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SELECTION AND CHARACTERIZATION OF A NOVEL FACTOR XI INHIBITING PEPTIDE BY USING PHAGE DISPLAY TECHNOLOGY

By

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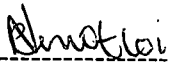
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DECLARATION

I, the undersigned, hereby declare that this dissertation submitted towards a M.Med.Sc degree is original, independent and has not in its entirety or part been submitted to any University or faculty for degree purposes.

All sources I have made use of or quoted have been acknowledged by complete references.



N.C. Motloi

November 2002.

THIS THESIS IS DEDICATED WITH LOVE TO MY PARENTS,
TLOKOTSI MOTLOI AND MOIPONE MOTLOI.

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Abbreviations

Ala	Alanine
Amp ^r	Ampicillin resistance
APC	Activated protein C
Arg	Arginine
AT III	Antithrombin
Ca ²⁺	Calcium
DNA	Deoxyribonucleic acid
E.coli	Escherichia coli
EDTA	Ethylene diamine tetra-acetic acid
ELISA	Enzyme-linked immunoabsorbent assay
FXIa	Activated factor XIa
FX	Factor XI
FX	Factor X
FIX	Factor IX
Gla	γ -carboxy glutamic acid
Gly	Glycine
HMWK	High molecular weight kininogen
H ₂ SO ₄	Hydrogen sulfate
Ile	Isoleucine
IPTG/XGAL	Isopropyl- β -D-thiogalactoside/5-bromo-4-chloro-3-indolyl- β -D-galactoside
LB-medium	Luria broth medium
LDL	Low density lipoprotein
Mr	Mass ratio
mRNA	Messenger ribonucleic acid
NaCl	Sodium chloride
NaOH	Sodium hydroxide
OD	Optical density
OPD	Ortophenylenediamine-dihydrochloride
pIII	Protein III
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethyleneglycol
PK	Prekallikrein
PL	Phospholipids
PN II	Protease nexin II
PT	Prothrombin time
PT	Partial thrombin time
pV	Protein VI

pVIII	Protein VIII
RF	Replicative form
RT	Room temperature
RT-PCR	Reverse transcriptase polymerase chain reaction
ScFV	Single chain variable fragment
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SM	Skimmed milk
TAFI	Thrombin activable fibrinolysis inhibitor
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
Thr	Threonine
TM	Thrombomodulin
tPA	Tissue plasminogen activator
Tris	Tris (hydroxymethyl) aminomethane
Vmax	Maximum velocity
vWD	von Willebrand disease

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CHAPTER 1

INTRODUCTION

The precise role of factor XI in physiological coagulation is still a matter of debate. In the "cascade" and "waterfall" theories of blood coagulation, factor XI was envisioned as playing a critical role in the initiation of coagulation through contact activation and the intrinsic pathway (Davie, 1991). The observation that patients individually deficient in factor XII, prekallikrein, or high molecular weight kininogen (HMWK) do not bleed casts doubt on the importance of contact activation for physiological blood coagulation (Schmaier et al, 1987). But, unlike factor XII deficiency, factor XI deficiency causes an injury related bleeding tendency (Minnema et al, 1999). This fact can be explained by the activation of factor XI by thrombin (Galiana and Broze, 1991). Therefore factor XI serves in a feedback loop involving thrombin, factor XIa, factor IXa, factor Xa and thrombin again to generate thrombin and sustain the coagulation process. The additional thrombin is capable of stabilizing the fibrin clot by protecting it from fibrinolysis (Von dem Borne, 1996, Meijers et al, 2000). This is done by the activation of thrombin activatable fibrinolysis inhibitor (TAFI) that down regulates fibrinolysis (Bouma and Meijers, 1999). Thrombin then activates fibrinogen to fibrin. Fibrin monomers spontaneously polymerize to form the insoluble fibrin blood clot and prevent the catastrophic loss of blood (Kondratovich, 2002, Bajzar et al, 1995). In this way, factor XIa produced in the contact phase of coagulation sets the initial conditions for the subsequent process of fibrin formation. Therefore, the dynamics of clot growth and final clot shape may significantly depend on the spatial distribution of contact activation factor XIa. (Kondratovich et al, 2002). The role of factor XI in hemostasis can be seen as a combination of a procoagulant action (the formation of fibrin) and an antifibrinolytic action (the protection of fibrin).

Thromboembolic disease, such as myocardial infarction, stroke and deep vein thrombosis, are a leading cause of death throughout the world (Vacca, 2000). It is

shown in the literature that with high levels of factor XI, the secondary generation of thrombin would be enhanced or sustained leading to a prolonged down regulation of fibrinolysis and therefore a risk of thrombosis (Meijers et al, 2000). Furthermore, under disease conditions associated with Disseminated Intravascular Coagulation (DIC), the continuous exposure to excess TF typically exhaust the available tissue factor pathway inhibitor (TFPI), leading to rampant thrombin generation and persistent feedback activation of factor XI by the generated thrombin (Østerud and Bjørliid, 2001). I thus argued that inhibitors of factor XI could stop this secondary generation of thrombin in thrombotic diseases. Such an inhibitor would also prevent or slow down the anti-fibrinolytic state of thrombi under these conditions. Furthermore, inhibitors of factor XI could be attractive candidates for anticoagulant therapy. The relatively mild bleeding associated with congenital factor XI deficiency suggest that drug induced blockade of factor XIa would be associated with relatively low bleeding risk compared to the standard anti-coagulants presently in use.

I thus decided on investigating the possibility of developing inhibitors against factor XI. There are no factor XI inhibitors commercially available as anticoagulants yet and no studies on this have been reported.

I selected possible inhibitors of factor XI using phage display technology. The technique of phage display allows for large numbers of phage clones to be screened. Greater than 10^9 different sequences can be screened which gives phage display a major advantage over other methods (New England Biolabs, 2000). The phage display technique is also faster (weeks/months) and less expensive than most other techniques that are used for drug discovery and it yields a product with practically indefinite stability (Jarolim, 2001).

I selected factor XI binding phages and then picked and grown up single phage clones and tested them for their ability to bind to and inhibit factor XI. I sequenced the factor XI-inhibitory clones and decided on one sequence to synthesize. A

linear 12-mer peptide with sequence "Gln-Gln-Val-Ala-Asn-Ala-Trp-Ser-Met-Ser-Pro-Ala" was synthesized and characterized by performing activated partial thromboplastin times (aPTT) and prothrombin times (PT). I selected a small peptide since small peptides have the advantage of being non-immunogenic (Markwardt, 1990).

CHAPTER 2

LITERATURE REVIEW

This study focuses on the selection and characterization of novel factor XI inhibitory peptides. It is therefore necessary to commence the literature review with a brief discussion on the mechanism of blood coagulation.

2.1 Blood Coagulation

Blood coagulation, together with platelet activation and fibrinolysis, is part of haemostatic response to injury. It serves to maintain the integrity of the vascular system and helps to prevent excessive blood loss through platelet-fibrin formation. The tissue factor pathway (Fig 2.1) of coagulation is initiated when damage to the blood vessels allows blood to be exposed to tissue factor (TF) that is produced constitutively by cells beneath the endothelium (Broze, 1995, Broze and Gailani, 1993). Factor VII or activated factor VII (factor VIIa) present in plasma binds to TF, and forms a factor VIIa/TF complex that activates limited quantities of factor X and factor IX (Broze, 1995, Broze and Gailani, 1993, Uszynski et al, 2001). A small amount of factor X is necessary to activate prothrombin to form thrombin. The remaining factor Xa is inhibited by tissue factor pathway inhibitor (TFPI), and so participates in feedback inhibition of the factor VIIa/TF complex. The initial factor Xa generation however provides sufficient thrombin to induce the local aggregation of platelets and activation of cofactor V and VIII, and factor XI. In order to sustain the coagulation process, additional factor Xa production must occur. This is done through activation of factor XI by thrombin. Factor XIa on its turn activates factor IX that activates factor X. The presence of variable but usually mild bleeding diathesis in a person with factor XI deficiency (sometimes called hemophilia C)

implies that, the initial quantity of factor IXa produced by factor VIIa/TF is insufficient for normal hemostasis and additional factor IXa generated by factor XIa is needed for normal hemostasis. This illustrates that the TFPI-induced feedback inhibition of factor VIIa/TF can explain the clinical need for both the tissue factor pathway and the contact system. Factor XIa is one of the key proteins of the contact system (see fig 2.1). Therefore, both these pathways are necessary to sustain blood coagulation (Broze, 1995, Meiring, 1996).

2.1.1 Tissue Factor Pathway

This pathway is initiated at the site of injury in response to the release of TF (Nemerson, 1992). TF is a specific transmembrane glycoprotein that plays a fundamental role in hemostasis by initiating blood coagulation. TF is expressed on the surface of many extravascular cells, most notably on cells in the adventitia, which forms a hemostatic envelope surrounding blood vessels (Peterson et al, 2000). It is usually absent from cells normally in contact with plasma (blood cells and the endothelium of vessels). Brain, lung, and placenta stain strongly for TF, as do peripheral nerves, autonomic ganglia, the epithelium of the skin and mucosa (Broze, 1992). The location of TF on plasma membranes of neutrophils and monocytes enables the initiation of a place of a coagulation to remain fixed at the site of injury (Nemerson, 1992, Hack, 2000). TF and factor VII (Fig 2.1), a plasma protein, have a high affinity for each other with the K_d being in the subnanomolar range (Nemerson, 1992, Rong et al, 2001, Keller et al, 2001). Neither component alone has procoagulant activity, but upon complex formation, they develop catalytic activity, that has a high degree of specificity (Nemerson, 1992).

When vascular injury occurs, TF and factor VII forms a one-to-one complex in the presence of calcium ions, and this facilitates the conversion of factor VII to a serine protease, factor VIIa by minor proteolysis (Davie et al, 1991, Wiiger and Prydz, 2000). The binding of factor VIIa to TF is tight (with dissociation constant in the

picomolar range) and essential for the protease activity. Binding of factor VIIa to transmembrane TF induces fluctuations in intracellular Ca^{2+} mobilization in a variety of cultured cells (Ahmad et al, 2001). This complex is due to the cleavage of a single internal Arg₁₅₂-Ile peptide bond in the precursor protein. The factor VIIa/TF complex activates factor X to a serine protease, factor Xa (Davie et al, 1991, Minnema et al, 2000). This complex also activates factor IX, by cleaving factor IX between Arg₁₄₅ - Ala and Arg₁₈₀ - Val and release a small activation peptide of 35 amino acids is released (Wolberg et al, 1997, Davie et al, 1991). Factor IXa is a vitamin K-dependent clotting factor (Nemerson, 1992, Gailani, 2001). The newly generated factor Xa forms a complex with factor Va in the presence of calcium ions and phospholipids (Davie et al, 2000, Vacca, 2000, Pellequer, 2000). Phospholipids provide a surface to assemble the clotting factors (Hack, 2000). The factor Xa/factor Va complex referred to as prothrombinase, converts prothrombin to thrombin, a serine protease composed of two polypeptide chains (Davie et al, 1991, Vacca, 2000, Pellequer, 2000). The activation of prothrombin is due to the hydrolysis of two internal peptide bonds (Arg₂₇₁-Thr and Arg₃₂₀-Ile) that reduces the molecular weight of the precursor from 71600 to 39000 (Davie et al, 1991). This result in the generation and release of thrombin, a serine protease, from the carboxyl terminal portion of the precursor while the Gla- and kringle-domain region from the amino terminal end of prothrombin stay attached to the phospholipid. Factor Va participates as a cofactor in prothrombin activation by increasing the V_{max} of the reaction about 1000-fold, whereas the phospholipid provided by the activated platelets reduces the K_m for prothrombin about 1400-fold (Davie et al, 1991).

When thrombin is formed it convert fibrinogen to fibrin by limited proteolysis. The formation of fibrin is due to the cleavage of a peptide bond in each of the two α -chains (Arg₁₈ - Gly) and each of the two β -chains (Arg₁₆ - Gly). Fibrin accelerates the conversion of factor XIII to factor XIIIa by thrombin in the presence of calcium ions. Factor XIIIa is an enzyme that cross-links fibrin monomers by forming ϵ -(γ -glutamyl) lysine bonds between two adjacent molecules (Davie et al, 1991). Factor

XIII also stabilizes the fibrin clot, because without FXIII a newly formed fibrin clot would easily be disrupted (Davie et al, 1991, McCance and Huether, 2000). This cross-linking reaction initially involves the γ -chains of fibrin followed by cross-linking of the α -chains. These covalent cross-links lead to the formation of a very strong fibrin clot (Davie et al, 1991).

2.1.2 Contact system

The Initiation Mechanism of the Contact System

Exposure of blood to a foreign surface with a negative charge (e.g. glass, kaolin, dextran sulfides, Collagen, sulphatide vesicles, long-chain saturated fatty acids) results in the activation of the contact system of coagulation (Ratnoff et al, 1961, Brunée, 1993, Hernandez and Raja, 1999, Mitropoulos, 1999). This first stage is called contact activation, which includes the following proteins: factor XII (Hageman factor), factor XI (Plasma thromboplastin antecedent), kallikrein (Fletcher factor) and high molecular weight kininogen (HMWK) (Fitzgerald, Williams or Flauejac factor) (Walsh, 1991, Brunée, 1993, Mauron, 2000, Neth et al, 2001, Basmadjian, 1997, Kramoroff and Nigretto, 2001) (see fig 2.1).

The first step in the contact phase is the binding of factor XII to a negatively charged surface. Upon binding, factor XII is activated to factor XIIa, which auto catalytically enhances its own production and catalyzes the conversion of prekallikrein to kallikrein and factor XI to factor XIa (Kondratovich, 2002, Merlo et al, 2002). Factor XI circulate in plasma in a complex with HMWK (Minnema et al, 1999, Keularts et al, 2001, Mitropoulos, 1999), and is present at a concentration of 3 to 6 $\mu\text{g/ml}$ (Wuillemin et al, 1995). HMWK links factor XI to the negatively charged surface where it is activated by surface bound factor XIIa (Sugi and Makino, 2000). The mechanism of action of factor XI will be described in full at a

later stage. Factor XIa then activates factor IX in the presence of calcium (Minnema et al 1999, Baglia et al, 2001, Komiyama et al, 1992). Factor IX in association with its cofactor VIIIa, activates factor X. The cofactors V and VIII are each activated by thrombin and dramatically increase the rate of activation of factor X and factor IX, respectively resulting in strong amplification of thrombin formation (Minnema et al, 1999). Factor XI can also be activated by thrombin resulting in the generation of additional thrombin via this pathway (Borne, 1996, Meijers et al, 2000). This activation is due to the cleavage of an Arg₃₆₉ – Ile peptide bond in each of the two subunits of factor XI by the newly generated thrombin. This leads to the formation of factor XIa a serine protease composed of two heavy chains and two light chains, these four chains are held together by three disulfide bonds (Davie et al, 1991).

The additional thrombin is capable of stabilizing the fibrin clot by protecting it from fibrinolysis (Von dem Borne, 1996, Meijers et al, 2000). This is done by the activation of thrombin activatable fibrinolysis inhibitor (TAFI) that down regulates fibrinolysis (Bouma and Meijers, 1999). Thrombin then activates fibrinogen to fibrin. Fibrin monomers spontaneously polymerize to form the insoluble fibrin blood clot and prevent the catastrophic loss of blood (Kondratovich, 2002, Bajzar et al, 1995). In this way, factor XIa produced in the contact phase of coagulation sets the initial conditions for the subsequent process of fibrin formation. Therefore, the dynamics of clot growth and final clot shape may significantly depend on the spatial distribution of contact activation factor XIa (Kondratovich et al, 2002).

2.2 Regulation of blood coagulation

Regulation of coagulation is exerted at each level of the pathway, either by enzyme inhibition or by modulation of the activity of the cofactors (See figure 2.2) (Dahlback, 2000). I hereby list the physiological inhibitors of coagulation.

2.2.1 Tissue Factor Pathway Inhibitor (TFPI)

TFPI inhibits the reactions involving TF and factor VIIa (Dahlback, 2000, Wachtfogel, 1994, Østerud and Bjørklid, 2001). TFPI is considered to be the primary regulator of TF-induced coagulation (Bajaj and Joist, 1999). It is mostly bound to the low-density lipoproteins (LDL) in plasma or to heparan sulphate when associated with endothelial cells (Dahlback, 2000, Wachtfogel, 1994). The mechanism of factor VIIa/TF inhibition by TFPI is complex and involves its binding first to factor Xa and then to factor VIIa in the factor VIIa/TF complex. TFPI under normal physiological conditions is synthesized by endothelial cells and not by hepatocytes. A majority of TFPI stays associated with the endothelium and serves to maintain, in part, the antithrombotic nature of the vascular endothelium (Bajaj and Joist, 1999). The lack of TFPI may not be compatible with life, since no deficiency states have been described in human beings (Dahlback, 2000, Wachtfogel, 1994).

