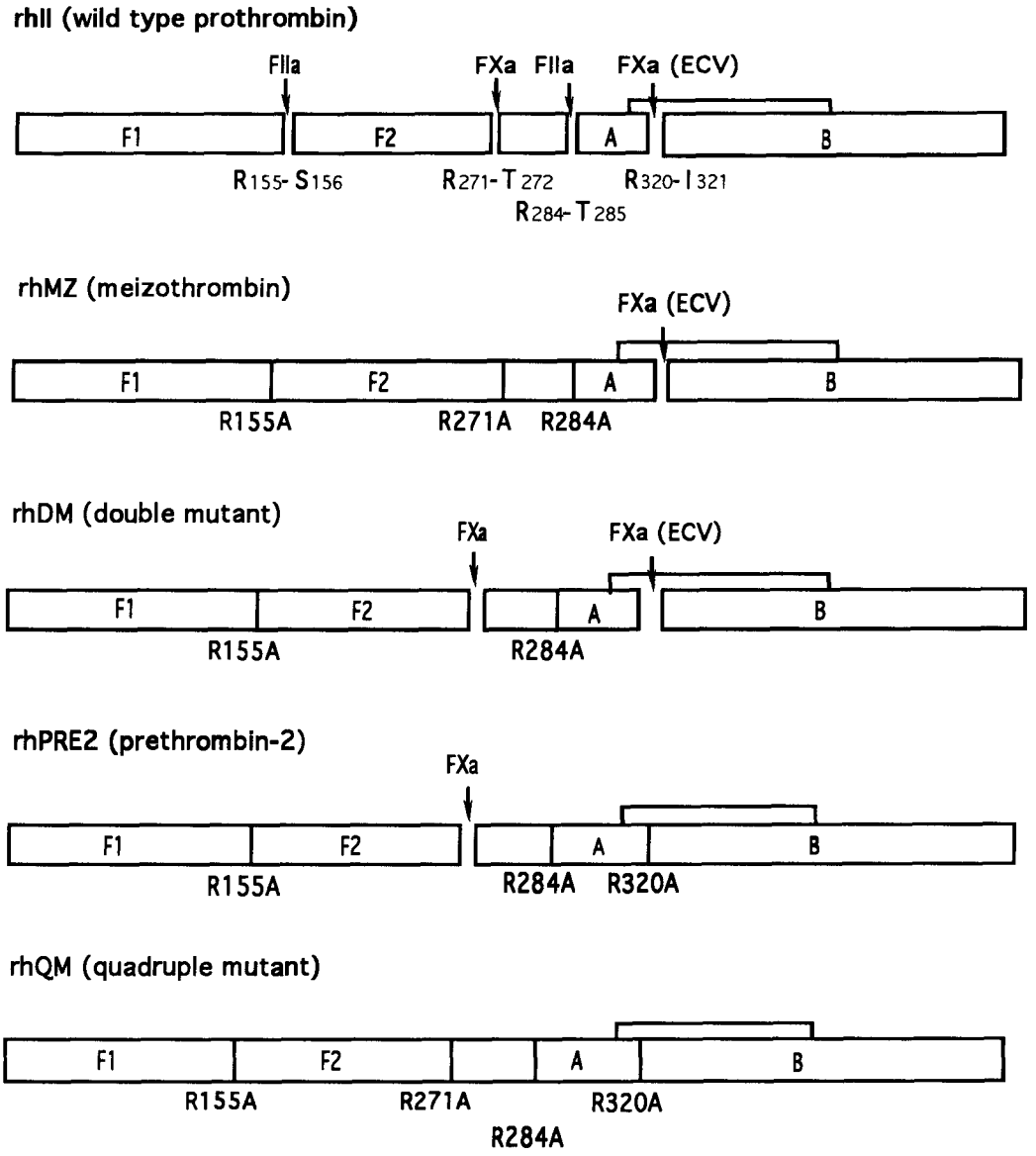


Figure 19. Description of the different prothrombin constructs.

rhII encodes the wild type human prothrombin cDNA; rhMZ encodes a triple mutant form (R155A, R271A, R284A) of the human prothrombin cDNA, disrupting two thrombin and one factor Xa cleavage sites; rhDM encodes a double mutant form (R155A, R284A) of the human prothombin cDNA, disrupting the two thrombin cleavage sites; rhPRE2 encodes a triple mutant form (R155A, R271A, R284A) of the human prothrombin cDNA, disrupting two thrombin and one factor Xa cleavage sites; rhQM encodes a quadruple mutant form (R155A, R271A, R284A, R320A) of the human prothrombin cDNA, disrupting all thrombin and factor Xa cleavage sites. Thrombin is indicated by FIIa, factor Xa is indicated by FXa and ecarin cleavage site is indicated by ECV.

hMZ which contains a mutations (R155A, R271A and R284A) destroying the factor Xa cleavage site between fragment 2 and prethrombin as well as the two thrombin cleavage sites, hDM (R155A, R284A) which retains only the two factor Xa sites, hPRE2 (R155A, R284A, and R320A) which can only be cleaved by factor Xa between fragment 1.2 and prethrombin, and finally hQM (R155A, R271A, R284A, and R320A) in which all the factor Xa and thrombin cleavage sites are mutated. In each case, the arginine residue preceding the scissile bond was mutated to an alanine. The substrate specificity of FXa and thrombin is dependent upon the presence of the arginine residue preceding the cleavage site.

Upon DNA sequence analysis of the hFII cDNA, two polymorphisms were identified. Asn^{121} which is encoded by AAC in the published human prothrombin cDNA sequence (Degen et al., 1983) is substituted to Thr (ACC) and Thr^{122} (ACG) is substituted to Met (ATG). Both changes are conservative and are encoded by exon 6 of the gene. Both the Asn->Thr polymorphism (Degen et al., 1983) and the Thr->Met polymorphism (Iwahana et al., 1992) have been reported previously. Exon 6 in the human prothrombin gene (corresponding fragment 2 in prothrombin) appears to be a highly polymorphic region. All the prothrombin cDNAs were ligated into the pNUT expression vector and transfected into BHK by the calcium-phosphate co-precipitation method. By using low confluence cells, and by increasing the stringency of the transfection and selection conditions, the number of resistant colonies obtained (~20 per dish) was markedly reduced. This made the isolation of clones easier and seemed to select for high secreting clones (presumably reflecting the selection of high copy number clones).

B. Selection of clones

For each construct, between 6 and 12 colonies were cloned and cultured, and 3 or more were analyzed for their level of expression/secretion. A first screen was performed

by Western blot analysis after which a high-secreting clone was chosen for large scale expression. The criteria used in determining the best cell line were the secretion level, the doubling time and the general phenotype of the cell. Because integration events might disrupt important genes and lead to unusual cell phenotypes, healthy-looking cells with a normal doubling time were preferentially chosen. Variations in secretion levels of 5 to 10 fold were observed between clones derived from a single construct and transfection.

Figures 20 and 21 illustrate these differences as shown by Western blot analysis of various clones of phII, phMZ and phQM. Also visible in Figures 20 and 21 are small variations in apparent molecular weight between the different clones. Recombinant hFII clones display a faster electrophoretic mobility than human plasma prothrombin while the rhMZ clones appear slower. The large number of rhQM clones in Figure 21 clearly demonstrates the variability in secretion level and in size observed between clones derived from a single transfection.

The cell lines chosen for large scale expression in roller bottle were: rhFII-4, rhMZ-11, rhPRE2-3, rhDM-8 and rhQM-10. During the culture in roller bottles, small aliquots were withdrawn at the same time the medium collected and levels of secretion were analyzed by ELISA. Secretion levels of approximately 20 μg/mL were detected for rhMZ, up to as much as an average of 400 μg/mL for rhQM (Figure 22,A). Over a period of 30 days (average lifetime of the roller bottle), up to 90 mg of rhMZ and more than 1.5 g of rhQM were accumulated (Figure 22,B). To our knowledge, expression-secretion levels as high as the one observed for rhQM has never been reported for a human protein expressed in mammalian cells.

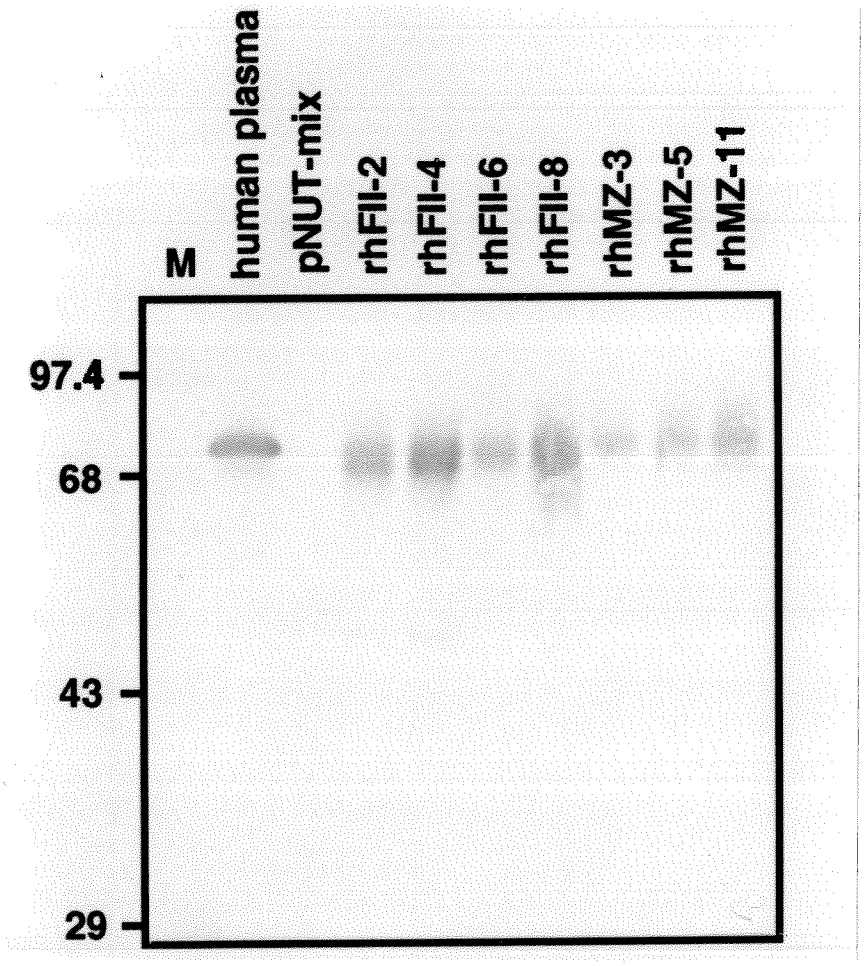


Figure 20. Selection of rhFII and rhMZ clones.

Following SDS-PAGE (10% acrylamide) under reducing conditions, the proteins were subjected to Western blot analysis using a sheep anti-human prothrombin polyclonal antibody. Lane 1, Prestained high molecular protein standard; lane 2, human plasma (1 μL); lane 3, medium from pNUT-transfected cells cultured in flask (20 μL); lanes 4-7, medium from cloned phFII-transfected cells cultured in flask (20 μL); lanes 8-10, medium from cloned phMZ-transfected cells cultured in flask (20 μL). In all cases, the medium was DMEM-F12/1% LPSR/10 μg/mL vitamin K.

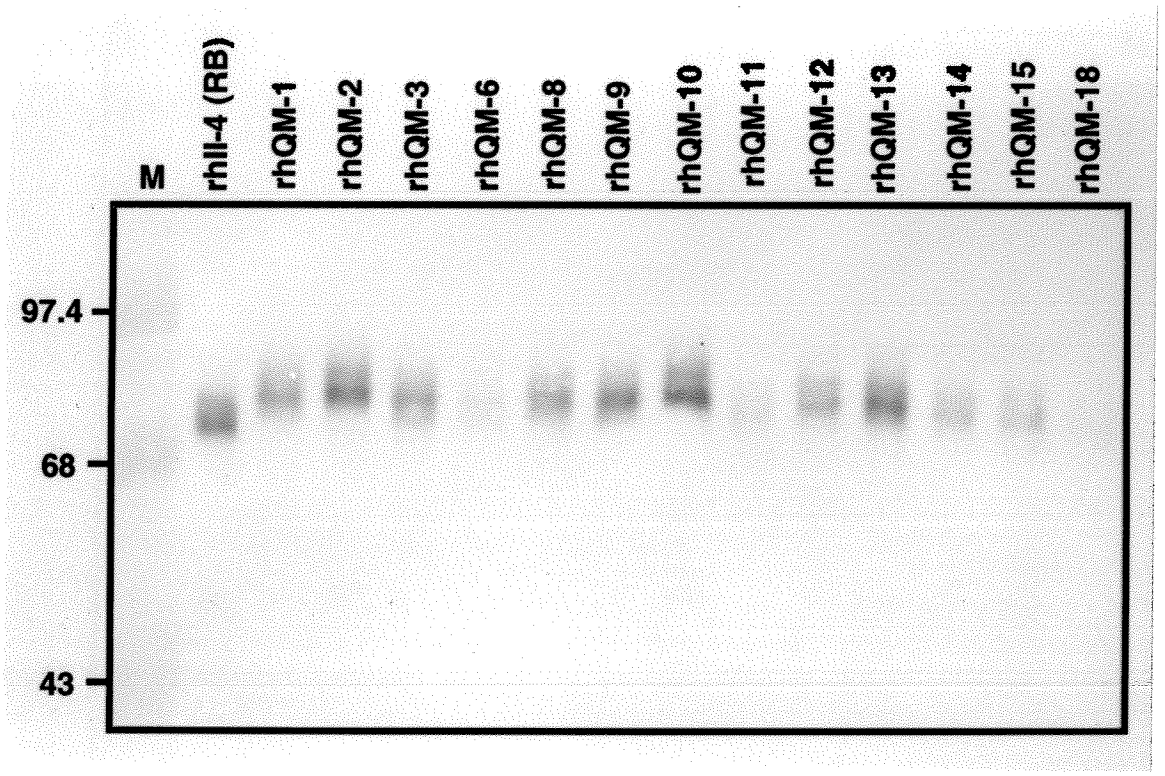


Figure 21. Selection of rhQM clones.