2.2.2 Antithrombin III (AT III)

Antithrombin III (AT III) belongs to the serpin (serine protease inhibitor) family and is the major physiological inhibitor of thrombin (Wuillemin et al, 1996). It can also inhibit coagulation factors XIa, IXa, Xa, and the factor VIIa-TF complex. This inhibition of serine proteases by ATIII is not rapid, and consequently the serine proteases in the coagulation cascade have ample opportunity to generate fibrin before they are inhibited. This inhibition is due to the formation of inactive one-to-

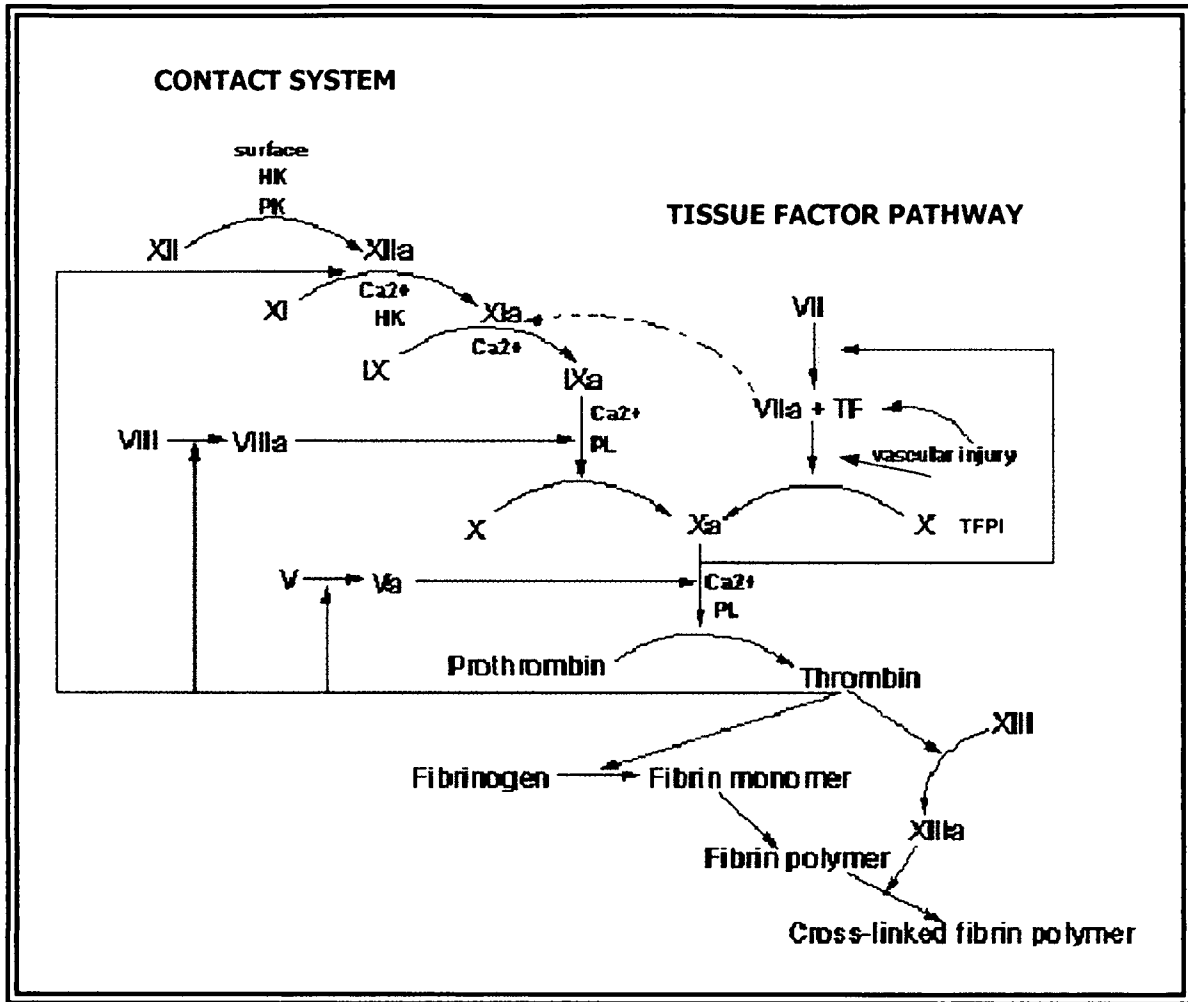


Figure 2.1: A schematic presentation of the coagulation cascade. The tissue factor pathway is initiated upon vascular injury, which leads to exposure of tissue factor (TF) to the blood. The contact system is initiated when contact is made between blood and negatively charged surface. The green dotted line represents a point of crossover between the contact system and tissue factor pathway. The two pathways converge at the activation of factor X to Xa (King, 1996).

one complexes of ATIII and the free enzymes (Davie et al, 1991). Enzymes that are part of the tenase or prothrombinase complexes are less accessible to inhibition (Dahlback, 2000). Antithrombin III is, in itself, an inefficient serine protease inhibitor, but heparin or similar sulfated glycoaminoglycans, that are present on the surface of endothelial cells stimulates its activity (Dahlback, 2000, Wachfogel, 1994, Davie et al, 1991, Wuillemin et al, 1996). Heparin, bound to AT III, induces a conformational change in AT III that facilitates the binding of AT III to the serine protease, which on its turn increases the rate of inhibition by about 100000 fold. Apart from the conformational change, heparin also seems to bring the inhibitor and protease together (Meiring, 1996). The physiological role of AT III is to limit the coagulation process to sites of vascular injury and to protect the circulation from liberated enzymes (Dahlback, 2000). AT III is in significant molar excess to its target enzymes and serves principally to quench enzyme activity once formed (Butenas et al, 1999).

2.2.3 Protein C and the Thrombin paradox

Protein C, the key component of the anticoagulant system, is a vitamin-K-dependent zymogen (Dahlback, 2000, Tanaka et al, 1999). It is activated on the surface of intact endothelial cells by thrombin that has bound to the endothelial cell membrane protein thrombomodulin (TM) (Dahlback, 2000, Tanaka et al, 1999, Esmon, 2000). TM is an integral membrane glycoprotein found on the luminal surface of blood vessel endothelium (Gailani and Broze, 1991). Thrombin when complexed to TM not only loses its ability to convert fibrinogen to fibrin but also gains the ability to activate protein C to activated protein C (APC) (Bajaj and Joist, 1999). APC in conjunction with protein S, efficiently inactivates the two important cofactors, factor Va and factor VIIIa and this decreases the generation of thrombin and therefore also inhibits the activation of TAFI (Bouma et al, 1998). The rate of clot lyses will therefore be increased (Bouma and Meijers, 1999, Minnema et al, 1998). The protein C/protein S complex cleaves factor Va and factor VIIIa even when they are part of fully assembled tenase and prothrombinase (Dahlback,

2000, Esmon, 2000). Thus, thrombin has the capacity to express both procoagulant and anticoagulant functions depending on the context under which it is generated (Dahlback, 2000, Tanaka et al 1999, Hack, 2000). The procoagulant process is mediated by an array of at least six plasma proteins (prothrombin, factor VII, factor IX, factor X, factor V, and factor VIII) and one tissue protein, TF. The anticoagulant process is governed by a minimum of four plasma proteins (AT-III, Protein C, Protein S, and TFPI) and by one membrane bound protein contributed by vascular tissue, TM (Butenas et al, 1999). The phenomenon is called the thrombin paradox. An explanation was provided by the observation that the mechanism of action of thrombin was dependent on its concentration.

Low levels of thrombin generate markedly increased levels of APC. *In vivo*, continuous activation of the coagulation mechanism at a basal level generates these low levels of thrombin, resulting in inhibition of coagulation by APC. Activation of coagulation by exposure to TF will result in higher levels of thrombin that overcome the anticoagulant effect of protein C resulting in clotting. It can be concluded that at high concentrations of thrombin not only becomes thrombin a procoagulant but also an antifibrinolytic, which enhances its thrombotic potential (Bouma et al, 1998). At sites of vascular disruption, the procoagulant effects of thrombin are fully expressed. In contrast, in an intact vascular system, thrombin has anticoagulant function since it binds to endothelial cell cofactor thrombomodulin and activates protein C also enhances activation of TAFI (Dahlback, 2000), TAFI is able to protect the fibrin clot from fibrinolytic attack (Bouma and Meijers, 1999). Thus on one hand thrombomodulin dampens the thrombin generation by enhancing the activation of protein C by thrombin, whereas on the other hand it makes these low concentrations of thrombin more effective in the activation of TAFI with an inhibition of the clot lysis as a result (Bouma et al, 1998, Bouma and Meijers, 1999).

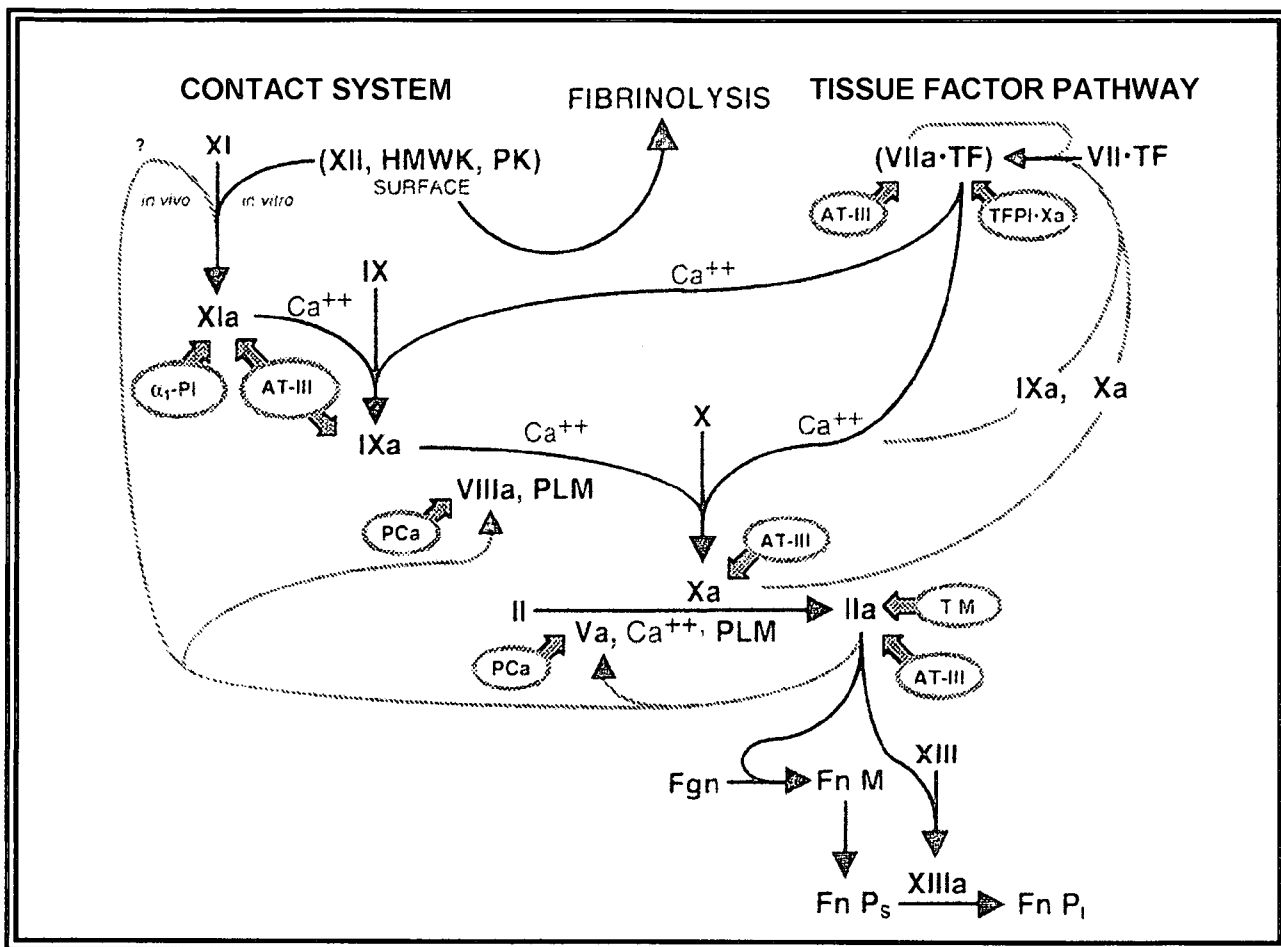


Figure 2.2: Regulation of blood coagulation. Current scheme of blood coagulation along with the natural inhibitors that inactivate clotting active serine proteases and activated cofactors. Antithrombin III (AT III) inhibits factors XIa, IXa, and Xa, FVIIa-TF and thrombin (IIa). TFPI inactivates Factor VIIa/TF complex. Factor XIa is inhibited by antithrombin. Thrombin when complexed with thrombomodulin gains the ability to activate protein C (PC) to activated protein C (APC), which in turn inactivates factors Va and VIIIa. The contact phase coagulation proteins represent the fibrinolysis, that is, conversion of plasminogen to plasmin (Bajaj and Joist, 1999).

Since this study focuses on the selection of factor XI inhibition, it is necessary to describe the biochemistry and the role of factor XI in haemostasis in detail.

2.3 Factor XI

2.3.1 Biochemistry

Factor XI is a plasma glycoprotein that was first identified by Rosenthal et al as a plasma coagulation factor (Rosenthal et al, 1953). It is the zymogen of a plasma serine protease (Fujikawa et al, 1986, Wolberg et al, 2000) that is composed of two identical polypeptide chains, which are linked by a disulfide bond each containing 607 amino acids (Fujikawa et al, 1986, Sun et al, 1999). Factor XI circulates in plasma in a complex with high molecular weight kininogen (HMWK) (Fujikawa et al, 1986, Mauron et al, 2000). The factor XI polypeptide is 80kDa in size and the mature molecule is synthesized in the liver. It has a N-terminal non-catalytic domain (heavy chain) and a C-terminal trypsin-like catalytic domain (light chain) (Ekdahl et al, 1999, Gailani, 2000). In contrast to other coagulation proteases, factor XI lacks the N-terminal calcium binding γ -carboxyglutamate (Gla) domain. The Gla domain is critical for protease interactions with phospholipids, and the absence of this domain may explain why factor XIa activity is not influenced by phospholipids (Gailani, 2001, Gailani, 2000). The proteolytic activation of FXI is achieved by cleavage of an internal Arg 369 - Ile 370 peptidyl bond to yield two heavy chains of 369 amino acids and two light chains of 236 amino acids.

A single disulfide bridge holds the heavy and light chain of each monomer together. The mass ratio of the native homodimer, as well as that activated factor XI, on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is 160 000 D under non-reducing conditions and, under reducing conditions it is 50,000 D and 30,000 D for the heavy and light chains, respectively (Baglia et al, 1996). Each light chain (Fig 2.3) contains an active site consisting of a catalytic

triad of His 413, Asp 462, and Ser 557 in the carboxyl terminal region of the molecule. A heavy chain consists of four repeat sequences or an apple domain called A1, A2, A3, and A4 and is 58 % identical to the corresponding region of prekallikrein. Each Apple domain contains six or seven cysteine residues that have a similar and characteristic disulfide-binding pattern in both factor XI and prekallikrein (Walsh, 1999). The A1 domain harbors the binding site for HMWK as well as the site for interaction with (pro)thrombin. The A2 domain contains a site necessary for the Ca^{2+} -dependent activation of factor IX by factor XI, although others claimed the binding site for factor IX on the A3 domain (Baglia et al, 1996). The A3 domain is capable of binding platelets in the presence of HMWK, and also contains a binding site for unfractionated heparin (Minnema et al, 1999). The A4 domain binds factor XII and mediates the dimer formation between the two polypeptide chains. (Minnema et al, 1999 Mitchell et al, 1999).

2.3.2 Genetics of Factor XI and Factor XI Deficiency

The gene coding for factor XI is located on the distal end on the long arm of chromosome 4. It is 23kb long and is composed of 15 exons and 14 introns (Minnema et al, 1999, Kadir et al, 1993, Sato et al, 2000, Ventura et al, 2000). The sequence and domain structure of Factor XI are depicted in Figure 2.3. Exons 3 to 10 encodes four tandem repeat sequences termed Apple domains, whereas exons 11 to 15 encodes the trypsin-like catalytic domain (Walsh, 1999). About 20 different mutations in this gene have been published, mostly resulting in decreased levels of factor XI and not dysfunctional factor XI proteins. The inheritance is autosomal recessive (Minnema et al, 1999, Sato et al, 2000, Economides et al, 1999, Sano et al, 1993), with varying expression in heterozygote. Autosomal recessive means that if the clotting defect is inherited from a parent, a child will be a genetic carrier of the condition but may or may not have symptoms. Although a cluster has been identified in northwest England (Minnema et al 1999, Kadir et al, 1993), factor XI deficiency, also called hemophilia C, is a rare disorder with

incidence in mostly people of Ashkenazi Jewish. The frequency of heterozygotes is calculated at 5.5 to 11 % and the homozygotes at 0.1 to 0.3 % (Martincic et al, 1999, Ginsberg et al, 1993). The bleeding tendency of factor XI deficient patients is difficult to understand. In general, factor XI deficiency presents itself as a mild bleeding disorder (Shirk et al, 2000 Lee, 1996, Bolton-Maggs, 1998, Smith et al, 1990). In contrast with hemophilia A and B, rare spontaneous bleedings occur and no bleeding into joints and muscles. (Shirk et al, 2000, Lee, 1996, Bolton-Maggs, 1998, Smith et al, 1990). Another distinctive contrast with hemophilia A and B is that the level of factor XI antigen or factor XI clotting activity does not predict bleeding risk. Factor XI deficiency occurs also in both males and females whereas in hemophilia A and B the genes for both factors are on the X-chromosome, which is why only males are affected and females are carriers of the disease (Shirk et al, 2000, Lee, 1996, Voorberg and Van den Brink, 2000, Dahlback, 2000). Although bleeding is observed more often in severe FXI deficiency, patients with nearly normal factor XI levels may also bleed excessive after trauma or surgery. The bleeding appears to be related to the genotype of the patient. Three genotypes of factor XI deficiency exists, they are genotype II/II, II/III and III/III. Patients with genotype III/III have less severe bleeding tendency than genotype II/II or II/III, because it has fewer injury related bleeding events (Shirk et al, 2000). On the other hand, several studies indicate that the site of injury or surgery is related to bleeding but it is independent of genotype. Also the presence of additional coagulation factor defects, most commonly von Willebrand disease (vWD) can influence the patient's bleeding tendency (Minnema et al, 1999, Kadir et al, 1999, Von dem Borne, 1995).