Following SDS-PAGE (10% acrylamide) under reducing conditions, the proteins were subjected to Western blot analysis using a sheep anti-human prothrombin polyclonal antibody. Lane 1, Prestained High molecular weight protein standard; lane 2, medium from clone rhFII-4 cultured in roller bottle (10 µL); lanes 3-15, medium from cloned rhQM cell lines cultured in flasks. In all cases, the medium was DMEM-F12/1% LPSR/10 µg/mL vitamin K.

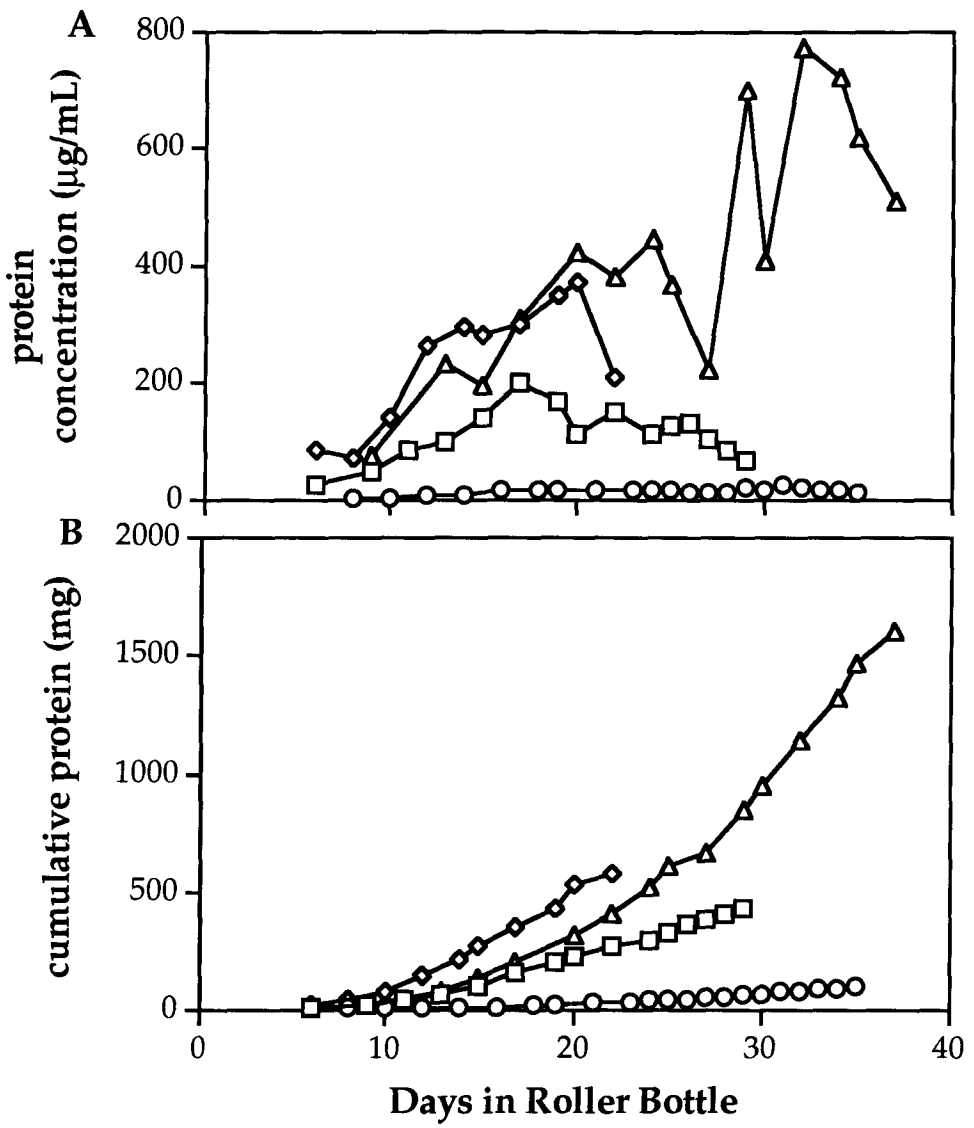


Figure 22. Production rate (A) and cumulative yield (B) of rhFII, rhMZ, rhPRE2 and rhQM produced by BHK cells cultured in roller bottle.

On the days indicated, 250 mL of medium was collected and assayed for recombinant protein by ELISA with a sheep anti-human prothrombin polyclonal antibody. rhFII is indicated by ❑, rhMZ is indicated by ○, rhPRE2 is indicated by ◊, and rhQM is indicated by Δ.

C. Isolation and characterization

After isolation by barium-citrate adsorption and ion-exchange FPLC, the recombinant prothrombins were homogeneous as judged by SDS-PAGE, but the various mutants showed slightly heterogeneous apparent molecular weights (Figure 23). Since all the constructs were derived from the same cDNA and because this difference in size cannot be explained by the mutations themselves, it is most likely due to variations in the glycosylation of the molecules. The glycosylation can differ in terms of the number of sites that are modified or in term of the sugar content of each polysaccharide, compared to the human plasma prothrombin.

Amino-terminal sequence analysis of the first five amino acids was performed on rhFII and rhMZ and indicated a sequence identical to that of human plasma prothrombin reflecting proper processing of the pre- and propeptides.

Figure 24A illustrates the purification of rhFII-4. The presence of the secreted protein in the medium is clearly visible before the barium-citrate adsorption. The protein eluted from the precipitate appears nearly homogeneous and the ion-exchange chromatography yields pure rhFII. The appearance of a second band (~50 kDa) is detected following concentration and storage of rhFII. This phenomenon is also observed if the rhFII-4 culture medium is kept at 4°C for longer than a few hours (Figure 24B), or upon freeze-thawing of the sample. N-terminal sequence analysis of the 50 kDa band (Ser-Glu-Gly-Ser-Ser) indicated that it resulted from partial cleavage of the fragment 1 region of the prothrombin, yielding prethrombin-1. This proteolytic activity could be attributed to a trace amount of thrombin present in the sample. Noticeably, this cleavage is not observed in rhMZ, rhPRE2 or rhQM in which Arg^{155} is mutated to alanine.

The variation in levels of secretion is clearly observed in Figure 24B. Following centrifugation, rhMZ and rhQM are completely adsorbed to barium-citrate and no antigen is detected in the supernatant; rhFII, however, is only partially recovered from the culture

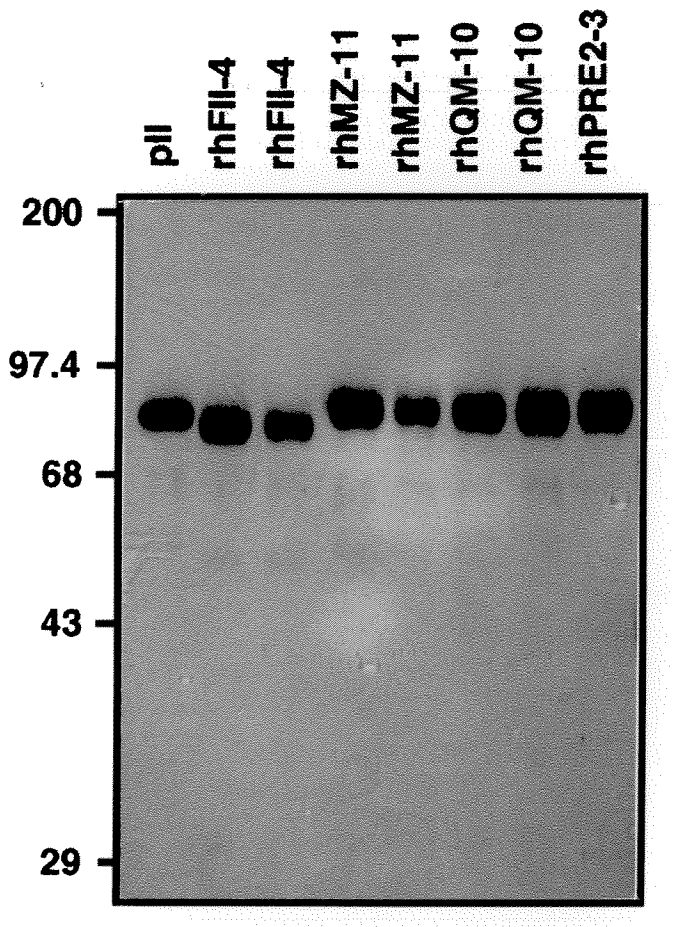


Figure 23. SDS-PAGE analysis of pure recombinant proteins.

On a 10% acrylamide gel were analyzed 5-10 μg of each protein, under reducing conditions. Lane 1, human plasma prothrombin; lanes 2 and 3, two preparations of rhFII-4, lanes 4 and 5, two preparations of rhMZ-11, lanes 6 and 7, two preparations of rhQM-10, lane 8, rhPRE2-3.

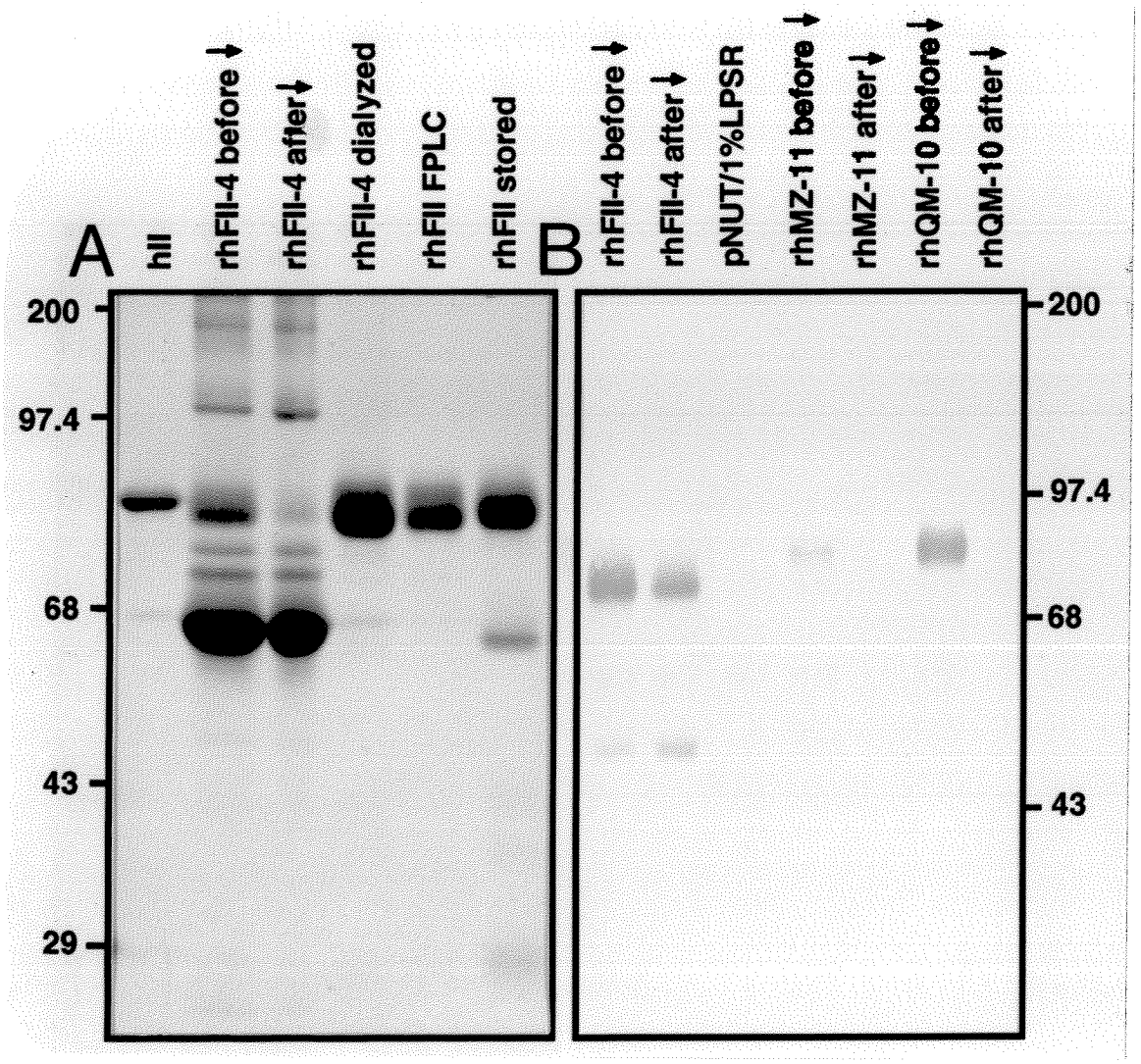


Figure 24. Purification of rhFII, rhMZ and rhQM.

A. SDS-PAGE (10% acrylamide) under non-reducing conditions. Lane 1, human plasma prothrombin (4 μg); lane 2, rhFII-4 culture medium before barium-citrate adsorption (40 μL); lane 3, rhFII-4 culture medium after barium-citrate adsorption (40 μL); lane 4, rhFII-4 after dialysis and concentration (~20 μg); lane 5, rhFII-4 after FPLC ion-exchange chromatography (~10 μg); lane 6, rhFII-4 after storage in 50% glycerol-H_20 at -20°C (~15 μg). B. Western blot analysis after SDS-PAGE (10% acrylamide) under reducing conditions, using a sheep anti-human prothrombin polyclonal antibody. Lanes 1 and 2, rhFII-4 culture medium before and after barium-citrate adsorption (40 μL); lane 3, culture medium from pNUT-transfected cells (40 μL); lanes 4 and 5, rhMZ-11 culture medium before and after barium-citrate adsorption (40 μL); lanes 6 and 7, rhQM-10 culture medium before and after barium-citrate adsorption (40 μL). In all cases the medium was DMEM-F12/1% LPSR/10 μg/mL vitamin K.

medium. The reason for this is unclear; the loss of fragment 1 which contains the Gla domain would prevent proper adsorption but some prothrombin is also detected in the supernatant.