Bleeding is observed more often in tissues with a high intrinsic fibrinolytic activity, such as the genital-urinary tract, the nasal and buccal mucosa, and tissues exposed to saliva (Minnema et al, 1999, Kadir et al, 1999, Von dem Borne, 1995). This is because the needed antifibrinolytic potential of the contact system pathway is not provided at these sites due to the absence of factor XI. In these patients tissue factor induced activation of the coagulation mechanism is expected to proceed

normally to fibrin formation. However due to the impaired initiation of the contact system pathway caused by the absence of factor XI, the secondary burst of thrombin formation which is normally provided by the amplification potential of the tenase and prothrombinase complexes is decreased. As a result the activation of TAFI is reduced and consequently the down regulation of the fibrinolytic system is insufficient in tissues with a high fibrinolytic activity. Women are exposed to a hemostatic challenge every month during their menstrual period. The other common challenge is childbirth (Kadir et al, 1999). Women with factor XI deficiency are more likely to have menorrhagia and postpartum hemorrhage than their unaffected relatives. However, factor XI deficiency does not seem to be associated with enhanced risk of miscarriage, as was also shown in a recently developed murine model factor XI deficiency (Minnema et al, 1999, Kadir et al, 1999).

2.3.3 Mutations of factor XI

Three independent point mutations in the factor XI gene were found in Ashkenazi Jews with factor XI deficiency (Seligsohn et al, 1993, Goldstein et al, 1999). The type I mutation, so far found in only one patient, is a G to A change at the spliced junction boundary of the last intron (intron N) of the factor XI gene. This mutation interrupts the coding region of the mRNA between amino acids Lys¹⁸⁵ and Gly¹⁸⁶ just before the active site Ser¹⁸⁸ of the light chain of factor XIa (Seligsohn et al, 1993).

The type II mutation involves the introduction of a stop codon in exon 5 with a change from GAA to TAA and presumably leads to premature polypeptide termination (Seligsohn et al, 1993). This produces a little or no circulating factor XI (Dossenbach-Glaninger et al, 2001), which may result in a decrease or instability of factor XI mRNA or the generation of truncated unstable molecules that may be degraded intracellular without secretion. These changes lead to the deficiency of

factor XI in plasma (Sato et al, 2000). This mutation has frequency of 2.17 % in Ashkenazi Jews, occurs with a frequency of 1.67 % in Iraqi Jews, and is present at lower frequencies in other Jewish populations. A common founder for this mutation has been indicated by the presence of a single background haplotype between both Ashkenazi and Iraqi Jews. This mutation is the first mutation to be reported with high frequency in both Ashkenazi and non-Ashkenazi Jewish populations (Goldstein et al, 1999).

The type III mutation is located in exon 9 and consists of a change of TTC, coding for Phe²⁸³, to CTC, coding for Leu. This missense residue at position 283 is at the fourth apple domain of the heavy chain of factor XI and is near the site of a disulfide bond which holds the factor XI dimer together (Martincic et al, 1999, Seligsohn, 1993). This mutation has an allele frequency of 2.54 % in Ashkenazi Jews but has not been observed in large samples of Iraqi and Sephardic (Goldstein et al, 1999). This substitution interferes with intracellular dimer formation and secretion of plasma factor XI, so that type III patients typically have approximately 10 % of normal plasma factor XI levels (Shirk et al, 2000). The type III mutation was recently formed in a recombinant mutant factor XI that was expressed in the baby hamster kidney cells and caused diminished secretion of factor XI, apparently due to defective intracellular dimerization.

Since factor XI deficient patients have been identified that have neither type I, or type II or type III mutations, additional genetic defects may cause factor XI deficiency. Indeed, three point mutations were recently observed in three heterozygous patients of English origin one in exon 5 (a nonsense mutation) and two in exon 12 (missense mutation) (Seligsohn, 1993).

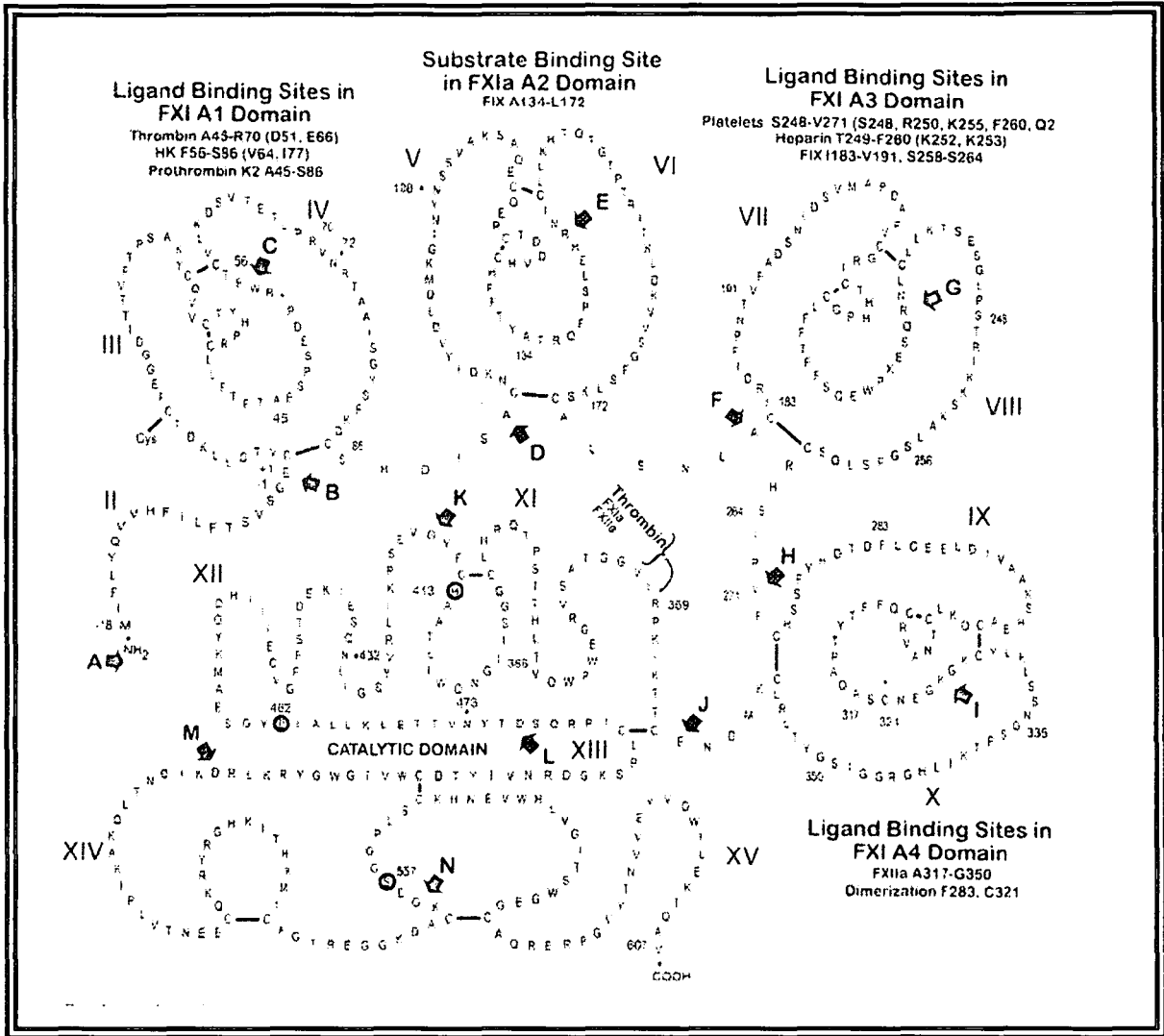


Figure 2.3: Amino acid sequences and primary structure of factor XI. Factor XI circulates in plasma as a homodimer connected by a single disulfide bond. Solid arrows show the 14 introns (A-N). The exons are denoted by II-XV amino to carboxyl terminus. The four apple domains (consisting of 90-91 amino acid) are labeled as A1, A2, A3, and A4. The three members of the catalytic triad (H413, D462, and S557) are circled in bold (Walsh, 1999).

2.3.4 Ethnic distribution and frequency

In 1958 Biggs et al suggested that factor XI deficiency might be more frequent among the Jews than among other populations. Several instances of severe factor XI deficiency in non-consanguineous Ashkenazi Jewish kindreds (due to homozygote-heterozygote mating) provided the first suggestion that the gene frequency in this population might be extremely high. Sporadic cases have also been reported among Italians, Germans, Japanese, Chinese, Koreans, Indians, American blacks and Arabs.

Recently, Bolton-Maggs et al have identified a second cluster of subjects with factor XI deficiency in 1992 among non-Jewish individuals residing in Northwest England. Two recent molecular genetic studies have addressed the question of the relative frequency of the defined mutation in patients with severe factor XI deficiency. It was found that type II and type III mutations account for most affected Jewish and English patients and that these two mutations are equally frequent among Ashkenazi Jews. No aggregation of geographic origins of ancestry was found for factor XI deficiency among Ashkenazi Jews. The extremely high gene frequency of factor XI deficiency among Ashkenazi Jews probably originates from genetic drifts caused by profound changes in population size, migration and founder effects rather than selection, since no advantage for carriers of factor XI deficiency has so far been demonstrated (Seligsohn, 1993).

2.4 Platelet Factor XI

Factor XI-like activity has been demonstrated in platelets. It contributes to 0.5 % of the plasma factor XI activity in sub cellular studies (Walsh et al, 1993). Platelet factor XI appears to differ structurally from plasma factor XI. It has an apparent mass ratio (Mr) of 220.000 Da (55,000 Da after reduction) whereas plasma factor

XI has a Mr of 160,000 Da (80,000 Da reduce) (Walsh, 2001, Shirk et al, 2001, Hu et al, 1998). This indicates that platelet factor XI probably exists as a disulfide-linked tetramer associated with the platelet membrane. Furthermore using reverse transcriptase PCR (RT-PCR) technique, factor XI mRNA could be amplified from platelets but not from other peripheral blood cells. Platelet factor XI is present in factor XI deficient patients and this correct the prolongation in thrombin activated coagulation assays.

A particular intriguing problem relating to the physiology of factor XI is the fact that the bleeding tendency in factor XI deficient patients is variable with 50 % of patients exhibiting excessive post-traumatic or post-surgical bleeding whereas the remainder appears to be hemostatically normal. A possible explanation for this variable in phenotype of factor XI deficiency is that the second form of factor XI found in the platelets might compensate for the absence of plasma factor XI, thus explaining the absence of bleeding complications in certain individuals with plasma factor XI deficiency. Factor XIa bound to platelets retains full coagulant activity as well as the capacity to activate factor IX and platelet-bound factor XIa appears to be protected from inhibition of both plasma and platelet-derived inhibitors (Minnema et al, 1999). Platelets are physiologic environments for reactions involving factor XI (Gailani et al, 2001). Activated platelets interact specifically with factor XI in the presence of high molecular weight kininogen (Sinha et al, 1984). When bound to activated platelets, the activation of factor XI by thrombin and factor XIIa is greatly accelerated. Furthermore, prothrombin can be used as a substitute for HMWK as a cofactor for factor XI/factor XIa binding to platelets, providing an explanation for the lack of excessive bleeding in patients congenitally deficient in HMWK (Gailani et al, 2001, Sharriat-Madar et al, 2001). A number of reports have demonstrated that washed platelets and isolated platelet membranes of factor XI-deficient individuals with normal hemostasis have normal quantities of platelet factor XI-like activity and normal behavior in the contact phase of coagulation, suggesting that platelet factor XI can substitute plasma factor XI in hemostasis (Walsh, 1999). Platelet factor XI mRNA is almost identical to the

mRNA for plasma factor XI. The only exception is the absence of exon V in platelet factor XI mRNA (Walsh, 2001, Shirk et al, 2000). Therefore platelet factor XI has been reported to be an alternative splicing product of the plasma factor XI gene lacking exon V (Walsh, 1999). Platelets, as well as factor XI participates in the contact system of blood coagulation leading to the local explosive generation of thrombin at sites of vascular injury with resulting hemostatic thrombus formation (Walsh, 2001).

2.5. Activation of Plasma Factor XI

The liver secretes most of the plasma factor XI, although mRNA has also been detected in pancreas and kidneys. The transfer of factor XI deficiency to the recipient further illustrates the hepatic origin after liver transplantation. In plasma, factor XI is non-covalently bound to HMWK at a 1:2 molar ratio. The plasma factor XI concentration is 4 to 6 g/L (approximately equal to 31 nmol/L). How factor XI is activated *in vivo* is presently under investigation. During contact activation, factor XII becomes autoactivated on binding to negatively charged surfaces where it can activate factor XI. HMWK is an important cofactor in these reactions, binding both factor XI and prekallikrein to the surface and stabilizing factor XI in a conformation facilitating its cleavage. Thrombin mediated factor XI activation was first described on non-physiological surface such as dextran sulphate (Walsh, 2001). Without such a surface, thrombin (and also factor XIIa) is a poor activator of factor XI. Thrombin does not remain bound to the cell membrane because the GLA-domain is removed during activation, but rather it binds to the fibrinogen and fibrin. Clot-bound thrombin is protected from inhibition by antithrombin but is still capable of activating other clotting factors and probably also factor XI, turning the fibrin clot into a localized, thrombogenic surface. During the conversion of prothrombin to thrombin, part of the prothrombin may be converted to intermediate meizothrombin.

In contrast to thrombin, meizothrombin may remain bound to phospholipid surfaces and is capable of activating factor XI. Once activated, factor XIa converts factor IX into a natural substrate. This reaction is dependent of membrane surface because the rate of factor IX activation is not affected by the binding of factor XIa to platelets. Factor IX is activated in the presence of calcium ions by cleavage of two peptide bonds one bond is located at Arg145-Ala145, the other at Arg180-Val181, to yield the enzymatically active factor IXa (Walsh, 2001).

2.6. Inhibition of Plasma Factor XIa

To prevent excessive activity of the clotting and other proteases in the blood, plasma contains a large excess of serine protease inhibitors. On interaction with proteases, serine protease inhibitors form very tight, covalently linked, complexes with plasma proteases. Two well-known inhibitors of factor XIa in human plasma are the serpins α -1-proteinase inhibitor and antithrombin III. However, both these inhibitors are characterized by low first-order rate constants of factor XIa inhibition and low affinity binding constants, making it unlikely that they are the major regulatory factors. Recently, another inhibitor of factor XIa has been discovered. Protease nexin II (PN II) is a truncated form of the transmembrane Alzheimer amyloid β -protein precursor. PN II is a 120,000Da kunitz-type serine protease inhibitor (Walsh et al, 1993 Keularts et al, 2001), which is secreted from platelet α -granules and accounts for 60 % of factor XIa inhibitory activity in platelet releasates. It is a specific, slow tight-binding inhibitor of factor XIa with a K_i of 35pM. Heparin increases the factor XIa inhibitory activity by ATIII about ten-fold. The maximum concentration of secreted PN II is about ten-fold higher than the K_i for factor XIa inhibition (Walsh et al, 1993).

2.7 Factor XI and Fibrinolysis

Patients with factor XI deficiency suffer from variable bleeding abnormalities, especially from tissues with high local fibrinolytic activity (e.g. urinary tract, nose, oral cavity, tonsils) (Martincic et al, 1999, Bouma and Meijers, 1999). The mechanism behind this clinical observation is unclear. When fixed concentration of thrombin was used to induce clotting, an antifibrinolytic effect of factor XI was observed. Trace amounts of activated factor XI were capable of completely inhibiting fibrinolysis. The inhibition of fibrinolysis was mediated by thrombin, which was generated in a factor XI dependent way by the contact system pathway. High concentrations of thrombin are necessary for the inhibition of clot lysis (Bouma and Meijers, 1999).

The feed-back loop in the contact system pathway, formed by thrombin-mediated factor XI activation, determines the amount of thrombin formed after clotting, thereby determining the fate of the clot during fibrinolytic attack. (Bouma and Meijers, 1999). Protection of the fibrin clot could be another distinctive function of thrombin in the regulation of coagulation. The inhibitory effect on the clot lysis by thrombin is caused by the activation of the carboxypeptidase B called thrombin activatable fibrinolysis inhibitor (TAFI) (Walsh, 1999, Minnema et al, 1993). TAFI also known as plasma procarboxypeptidase B provides a connection between thrombin generation and fibrinolysis inhibition. Activated TAFI exhibits substrate specificity similar to carboxypeptidase B and is hypothesized to inhibit fibrinolysis by removing the carboxy-terminal lysine residues from fibrin (Mosnier et al, 1999, Van Gorp et al, 2001).

The mechanism of factor-XI-dependent inhibition of fibrinolysis is therefore through the generation of thrombin via the contact system pathway, and is dependent on TAFI (Van Gorp et al, 2001). As described earlier, a high concentration of thrombin is needed to inhibit clot lyses and is also needed for the activation of

TAFI, in contrast to the small amounts of thrombin that are sufficient for the formation of fibrin and platelet activation (Van Gorp et al, 2001, Bouma and Meijers, 1999). Activated TAFI then is able to protect the fibrin clot from fibrinolytic attack. Therefore activation of TAFI in the clot is dependent on the presence of factor XI that cause thrombin generation after initial clot formation has taken place and that this thrombin is capable of activating TAFI (Minnema et al, 1998, Van Gorp, 2001).

Any disturbance in the contact system pathway therefore resulted in an increased clot lyses because of reduced thrombin formation. These regulatory roles of both factor XI and TAFI may explain the bleeding tendency that occasionally accompanies deficiency states of these proteins (Van Gorp et al, 2001). There is a correlation between TAFI concentration and clot lyses time in plasma of normal individuals. There was no correlation when clot lyses were performed in the presence of an antibody blocking factor XI feedback loop. This shows that there is a link between FXI-dependent thrombin generation and fibrinolysis since the generation of thrombin is increased in the presence of FXI and these higher concentrations of thrombin are needed for the activation of TAFI and TAFI down-regulates fibrinolysis (Bouma and Meijers, 1999).