Recombinant hMZ was subjected to a pseudo-affinity chromatography procedure that was developed to resolve variously γ-carboxylated forms of recombinant Protein C on the basis of their Ca^{++}-binding properties (Yan et al., 1990). Plasma-derived prothrombin eluted as a single homogeneous peak at 15 mM $CaCl_2$ on the gradient (Figure 25A). A subsequent salt gradient (0 to 1 M NaCl) failed to elute any more material from the column. The same procedure applied to rhMZ showed a major peak at 15 mM $CaCl_2$ followed by a minor peak at 18-25 mM $CaCl_2$ (Figure 25B). These results suggest incomplete γ-carboxylation of the molecule. As was the case for plasma derived prothrombin, all material eluted on the Ca^{++} gradient. The first and second peak fractions were pooled and are hereafter referred to as rhMZ(I) and rhMZ(II).

A small amount of rhQM was also submitted to the Ca^{++} gradient and a similar elution profile was observed although the ratio of the first peak (rhQM(I)) to the second peak (rhQM(II) was much lower (Figure 26). This result indicated that for rhQM, the incompletely γ-carboxylated species represented a greater percentage of the total protein. rhQM-10 secretion level is approximately 20-30 fold higher than that of rhMZ-11. Taken together, these results indicate that adsorption of the proteins to barium-citrate does not reflect complete and homogenous γ-carboxylation of the recombinant proteins, but rather their ability to interact with Ba^{++} ions.

The yield of the purified protein varied greatly between mutants. For rhMZ, a 50-60% recovery was routinely observed after ion-exchange chromatography. Despite a very high secretion level, rhQM however, was only recovered at 20-25%. In addition to loss by adsorption to membranes and other surfaces during the course of purification, most of the protein was lost during the washing of the barium-citrate pellet. This step

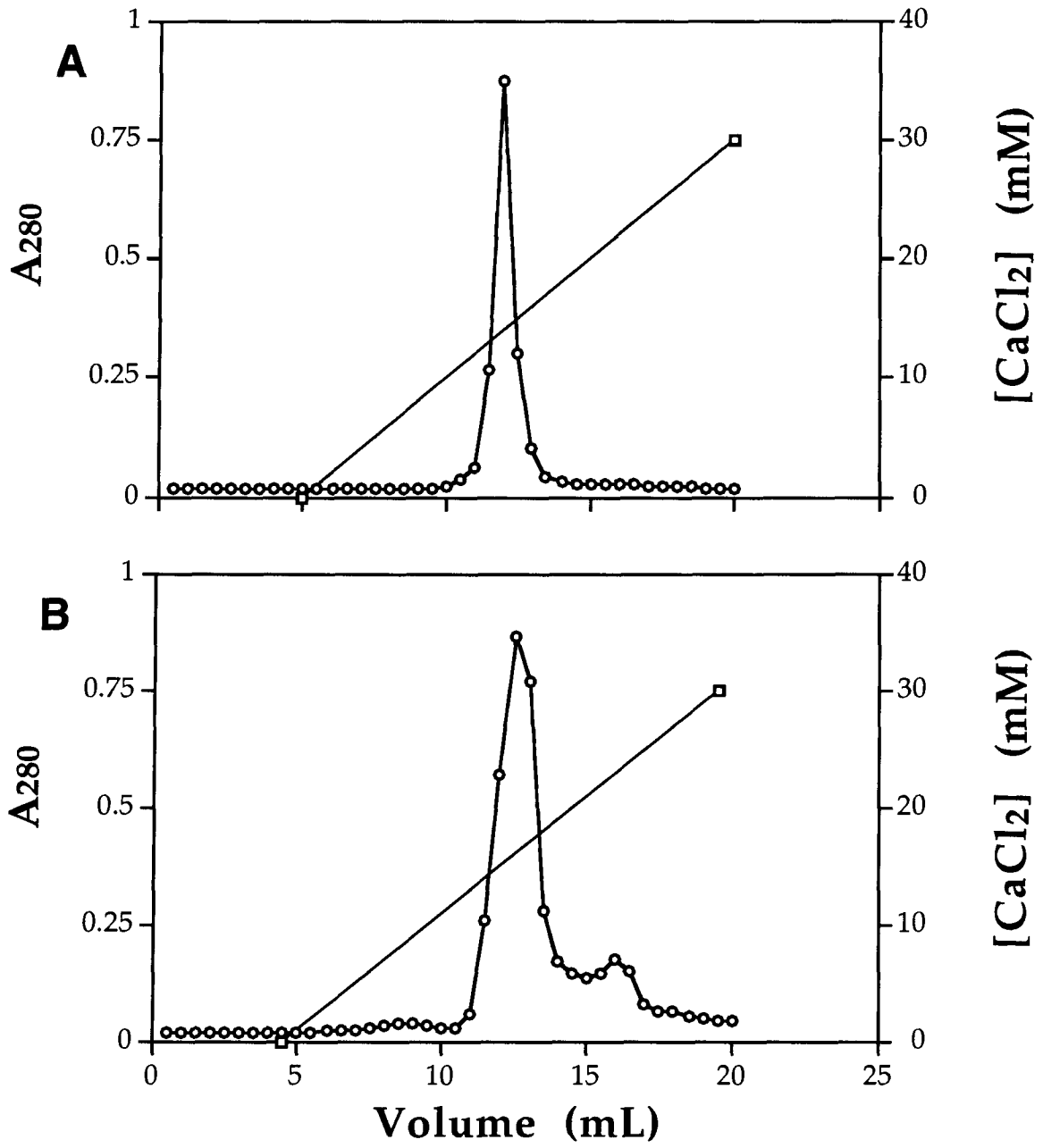


Figure 25. Elution profile of plasma-derived prothrombin (A) and rhMZ (B) during FPLC on a column of Mono Q (anion exchange).

The column was equilibrated at 4°C in 20 mM Tris-HCl, 0.15 M NaCl pH 7.4 and the protein sample (O) was eluted with a 0 to 30 mM $CaCl_2$ gradient (❑) in the same buffer.

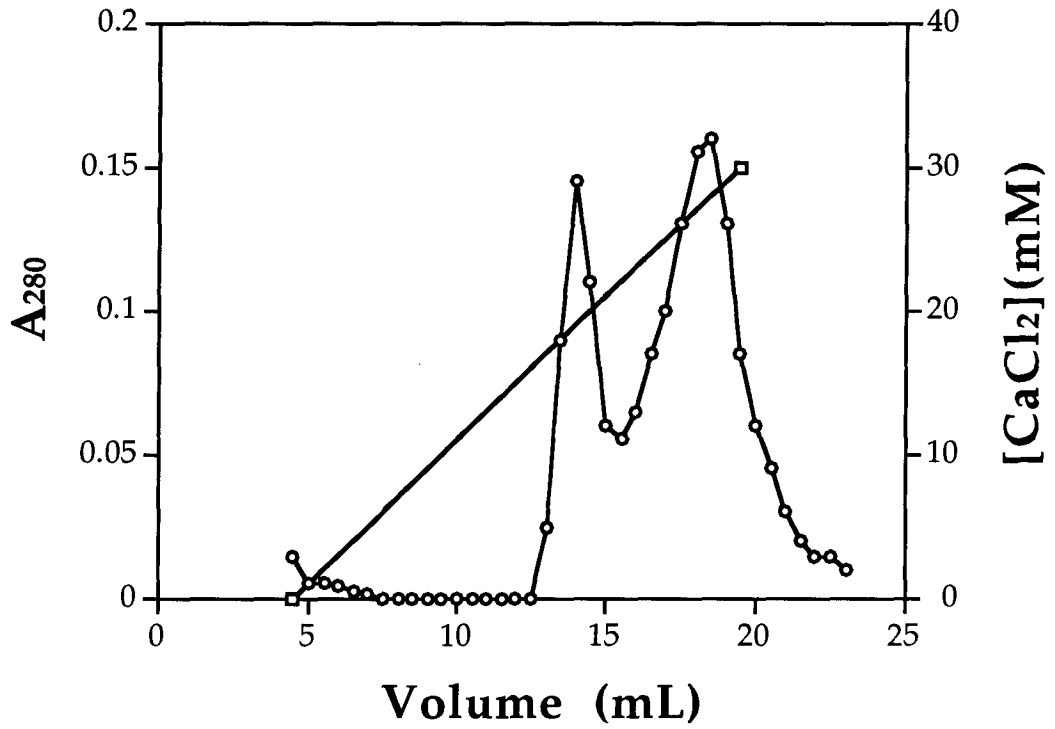


Figure 26. Elution profile of rhQM during FPLC on a column of Mono Q.
The column was equilibrated at 4°C in 20 mM Tris-HCl, 0.15 M NaCl pH 7.4 and the protein (O) was eluted with a 0 to 30 mM $CaCl_2$ gradient (❑) in the same buffer.

was performed under very stringent conditions, at room temperature. Although all the antigen adsorbed to barium-citrate in the culture medium, the washing and mixing may disrupt weak interactions. Other mutants were recovered between 25 and 40% approximately.

D. Ca^{++} and phospholipid binding properties of rhMZ

The Ca^{++} binding properties of the two peaks of rhMZ obtained by Ca^{++} gradient chromatography were assessed by intrinsic fluorescence and right angle light scattering, respectively. The results were compared to those obtained with plasma-derived prothrombin. rhMZ(I) and rhMZ(II) yielded increments of intrinsic fluorescence that were 76% and 58% of the value obtained with plasma-derived prothrombin (Figure 27A). Whether these differences imply the existence of sub populations which do not undergo a Ca^{++}-dependent conformational change, or an intrinsic difference in the molecules of the entire population can not be ascertained from the present data. Clearly, however, the materials from both peaks (rhMZ(I) and rhMZ(II)) of the Ca^{++} gradient can undergo similar, but not identical, Ca^{++}-dependent changes of conformation.

In contrast, the phospholipid binding properties of the two fractions differed substantially. The increments in scattering intensity upon the addition of Ca^{++} with rhMZ(I) and rhMZ(II) were 88% and 27%, respectively, of that observed with plasma-derived prothrombin (Figure 27B). These data suggest that both populations recovered in the Ca^{++} gradient retained Ca^{++} binding properties, but the population eluted late in the second peak of the gradient did not retain the phospholipid binding properties of plasma derived prothrombin. These data also suggest that the chromatography method appears to distinguish and dissociate the Ca^{++} binding from the phospholipid binding properties of rhMZ and probably prothrombin.

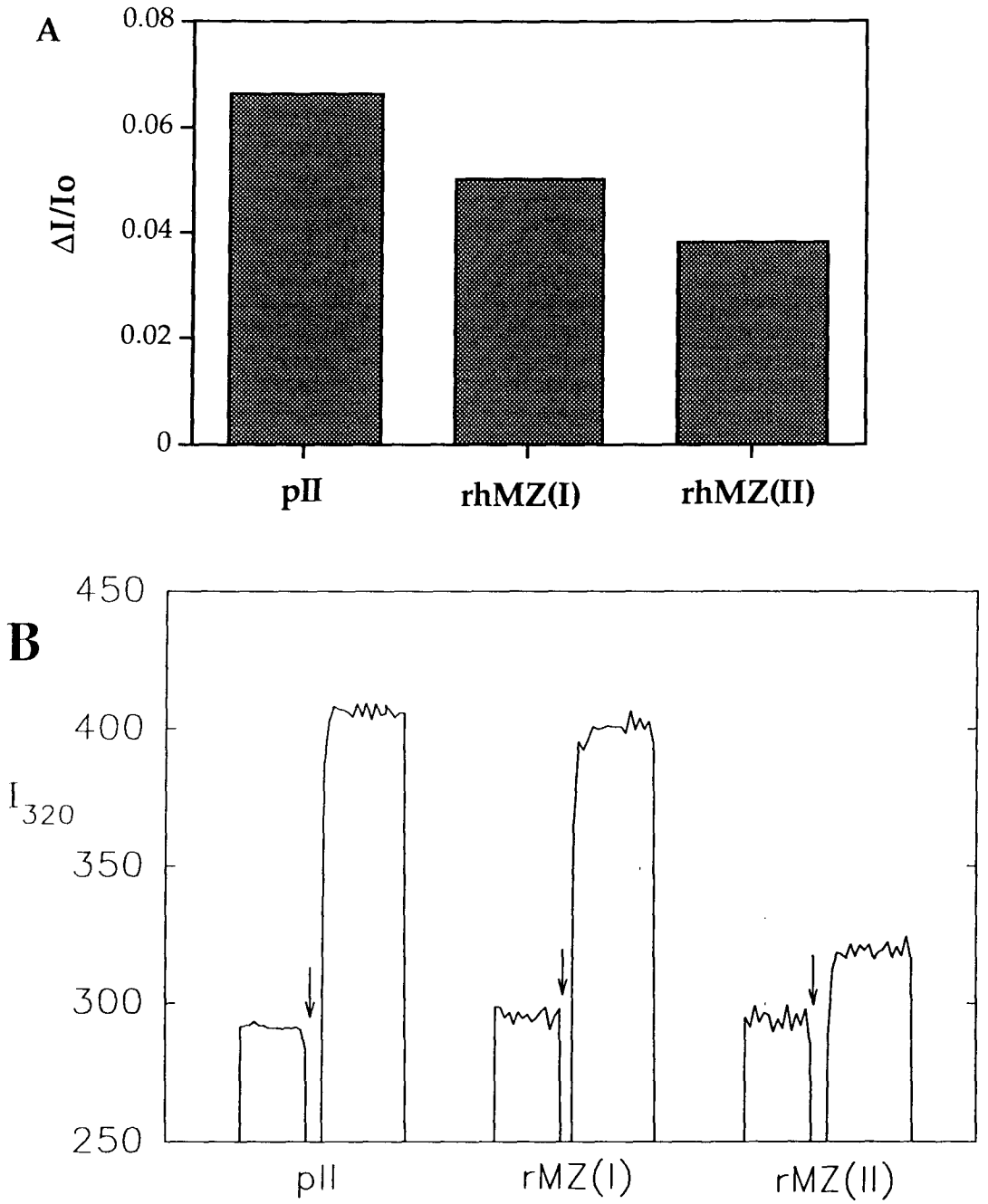


Figure 27. Fluorescence change in response to calcium ions (A) and light scattering intensity in response to PCPS vesicles binding (B).