2.9 Phage Display

2.9.1 Introduction

The technique of phage display is used in this study. I will discuss this technique in detail.

Phage display has proven to be a powerful technique for obtaining libraries containing millions or even billions of different peptides or proteins. This technique has been used for affinity screening of combinatorial peptide libraries to identify ligands for peptide receptors, define epitopes for monoclonal antibodies, select enzyme substrates and screen cloned antibody repertoires (Smith, 1985, Benhar, 2001). In many cases, it is possible to identify peptides with a sequence that closely resembles segments (epitopes) of the natural interacting partner of a protein by using this technique (Devlin et al, 1990, Kay et al, 2000, Rodi et al, 2001).

A practical consequence of this phenomenon, termed "convergent evolution" is that one can search whole genome databases for proteins containing segments that consensus sequences shared by the selected peptides, and experimentally determine whether or not they interact with the target (Devlin et al, 1990, Rodi et al, 2001). One of the most successful applications of phage display has been the isolation of monoclonal antibodies, and fragments thereof, using large phage antibody libraries. The most widely used library method is based on the use of the filamentous phage, a bacteriophage that infects male *Escherichia coli* (*E.coli*). Phage display is based on the cloning of DNA fragments encoding millions of variants of certain ligands (e.g. peptides, proteins or fragments thereof) into the phage genome, fused to the gene encoding one of the phage coat proteins (usually pIII, but also pIV, pVI or pVIII) (Gram et al, 1993, Benhar, 2001). This technique relies on the ability of certain phage to allow additional peptide sequences to be

incorporated into the coat proteins without disrupting their structure and function by linking the DNA sequence encoding the protein and the peptide itself (Gaskin et al, 2001). Upon expression, the coat protein fusion is incorporated into new phage particles that are assembled in the periplasmic space of the bacterium. Expression of the gene fusion product and its subsequent incorporation into the mature phage results in the ligand being presented on the phage surface, while its genetic material resides within the phage particle (Gram et al, 1993, Benhar, 2001). The physical linkage of phenotype and genotype intrinsic to a phage displayed library makes it possible for the binding properties of the displayed elements to be identified, modified and/or optimized in a matter of weeks with standard laboratory resources (Rodi et al, 2001, Sche et al, 1999).

Phages that display a relevant ligand are retained by virtue of their binding to the target, while non-adherent phages are washed away. Bound phage can be recovered from the surface, used to reinfect bacteria and reproduced for further enrichment, and eventually for analysis of binding. The success of ligand phage display hinges on the combination of this display and enrichment method, with the synthesis of large combinatorial repertoires on phage (Saggio et al, 1995). Phage selection is not limited to the isolation of antibodies or short peptides. As listed on table 2.1, this approach has also been instrumental in studies and manipulation of a variety of other biologically active molecules e.g. cytokines, receptors, enzymes, substrates, enzyme inhibitors, etc (Benhar, 2001). Phage display is by far the major tool for the isolation and engineering of recombinant antibodies (McCafferty et al, 1990, Sche et al, 1999). Antibodies in the form of recombinant antibody fragments were the first proteins to be successfully displayed on the surface of a phage. A phage antibody selection involves the sequential enrichment of specific binding phage from a large excess of non-binding ones. Multiple rounds of phage binding to the target and washing to remove non-specific phage elution to retrieve specific binding phage achieve this. Any method that separates phage that binds from those that do not, can be used for phage selection. The most popular selection method includes affinity selection (also called biopanning see fig 2.4) (Clackson et al, 1991, Marks et al, 1991, Jarolim, 2001).

Table 2.1: Phage display systems (Benhar, 2001)

Phage	Coat Protein	Displayed molecule	References
M13	pIII (minor)	Peptides Antibodies Cytokines Receptors Enzymes Enzyme inhibitors Catalytic antibodies DNA-binding proteins Cellulose-binding Proteins	Numerous reports. Recent reviews: Daniels and Lane, 1996; Lowman, 1997; Rodi and Makowski, 1999; Calliby, 1999 Numerous reports. Recent reviews: Winter et al., 1994; Burton and Barbas, 1994; Rader and Barbas, 1997; Hoogenboom, 1997; Hoogenboom et al., 1998; Griffiths and Duncan, 1998 Gram et al., 1993; Saggio et al., 1995; Buchli et al., 1997 Lowman et al., 1991; Scarselli et al., 1993 McCafferty et al., 1991; Soumillion et al., 1994 Pedersen et al., 1998; Demartis et al., 1999 Forrer et al., 1999 Roberts et al., 1992; Pannekoek et al., 1993; van Meijer et al., 1996; Huang et al., 1998 Janda et al., 1994; Sastry et al., 1995; Baca et al., 1997 Fujii et al., 1998 Rebar and Pabo, 1994; Jamieson et al., 1994 Choo and Klug, 1994; Choo et al., 1997 Smith et al., 1998; Berdichevsky et al., 1999
M13	pVI	Enzyme inhibitors Enzymes cDNA libraries	Jespers et al., 1995 Hufton et al., 1999 Hufton et al., 1999
M13	pVIII (major)	Peptides Antibodies Enzymes Enzyme inhibitors	Numerous reports. Recent reviews: Felici et al., 1995 Ladner, 1995; Cortese et al., 1996; Lowman, 1997; Wilson and Finlay, 1998 Kang et al., 1991; Wang et al., 1997 Corey et al., 1993 Markland et al., 1991, 1996
M13	pVII/pIX	Antibodies	Gao et al., 1999
λ	D (Head protein)	Peptides IgG-binding protein Enzymes Protein A cDNA libraries	Sternberg and Hoess, 1995 Sternberg Hoess, 1995 Mikawa et al., 1996 Mikawa et al., 1996 Santini et al., 1998
	pV (Tail protein)	Peptides Enzymes	Maruyama et al., 1994; Kuwabara et al., 1997 Maruyama et al., 1994
P4	Psu capsid protein	Peptides	Lindqvist and Naderi, 1995
T7	10B capsid protien	Peptides cDNA	Houshmand et al., 1999 Yamamoto et al., 1999
T4	Hoc capsid protein	Peptides Antibodies	Jiang et al., 1997 Ren and Black, 1998
	Soc capsid protein	Peptides Antibodies	Jiang et al., 1997 Ren and Black, 1998
	Internal protein III	Enzymes Green flourescent protein	Mullaney and Black, 1996, 1998 Mullaney and Black, 1996
MS2	Coat protein	Peptides	Mastico et al., 1993; Hael et al., 1999

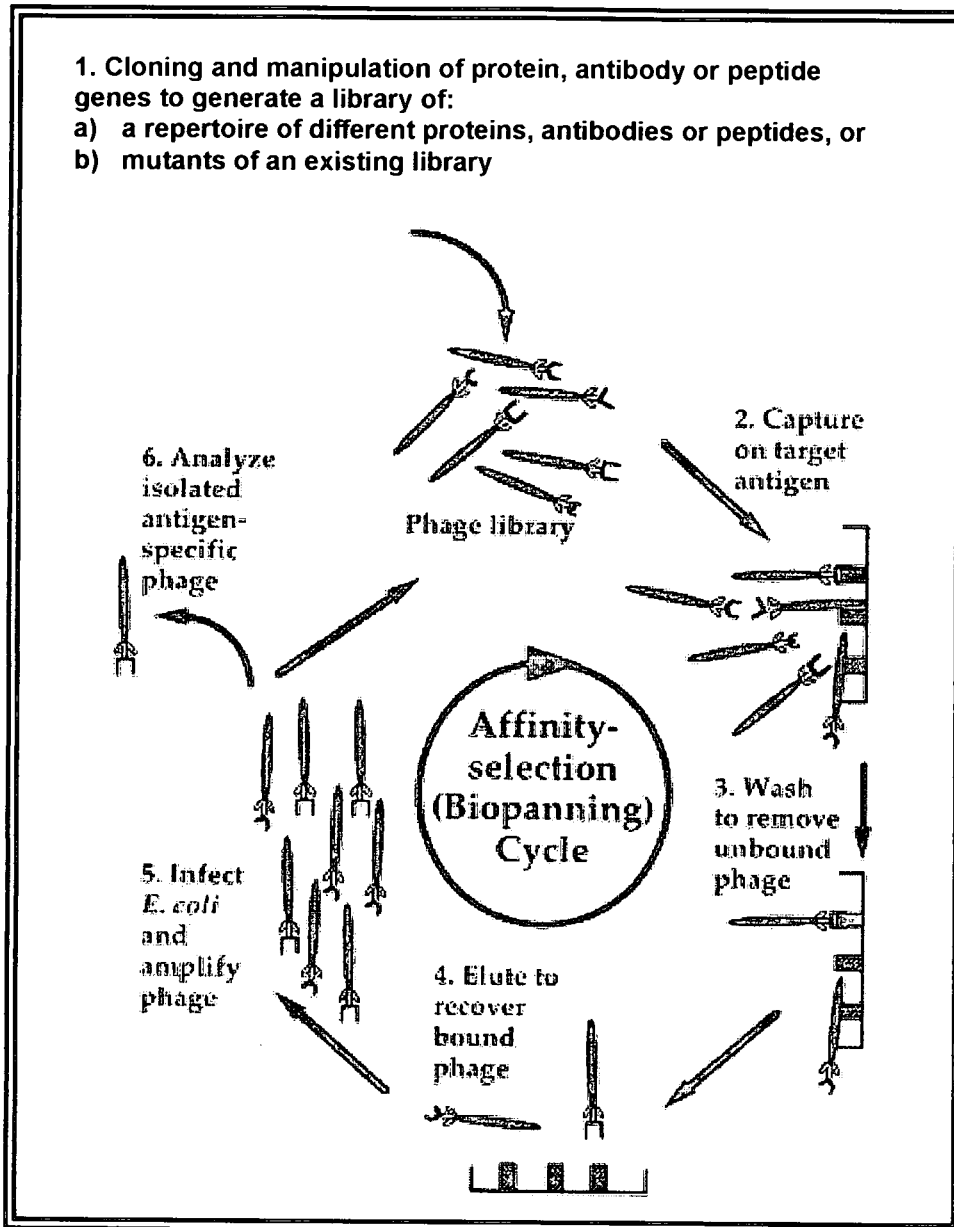


Figure 2.4 The phage affinity selection (biopanning cycle). Recombinant DNA techniques are used to generate a library consisting of millions (10^9) of different antibodies, or of variants (mutants) of an existing antibody. The resulting phage library is subjected to several cycles of affinity selection including capture of phage with antigen, washing to remove unbound phage, elution to release antigen-bound phage, and amplification in *E. coli* (Benhar, 2001).

Phage are prepared from the library and selected against the antigen. These binding phages are reinfected into *E.coli* and amplified for further rapid rounds of affinity selection. Enzyme-linked immuno-absorbent assay (ELISA) screening can confirm binding of individual clones (Devlin et al, 1990, Mullaney and Pallavicini, 2001). The individual isolated binding phage always contains the DNA sequence encoding the displayed protein, which allows direct physical linkage of phenotype and genotype are linked by means of the phage surface (Smith, 1995, Mullaney and Pallavicini, 2001). The linkage of genotype and phenotype in phage libraries has two immediate consequences: First, it allows the selection and the amplification of particular clones from pools of millions. With filamentous phage, the amplification step is accomplished simply by infecting male *E.coli*, which then produce many more phage. Second, the amino acids sequence of a peptide sequence displayed on a phage (which has been selected, e.g. to bind to a receptor) can readily ascertained by deciphering the DNA sequence of the relevant selection of the phage genome (Oldenburg et al, 1992, Barbas, 1993).

2.9.2 Filamentous Bacteriophage

The filamentous bacteriophage (Genus Inovirus) is a group of viruses that contain a circular single-stranded DNA genome encased in a long protein capsid cylinder. Many use some type of bacterial pilus to facilitate the infection process (Wilson and Finlay, 1998, Webster, 2001). The Ff class of the filamentous phages (f1, fd, and M13) has been the most extensively studied. As the name implies, these bacteriophage use the tip of the F-conjugative pilus as a receptor and thus are specific for *E.coli* containing the F-plasmid (Devlin et al, 1990, Wilson and Finlay, 1998, Webster, 2001). The DNA sequence of these three phages (f1, fd, and M13) shows them to be 98 % homologous; consequently the protein sequences of the gene products are practically the same. The Ff phages do not kill their host during productive infection.

The single-stranded viral DNA is replicated via a double-stranded intermediate by a mixture of bacterial and phage encoded components (Webster, 2001). The result of this replicative process is a newly synthesized viral single-stranded DNA in a complex with many copies of a phage encoded single stranded DNA binding protein (Webster, 2001). Assembly occurs at specific sites in the bacterial envelope where the cytoplasmic and outer membrane are close contact. During the assembly process the Ff phage are continuously extruded through the host cell envelope in the process that couples assembly with export, where the phage DNA-binding proteins are removed and the capsid proteins are packaged around the DNA. This process continues until the end of the DNA is reached (Webster, 2001, Wilson and Finlay, 1998). The filamentous bacteriophage M13, however has been the platform of choice by far, both from a historical perspective as the first and the best-characterized library display vector and as the source of the majority of successful screening. I will concentrate on this phage type in further discussion (Rodi and Makowski, 1999).

2.9.3 Structure of Filamentous Bacteriophage (M13).

The M13 phage is approximately 6.5 nm wide and 930 nm in length (Webster, 2001). The length depends on the size of the DNA and on the positive charge density along the inside surface of protein tube (Marvin and Hale, 1994). The mass of the phage is approximately 16.3 MD of which 87 % is contributed by protein (Webster, 2001). The genome of the M13 phage is a single-stranded DNA molecule of about 6400 nucleotides that is encased in a somewhat flexible protein cylinder (Webster, 2001, Mullaney and Pallavicini, 2001). Multiple copies of major and minor coat proteins surround this DNA molecule. This phage is a bacterial virus that infects *E.coli* and replicates within the host cell (Mullaney and Pallavicini, 2001). Each phage particle consists of approximately 2700 copies of pVIII major capsid protein, and 5 copies each of pIII and pVI (infection and assembly proteins)

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located at the terminal tip (fig 2.5) (Mullaney and Pallavicini, 2001, Dunn, 1996, Rodi and Makowski, 1999, Wilson and Finlay, 1998).

All of the coat proteins contribute to the structural stability of the phage particle, but pIII is also necessary for host cell recognition and infection (Sidhu, 2001). Electron micrography has shown that pIII appears as a nodule linked to the phage by a flexible tether that contains a series of Gly-Gly-Gly-Ser repeats (Wells and Lowman, 1992). Consequently, pIII is the largest and most complex of the coat proteins and it contains three distinct domains. The N-terminal domain initiates translocation of the viral DNA into *E.coli* during infection while the second domain confers host cell recognition by binding to the F pilus on the *E.coli* surface (Sidhu, 2001).

The C-terminal domain interacts with other phage coat proteins, and thus responsible for the integration of pIII into the phage coat. (Sidhu, 2001). Delays in supplying pIII lead to production of multilength viral particles (polyphage) containing two or more unit length phage genomes. Mutants producing pIII that lacks a substantial portion of N-terminal domain produce normal numbers of non-infectious unit length virions (monophage). But mutants unable to produce at least C-terminal portion of pIII are host lethal or produce relatively less stable non-infectious polyphage (Wilson and Finlay, 1998). A phage display library consists of a large collection of bacteriophage display a vast number of unique protein sequences. These proteins include peptides, antibody fragments, and protein domains corresponding to gene fragments or cDNA (Mullaney and Pallavicini, 2001).

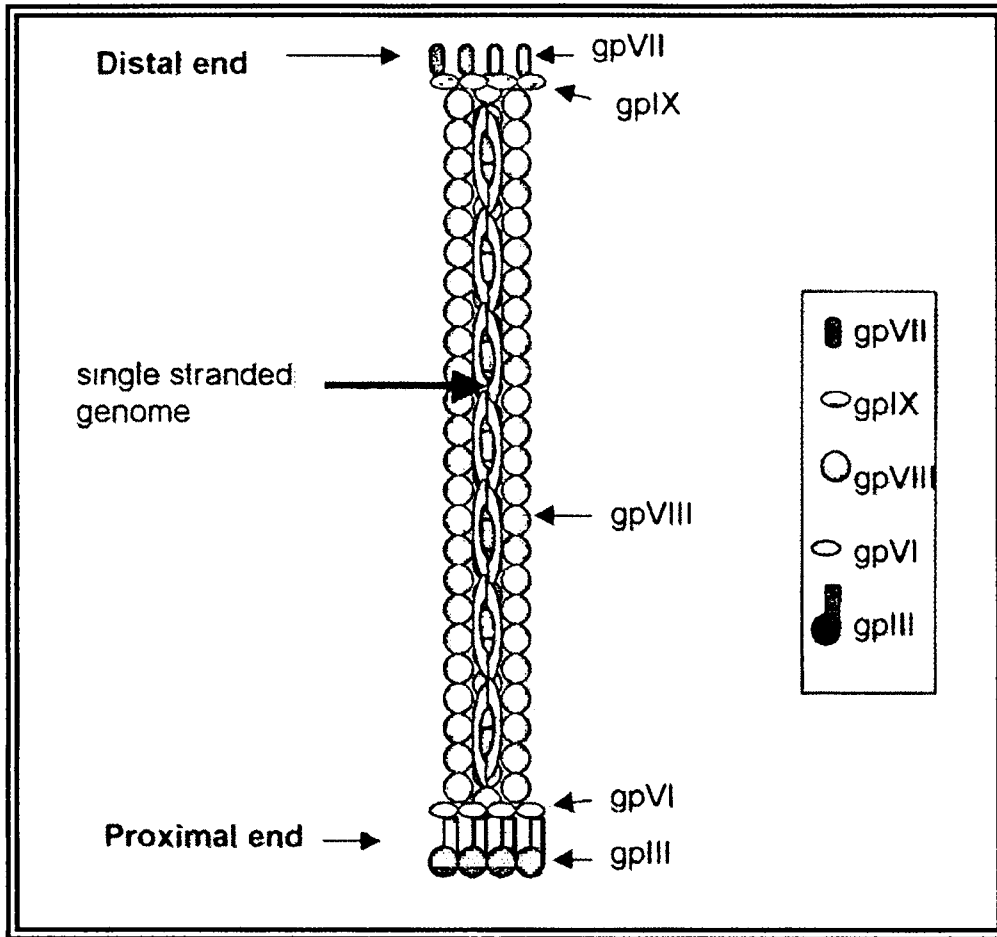


Figure 2.5. Structure of the M13 bacteriophage with coat proteins and single stranded DNA genome (ssDNA) (Wilson and Finlay, 1998)

2.9.4 Phage display systems

Phage display systems can be classified according to the arrangement of the coat protein genes. The display sites most commonly used are within genes III and VIII, although there have been attempts at cloning genes VI, VII and IX (Armstrong et al, 1996, Smith and Petrenko, 1997).