A. Decrement of intrinsic fluorescence in response to 5.0 mM Ca^{++} of pII, and the material of the two peaks obtained with rhMZ upon chromatography in the Ca^{++} gradient (rhMZ(I) and rhMZ(II)) (Figure 25,B). B. Increment in light scattering upon addition of Ca^{++} to the solutions of the proteins and PCPS vesicles.

E. Activation

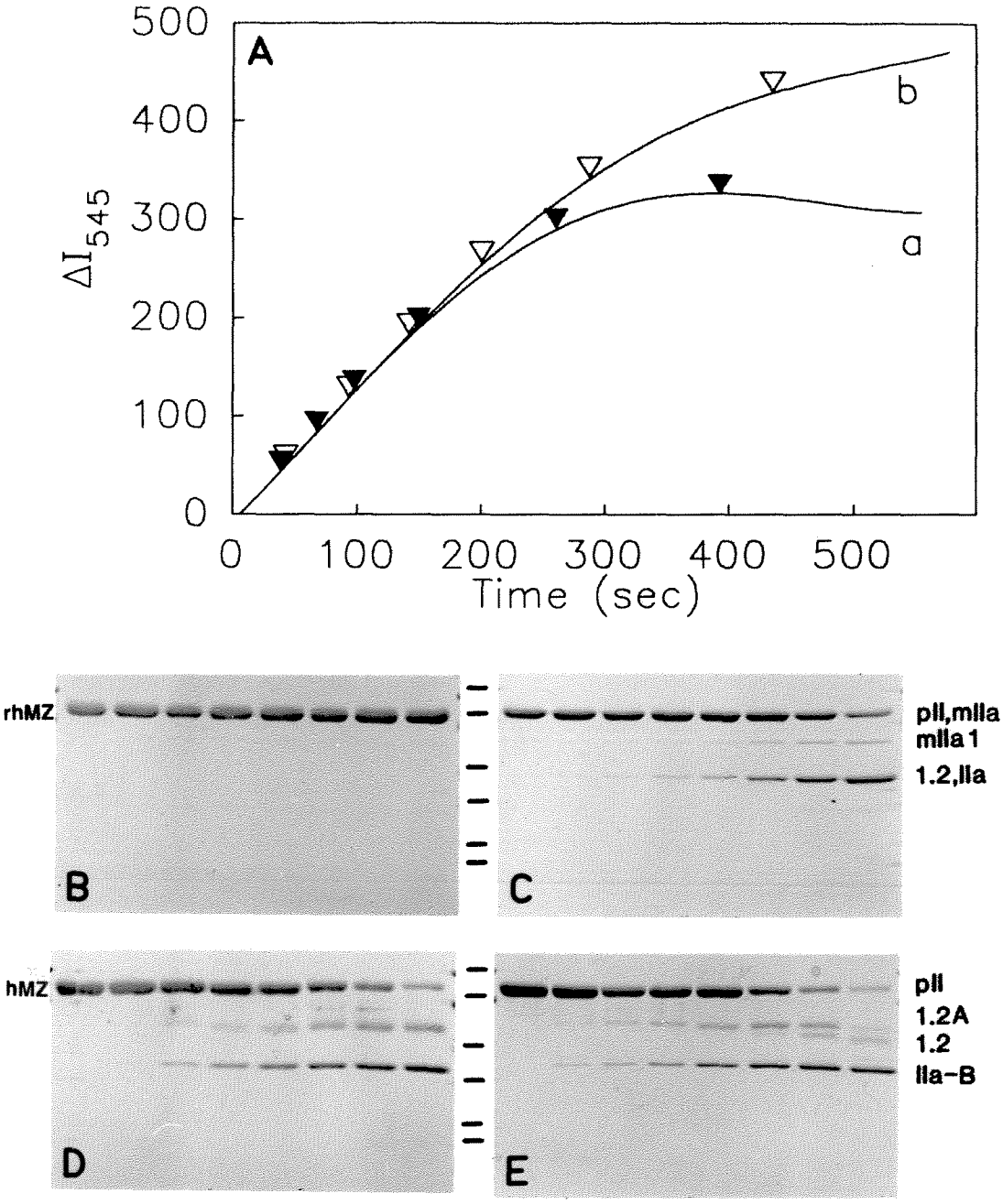
1. By the human prothrombinase complex in the presence of DAPA

The time courses of product formation upon activation of pII (trace a) and rhMZ(I) (trace b), as indicated by DAPA fluorescence, are shown in Figure 28A. Activation of pII was characterized by the appearance of a maximum at approximately 300 seconds and subsequent progression to a lower stable plateau. This profile indicates the transient formation of meizothrombin as an intermediate in the reaction, since meizothrombin-DAPA fluoresces 1.5 fold more intensely than thrombin-DAPA (Krishnaswamy et al., 1986). In contrast, the profile with rMZ(I) increased monotonically and approached, at approximately 600 seconds, a plateau that was 1.54 fold greater than the plateau obtained with pII. In addition, the profiles were coincident for the first 160 seconds of the reaction, suggesting that initial rates of meizothrombin formation were similar with both substrates and that meizothrombin is the sole intermediate of prothrombin activation, as concluded by Krishnaswamy, et al.

Samples were withdrawn at times indicated by the inverted triangles in Figure 28A and were subjected to SDS-PAGE. Results with pII are shown in Figures 28C and E. The gels indicated the presence of fragment 1.2 and thrombin which co-migrated under non reducing conditions (Figure 28C). Fragment 1.2, fragment 1.2A chain and the thrombin B chain were observed under reducing conditions (Figure 28E). Minor quantities of prethrombin-1 or meizothrombin-des F1 also are evident in Figure 28C. These patterns indicate the conversion of prothrombin to thrombin via meizothrombin and provide no evidence for accumulation of prethrombin-2 as an intermediate. The results with rhMZ(I) are shown in Figures 28B and D.

Figure 28. Prothrombinase-catalyzed activation of pII and rhMZ(I) monitored by fluorescence change in the presence of DAPA (A) and SDS-PAGE (B-E).

The reaction was initiated by the addition of FXa and fluorescence was monitored at 545 nm (A). Samples of rhMZ(I) and pII were withdrawn from ongoing activation (at the times indicated by the inverted triangles) and subjected to SDS-PAGE under non-reducing (B and C) and reducing (D and E) conditions. Sampling with pII from left to right were 0.0, 0.65, 1.12, 1.62, 2.50, 4.35, 6.57, and 9.28 minutes (C and E), and with rhMZ(I) were 0.0, 0.73, 1.57, 2.38, 3.37, 4.82, 7.28, and 14.42 minutes (B and D) after the addition of FXa. The abbreviations used are: pII, human plasma prothrombin; 1.2.A, fragment 1.2-A chain; 1.2, fragment 1.2; mIIa, plasma meizothrombin; mIIa 1, plasma meizothrombin(desF1); IIa, thrombin; IIa-B, thrombin B chain.


A
b
a
500
400
300
200
100
0
ΔI_{545}
0
100
200
300
400
500
Time (sec)
rhMZ
B
C
pII,mIIa
mIIa 1
1.2,IIa
hMZ
D
E
pII
1.2A
1.2
IIa-B

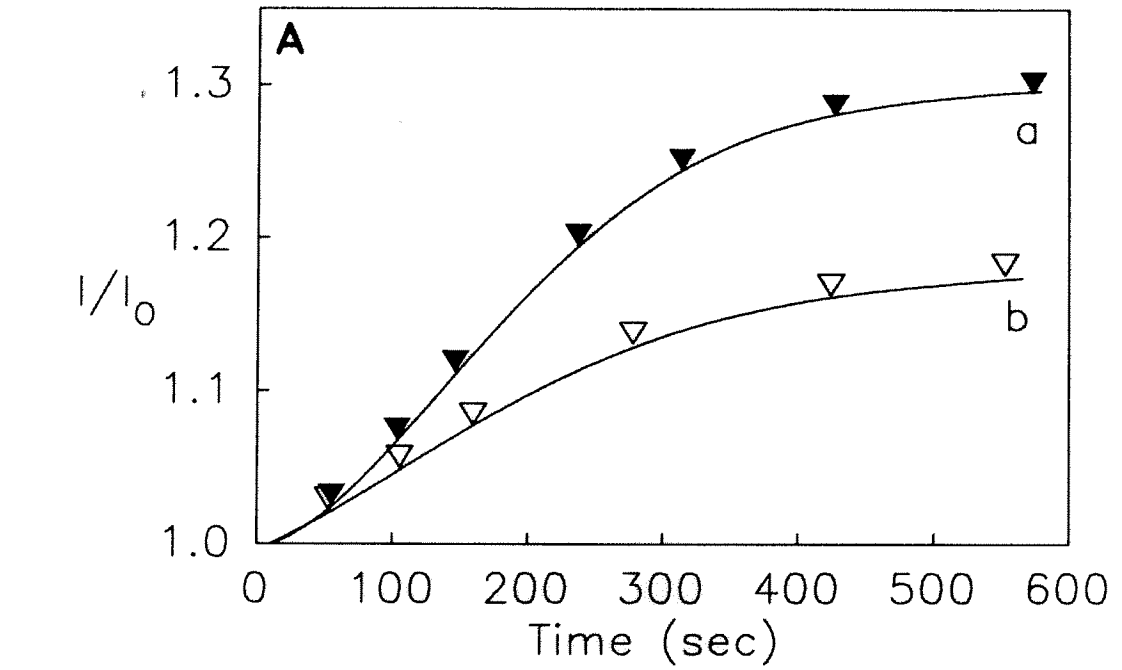
Under non reducing conditions, a single band that co-migrates with prothrombin was observed at all sample times. Upon reduction, however, the rhMZ(I) band progressively decreased in intensity and was replaced by bands migrating to the positions of the fragment 1.2 A chain and thrombin B chain. At the time of the last sample, consumption of rhMZ(I) was substantial but not complete. The patterns indicate that a single prothrombinase catalyzed cleavage of rhMZ(I) at Arg^{320} - Ile^{321} yielded the activated species rhMZ(I)a, and that no further proteolysis catalyzed by rhMZ(I)a occurred in the presence of DAPA.

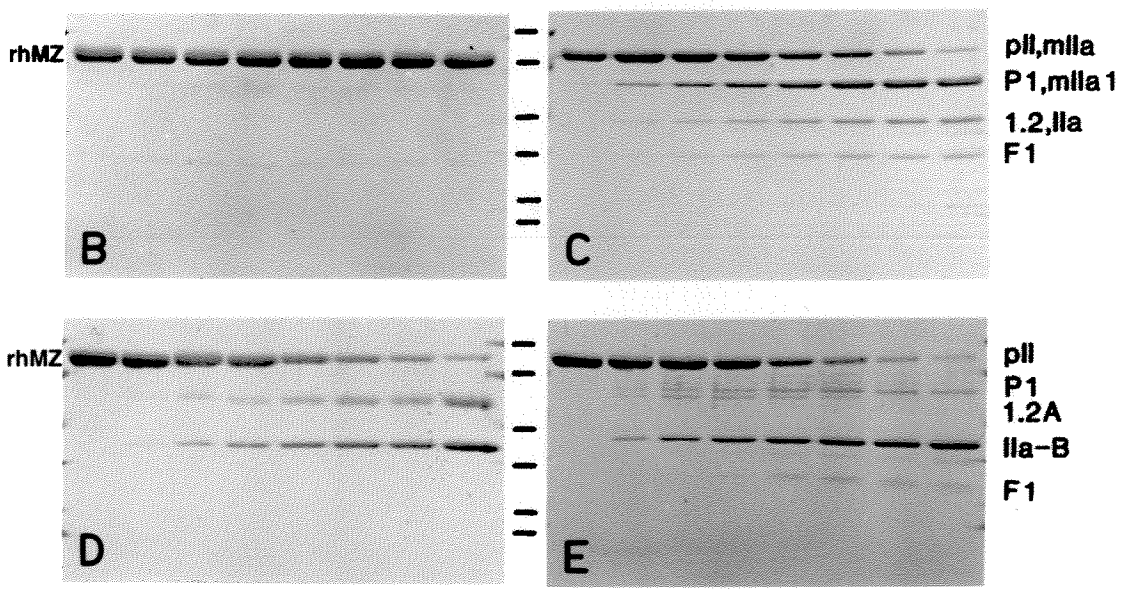
2. By the human prothrombinase complex in the absence of DAPA

Because of DAPA, the data of Figure 28 do not allow evaluation of the intrinsic stability or lack thereof of the intermediates to thrombin and meizothrombin catalyzed feedback cleavages. Thus, the experiments of Figure 29 were performed in the absence of DAPA and activation was monitored by intrinsic fluorescence. Figure 29A indicates that activation of both pII (trace a) and rhMZ(I) (trace b) are characterized by enhanced intrinsic fluorescence as observed previously (Stevens and Nesheim, 1993). Although the relative increment with pII is greater than that of rhMZ(I), the absolute values at the end of the reactions were similar (data not shown). The lower relative change with rhMZ(I) reflects an initial value that exceeded the value with pII by a factor of 1.07 at identical substrate concentrations. Samples were withdrawn at times indicated by the inverted triangles and were subjected to SDS-PAGE under non-reducing and reducing conditions. Gels with plasma prothrombin indicated consumption of prothrombin, formation of thrombin and extensive thrombin feedback, as evidenced by the accumulation of meizothrombin(desF1) and fragment 1 (Figure 29C and E). In contrast, results with rhMZ(I) indicated a single band in all samples under non-reducing conditions (Figure 29B) and the sole formation of fragment 1.2 A chain and thrombin B chain under reducing conditions (Figure 29D).

Figure 29. Prothrombinase-catalyzed activation of pII and rhMZ(I) monitored by intrinsic fluorescence (A) and SDS-PAGE (B-E).

The reaction was initiated by the addition of FXa and fluorescence was monitored at 340 nm (A). Samples of rhMZ(I) and pII were withdrawn from ongoing activation (at times indicated by the inverted triangles) and subjected to SDS-PAGE under non-reducing (B and C) and reducing (D and E) conditions. Sampling with pII from left to right were 0.0, 0.92, 1.73, 2.58, 3.95, 5.25, 7.13, and 9.57 minutes (C and E) and with rhMZ(I) were 0.0, 0.95, 1.8, 2.73, 4.67, 7.15, 9.33, and 16.1 minutes (B and D) after the addition of FXa. The abbreviations used are the same as in the legend of Figure 28. Included also are P1 for prethrombin-1 and F1 for fragment 1.


A
1.3
1.2
1.1
1.0
I/I0
a
b
0
100
200
300
400
500
600
Time (sec)


rhMZ
B
C
pII,mIIa
P1,mIIa1
1.2,IIa
F1
rhMZ
D
E
pII
P1
1.2A
IIa-B
F1

These latter data indicate that rhMZ(I), unlike pII, is stable with respect to feedback proteolysis. The gels were analyzed by laser densitometry and the results showed that the initial rate of consumption of plasma prothrombin was 1.5 fold greater than that of rhMZ(I).

The two mutants rhPRE2 and rhQM purified by ion-exchange chromatography were activated by the prothrombinase complex, in the absence of DAPA. Again, samples were withdrawn and subjected to SDS-PAGE under reducing conditions. Results with rhPRE2 (Figure 30A) indicated the formation of fragment 1.2 and prethrombin-2 solely. The reaction however proceeded very slowly as most of the protein is still intact after ~15 minutes. Partial γ-carboxylation of rhPRE2 might impair the formation of the prothrombinase complex on the PCPS vesicles and explain this lack of reactivity. The slow cleavage of rhPRE2 by FXa might also reflect a preference of FXa for cleaving Arg^{271}-Thr^{272} after the Arg^{320}-Ile^{321} cleavage. More experiments would be required to verify this hypothesis. Activation of rhQM under the same condition did not result in any cleavage of the molecule as indicated by a single band in all samples (Figure 30C). The mutant prothrombin rhDM was not yet available at the time these experiments were carried out.

3. By ecarin

The activation of pII or rhMZ(I) by the prothrombin activator of *E. carinatus* venom, ecarin, was monitored by intrinsic fluorescence in the absence of DAPA. The time courses of intrinsic fluorescence are exhibited in Figure 31 (trace a, pII; trace b, rhMZ(I)). In both instances the reactions were marked by an increase in signal, similar to those observed with prothrombinase (Figure 29). Samples were removed at various times (inverted triangles) and subjected to SDS-PAGE under reducing and non-reducing conditions. With pII, the predominant species were meizothrombin(desF1) (Figure 31C)

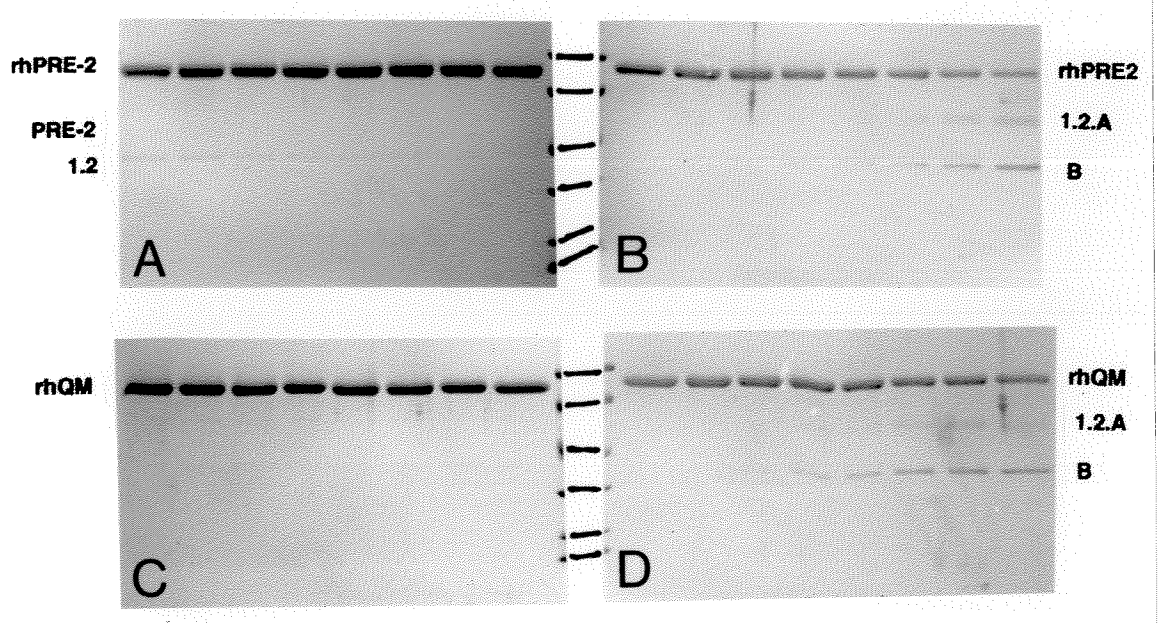
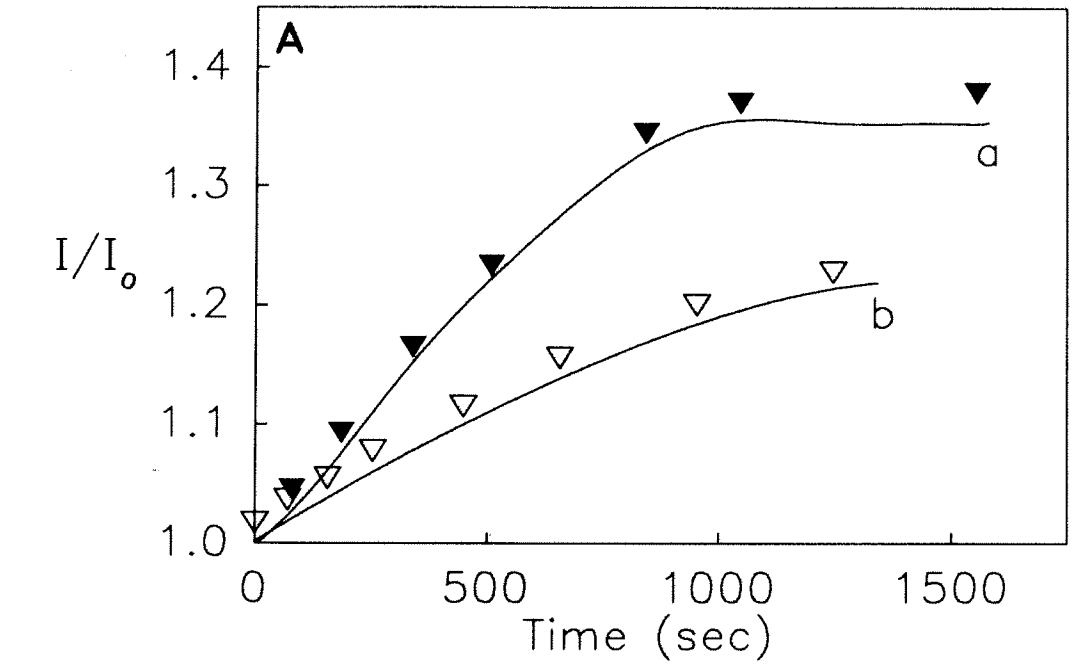


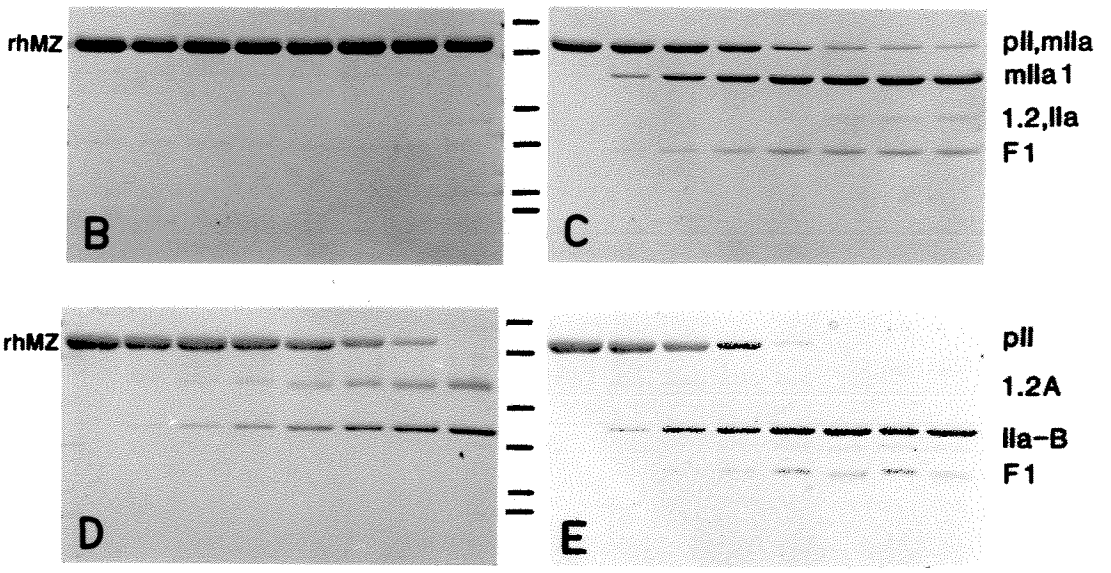
Figure 30. Prothrombinase and ecarin-catalyzed activation of rhPRE2 and rhQM monitored by SDS-PAGE.

The reaction was initiated by the addition of FXa (A and C) or ecarin (B and D). Samples of rhPRE2 and rhQM were withdrawn from ongoing activation and subjected to SDS-PAGE under reducing conditions. Sampling from left to right (except gel A, from right to left) were between 0.0 and 15.0 minutes after the addition of FXa. The abbreviations used are the same as in the legend of Figure 28. Also included is PRE-2 for prethrombin-2.

Figure 31. Ecarin-catalyzed activation of pII and rhMZ(I) monitored by intrinsic fluorescence (A) and SDS-PAGE (B-E).

The reaction was initiated by the addition of ecarin and fluorescence was monitored at 340 nm (A). Samples of pII and rhMZ(I) were withdrawn from ongoing activation (at times indicated by the inverted triangles) and subjected to SDS-PAGE under non-reducing (B and C) and reducing (D and E) conditions. Sampling with pII from left to right were 0.0, 2.0, 5.58, 8.38, 13.0, 16.4, 19.0, and 23.7 minutes (C and E) and with rhMZ(I) were 0.0, 1.22, 2.62, 4.23, 7.50, 9.33, 14.92, and 24.58 minutes (B and D) after addition of ecarin. The abbreviations used are as in the legend of Figure 29


A
1.4
1.3
1.2
1.1
1.0
I/I$_o$
a
b
0
500
1000
1500
Time (sec)


rhMZ
B
C
pII,mIIa
mIIa1
1.2,IIa
F1
rhMZ
D
E
pII
1.2A
IIa-B
F1

and thrombin B chain plus fragment 1 (Figure 31E), indicating substantial feedback proteolysis. With rhMZ(I), the products were indistinguishable from those obtained with prothrombinase, and were similarly stable. Laser densitometry of the gels showed that the initial rate of consumption of plasma prothrombin exceeded that of rhMZ(I) by a factor of 1.24.

rhPRE2 and rhQM were also activated in the presence of ecarin. The reactions were analyzed on SDS-PAGE under reducing conditions. Surprisingly, unlike factor Xa, ecarin appears capable of recognizing the mutated cleavage site to a certain extent as indicated by the slow appearance of thrombin B chain and fragment 1.2.A in both cases (Figure 30B and D). The activation, however, is markedly slower than the one observed with pII or rhMZ(I) under identical conditions.

F. Functional properties

1. rhMZ(I)

rhMZ(I) was activated by either prothrombinase or ecarin and assayed for esterase, amidolytic and fibrinogen clotting activities. Results were compared to samples of plasma prothrombin that were activated under identical conditions. The data are presented in Table 8. rhMZ(I) activated by either prothrombinase or ecarin demonstrated 6.8 % of the clotting activity of plasma prothrombin. Plasma prothrombin activated with ecarin (to yield predominantly meizothrombin), and rhMZ(I) activated with either prothrombinase or ecarin, exhibited identical TAME esterase activities. These were two-fold greater than that obtained with plasma prothrombin activated with prothrombinase. rhMZ(I) activated with either prothrombinase or ecarin yielded similar amidolytic activities, which were approximately one half the activity obtained with plasma prothrombin treated with either activator.

rhPRE2 and rhQM activated by the prothrombinase complex did not exhibit any amidolytic activity (data not shown).