Phage display systems can be differentiated on the basis of the coat protein used for display (e.g. pIII or pVIII), whether the protein to be displayed can be fused to all copies of pIII or pVIII, or to only a fraction of them, and whether the recombinant fusion is encoded on the phage genome or on a separate genome. This classification was made by George Smith (1993).

The simplest phage display vectors are type 3 and type 8. Their basic functions have not been altered. The gene for the coat protein/peptide that will be used as the scaffold for display is modified to carry restriction sites for cloning the protein or peptide to be displayed (Scott and Barbas, 2001). In the type 3 vector, there is a single phage genome bearing a single gene III, which accepts foreign DNA, inserts and encodes a single type of pIII molecule (see figure 2.6). The foreign peptide encoded by the insert is theoretically displayed on all five pIII molecules on a virion (though in practice normal proteolytic enzymes in the host bacterium often remove the foreign peptide from some or even most copies of pIII, especially if the foreign peptide is large) (Smith and Petrenko, 1997). This displayed peptides and variety of proteins are displayed at the N-terminus of mature pIII. The same applies to type 8, display foreign peptides on every copy of pVIII. Only short foreign peptides can be displayed on every copy of pVIII, this may be due to the close packed nature of the viral surface. It does not matter if it is short peptides because the peptide comprises a substantial fraction of the virion's mass and can dramatically change its physical and biological properties (Armstrong et al, 1996, Smith and Petrenko, 1997). Some of the sequences are not well displayed on the surface of M13 phage, due to defects in viral particles, assembly, stability, and infectivity. This has led to the development of other systems, type 33 and type 88 (Armstrong et al, 1996).

In these systems, 33 and 88 the phage genome bears two genes III or VIII and the encoding two different types of coat proteins. Only one of these genes III or VIII is a recombinant bears a foreign DNA insert and the other is wild type (see figure 2.6).

In another type vector, 3+3 and 8+8 there are two different copies of the genes, but in this case the genes occur on separate genomes. The wild type version is on a phage usually called the helper phage, while the recombinant version is on a special kind of plasmid called a phagemid (Smith and Petrenko, 1997). A phagemid is a plasmid, which contains DNA sequences that allow packaging into phage particles. The phagemid carries the recombinant version of the type 8+8, 3+3, and 6+6 systems. The recombinant pIII encoded by a type 3+3 phagemid is usually missing the N-terminal domain, since the cells expressing this domain are resistant to superinfection by helper phage. The phagemid replicates in *E. coli* as a double stranded plasmid, but co-infection with a helper phage results in the production of single-stranded phagemid helper phage provides all the proteins necessary for phage assembly, including wild type copies of the coat proteins. For phage display, the phagemid contains a gene that encodes an additional copy of one of the coat proteins fused to polypeptide that is to be displayed. The resulting phage contains both wild type coat proteins from the helper phage and also the fusion coat protein from the phagemid (Sidhu, 2001). Library members are cloned in-frame at the N-terminus of the pIII gene. Tens of millions of unique sequences may be cloned to create a diverse molecular library. The phagemid plasmid contains only a single phage gene; thus, infection with a helper phage is required to provide the remaining machinery for replication. While the power of the library in its diversity, the essential feature is the ability to select members with desired properties. The basis of phage selection is an affinity interaction between the displayed protein and its target (e.g. antigen) (Mullaney and Pallavicini, 2001). In these types, the displayed peptide is monovalent, thus both wild type and phagemid will have one copy of the peptide. The advantage of the monovalent display is that the expression is on the phagemid and helper phage then supplies large excess of the wild type coat protein (Armstrong et al, 1996).

Foreign peptides have also been fused to pVI this fusion however, takes place at the C-terminal of coat protein VI (Jespers et al, 1995). A major advantage of pVI

display is that pVI is not involved in phage infection and the C-terminus of the coat protein is exposed on the surface, therefore the presence of stop codons will not prevent the display of proteins (Hufton et al, 1999). Protein VI is also suitable for cDNA expression. There are also the different types of gVI display. The types are 6, 66 and 6+6 (Smith and Petrenko, 1997).

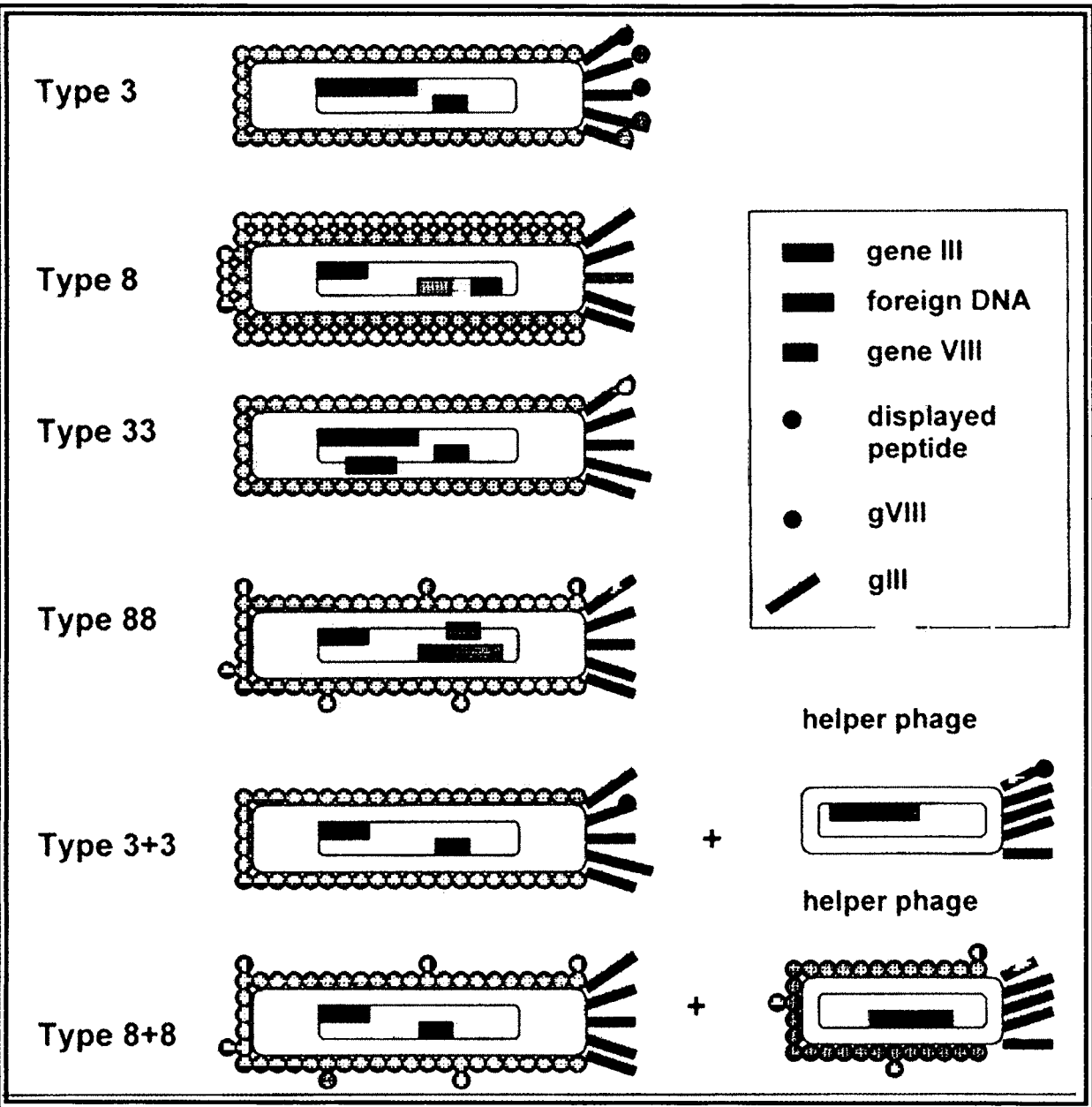


Figure 2.6 Types of phage display systems. Type 6, 66, and 6+6 systems are not shown (Smith and Petrenko, 1997)

2.9.5 Life cycle of M13 phage

The life cycle of M13 is initiated when the single strand genome (designated '+' strand) which is attached to the F-pilus, enters the *E.coli* cells (Blaber 1998). This is done by the binding of the tip of the F-pilus of the *E.coli* to the amino-terminus of the phage pIII protein (see figure 2.7). The exact binding on the amino-terminus is not known (Webster 2001). After the phage binds to the pilus, the pilus retracts and the coat protein, pIII, remains attached and guides the phage in the infection process. The major coat protein, pVIII, is then stripped off (Webster 2001, Blaber 1998). It is not known whether this retraction of the F-pilus is the result of normal assembly-retraction cycles inherent to the pilus or whether the attached phage triggers this retraction process. The major coat protein, pVIII then disassemble into the cytoplasmic membrane as the phage DNA is translocated into the cytoplasm of the *E.coli* (Webster, 2001). The replicating enzymes of host cell convert single strand (+) genome to double stranded circular DNA called the replicative form (RF form) (Blaber 1998).

The next phase of the life cycle is the replication phase where transcription starts. Series of promoters provides a gradient of transcription, such that the gene nearest the two transcription terminators is transcribed the most. Two terminators of transcription are located at the ends of genes VIII and IV. Transcription always occurs in the same direction (Blaber 1998).

In the next phase, the gene II product is synthesized after completion of the synthesis of the complementary strand. This protein is responsible for introducing a "nick" in the (+) strand at a specific place in the intergenic region (see figure 2.7). The resulting 3'hydroxyl end then act as a primer for the extending (+) strand (Blaber 1998, Webster 2001). Gene II introduces a nick after one trip around the genome in the (+) strand. This separates the parental (+) strand from the newly synthesized (+) strand (Blaber 1998), and it also circularizes the displaced viral (+)

strand DNA. This (+) strand is then converted to a covalently closed, supercoiled, double-stranded RF molecule. In this way, pools of progeny double-stranded RF molecules are produced (Webster 2001). The pV levels increase as the RF molecules accumulate in the cell. This protein binds to the (+) strand and prevents it to convert to the RF form. This leads to build up of single stranded (+) DNA.

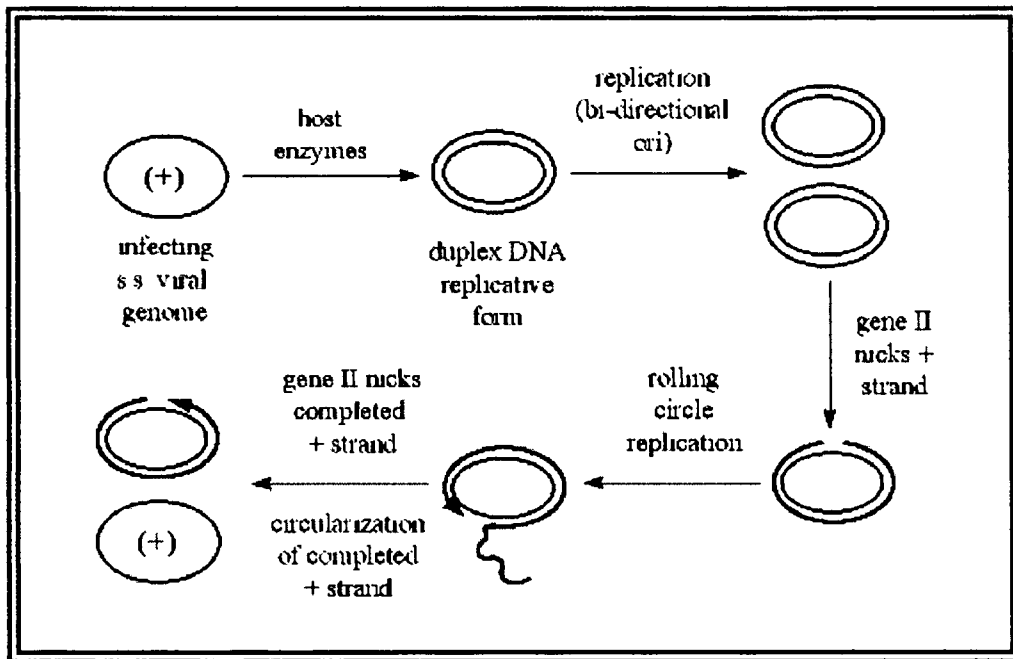


Figure 2.7 Life cycle of M13 phage (Blaber, 1998)

This complex of single stranded (+) DNA and pV now moves to the membrane where the pV is replaced and pVIII covers the phage DNA again as it is being extruded out (Webster, 2001, Blaber, 1998). The release of the phage does not require the F-pilus (Blaber, 1998).

2.9.6 *In vivo* phage display

In vivo phage display systems are extremely limited, because the selections require that an *in vivo* assay be linked to the single functional property of interest (Wells and Lowman, 1992). While most of the peptides and the antibody phage selection strategies are carried out using *in vitro* assays, *in vivo* phage display can select and identify peptides that interact with tissues and cells in their native three dimensional micro-environment (Mullaney and Pallavicini, 2001).

Endothelial cells from different tissues express unique receptors for trafficking. Injecting a peptide phage library into the tail vein of a mouse and allowing the phage to circulate has led to identification of peptides involved in endothelial cell interactions. Phage can be removed from target organs (e.g. brain and kidney), reinfect into *E.coli*, and enriched in a second *in vivo* round. Peptides directed against integrins localized on endothelial cells. Phage targeted to tumors may also be candidates for drug delivery (Mullaney and Pallavicini, 2001).

2.10.7 Comparison of phage display to other techniques

2.10.7.1 Phage Display and the two-hybrid technique

The yeast and other two hybrid technologies offer an alternative strategy for mapping protein-protein interactions. The two-hybrid technique shares a common shortcoming with phage display technique. They are both limited by the biology of the system underlying their implementation (Rodi and Makowski, 1999).

Although the yeast two hybrid has a proven track record in identifying candidate interacting partners in any given proteome, phage display has some advantage in mapping protein-protein interactions due, to the enormous diversity of sequences

capable of being displayed by bacteriophage. Thus a phage display combinatorial peptide library has enormous coding potential, far greater than most yeast two-hybrid cDNA libraries, from which one can select a "molecular needle in a haystack". Although it has been possible to generate and screen combinatorial peptide libraries by the yeast two-hybrid screening, the libraries are of low complexity and are not useful in mapping protein-protein interactions. Phage displayed peptides are not biased towards the frequency of any mRNA, nor are they affected by the difficulty of expressing and localizing certain proteins, such as transmembrane proteins. A list of differences between phage display technology and the two-hybrid technique is outlined in table 2.2. With phage display technology it is possible to screen combinatorial peptide libraries for peptide ligands to target while it is not traditionally used in the yeast two-hybrid screening. Also the *in vitro* nature of phage library screening offers more versatility in screening than yeast two-hybrid screening, assuming that the target protein is readily available in native conformation in sufficient amounts. (Rodi and Makowski, 1999).

2.9.8 Limitations of phage display technology

There are a number of very important limitations to create complete libraries of structural diversity by using phage display technology. There is a limit to the natural set of L-amino acids, which can be incorporated, in an *in vivo* system. Furthermore, the protein that is displayed as a fusion to a coat protein must be expressed and secreted from *E.coli* in a functional manner. This is by no means assured, since proteases and other chemical modifications (e.g. oxidation, deamidation, etc) may make full-length protein expression and folding difficult. If the carboxyl part of the protein of interest is crucial for binding, to the target molecule, then this binding will be severely reduced, because the protein is fused to pIII at it's carboxyl end. Even though the sizes of the phage libraries appear large (10^7 - 10^8), they are small in comparison to the total sequence diversity of even

small proteins of ~100 residues (20^{100}) (Wells and Lowman, 1992). For example, the creation of all possible mutations simultaneously in just five codons using random DNA (which generate all possible amino acids in 32 codons) requires the library to have 2×10^8 independent transformants (for 95 % confidence that a given sequence will be represented). This problem can be mitigated by homing in on important regions for binding on the basis of previous structural or mutational analyses, and then applying phage display to optimize those specific regions (Wells and Lowman, 1992).