Table 8. Esterase, amidolytic and fibrinogen clotting activity of plasma prothrombin and rhMZ(I)

sample	TAME (mol TAME/ mol ENZ/sec)	S-2238 (mol pNA/ mol ENZ/sec)	relative clotting activity
pII + PASE#	16 ± 2	129 ± 3	1.000
rhMZ(I) + PASE	30 ± 4	60 ± 1	0.068
pII + ECARIN	30 ± 4	119 ± 12	N.D.*
rhMZ(I) + ECARIN	31 ± 2	76 ± 5	0.068

* not determined

prothrombinase complex

The esterase and amidolytic activity assays (Table 8) were performed in the absence of calcium ions. Upon addition of Ca^{++}, the amidolytic activity of rhMZ(I)a noticeably increased. A titration of the effect of Ca^{++} on the amidolytic activity of rhMZ(I)a and human thrombin is presented in Figure 32. While thrombin is mostly unaffected by Ca^{++}, rhMZ(I)a demonstrates a Ca^{++}-dependent increase in activity, with the sharpest rise observed around 2 mM $CaCl_2$, the physiological Ca^{++} concentration. When the amydolytic assay are performed in the presence of 2 mM $CaCl_2$, rhMZ(I)a and thrombin display identical activity.

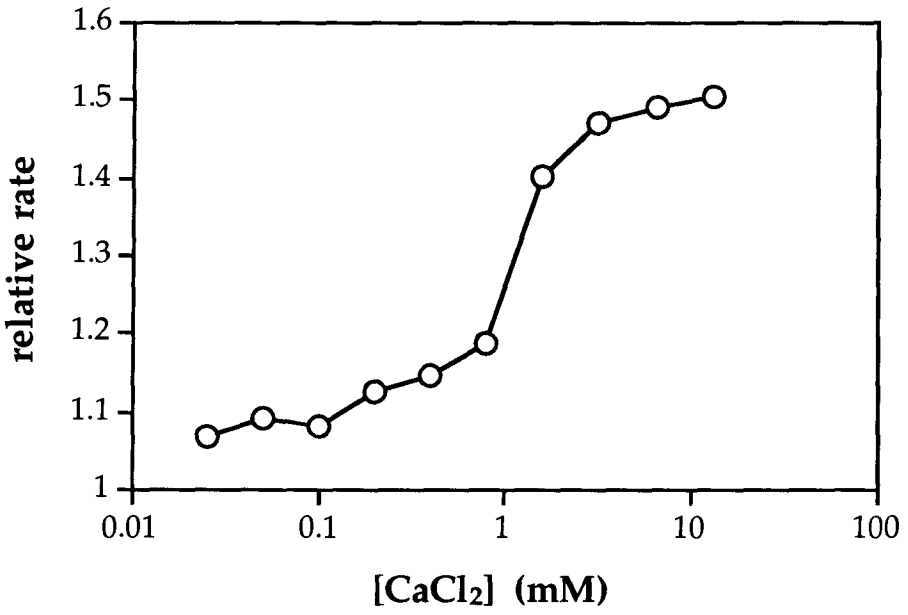


Figure 32. Ca^{++} titration of the amidolytic activity of rhMZ(I)a.
Relative rate of amidolytic activity of rhMZ(I)a toward the chromogenic substrate S-2238, in the presence of increasing concentration of $CaCl_2$.

2. Other mutants

The clotting activity of all the recombinant proteins was assayed using human prothrombin deficient plasma, and compared to that of plasma prothrombin (Table 9). Recombinant hFII demonstrates 57.4% of the clotting activity of plasma prothrombin. A lower activity was expected for rhFII due to the partial conversion to prethrombin-1 and the incomplete state of γ-carboxylation of the protein, which might lead to incomplete activation. As determined by fibrinogen clotting assay, rhMZ(I) and (II) show a weak clotting activity (7-9%). The rhDM mutant activity is very similar to that of rhFII, at 54% of the prothrombin activity. Although rhDM is probably undercarboxylated, the formation of prethrombin-1 cannot occur. The activation of rhDM was not analyzed by SDS-PAGE, but it is presumed that a portion of the activated material would correspond

Table 9. Clotting activity of plasma prothrombin and of the various recombinant human prothrombin.

sample	clotting time (sec)	relative clotting activity
pII	33.3	1.000
rhFII	43.0	0.574
rhMZ(I)	101.5	0.089
rhMZ(II)	112.8	0.071
rhDM	44.2	0.540
rhPRE2	219.5	< 0*
rhQM	218.7	< 0*
HBS#	181.9	0.000

* clotting time longer than buffer control

\# control with 20 mM HEPES, 0.15 M NaCl pH 7.4

to meizothrombin. Both rhPRE2 and rhQM inhibited the reaction, leading to a clotting time longer than the one observed in the absence of protein. Although their level of γ-carboxylation is probably not complete (comparable to rhMZ(II)), the mutant proteins can interact with the prothrombinase complex, thereby occupying sites and decreasing the rate of the reaction.

G. Stability of rhMZ(I)a

A sample of rhMZ(I) was activated with prothrombinase and the reaction mixture was subsequently stored at 4°C. After 3, 5, 8, 11, 15, 23 and 28 days, aliquots were withdrawn and prepared for SDS-PAGE. On day 28, electrophoresis was performed and

the remaining solution was assayed for amidolytic (S-2238) and esterase (TAME) activities.

Results of electrophoresis under non-reducing and reducing conditions are shown respectively in Figures 33. The gels show that rhMZ(I)a is highly resistant to thrombin-like feedback proteolysis over the 28 day period, although after about 8 days of storage low amounts of unidentified degradation products were visible and these accumulated over time. The functional assays showed that the esterase and amidolytic activities after 28 days were respectively 81 % and 53 % of the activities measured immediately following activation.

H. Inhibition of prothrombinase by rhQM

rhQM inhibited clotting in a prothrombin deficient plasma clotting assay. To further investigate this observation, the activation of prothrombin by the prothrombinase complex was monitored by DAPA fluorescence, in the presence of rhQM (Figure 34). The profile of prothrombin activation in the absence of rhQM (ratio 1:0) demonstrates the characteristic increment in fluorescence (at approximately 300 sec) followed by a progression toward a stable plateau, as observed previously (Figure 28). Upon addition of various amounts of rhQM to the reaction, a decrease in the rate of activation is observed, but a maximum fluorescence is still reached at approximately 370 seconds. As more rhQM is added to the reaction, the rate of conversion is further decreased and the profile changes. The increase in fluorescence becomes monotonic and approaches a plateau that is lower than that obtained with prothrombin alone. The initial rate of reaction appears to be directly proportional to the concentration of rhQM in the reaction. Although rhQM clearly inhibits the activation of prothrombin by the prothrombinase complex, the mechanism of the inhibition is unclear.

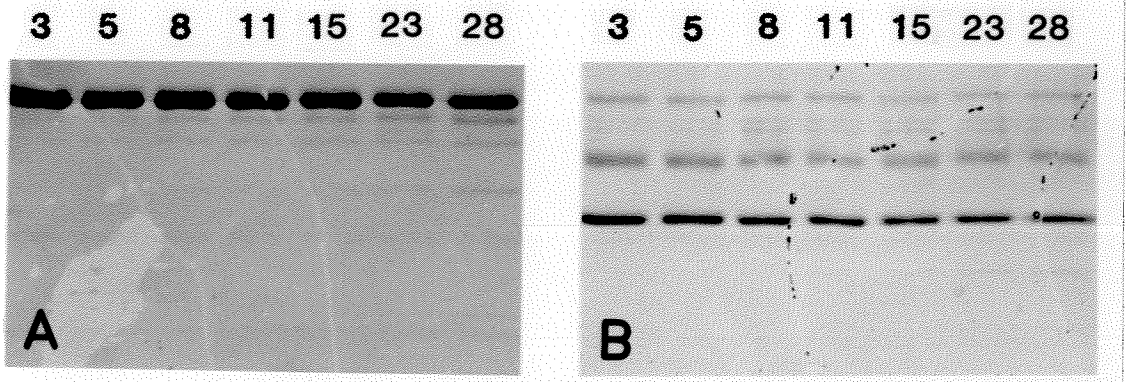


Figure 33. Stability of rhMZ(I)a.

A sample of rhMZ(I)a activated with the prothrombinase complex as described in Figure 29 was stored at 4°C. Samples were withdrawn 3, 5, 8, 11, 15, 23, and 28 days later were analyzed by SDS-PAGE under non-reducing (A) and reducing (B) conditions.

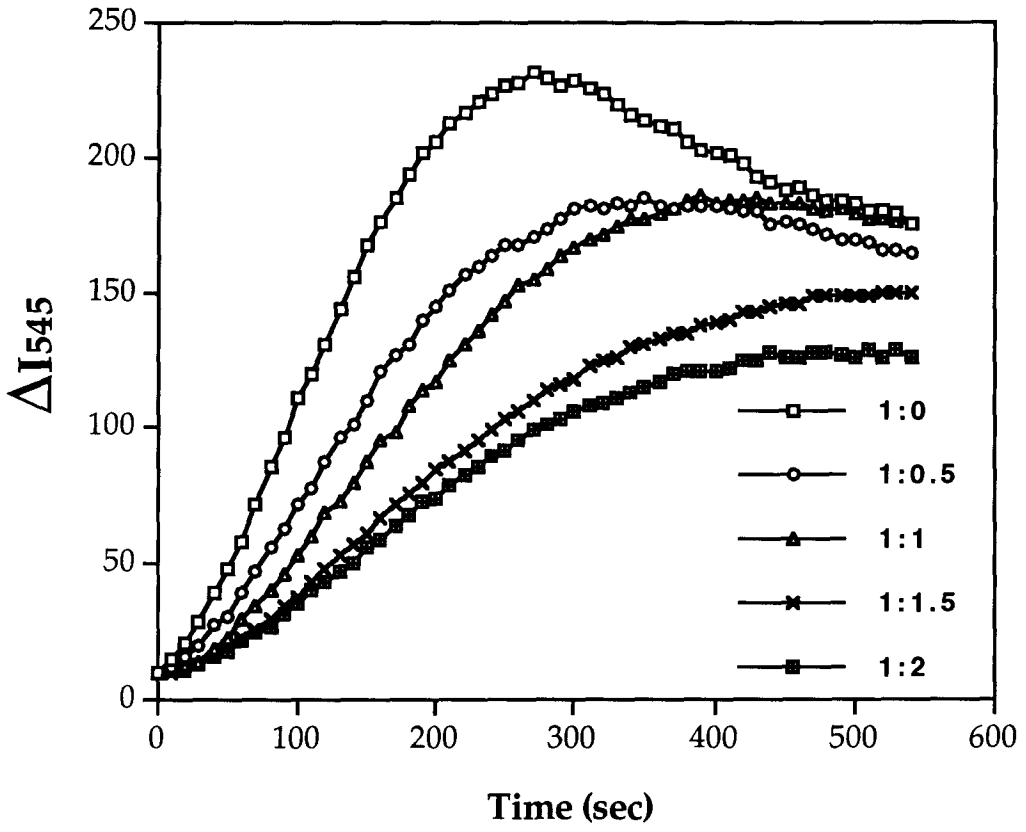


Figure 34. Inhibition of prothrombin activation by rhQM, in the presence of DAPA.

The reaction, in the presence of rhQM at various molar ratio, was initiated with the addition of FXa and fluorescence was monitored at 545 nm. The ratio pII:rhQM are indicated in the figure legend.

The experiment was repeated in the presence of prothrombin fragment 1. F1 contains the Gla domain and the first kringle of prothrombin. If the inhibition by rhQM is simply due to competition between prothrombin and rhQM for interaction with the complex through the Gla domain, F1 should affect the reaction similarly. Figure 35 illustrates the time course of prothrombin activation in the presence of equimolar amount of rhQM or F1, at a 2:1 ratio with prothrombin. Because F1 does not inhibit the activation of prothrombin by the prothrombinase complex as much as rhQM, the interaction of rhQM with the complex is not solely mediated by the Gla domain but involves other region(s) of the molecule.

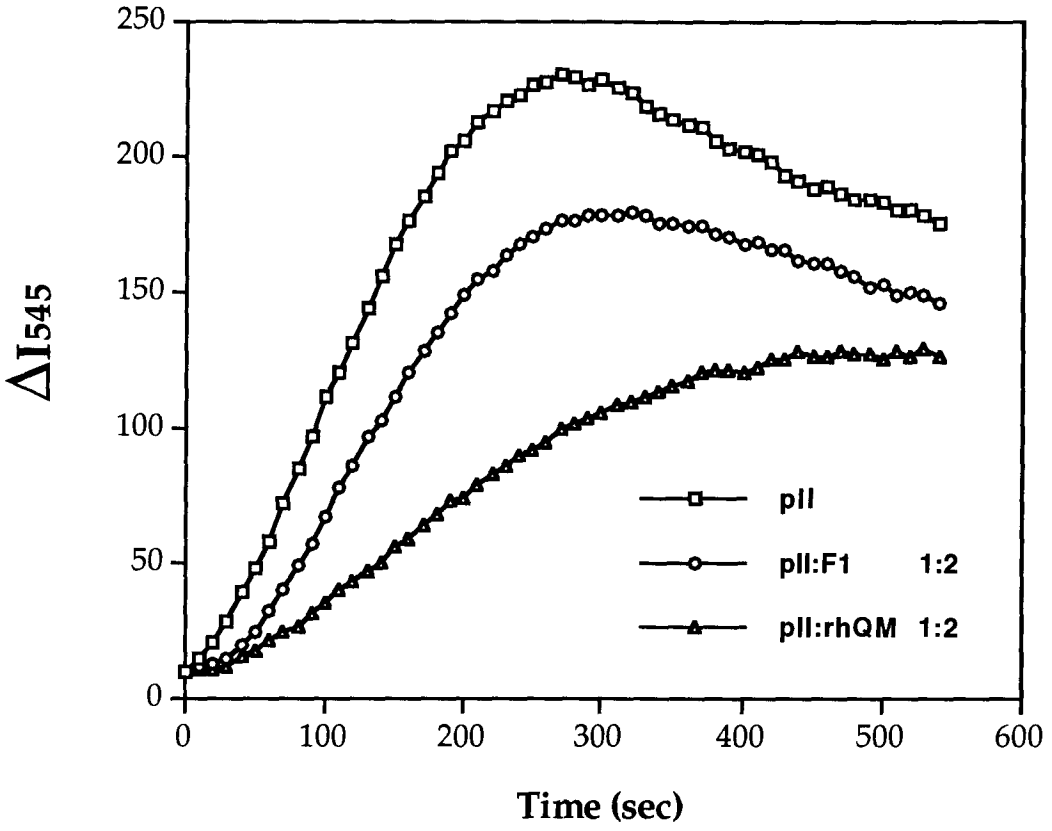


Figure 35. Inhibition of pII activation by rhQM and F1, in the presence of DAPA.