Table 2.2: Phage display versus yeast two-hybrid technology (Rodi et al, 2001)

Phage display versus yeast two-hybrid technology		
	Phage display	Yeast two-hybrid
Screening population	Combinatorial peptides cDNA fragments	Combinatorial peptides cDNA fragments
Sequence diversity of library	Peptides: high cDNA fragments: dependent upon expression levels, unless normalized	Peptides: low cDNA fragments: dependent upon expression levels, unless normalized
Targets	Proteins: available through purification or Overexpression Cell surfaces or tissues Small organic molecules (such as drugs or natural products)	Proteins: bait is synthesized by the yeast cell Possibility of being folded properly by chaperones
Screening format	<i>In vitro</i> (can vary screening conditions, such as salt and detergent concentrations or on/of rates)	<i>In vivo</i> (needs to be transported to yeast nucleus)
High-throughput	Billions of clones can be screened in one week	Millions of clones can be screened in two to four weeks
Range of interactions detected	Below 100 μ M	Below 50 μ M
Positive outcomes	From peptide libraries: isolate peptide ligands, map region of interaction to short peptide sequences, predict interacting protein From cDNA fragment libraries: identify putative interacting proteins	From peptide libraries: isolate peptide ligands From cDNA fragment: identify putative interacting proteins
False positives	Plastic-, GST-, six histidine-, and streptavidin binding sequences	Transcriptional-activating sequences
False negatives	Mimotopes with no homologs in nature UTRs, staggered reading frame products, promiscuous binding by exposed regions of misfolded proteins	Bait or prey proteins fail to fold properly or enter the nucleus, UTRs, staggered reading frame products, promiscuous binding by exposed regions of misfolded proteins.
Set-up time	Weeks	Months

2.9.9 Challenges in phage display technology

Phage display requires specialized resources and skills. A high-quality, large-diversity display library is an essential requirement for experimental success. A useful do-it-yourself immune antibody library requires a library containing about 10^6 single chain variable fragments (scFv); however generating large naïve libraries (above 10^8 clones) is a technical challenge. Well-characterized libraries from an academic collaborator are the best starting point. A well-equipped laboratory for phage display includes shakers, incubators, and centrifuges for bacterial growth, ELISA method for clone screening, PCR and DNA sequencing for clone analysis, and methods for high scale protein expression. Additional techniques must minimize phage contamination of glassware. Other challenges may arise after identification of candida scFv clones. Low levels of scFv protein expression, immunogenicity of the antibody and low antibody affinity may be problematic for some types of applications. The affinity of phage driven antibodies is typically 1-2 orders of magnitude less than conventional monoclonal antibodies. Affinity maturation by phage display involves construction of new phage display libraries whose members are based on mutations of the original protein or antibody sequence.

A final challenge occurs when phage display generates either too many candidates to fully characterize or no binders against target antigen. In the former case, more selective and specific screens need to be developed, whereas in the latter case less stringent selection criteria are likely to be beneficial (Mullaney and Pallavicini, 2001).

2.9.10 Applications of phage display

2.9.10.1 Phage-peptide application

The random peptides that are displayed on filamentous bacteriophage as fusion to either pIII or pVIII coat proteins, has allowed scientist to identify peptides that bind specifically to different targets including antibodies, streptavidin, ribonuclease S, and concanavalin A (Azzazy and Highsmith, 2002). Peptides selected against a particular target that had similar sequences played a role in identifying a sequence motif necessary for binding. In cases where the selected peptides did not resemble the natural peptide ligand, they were termed mimotopes (Geysen et al, 1986, Smith and Scott, 1990). Mimotopes are small peptides that specifically bind to a receptors binding site (and in that sense mimic the epitope on the natural ligand) without matching the natural epitope at the amino acid sequence level. The definition includes cases where the natural ligand is not a protein. In most cases it is highly desirable to "map" the epitope to a confined portion of the natural protein ligand. If the epitope is (or might be) continuous and not conformation dependent, random peptide libraries provide a cheap and easy approach to this goal (Geysen et al, 1986).

A receptor can be used to affinity select random peptide ligands, and the sequence motif in the selected peptides can then be compared to the amino acid sequence of the natural ligand (Smith and Petrenko, 1997). Peptides selected against a particular target that had similar sequences played a role in identifying a sequence motif necessary for binding. Peptide sequences identified by phage display have been shown to act as agonists and antagonists of receptors. Random peptide libraries can be used for mapping epitopes of monoclonal and polyclonal antibodies identifying peptide ligands, and developing "substrate phage" to define substrate sites for different enzymes. Display of small peptides on the surface of phage particles can increase their immunogenicity and consequently their potential as vaccine candidates (Azzazy and Highsmith, 2002).

In the field of thrombosis and haemostasis, phage display of peptides has been used in the isolation of a peptide antagonist to the thrombin receptor (Doorbar and Winter, 1994). A constrained peptide library was used to isolate ligands of the $\alpha_{11b}\beta_3$ integrin, the platelet receptor for fibrinogen. This was done by flanking a library of hexapeptides by cysteine residues to introduce a degree of conformational constraint into random peptides (O'Neil *et al.* 1992). A random cyclic heptapeptide library was used to characterize the peptide binding specificity of the $\alpha_5\beta_1$ integrin, the fibronectin receptor on platelets (Koivunen *et al.* 1994).

2.9.10.2 Phage antibody application

Generation of a large natural display library from variable gene repertoires can eliminate animal immunization and large scale cell culture for hybridoma development and allow isolation of antibodies. Phage display is particularly useful in cases where monoclonal antibodies could not be obtained by classical hybridoma technique such as antibodies against nonimmunogenic or toxic antigens. Phage displayed antibodies have stable genetic source. Phage antibody technology can also be used to clone and rescue monoclonal antibodies from genetically unstable hybridomas. It is easy to sequence, mutate, and screen phage antibody genes to improve antigen binding. One can search antibody libraries for catalytic antibodies by selecting the actual catalysis rather than for binding activity only (Azzazy and Highsmith, 2002).

2.9.10.3 Drug discovery

Many of the receptors used in affinity selection are targets of drug discovery programs, and the peptide ligands selected by them are therefore potential leads to new drugs. Such peptides might act as receptor agonists and antagonists (for

example, of enzymes or hormone receptors) or otherwise modulate the receptor's biological effect. For most pharmaceutical applications, peptides have poor pharmacological properties, being generally orally unavailable and subject to rapid degradation in the body by naturally occurring enzymes. Synthesizing peptidomimetic compounds that mimic the essential pharmacological features of bioactive peptides on a non-peptide scaffold. Developing peptidomimetics is an arduous and chancy project. But with the contribution of phage display to drug discovery will confine to applications where peptides themselves can serve as plausible therapeutics. Peptides composed of D-amino acids are much less susceptible to degradation in the body than peptides composed of the natural L-amino acids. Phage display was used to identify D-amino acid peptide ligands for target receptors. They synthesized chemically the D form of an SH3 domain and used it to affinity select ligands from a random peptide library, whose amino acids are of course the natural L isomers. The D forms of these peptides are therefore ligands for the natural L form of the receptor the form that would be the actual target of drug discovery (Smith and Petrenko, 1997).

There are several examples of studies where phage display technology was used to identify peptide ligands for receptors. Ligands were identified for the antigen-binding site of the surface immunoglobulin receptor of the human Burkitt lymphoma cell line SUPB8. Potent ligands were identified for the human urokinase receptor and phage display was also used to localize epitopes for the binding protein, somatostatin (Renschler *et al.* 1994; Goodson *et al.* 1994; Wright *et al.* 1995).

Furthermore phage display was also successfully used to isolate three peptides that interacted with the HIV-1 nucleo-capsid protein (NCp7) (Lener *et al.* 1995). All these examples show the broad spectrum of applications for the phage display technique

CHAPTER 3

MATERIALS AND METHODS

3.1. Phage display

3.1.1. Phage display peptides libraries

I used two peptide library kits. A cyclic 7-mer phage display library kit (Ph.D-C7C, cat # 8120) and the linear 12-mer phage display library kit (Ph.D-12, cat # 8110). The kits were purchased from New England Biolabs, Beverly, MA, USA. Both libraries are pIII fusions with 5 copies of the fused peptide per phage. The cloning vector for both is M13KE (New England Biolabs, 2000).

The Ph.D-C7C peptide library kit includes random peptide sequences, which are flanked by a pair of cysteine residues. This cyclic-7-mer library is therefore also a combination library of random 7-mer peptides fused to pIII. The cysteine residues will spontaneously form a disulphide cross-link under non-reducing conditions. This results in the display of cyclic peptides, in contrast to the linear peptides displayed in the 12-mer Ph.D-12 library. The cyclic peptides are also displayed at the amino terminus of pIII. This library contains approximately 3.7×10^9 electroporated sequences. These were also amplified once to yield about 50 copies of each sequence in 10 μ l of the phage library (New England Biolabs, 1998a).

The Ph.D-12 peptide library kit includes a library of linear random 12-mer peptides fused to the minor coat protein, pIII, of M13 phages. The 12-mer peptides are expressed at the N-terminus. The 12-mer library contains approximately 2.7×10^9

electroporated sequences. The sequences were amplified once to yield about 55 copies of each sequence in 10 μ l of the phage library (New England Biolabs, 1998b). The *E.coli* host strain, ER2537 was supplied with the library kits. This strain is a robust F+ strain with a rapid growth rate.

3.1.2 Biopanning method for selection of phages

I coated two immuno tubes (NUNCTM Brand products denmark,USA) with 10 μ g of purified factor XI each (kind gift from Dr Andras Gruber, Emory University, USA) in 1ml PBS (10 μ l FXI/1ml PBS), rotated it for 1 hour and incubated overnight at 4°C. A pre-culture of *E.coli* cells (ER 2537 strain, New England Biolabs, USA) was prepared by adding the *E.coli* cells from the minimal plate to 10ml Luria Broth medium (LB-medium,DIFCO Laboratories, Detroit, USA), and shaking overnight at 37 °C.

The next day, I discarded the coating solution from the immuno tubes and added 4ml of 4 % Skim milk (SM) (Difco laboratories, USA) in phosphate buffer saline (PBS), to the tube and rotated it for 2 hours at room temperature (RT). The SM acts as a blocking buffer to block the vacant binding sites on the immuno tube. After the 2 hours of incubation, the blocking buffer was discarded and the tubes washed three times with 0.1 % Tween-20 (Merck-Schuchardt, Hohenbrunn). I added 2×10^{12} phages of each of the linear phage library and the cyclic phage library (New England Biolabs, USA) to 1ml of 2 % SM into each of the two tubes and rotated it for 2 hours at RT. The non-binding phages were then removed from the tubes and stored in an eppendorf tube. This was called the "INPUT" phages. The tubes were washed 10 times with 0.1 % Tween-20 (Merck- Schuchardt, Hohenbrunn) in PBS. A wash step was done by vortex the tube for 2 seconds, discard the contents and add 2ml of washing buffer to the tube. Only the phages that bind to factor XI will stay bound to the immuno tube and the unbound phages were therefore washed away. The bound phages that have a high affinity for factor

XI were eluted non-specifically with 1ml of 0.1 M glycine (0.2 M, pH 2.2). To neutralize the low pH of the glycine, 125 μ l of Tris (1M, pH 8) was added. These eluted phages are the "OUTPUT" phages. The INPUT and the OUTPUT phages were stored at -20°C with 5 % glycerol for further use.

Simultaneously, the pre-culture of the ER 2537 *E.coli* cells was diluted 1:100 in 40 ml LB-medium (DIFCO, Laboratories, Detroit, USA). The culture solution was shaken for 2½ hours at 37 °C until the *E.coli* cells were in the log-phase of growth, i.e. an OD₆₀₀ of between 0.6 and 1. I used 2ml of the diluted culture to make dilutions of the "INPUT" and "OUTPUT" phages. I first diluted the "INPUT" phages 10 fold up to 10⁻⁹ and "OUTPUT" phages up to 10⁻⁴ in LB-medium. I then added 10 μ l of the last two 4-dilutions of the "INPUT" and "OUTPUT" phages to new eppendorf tubes and added 200 μ l of the diluted culture cells to it. I added the dilutions to 3ml agarose and plated it out on isopropyl- β -D-thiogalactoside/5-bromo-4-chloro-3-indolyl- β -D-galactoside plates (IPTG/XGAL plates). IPTG/XGAL plates were used to ensure phage clones that contain the random DNA inserts, were picked (in the next section), because phages with inserts contains the lac Z gene and such clones color blue in the presence of X-Gal. Infecting 40ml of log-phase *E.coli* cells and incubating it overnight at 37°C amplified the "OUTPUT" phages.

The next morning, I poured the infected cell culture into a centrifuge tube (Beckman Instruments, California USA) and centrifuged it for 15 minutes at 23000g at 4 °C to remove the *E.coli* cells. The phages in the supernatant were poured into another centrifuge tube and precipitated on polyethyleneglycol / sodium chloride (PEG/NaCl, PEG: 0.03M, NaCl: 2.5M) for 2 hours on ice (4 °C). It was then centrifuged for 20 minutes at 23000g at 4 °C. The supernatant was discarded and, the pellet dissolved in 1ml PBS and poured into an eppendorf tube and centrifuged for 3 minutes at 18000g to remove the remaining *E.coli* cells. The supernatant was poured into a clean tube, and the phages precipitated again on 20 % PEG/NaCl for

30 minutes at 4 °C. After centrifugation for 10 minutes at 18000g the amplified phages were finally dissolved in 500µl PBS. I used 2×10^{11} of the purified phages for the next round of selection. Four rounds of selection were performed to purify the factor XI-binding phages. The phage concentration after each selection round was determined by reading the OD at 260nm and the phage concentration (phages/ml) was calculated as follows: Amount phages/ml = $OD_{260} \times \text{dilution} \times \text{constant}$ where the constant is 2.214×10^{11} since the OD_{260} of 2.214×10^{11} phages is equal to one on the spectrophotometer (GeneQuant Pro RNA/DNA Calculator, Amersham Biosciences, UK). I used a 1:10 and 1:20 dilution.

3.1.3 Global-ELISA

A global ELISA was performed on the different libraries (selection round 0) and on the amplified phages of each selection round. Different phage concentrations of the amplified phages of each selection round were added to factor XI coated wells. Half of an ELISA plate (Nunc™ Brand products, Denmark) was coated with 50µg factor XI per well and incubated overnight in the fridge at 4 °C. The next day I discarded the factor XI solution from the plate and blocked the plate with 200µl of 4 % SM per well in PBS for 2 hours at RT. The plate was then washed three times with 0.1 % Tween/PBS. I added $5 \cdot 10^{10}$ phages of each selection round to the first well of each column of the coated and non-coated part of the ELISA plate in a final concentration of 2 % SM and diluted it 7 times 1:2 into other wells of the columns. No phages were added to the last well of each column since these wells served as a negative control. I added the same amount of phages to the non-coated wells as to the coated wells to make sure that I have not selected plastic-binding phages instead of FXI-binding phages. The ELISA plate was then incubated for 2 hours at RT, and then washed nine times with 0.1 % Tween in PBS. I added 100µl of a horseradish peroxidase conjugated anti-M13 phage antibody (500 X diluted) per well, incubated it for one hour, then washed the plate twelve times with 0.1 %

Tween in PBS. The horseradish peroxidase conjugated M13 phage antibody was purchased from (Amersham Pharmacia Biotech, UK). To develop a colour reaction, I added 90µl of the substrate ortho-phenylenediamine-dihydrochloride (OPD) and peroxidase (H₂O₂) per well. The reaction was stopped after 15 minutes with 30µl of 1M H₂SO₄ per well. The optical density readings were taken at 490nm on an EL312e Microplate Bio-kinetics reader (Bio-tek instruments, Vermont, USA). The global ELISA indicated that I have selected the highest concentration of FXI-binding phages in round 4, since the OD₄₉₀ of round 4 was the highest. I thus grew single clones of the fourth selection round of panning with each library, to search for displayed peptides that bind to and inhibit FXI.

3.1.4 Grow single clones

I picked forty-eight blue clones of each library from the IPTG/XGAL plates of the fourth selection round. Since the library phage are derived from the common cloning vector M13mp19, which carries the lac Z gene, phage plaques (with the peptide inserts) appear blue when plated on media containing Xgal and IPTG. They were grown in 2 ml of a 1:100 dilution of a pre-culture of *E.coli* cells and incubated (shaking) overnight at 37°C. Half of the cultures were centrifuged and the supernatant containing the amplified phages was used in an ELISA to determine which clones bind to FXI (binding-ELISA of single clones). A cell stock was prepared with the other half of the culture, by mixing it with glycerol (35 % final concentration) and frozen for further use.

3.1.5 Binding-ELISA of single clones

In order to distinguish between actual FXI binders and plastic binders, each clone was tested in an FXI-coated and non-coated well. An ELISA plate (NUNC™ Brand

Products, Denmark) was coated with 50µg factor XI per well and incubated overnight at 4 °C. The next day I discarded the factor XI solution from the plate and blocked the plate with 200µl of 4 % SM in PBS per well. I incubated it for 2 hours at RT and then washed the plate 3 times with 0.1 % Tween / PBS. I added 100µl of the supernatant of each grown clone of selection round four to a coated and non-coated well of the ELISA plate in final concentration of 2 % SM. I also added 100µl of the supernatant of a non-FXI binding phage clone that I grew up together with FXI binding clones. It was then incubated for 2 hours at room temperature, and then washed nine times with 0.1 % Tween in PBS. I added 100µl of the horseradish peroxidase conjugated anti-M13 phage antibody (Amersham Pharmacia biotech, NJ, USA) per well , incubated for one hour, then washed the plate twelve times with 0.1 % Tween in PBS. The substrate, orthophenylenediamine-dihydrochloride (OPD) and peroxidase was added to each and incubated for 10 minutes at RT to develop a colour reaction. The reaction was stopped with 30µl of 1M H₂SO₄ per well. The optical density readings were taken at 490nm on an EL312e Microplate Bio-kinetics reader (Bio-tek instruments, Vermont, USA). Wells with the most intense colour indicate the strongest binding phages or a high concentration of weak factor XI binding phages.

I found only two clones with high colour intensity. They were clone 27 and 45. Both were from the linear 12-mer phage library. These strongest binding clones were grown from the cell stock and tested for concentration dependent binding to and for inhibition of FXI in a dilution- and inhibition-ELISA respectively. The phage clones that did not bind to FXI shows no colour reaction in this ELISA.