The reaction, in the absence (❑) or in the presence of rhQM (Δ) or prothrombin fragment 1 (O) at a 2:1 ratio with pII, was initiated by the addtion of FXa and fluorescence was monitored at 545 nm.

DISCUSSION

I. HUMAN FACTOR XII STUDIES

A. Epitope mapping

It is believed that monoclonal antibodies raised against native proteins will recognize conformational epitopes rather than linear ones (Benjamin et al., 1984). Although numerous studies (e.g., Green et al., 1982) claimed to have localized epitopes on native proteins by studying synthetic peptides corresponding to short linear sequences within the protein, the issue is controversial (for review see Laver et al., 1990), and opinions are divided. In the case of linear epitopes or conformational epitopes contained within a short region of the protein, this approach can provide useful information.

Screening of a factor XII cDNA expression library with two monoclonal antibodies (B7C9 and P-5-2-1) which inhibit the activation of FXII by negatively charged surfaces (Pixley et al., 1987; Saito et al., 1985) resulted in the isolation of 16 independent bacteriophage clones. All these clones contained cDNA encoding the first 31 amino acids of factor XII (Figure 13 and 14). Hydropathic analysis of this region of the FXII polypeptide (Clarke et al., 1989) revealed a highly hydrophilic cluster between amino acids 5 and 15, with positively charged lysine residues at positions 8, 11 and 13. The marked hydrophilic nature of this area fulfill the predicted criteria for epitope antigenicity in polypeptides (Hopp and Woods, 1981). This model locates protein antigenic determinants based on regions of greatest local hydrophilicity. Because of the N-terminal location and hydrophilic nature of the region, it is also likely to exhibit relative mobility and to lie close to the surface of the molecule. Accessibility and mobility also correlate with antigeneicity (Benjamin et al., 1984; Berzofsky, 1985; Getzoff et al., 1987).

Another study (Pixley et al., 1987) of B7C9 mAb suggested that the surface-binding site of factor XII lies between amino acids 134 and 153 on the heavy chain of FXII, spanning part of the type I fibronectin homology and part of the EGF homology. This result was based on affinity isolation using B7C9 and N-terminal sequencing of peptides produced after cleavage by kallikrein. None of the peptides isolated contained sequences covering the region from 1 to 14 of factor XII.

To support our epitope mapping results, a set of nested peptides between amino acids 1 and 28 of FXII were synthesized and their reactivity was tested with mAb B7C9. These experiments strongly implicated the first 14 amino acids of FXII in antibody binding (Figure 14). Binding of peptides 1-14 and 1-28 was readily detectable at peptide concentrations two orders of magnitude below those employed for peptide 134-153 (Pixley et al., 1987). However, it is possible that the B7C9 antibody recognizes two discontinuous sequences of factor XII as has been reported for other protein-antibody complexes (Mohri et al., 1988; Amit et al., 1986).

The N-terminal 19 amino acids of plasma FXII are encoded by the second exon in the FXII gene (Cool and MacGillivray, 1987). In contrast to the sequence homologies between FXII and other plasma proteins (Figure 9), the region encoded by exon 2 is unique within the blood clotting factors (Cool et al., 1985) and does not share any sequence identity with either tPA or uPA in exon 2. Interestingly, the tetrapeptide His-Lys-X-Lys between amino acids 10 and 13 of FXII (X=Tyr) is also found in two locations in bovine HMWK (Kitamura et al., 1983) and in three locations in human HMWK (Takagaki et al., 1985), where X=Asn, His or Phe. These positively charged peptide fragments have been implicated in the binding of HMWK to negatively charged surfaces (Berretini et al., 1987). A recent study (Samuel et al., 1993) based on chemical modification of histidine residues in human FXII concluded that two histidine residues of the molecule play a key role in the surface-binding activity. The His-Lys-X-Lys

sequence may therefore be involved in binding of FXII and HMWK to negatively charged surfaces.

Following the same reasoning applied to mAb B7C9, another anti-FXII monoclonal antibody, K0K5 was studied using the FXII expression library. K0K5 was known to inhibit the clotting activity of FXII without affecting its amidolytic or proteolytic activity. The common region encoded by all 29 strongly positive bacteriophage spanned the fibronectin type II homology of factor XII. Two cysteine residues involved in a disulfide bond appeared necessary for binding. Because K0K5 did not react with reduced FXII, it was assumed that a specific conformation was required for binding of the antibody to the antigenic determinant. Peptide binding studies were not pursued due to the size of the region of interest (45 amino acids) and because of the presence of two disulfide bonds within this region. The fibronectin type II homology is also found in tPA and type IV collagenase. Little is known about the function of this structure except that in collagenase, it has been implicated in gelatin binding (Collier et al., 1992). In tPA, deletion of the type II homology does not affect plasminogen activation nor fibrin binding (van Zonnefeld et al., 1986).

The third anti-FXII antibody subjected to the library screen was C6B7. This antibody inhibits activation of factor XII in plasma (Pixley et al., 1993). The few bacteriophage clones reacting with C6B7 encoded a common region between amino acids 336 to 364 which comprises two of the three proteolytic cleavage sites that give rise to FXIIf. The third cleavage site at Arg^{334}-Asn^{335} is adjacent to this region.

Several monoclonal antibodies complexed with protein antigens have been structurally analyzed. These include three complexes with lysozyme (Amit et al., 1986; Sheriff et al., 1987; Padlan et al., 1989) and two complexes with the neuraminidase from influenza virus (Colman et al., 1983; Colman et al., 1987). A feature of these interactions was the shape complementarity of the two surfaces, as revealed by the almost total exclusion of water molecules from the interface. The interface covered an area of about

700 Å^2 for both the antibody and the antigen, and involves 15-22 amino acid residues on several surface loops (for review see Davies and Padlan, 1990). This tight interaction of C6B7 with the region of FXII normally proteolytically cleaved by kallikrein could explain the lack of activation of FXII observed *in vitro* as well as *in vivo* (Pixley et al., 1993).

The last monoclonal antibody studied was F1. This monoclonal anti-FXII antibody was shown to bind FXIIa but the presence of a negatively charged surface was needed for epitope accessibility (Nuijens et al., 1989). In the presence of FXII, PK and HMWK, mAb F1 induced activation of the contact system. It was then proposed that F1 could induce a conformational change in FXII promoting the activation process of the molecule. Screening of the expression library with F1 was difficult and few positives were isolated. Furthermore, the reacting clones did not all share a common region. F1 probably reacts with various regions of the molecule brought together in the tertiary structure; these regions might be too far apart to be encoded by a 200-400 bp fragment of cDNA. This molecular biology mapping technique was judged unsuitable for this antibody.

B. Recombinant FXII expression

Once a region of interaction between antibody and antigen is determined, one approach to relate the function affected by antibody binding with the structure involved in the interaction is to express mutated forms of the protein of interest and assay for loss of function. For this purpose, BHK cells were transfected using the pNUT expression vector, to express and secrete recombinant human factor XII. The 5-10 µg/mL levels of secretion obtained were similar to those observed with other recombinant coagulation and fibrinolytic proteins. Qualitative characterization showed that rhFXII exhibited clotting and amidolytic activity and N-terminal sequencing agreed with the sequence found in mature human plasma FXII. Although the rhFXII was purified using an affinity column

prepared with an antibody that inhibits FXII activation, cleavage occurred rapidly following elution from the column. A better method of purification must be developed. The use of protease inhibitors appears essential to prevent activation of this very "activable" molecule.

Two mutant factor XII constructs studied in this system contained a deletion of the region identified as the epitope of mAb B7C9, putatively a region of FXII involved in binding to negatively charged surfaces (amino acids 1-20 and 5-20). Although the mutant proteins were expressed in the BHK cells, they were not secreted into the medium. Immunocytochemistry studies showed that the recombinant proteins accumulated in the ER, probably because of uncleaved signal peptides. Relatively little is understood about mammalian signal peptidases and their cleavage site recognition. The "signal hypothesis" (Blobel and Dobberstein, 1975; Blobel, 1980) involves the binding of the signal peptide to the endoplasmic reticulum which initiates transport before translation is completed (Blobel and Dobberstein, 1975). In this hypothesis, the signal peptide must be transient, as is the case for FXII, and is cleaved from the nascent polypeptide during or immediately after transport across the ER membrane. Initiation of this process involves the signal recognition particle (SRP) (Walter and Blobel, 1981), which consists of six polypeptide chains and a 7S RNA (Walter and Blobel, 1982), and interacts with ribosomes carrying nascent secretory polypeptide chains (Kurchalia et al., 1986). SRP functions *in vitro* by selectively stopping the translation of nascent secretory proteins (Walter and Blobel, 1981). Translation proceeds when the initiated ribosomal complex has made contact with the correct membrane, that is, the one containing the "docking protein" of the ER (Meyer et al., 1982).

Numerous studies have attempted to reconcile the high specificity of the cleavage reaction with the very limited degree of sequence homology found amongst different signal peptide sequences (von Heijne, 1984; Watson, 1984; Perlman and Halvorson, 1983; von Heijne, 1983). Although some consensus has been found in the signal peptide

sequence itself, little attention has been devoted to the importance of residues at the N-terminal end of the mature protein. The position +1 does not influence the signal peptidase cleavage and seems to accommodate almost any residue with the exception of proline (von Heijne, 1983). The fact that the wild type FXII construct allowed for protein secretion but that neither Δ1-20 nor Δ5-20 showed secretion indicates that these regions influence the signal peptidase recognition of its target or the process of translocation through the ER..

A naturally occurring mutation in the signal peptide (not propeptide) of human coagulation factor X has been reported recently which resulted in severe FX deficiency (Racchi et al., 1993). The mutation was characterized as a substitution of Arg for Gly at position -3 of the signal peptide. This mutation did not affect targeting and translocation to the ER but did block cleavage by the signal peptidase. Another mutation at codon -20 of the signal peptide of factor X San Domingo (Watzke et al., 1991), near the presumed signal peptidase cleavage site, resulted in lack of secretion. A similar outcome was observed with the Δ1-20 and Δ5-20 mutants of FXII. Another mutation was found at position -3 (Val->Glu) of antithrombin Dublin (Daly et al., 1990). In this case, the mutant protein showed an aberrant signal peptidase cleavage site two amino acids into the mature protein sequence but that did not affect its activity.

The wild type recombinant factor XII expressed in the vaccinia virus system (Citarella et al., 1992) was secreted at 2.85 μg/mL with a specific activity of 2.33 U/nmol which is considered within the normal range (Wuillemin et al., 1991a). However, a deletion of amino acids +3 to +319 (or +2 to +318) which only leaves intact the first two amino acids did not inhibit secretion in that system. HepG2, a human hepatoma cell line might produce a signal peptidase capable of recognizing the new cleavage site better than the one produced in hamster kidney cells. The concentration of the mutant FXIIΔ3-319 in the medium was determined to be 0.175 μg/mL and its specific activity was shown to be 1.5-2 fold higher than the native and recombinant full-length FXII (Citarella et al.

1992). Furthermore, the deleted mutant was reported to bind to negatively charged surfaces. The 16 fold difference in secretion level between the wild type and the mutant protein might reflect some impairment of the secretory pathway. The recombinant proteins were not subjected to N-terminal sequencing. Considering that the protein was impure and very dilute, and that HepG2 cells used in this expression system most probably secrete endogenous human factor XII (Knowles et al., 1980; Gordon et al., 1990), it would be interesting to attempt the reproduction of those results.

The third mutant expressed, FXII Δ28-69 appeared to be secreted normally. The activity of the recombinant mutant was not assessed but the level of secretion was similar to the wild type and the electrophoretic mobility was as expected.

In this system, the integrity of the N-terminal region of the FXII protein is necessary for processing such as signal peptide cleavage and secretion. The presence of the first 4 amino acids is not sufficient for processing but that of the first 28 is. It would be interesting to generate more deletion mutants and determine the requirements for secretion.

To overcome the secretion blockage in the study of the surface-binding domain of FXII, it would probably be preferable to substitute single amino acids such as His^{10}, Lys^{11}, Lys^{13} and His^{17}. Further proof that the 1-28 area makes up part of the binding site would require demonstration that the 1-28 peptide interferes with the binding of FXII to negatively charged surfaces.

II. HUMAN PROTHROMBIN STUDIES

A. rhMZ

The activation of prothrombin has been studied extensively (for review see Doyle and Mann, 1990). After much debate regarding the physiological intermediates of the activation, meizothrombin is now accepted as the main intermediate observed during the

activation of prothrombin by the complete prothrombinase complex (Krishnaswamy et al., 1986; Rosing and Tans, 1988).