3.1.5.1 Dilution-ELISA

I diluted the two clones 1:2 and tested them for concentration dependent binding to FXI. Each clone was also diluted on non-coated wells to distinguish between

factor XI binding phages and non-specific plastic binding phages. The highest phage concentration was 5×10^{10} and I diluted it 1:2 as in the global-ELISA. The rest of the ELISA was done the same way as the global-ELISA. I repeated this ELISA three times in three independent experiments.

3.1.5.2 Inhibition-ELISA

This ELISA was performed to determine whether factor IX and/or thrombin were able to prevent these two strong binding clones from binding to factor XI.

An ELISA plate was coated with $50\mu\text{g}$ factor XI per well and incubated overnight at $4\text{ }^{\circ}\text{C}$. The next day I discarded the factor XI solution from the plate and blocked the plate with $200\mu\text{l}$ of 4 % SM per well in PBS, for two hours at room temperature. Different concentrations of factor IX (Sigma-Aldrich, Deisenhofen, Germany) were added to the factor XI coated wells. The concentration ranging from 0 to $25\mu\text{g}$ factor IX per well. I added different thrombin concentrations ranging from 0 to $50\mu\text{g}$ thrombin per well to the other factor XI coated wells. After incubation for 15 minutes at RT, I added 5×10^{10} phages from the two clones in $50\mu\text{l}$ of 2 % SM each well and incubated it for 2 hours at RT, then washed six times with 0.1 % Tween in PBS. The bound phages were detected after one hour of incubation with the anti-M13 antibody. Observation was done by adding the substrate, OPD and H_2O_2 per well and left it for 10-15 minutes to develop a colour reaction. The reaction was stopped with $30\mu\text{l}$ 1M H_2SO_4 per well. The optical density readings were taken at 490nm. I repeated the inhibition ELISA three times in three independent experiments.

3.1.6 Activated Partial thromboplastin time (aPTT)

The effect of a dilution series of the two strongest factor XI-binding phage clones on the aPTT was determined. To prepare platelet poor plasma, 10ml of normal human blood was drawn in 3.2 % sodium citrate and centrifuged at 2000g for 10 minutes. For the control aPTT, I incubated 50 μ l of plasma with 100 μ l PBS and 50 μ l aPTT reagent (Dade Behring Marburg, Gmbh, Marburgl Germany) for 3 minutes at 37°C, and then added 50 μ l Ca²⁺ (Dade Behring Marburg, Gmbh, Marburgl Germany). The clotting time was measured using the SStart[®]4 coagulation timer (Diagnostica stago, Asnieres, France). The aPTT was determined by incubating 50 μ l of plasma with 100 μ l of different concentrations of phages from the two strongest factor XI-binding phages (clone 27 and 45) and 50 μ l of aPTT reagents. The phage concentration ranges from 0 to 2 x 10¹⁰ phages per reaction. After incubation of three minutes at 37°C, 50 μ l Ca²⁺ was added to start the reaction. All reactions were done four times.

3.1.7 Prothrombin Time (PT)

I also did PT's on the two strongest factor XI-binding phages. The PT's was done by incubating 100 μ l of plasma with 50 μ l of different phage concentrations for 10 minutes, using PBS as control. Two hundred μ l of Dade Innovin (Dade Behring, Marburg, Germany) was added to start the reaction. The clotting time was also measured on the SStart[®]4 coagulation timer (Diagnostica stago, Asnieres, France). I repeated the PT also four times with each of the two clones.

3.1.8 Sequencing of FXI-binding phages

3.1.8.1 Isolation of DNA

I sequenced the two phage-clones that bind strongly to and inhibited FXI (clone 27 and 45). DNA of these 2 clones was prepared to sequence the phages. I precipitated 4×10^{11} of phages of the two clones with 20 % PEG/NaCl ice for 1 hour. I then centrifuged it for 10 minutes at 18000g and dissolved the pellet in 100 μ l of 4M sodium iodide buffer (10mM Tris, 1mM EDTA, 4M NaI). I added 250 μ l of 100 % Ethanol, incubated for 10 minutes at room temperature and centrifuged it again for 10 minutes to pellet the DNA and discard the supernatant. The pellet was washed with 70 % ethanol and the DNA dried for 2 minutes in vacuumdryer at medium speed. The DNA was rehydrated in 20 μ l of distilled water (DH₂O) and stored at -20 °C until use. The DNA concentration was determined by preparing a 10x and 20x dilution and reading the OD at 260 nm. The DNA concentration was calculated as follow: $[\mu\text{g/ml}] = \text{OD}_{260} \times \text{dilution} \times \text{constant}$. The constant of a single stranded DNA is 37. This is the amount of single stranded DNA at an OD₂₆₀ of 1 (Sambrook and Russell, 2001)

3.1.8.2 Polymerase Chain Reaction (PCR)

I used the DYEnamic ET Terminator Cycle Sequencing Premix Kit (Amersham Pharmacia Biotech Inc, NJ, USA) for the sequencing reactions. A PCR mixture was prepared by using 2 μ g of DNA of each of the 2 clones, 8 μ l Premix reagent (Amersham Pharmacia Biotech Inc, Piscataway NJ, USA) and 0.5 μ l Primer (5pmol, 5'-^{HO}CCC TCA TAG TTA GCG TAA CG-3'). The total volume of the reaction was 20 μ l. The PCR was performed on the Gene Amp PCR system (Applied Biosystems, CA, USA). One cycle was performed at 94°C for 1 minute, 25 cycles were performed at 94 °C for 30sec, 45 °C for 30sec, and 60°C for 4min. The holding temperature was at 4°C. After the PCR, the DNA was extracted by adding

2 μ l of 3M sodium-acetate 76 μ l of 100 % ethanol to the PCR product, and incubated it for 15 minutes at 4°C. It was then centrifuged for 15 minutes at 18000g to pellet the DNA. To wash the DNA pellet, 300 μ l of 70 % Ethanol (BDH Ltd, Poole England) was added to the DNA pellet and centrifuged for 1 minute. The supernatant was carefully removed and the pellet was dried for 2 minutes in a vacuumdryer at medium speed. I then added 25 μ l of the template suppression reagent (TSR, Applied biosystems Prism, Foster City CA, USA) to the dried DNA and mixed well. I denatured the DNA at 95°C for 3 minutes and placed it on ice. Sequencing was performed on the ABI Prism 310 Genetic Analyser (Applied Biosystems, CA, USA).

This genetic analyzer uses the BigDye Terminator cycle Sequencing Ready reaction format, and performs fluorescence-based cycle sequencing reactions on single-stranded or double-stranded DNA templates, on PCR-fragments and on large templates. The advantage of using Cycle sequencing is that the protocols are robust and easy to perform and requires much less template DNA. The single temperature extension methods is also more convenient than traditional single-temperature labeling that require a chemical denaturation step of double-stranded DNA and the same protocol is used for double and single-stranded DNA. The protocol also works well for direct sequencing of PCR products. Difficult templates such as bacterial artificial chromosomes (BACs) can also be sequenced (Applied biosystems, 1998).

I compared the peptide sequences displayed on the two FXI-binding clones to that of thrombin, factor IX, high molecular weight kininogen and factor XIII. Since clone 27 was prevented from binding to factor XI by thrombin and factor IX, I decided to synthesize a peptide with this sequence. The sequence is given in the results section. Ansynth Services in Roosendaal, the Netherlands synthesized this peptide.

3.2 Tests performed on the synthesized peptide.

3.2.1 Activated Partial Thromboplastin Time (aPTT) and Prothrombin Time (PT)

The effect of the peptide on aPTT and PT was measured in normal pooled human plasma. The aPTT was measured as described (see 3.1.6). The final peptide concentrations ranged from 0 to 14,313 μM . The control aPTT was determined by adding PBS to plasma. For negative control, I added a peptide that does not bind to factor XI.

The PT was measured as described (see 3.1.7). The final peptide concentrations ranged from 0 to 14,313 μM . I also used PBS as a control and a peptide that does not bind to factor XI as a negative control. This peptide is a factor VIIa binding peptide that does not have an effect on the aPTT. I repeated the aPTT and PT four times.

CHAPTER 4

RESULTS

4.1 Biopanning of Factor XI

The concentration of the rescued phages after each round of panning was as follows. The phage concentrations were 1.895×10^{12} for round one, 2.478×10^{12} for round two, 2.324×10^{11} for round three and 2.312×10^{12} for round four for the linear 12-mer phage library.

The phage concentrations were 1.549×10^{12} for round one, 1.947×10^{12} for round two, 1.735×10^{11} for round three and 2.525×10^{12} for round four for the cyclic 7-mer library.

The global ELISA indicates that I have selected the highest concentration of factor XI binding phages in selection round four of panning with both libraries. I therefore decided to use the phages from rounds four of both libraries for binding studies. In figure 4.1 I plotted the OD_{490} of $5 \cdot 10^{10}$ phage of each selection round of the selection with the two libraries. This figure also shows that round four of both libraries contains the most factor XI binding phages.

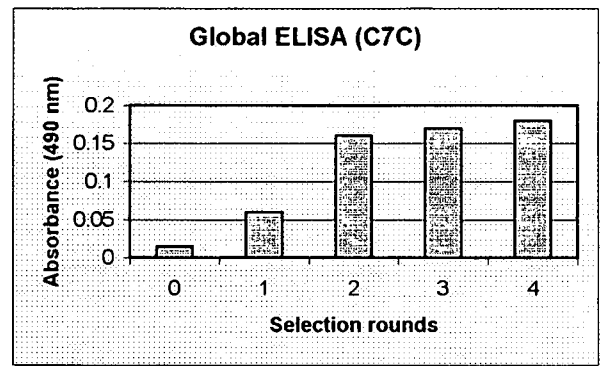
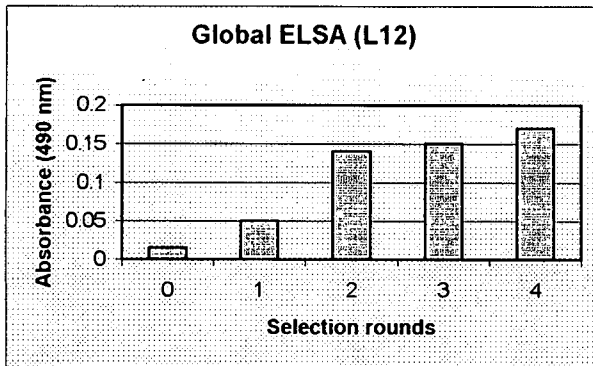


Figure 4.1 Shows the binding affinity to factor XI of 5×10^{10} phages of all selection rounds from both L12 and C7C libraries. Round 4 from both libraries show the highest binding to factor XI.

With the binding ELISA of the single clones, I only found two phage clones that bind strongly to FXI. Both clones were from the linear 12-mer library. They were clone 27 and clone 45. Because I had limited quantities of factor XI, I did not search for more binding clones. The bindings of these clones to factor XI are shown in figure 4.2.

The OD values in the next two figures (4.2 and 4.3) are the difference between the factor XI binding phages of the different panning rounds on the coated and non-coated parts of the ELISA plate. The OD values of the controls were less than 0,001.

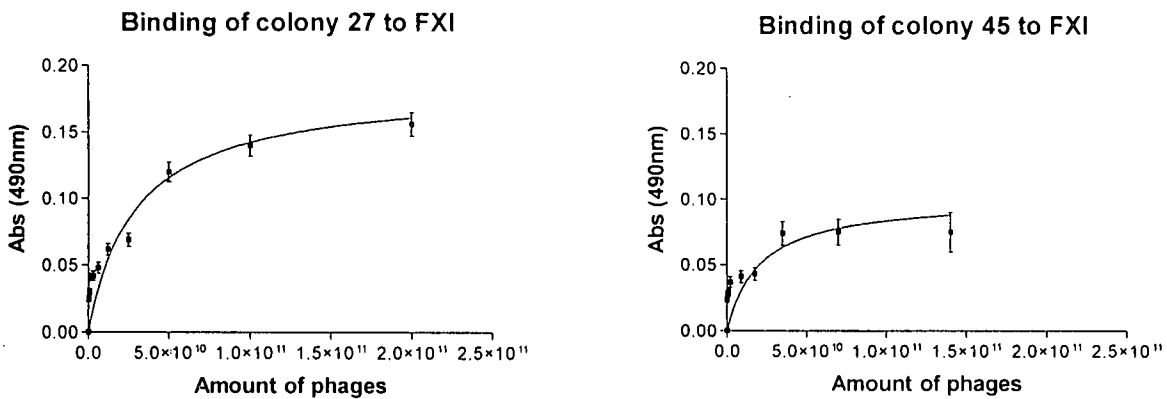
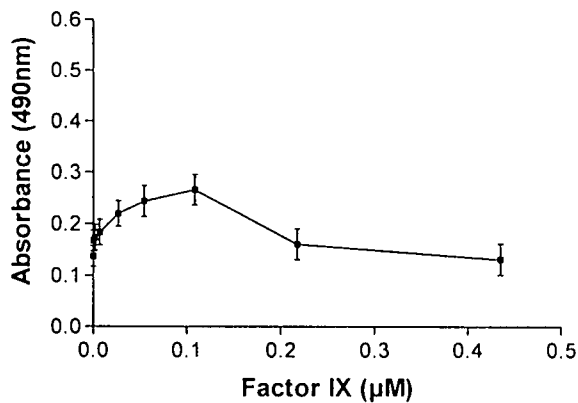


Figure 4.2. Dilution ELISA of clone 27 and 45. Both clones bind to factor XI. Clone 27 binds with a higher affinity to factor XI than clone 45. The OD₄₉₀ increased with increasing concentration of phages. Clone 27 binds stronger to factor XI than clone 45. (n = 3).

An inhibition ELISA was also performed to determine if factor IX and thrombin prevent the binding of these two clones to factor XI. Different concentrations of factor IX and thrombin were added to a factor XI-coated plate. After 10 minutes of incubation I added 5×10^{10} phages from clone 27 and 45 to each well of the plate, and I detected the binding of the phages to factor XI by using the anti-M13 phage antibody that is linked to peroxidase. At higher concentrations of factor IX, both clones were prevented or blocked from binding to factor XI by factor IX (see figure 4.3).

Inhibition ELISA of colony 27



Inhibition ELISA of colony 45

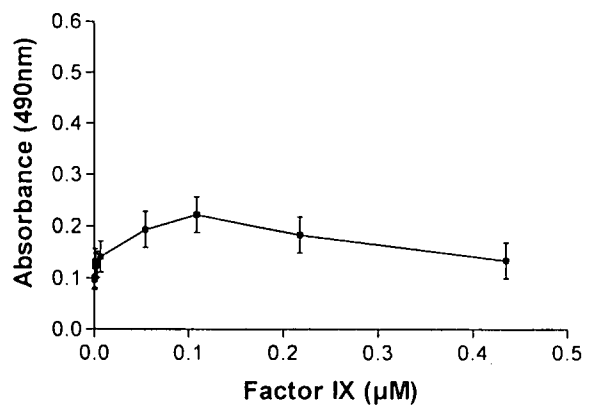


Figure 4.3. Inhibition ELISA of both clone 27 and 45 at different factor IX concentrations. Factor IX concentrations ranging from 0 to 0.5 µM were added to factor XI coated wells and incubated for 15 minutes. 5×10^{10} phages of clone 27 and 45 were added to the wells and incubated for 2 hours where after the factor XI bound phages were detected using an anti-phage antibody ($n=3$).

At high concentrations of thrombin only clone 27 inhibits the binding of thrombin to FXI. Thrombin has no effect on the binding of clone 45 to factor XI. This is shown on figure 4.4.

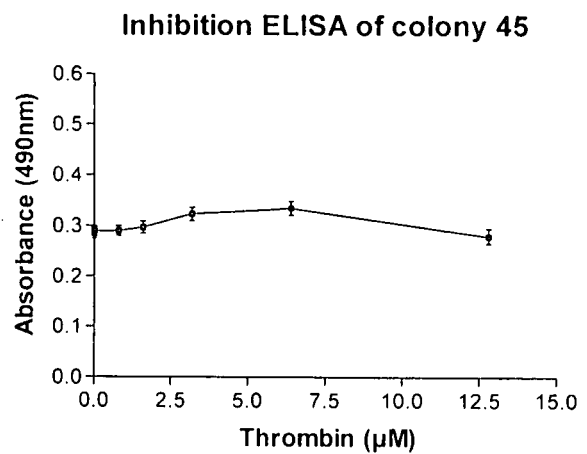
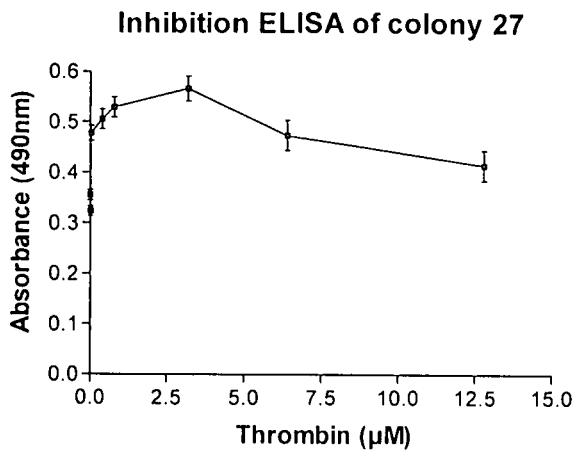


Figure 4.4. Inhibition ELISA of clone 27 and 45. Thrombin concentrations ranging from 0 to 15 μM were added to factor XI coated wells and incubated for 15 minutes. 5×10^{10} phages of clone 27 and 45 were added to the wells and incubated for 2 hours where after the factor XI bound phages were detected using an anti-phage antibody ($n=3$).

I tested the effect of these clones on the aPTT and PT. Both clones lengthened the aPTT. The lengthening in aPTT was concentration dependent, the higher the concentration the more lengthening in aPTT. Clone 45 appeared to lengthen the aPTT a little bit more than clone 27. The results of this aPTT can be seen in figure 4.5. There was no effect on PT's as it has been expected. These results are not shown.