To overcome the difficulty of isolating meizothrombin free of meizothrombin(desF1) and/or trace amounts of thrombin, a triple mutant form of the human prothrombin cDNA was expressed. Three cleavage sites, two recognized by thrombin and one by factor Xa were disrupted. Arginine residues preceding the normally hydrolyzed bond were substituted with alanine. Because alanine is small and uncharged, it was expected that these changes would not disrupt greatly the tertiary structure and function of the molecule but that the cleavage sites would not be recognized by the specific proteases.

Levels of secretion averaging 20 μg/mL were achieved in BHK cells cultured in roller bottles. This level was higher than any previously reported for the secretion of human prothrombin (Jorgensen et al., 1987a; Le Bonniec et al., 1991; Falkner et al., 1992).

Slight differences in electrophoretic mobility were observed between the recombinant proteins and the plasma-derived human prothrombin. These were probably due to heterogeneous glycosylation of the molecule. The role of carbohydrates in prothrombin is unknown. In tPA, glycosylation at Asn^{184} has been shown to inhibit the conversion of single-chain to two-chain tPA by plasmin (Wittwer and Howard, 1990), while N-linked carbohydrates appear to modulate the activation of coagulation factor X (Sinha and Wolf, 1993). In contrast, unglycosylated prourokinase expressed in *E. coli* showed greater activity than the Asn^{302}-glycosylated form expressed in mammalian cells (Lenich et al., 1992).

The rhMZ protein was purified by barium-citrate adsorption from the medium. Although all the antigen was adsorbed to barium-citrate, the pure protein appeared partially γ-carboxylated as 10-15% would not activate in the presence of the prothrombinase complex. This suggested that barium-citrate adsorption on its own did

not reflect complete γ-carboxylation of the molecule as 8 or 9 out of 10 modified glutamate residues might suffice for efficient adsorption to barium-citrate. Therefore, rhMZ was purified further by pseudo-affinity chromatography based on Ca^{++} binding properties. The incompletely γ-carboxylated protein (rhMZ(II)) recovered by barium-citrate adsorption, did undergo substantial Ca^{++}-dependent conformational change but interacted differently than rhMZ(I) with the elements of the prothrombinase complex including the phospholipid surface and possibly factor Va. This was illustrated by rhMZ(II) which was purified on the basis of its barium citrate binding properties, yet hardly bound to phospholipid vesicles. It has been reported (Jorgensen et al., 1987a) that when prothrombin expression levels were amplified by subculturing the transfected cells at a high methotrexate concentration, higher levels of total antigen were secreted but that 10 to 15 % of it did not interact with Ca^{++}-dependent conformation-specific antibodies. rhMZ(II) represents approximately 10 % of the total antigen but our expression level was three to four fold higher than that reported by Jorgensen et al.

The rhMZ(I)a-DAPA complex exhibited greater fluorescence intensity than the thrombin-DAPA complex. This confirmed the observation that the maximal fluorescence intensity routinely observed during prothrombin activation in the presence of DAPA reflects the presence of meizothrombin-DAPA complex and that a change in environment of the bound DAPA, as a consequence of the Arg^{271}-Thr^{272} bond cleavage, decreases the fluorescence intensity. The lower relative intrinsic fluorescence increment recorded during meizothrombin activation by either ecarin or the prothrombinase complex, and the slightly greater quantum yield of rhMZ(I) compared to plasma prothrombin suggested that rhMZ(I) exists in a conformation slightly different from that of prothrombin. The activation of rhMZ(I) by the prothrombinase complex appeared to be 33 % slower than that of plasma prothrombin under identical conditions. Similarly, an active site mutant of bovine prothrombin activated more slowly (4-fold) than plasma prothrombin (Pei et al., 1991). The non-cleavable bond between F1 and F2 might affect the rate of activation as

it was demonstrated that the thrombin-catalyzed feedback cleavage at Arg^{154}-Ser^{155} promoted the release of thrombin from the catalytic surface during the activation of bovine prothrombin (Nesheim et al., 1988).

Recombinant meizothrombin proved to be extremely stable and resistant to further degradation. Neither ecarin nor factor Xa was capable of catalyzing the hydrolysis of the modified cleavage sites, even over long periods of time. rhMZa can therefore be stored for extended periods of time. This eliminates the ambiguities surrounding the possible presence of meizothrombin(desF1) or thrombin in the meizothrombin preparation, and potential effects of inhibitors such as DAPA in studies of the functional properties of meizothrombin generated from plasma prothrombin. The only ambiguity remains the effect of replacing three charged residues by alanine on the conformation of the molecule; because the activation sites are probably on the surface of the zymogen molecule (and accessible to proteases), these minor changes would be expected to have little or no effect on the overall conformation of the zymogen.

Several groups reported that plasma-derived meizothrombin has very little clotting activity (Franza et al., 1975; Rosing et al., 1986; Doyle and Mann, 1990). This property was also exhibited by rhMZ(I)a. The amidolytic activity observed in the absence of Ca^{++}, however, was less than that of thrombin. This was in contrast to the similarities in amidolytic activities reported previously for bovine plasma-derived meizothrombin in the presence of 2 mM $CaCl_2$ (Doyle and Mann, 1990) or 20 mM EDTA (Rosing et al., 1986). Further investigation revealed that while thrombin activity toward chromogenic substrates was largely Ca^{++}-independent, rhMZ(I)a was Ca^{++}-dependent with similar activity observed for both molecules at 2 mM $CaCl_2$ (Figure 32). The presence of the fragment 1 domain containing the Gla region might play a role in this interaction. However, it has been shown that the conformation of the active site of bovine meizothrombin was changed when Ca^{++} ions bound to the molecule, and that the

binding of Ca^{++} elicited a conformational change that extended beyond the fragment 1 domain into the protease domain (Armstrong et al., 1990).

On the other hand, the esterolytic activity of rhMZ(I)a was greater than that of thrombin. This was true whether meizothrombin was generated from rhMZ(I) or plasma prothrombin. It has previously been observed that bovine fragment 2 enhanced the esterolytic activity of both human and bovine α-thrombin, but it was found that human F2 did not enhance this activity for either human or bovine α-thrombin (Myrmel et al., 1976).

Thus, the availability and stability of rMZa should make a valuable model for studies of the function of meizothrombin. It could fruitfully be employed in further studies of the activity of meizothrombin toward activation of factor V, factor VIII and platelets. rhMZa should also prove useful in resolving the dilemma as to whether or not meizothrombin can bind TM.

Equilibrium binding studies of human thrombin and meizothrombin (derived from ecarin-activated prothrombin S205A) to human recombinant TM concluded that meizothrombin was unlikely to be an important TM-dependent protein C activator (Wu et al., 1992). One explanation for the lack of clotting activity of meizothrombin is that the extended fibrinogen-binding pocket is not yet available, because the activation fragment (F2) masks the pocket referred to as anion-binding exosite (Liu et al., 1991b). However, several studies have demonstrated that hirugen, a synthetic dodecapeptide corresponding to the carboxyl-terminal amino acids 53-64 of hirudin, inhibits fibrinogen clotting activity without inhibiting hydrolytic activity toward small chromogenic substrates (Naski et al., 1990; Jakubowski and Maragonore, 1990). Crystallographic studies have proven that hirudin binds to the anion exosite (Grutter et al., 1990; Rydel et al., 1990; Rydel et al., 1991). Furthermore, the proteolytic formation of either of the two prothrombin activation intermediates (prethrombin-2 or meizothrombin) results in formation of a hirugen-binding site (Liu et al., 1991b). It is widely accepted that TM, hirudin and fibrinogen

(Tsiang et al., 1990) and possibly factor V and platelets (Suzuki and Nishioka, 1991) share the same binding site and compete for binding. By comparison with hirugen, the region of TM responsible for binding to the anion-exosite and altering the specificity of thrombin, was identified as the fifth and sixth growth factor-like domains (Ye et al., 1992).

If rhMZa can activate protein C to aPC, as was reported for bovine meizothrombin/DAPA in the presence of TM (Doyle and Mann, 1990), it would support the suggestion that the initial generation of meizothrombin may protect against restriction of blood flow due to clotting in small vessels. In major vessels, because the TM concentration is low, the thrombin generated favors fibrinogen binding over TM binding. Under these conditions, rhMZa would act as an anticoagulant.

More studies with rhMZa could also shed light as to why inhibition of human meizothrombin(desF1) and bovine meizothrombin by AT-III is not promoted by heparin (Schoen and Lindhout, 1987; Lindhout et al., 1986) . Other thrombin activities such as the activation of factor XIII and the vasoconstrictive and chemotactic effects should also be investigated to elucidate further the role of meizothrombin. The action of rhMZa on the thrombin receptor and platelet aggregation should be investigated. Finally, studies are also needed to determine whether activities observed *in vitro* in purified systems are representative of physiological processes *in vivo.*

B. rhDM and rhPRE2

rhMZ and rhPRE2 represent the 2 possible prothrombin activation intermediates in their stable form and rhDM mimics wild type prothrombin without the possibility of autolysis. As such, these three molecules should provide a good and simple model for studies on the kinetics of the activation of prothrombin by factor Xa and on the factors and conditions influencing those kinetics. The biological activity of these two mutants was not investigated in detail. However, SDS-PAGE analysis showed the expected

activation pattern and generation of prethrombin-2 from rhPRE2 (Figure 30), and the rhDM clotting and amidolytic activities were demonstrated after activation by the prothrombinase complex (Table 9).

C. rhQM

The quadruple mutant prothrombin showed the highest level of expression ever reported for a fibrinolysis or coagulation protein, in a mammalian expression system . Interestingly, rhQM and rhPRE2, the two mutant forms that should not generate proteolytic activity showed the first and second highest level of secretion of all the prothrombin constructs.

Expression of recombinant human plasminogen in mammalian cells led to cytotoxicity toward the cells and resulted in low cell survival, low secretion levels and intracellular degradation of the protein (Busby et al., 1991). This was due to generation of plasmin within the cells, from its precursor plasminogen, by an endogenous plasminogen activator. Co-expression of plasminogen with the α_2-antiplasmin inhibitor prevented the toxic effect and increased the synthesis and secretion of native human plasminogen (Busby et al., 1991). Similarly, although thrombin-like activity might not be as detrimental to the cells as plasmin activity, it could somehow affect them.

The rhQM construct was originally designed to provide a control for the effect of the presence of the Gla domain in meizothrombin. As was expected, the quadruple mutant was not cleaved by FXa, and it did not demonstrate any proteolytic or clotting activity. The first functional assay (Table 9) revealed that rhQM increased the clotting time of prothrombin deficient plasma Because rhQM is structurally similar to prothrombin, it probably interacted with the prothrombinase complex and slowed further reaction. Since rhQM will not be cleaved, it may not dissociate as readily as thrombin. The greater inhibition of prothrombin activation by rhQM than fragment 1 revealed that the presence of the Gla domain was not the only component of the interaction (Figure

35). Previous studies have shown that activation peptides prothrombin fragment 1 and fragment 1.2 inhibit the activation of bovine prothrombin by factor Xa (Govers-Riemslag et al., 1985). The fact that in the presence of the complete prothrombinase complex this inhibition is greatly reduced showed that factor Va protects prothrombin against inhibition by its own activation peptides. This study suggested that the inhibition is due to the Gla region of the activation peptides which compete with prothrombin and factor Xa for binding sites at the phospholipid surface (Govers-Riemslag et al., 1985). However, bovine factor V heavy chain interactions with bovine prothrombin or prethrombin-1 (prothrombin desF1) exhibit the same dissociation constant (Luckow et al., 1989). This indicates that the fragment 1 portion of prothrombin does not influence the association with FVa. The interaction between rhQM and the prothrombinase complex might therefore involve multiple associations which further inhibit the activation of prothrombin to thrombin. This inhibition of prothrombin activation by rhQM could be interpreted as anticoagulant activity by mean of preventing coagulation.

rhQM should prove a useful tool to study the protein-protein and protein-surface interactions taking place within the prothrombinase complex, by virtue of rendering the complex somewhat static.

III. FUTURE WORK

Prothrombin has never been successfully crystallized, despite repeated attempts. Prothrombin is a fairly large polypeptide and analysis of long polypeptides is more complicated but feasible. It could be hypothesized that prothrombin does not crystallize because of heterogeneity caused by post-translational modifications of the polypeptide chain. Such modifications include γ-carboxylation and glycosylation. However, crystallization of prothrombin F1 was achieved despite the fact that F1 is carboxylated and contains 2 N-linked carbohydrate chains (Park and Tulinsky, 1986; Soriano-Garcia et al., 1989; Soriano-Garcia et al., 1992). Thrombin was crystallized but only in the

presence of inhibitors such as PPACK or hirudin (Bode et al., 1989, Rydel et al., 1990; Rydel et al., 1991; Bode et al., 1992). It is likely that proteolytic degradation interferes with the long process of crystal formation. rhQM, although it may show some glycosylation heterogeneity, could be suitable for crystallization, because of its extreme stability. Similarly, rhMZa which was shown to be highly resistant to degradation might also crystallize. The knowledge of rhQM and/or rhMZa three-dimensional structure combined with the information already available on thrombin would add to the understanding of how activation of coagulation and fibrinolytic proteins generates proteolytic activity and biological function.

Preliminary experiments are presently under way to determine the activity of rhMZ(I)a toward factor V, factor VIII, Protein C with and without TM, and platelet aggregation. The regulation of rhMZ(I)a by AT-III with and without heparin is also being investigated. Approximately 50 mg of rhQM was sent to Dr. M. James at the University of Alberta for investigation of crystallization conditions.

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