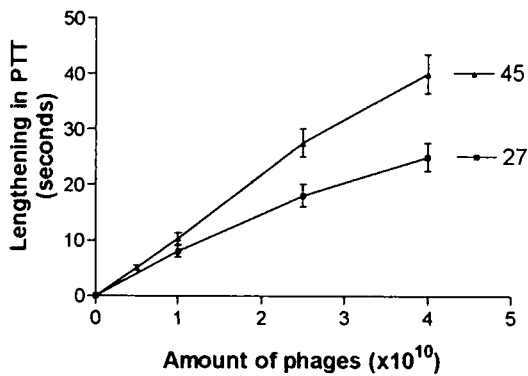


Figure 4.5. Lengthening in aPTT of increasing concentration of both clone 27 and 45. Clone 45 lengthened the aPTT a little bit more than clone 27. The lengthening in aPTT was dose dependently ($n=4$).

4.2. Sequences of phage clones.

I sequenced both clones. The amino acid sequences displayed on the phages were as follows.



Both the clones contain three amino acid sequences of High Molecular Weight Kininogen (HMWK) and Thrombin and clone 27 also contains a three amino acid sequence of factor XII.

Since clone 27 was prevented from binding to factor XI by thrombin and factor IX and because it binds stronger to factor XI than clone 45, I decided to synthesize a linear peptide with the sequence similar to the peptide sequence displayed on clone 27.

4.3 Effect of this peptide on the aPTT and the PT.

aPTT and PT were done on pooled human plasma. There was no effect on PT as expected. This peptide lengthened the aPTT dose dependently. Figure 4.6 the prolongation of aPTT of this peptide. The peptide I used, as a negative control did not lengthened the aPTT at all

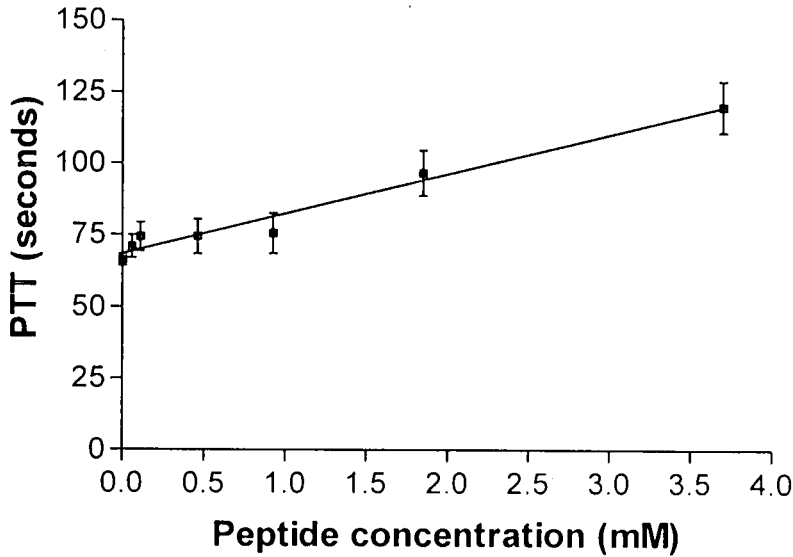


Figure 4.6. Prolongation of aPTT in human plasma. Different peptide concentrations were added to normal pooled human plasma and incubated for 15 minutes before the aPTT test were done. At the highest concentration of 4.0mM, the aPTT was prolonged with 125 seconds.

CHAPTER 5

DISCUSSION

The precise role of factor XI in physiological coagulation is still a matter of debate. In the contact phase of coagulation, factor XIIa in the presence of high molecular weight kininogen and a negatively charged surface activate factor XI (Movat and Ozge-Anwar, 1974). Factor XI then activates factor IX, which together with factor VIIIa on phospholipid surfaces activate factor X (Dahlback, 2000). The observation that factor XII deficiency does not cause a hemorrhagic disease casts doubt on the importance of contact activation for physiological blood coagulation (Schmaier et al, 1987). But, unlike factor XII deficiency, factor XI deficiency does cause an injury related bleeding tendency (Minnema et al, 1999). This fact is not explained by the classical contact activation mechanism. It is now known that factor XI can be activated by trace amounts of thrombin (Galiana and Broze, 1991). Therefore factor XI serves in a feedback loop involving thrombin, factor XIa, factor IXa, factor Xa and thrombin again to sustain the coagulation process. In addition, factor XI is required for the generation of lysin resistant clots (Von dem Borne et al, 1995), because factor XI increases the generation of thrombin activatable fibrinolysis inhibitor (TAFI) at low concentrations of tissue factor and high concentrations of thrombin. This is done through the feed back mechanism where the thrombin concentration increases and thrombin then activates TAFI. Once activated, TAFI inhibits fibrinolysis by removing carboxy-terminal lysine residues from fibrin, which are essential for the binding and activation of plasminogen. The role of factor XI in hemostasis can be seen as a combination of a procoagulant action (the formation of fibrin) and an antifibrinolytic action (the protection of fibrin). It is shown in the literature that with high levels of factor XI, the secondary generation of thrombin

would be enhanced or sustained leading to a prolonged down regulation of fibrinolysis and therefore a risk of thrombosis (Meijers et al, 2000). Furthermore, it has been shown that under disease conditions associated with Disseminated Intravascular Coagulation (DIC), the continuous exposure to excess TF typically exhaust the available tissue factor pathway inhibitor (TFPI), leading to rampant thrombin generation and persistent feedback activation of factor XI by the generated thrombin (Østerud and Bjørliid, 2001). One can thus argue that inhibitors of factor XI could stop this secondary generation of thrombin in thrombotic diseases. Such an inhibitor would also prevent or slow down the anti-fibrinolytic state of thrombi under these conditions. Furthermore, inhibitors of factor XI could be attractive candidates for anticoagulant therapy. The relatively mild bleeding associated with congenital factor XI deficiency suggest that drug induced blockade of factor XIa would be associated with relatively low bleeding risk compared to the standard anti-coagulants presently in use.

The aim of this study was therefore to select peptide inhibitors to factor XI. I chose small peptides, because they are non-immunogenic and non-toxic (Markwardt, 1990).

I used phage display technique to select peptide inhibitors to factor XI. Phage display is a powerful technique for obtaining libraries containing millions or even billions of different peptides or proteins and also for selecting and engineering peptides with desired binding specificities (Sidhu, 2001). DNA sequences of interest are inserted into a location in the genome of filamentous bacteriophage such that the encoded protein is expressed or displayed on the surface of filamentous phage as a fusion product to one of the phage coat proteins. Therefore, instead of having to genetically engineer proteins or peptide variants one-by-one and then express, purify, and analyze each variant, phage display libraries containing several billion variants can be constructed simultaneously. These libraries can then be easily used to select and purify specific phage particles bearing sequences with desired binding specificities from the non-binding variants

(Azzazy and Highsmith, 2002). The phage display technique therefore creates a physical linkage between a vast library of random peptide sequences and the DNA encoding each sequence allowing rapid identification of peptide ligands for a variety of target molecules (Smith, 1985). It has advantages over other techniques in that it is faster and less expensive. It yields a product with practically indefinite stability (Jarolim, 2001). It also has some advantages in mapping protein-protein interactions due, in part, to the enormous diversity of sequences capable of being displayed by bacteriophage and its coding potential is enormous compare to the other techniques from which one can select a "molecular needle in a haystack" (Rodi et al, 2001).

I started the phage display selection by biopanning in immuno-tubes. To achieve this, I coated the immuno-tube with factor XI and incubated the phage libraries with it. To select as many factor XI binders possible, I eluted the factor XI binding phages non-specifically from the immuno-tube. I did four selection rounds, to enrich the factor XI binding phages. Round four of each library contains the most factor XI binding phages. This is shown in the global ELISA (see figure 4.1).

I found only two strong factor XI binding phage clones from the linear 12-mer phage library. They were clones 27 and 45. Because I had limited quantities of factor XI, I had to be economical and this did not allow us to search for more binding clones. Both phage clones bound with a high affinity to factor XI (figure 4.2). Clone 27 however binds stronger to factor XI than clone 45. The binding of these clones to factor XI was also dose dependently. The higher the phage concentration, the more phages bind to factor XI.

Both clones were prevented from binding to factor XI by high concentrations factor IX. This inhibition is however not significant because the point with the highest concentration of factor IX is not significant lower than the zero factor IX point. Therefore, one may speculate that the clones may bind to factor XI at the same binding site as factor IX, therefore the A2-domain of factor XI. Only clone 27 was

prevented from binding to factor XI by high α -thrombin concentrations (see figure 4.4). This inhibition is also not significant. One can however argue that since thrombin binds to the A1 domain of factor XI, it may be possible that clone 27 binds to the A1 domain as well. However further binding studies with a peptide containing the same amino acid sequence as the peptide sequence displayed on clone 27 needs to be done to determine exactly where on factor XI, this peptide binds. The reason why the peptide should be used in binding studies is that only limited numbers of phages can be used in any assay because of the inherent size restrictions of the phages. I however determine the effect of both clones 27 and 45 on the PTT and PT.

Both clones lengthened the aPTT dose dependently. Clone 45 however lengthened the aPTT a little bit more than clone 27 (see figure 4.5). It is known that patients with factor XI deficiency typically exhibit an abnormally high aPTT, and normal PT (Hernandez and Raja, 1999). This is because the aPTT is sensitive to a deficiency of any one of the coagulation factors except factors VII and XIII, and it is most sensitive to a deficiency of or inhibition of contact system factors. The aPTT becomes abnormal when contact system factor levels fall below about 20 to 50 % of normal. The PT on the other hand is sensitive to abnormalities in the tissue factor phase and common phase of blood coagulation. It is not sensitive to deficiencies in the contact phase coagulation factors, factor XI, factor IX and factor VIII, since excessive amounts of tissue factor are used in the assay (Bajaj and Joist, 1999).

I determined the amino acid sequence of the peptides displayed on both the factor XI binding clones. Both peptides contain three amino acid sequences of HMWK and thrombin. Clone 27 also contains a three amino acid sequence of factor XII. None of them contains a three amino acid sequence of factor IX. Since clone 27 contains amino acids sequences present in HMWK and thrombin and is also prevented from binding to factor XI by thrombin, one can suggest that this clone binds to the A1 domain of factor XI, therefore the binding domain of thrombin and

HMWK on factor XI (Baglia et al, 1996). However this is only suggestion, since the exact binding site of this peptide sequence to factor XI still needs to be determined. Clone 45 is not prevented from binding to factor XI by thrombin and therefore, one cannot suggest that it bind to the thrombin-binding site of factor XI. Both clones 27 and 45 were prevented from binding to factor XI by factor IX, but neither of them contains sequences of factor IX. I therefore cannot make suggestion on the involvement of the A2 domain (the binding site for factor IX to factor XI) in the binding of these two clones to factor XI. Since clones 27 binds with a higher affinity to factor XI than clone 45 (fig 4.2) and both factor IX and thrombin prevents this clone from binding to factor XI (fig 4.3 and 4.4) I synthesize a linear peptide with the corresponding sequence as the peptide sequence displayed on clone 27.

I characterized the peptide by studying its effect on the aPTT and PT. This peptide lengthens the aPTT dose-dependently (see figure 4.7). A concentration of 3.7mM peptide lengthened the aPTT about two fold (from 66 seconds to 120 seconds). There was no lengthening in PT as expected because the PT is not sensitive to inhibitors of factor XI, since excessive amounts of TF are used in the assay. The lengthening in aPTT indicates that this peptide may inhibit the factor XI levels probably more than about to 20 to 50 % of normal, since the aPTT becomes abnormal when contact system factor levels falls below 20 to 50 % normal (Bajaj and Joist, 1999). Reports of inhibitors to factor XI are rare. A case report of an acquired factor XI inhibitor in a factor XI deficient patient is reported. The aPTT was lengthened from 60 seconds (normal) to 120 seconds where the inhibitor titer was 300 units and factor XI level 4 % (Ginsberg et al, 1993). No information about the effect of the natural factor XI inhibitors such as ATIII, Protease Nexin II, and platelet coagulation factor XIa inhibitor on the aPTT is described in the literature. However the aPTT is not a good screening test for example heterozygotes with partial factor XI deficiency since it overlap with the normal range (Seligsohn, 1993). The sensitivity of this test for factor XI deficiency is also in the range of 30-50 units/dl where the normal reference range is 60-160 units/dl (Bajaj and Joist, 1999). The aPTT is therefore not the optimal test for studying the effect of factor XI

inhibitors on haemostasis. A more reliable test would be a chromogenic assay to determine the strength of the inhibition, if inhibition does occur. Because of our limited amount of factor XI, I did not perform such a test. The lengthening in aPTT of our peptide however indicates that I have selected an inhibitor of the contact system factors of coagulation, which is effective.

With this study, I have shown that the phage display can be used to select novel factor XI inhibitors from random peptide libraries. I have indicated that by using a biopanning method where I coated immuno tubes with the target protein, factor XI, and eluted the binding phages non-specifically, I could select factor XI binding phages. These phages may also bind to the factor IX and/or thrombin binding sites on factor XI, because they were prevented from binding to factor XI by factor IX and/or thrombin. These phages are inhibitory to factor XI because they lengthen the aPTT. The peptide I have synthesized which has the same sequence as the peptide displayed on one of the phage clones also lengthens the aPTT.

Further studies would involve the further characterization of our peptide. This would include kinetic studies to determine the strength of the inhibition and X-ray crystallography to determine exactly where this peptide binds. It would also be interesting to determine the *in vivo* effect of this peptide in animal models.

CHAPTER 6

ABSTRACT

The role of factor XI in hemostasis can be seen as a combination of a procoagulant action (the formation of fibrin) and an antifibrinolytic action (the protection of fibrin). High levels of factor XI lead to a prolonged down regulation of fibrinolysis and therefore a risk of thrombosis (Meijers et al, 2000). Under disease conditions associated with Disseminated Intravascular Coagulation (DIC), the continuous exposure to excess TF typically exhaust the available tissue factor pathway inhibitor (TFPI), leading to rampant thrombin generation by factor XI feedback and therefore also a risk of thrombosis (Østerud and Bjørliid, 2001).

I selected possible inhibitors of factor XI using phage display technology. I started the phage display selection by biopanning in immuno-tubes and eluted the factor XI binding phages non-specifically from the immuno-tube. I did four selection rounds, to enrich the factor XI binding phages. I found only two strong factor XI binding phage clones from a linear 12-mer phage library. Both phage clones bound dose dependently and with a high affinity to factor XI. Both clones also lengthened the partial thromboplastin time (aPTT) dose dependently.

The amino acid sequences of the peptides displayed on these two clones indicate that both peptides contain three amino acid sequences of HMWK and thrombin. One clone also contains a three amino acid sequence of factor XII. None of them contains a three amino acid sequence of factor IX. I synthesize a linear peptide with the corresponding sequence as the peptide displayed on the clone that was prevented from binding to factor XI by both factor IX and thrombin.

I characterized the peptide by studying its effect on the aPTT. This peptide lengthens the aPTT dose dependently. The lengthening in aPTT of our peptide however indicates that I have selected an inhibitor of the contact system factors of coagulation.

In summary, this study shows that the phage display can be used to select novel factor XI inhibitors from random peptide libraries. With further studies, this peptide may be developed as an antithrombotic.

ABSTRAK

Die rol van factor XI in trombose kan beskou word as 'n kombinasie van pro-stollings- (vorming van trombien) en teen-fibrinolise- (beskerming van die fibrien stolsel teen afbraak) funksies. Verhoogde factor XI-vlakke in plasma lei tot 'n vertraging van fibrinolise en daarom 'n hoë risiko vir trombose (Meijers et al., 2000). Die voortdurende blootstelling van weefselfaktor in siektetoestande soos verspreide intravaskulêre stolling, put die beskikbare weefselfaktorbaan-inhibeerder uit. Dit lei dan tot 'n toenemende trombien generasie deur die factor XI terugvoer-baan en daarom ook 'n verhoogde risiko vir trombose (Østerud en Bjørlid, 2001).

In hierdie studie het Ek inhibeerders teen factor XI d.m.v. peptiedblootlegging op fage geselekteer. Herhaalde seleksie van fage vanaf 'n sikliese heptapeptied faag biblioteek asook 'n lineêre 12-aminosuur faag biblioteek het gelei tot die vermeerdering van fage wat spesifiek aan factor XI bind. Ek het immuunbuise in die seleksieproses gebruik en die factor XI-bindende fage nie-spesifiek van die ongebonde fage ge-elueer. Ek het twee klone wat sterk aan factor XI bind geselekteer. Bedie klone het ook die gedeeltelike tromboplastien tyd (PTT) dosis afhanklik verhoog.

Die aminosuurvolgorde van die peptiede wat blootgelê is op die fage van hierdie twee klone, bevat 3-aminsuur volgordes van hoë molekulêre gewig kininogeen (HMWK) en van trombien. Die een klone bevat ook 'n 3-aminsuur volgorde van factor XII. Nie een van die peptiede bevat aminosuurvolgordes van factor IX nie. Ek het 'n lineêre peptied met 'n ooreenstemmende aminosuur volgorde as die peptied wat blootgelê is op die klone wat deur factor IX en trombien verhoed is om aan factor IX te bind, laat sintetiseer. Hierdie peptied verleng die PTT dosis-afhanklik. Dit dui dus daarop dat Ek 'n inhibeerder van die kontakstelsel suksesvol geselekteer het.

Ter opsomming kan ons sê dat die tegnologie van peptied blootlegging op fage gebruik kan word om nuwe factor XI inhibeers vanaf lukraak peptiedbiblioteke te selekteer. Met verdere studies kan hierdie peptied as 'n antistolmiddel ontwikkel word.

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