PHAGE DISPLAY SELECTION OF PEPTIDE INHIBITORS OF FVIIa AND THEIR FUNCTIONAL CHARACTERISATION

by

Catharina Elizabeth Roets

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In the Faculty of Health Sciences
Department of Haematology and Cell Biology
at the University of the Free State
Bloemfontein
South Africa

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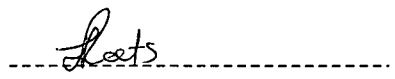
Promoter: Dr. S.M. Meiring

Declaration

Hereby I declare that the script submitted towards a Ph.D. degree at the University of the Free State is my original and independent work and has never been submitted to any other university or faculty for degree purposes.

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



C.E. Roets

June 2002

Dedicated to my two beautiful children, Lize-Louise and Carlize

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Abbreviations

ADP	adenosine-5'-diphosphate
Apo(a)	apolipoprotein a
Arg	arginine
Asp	aspartic acid
ATIII	antithrombin III
Ca^{2+}	calcium
Cys	cysteine
DNA	deoxyribonucleic acid
E coli	Escherichia coli
EDTA	ethylenediaminetetra-acetic acid
EGF	epidermal growth factor
ELISA	enzyme-linked immuno-sorbent assay
FITC	fluorescein isothiocyanate
FVII	factor VII
FVIIa	activated factor VII
Gla	γ-carboxy glutamic acid
Gp	glycoprotein
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
His	histidine
HMEC-1	human microvascular endothelial cells, type 1
HRP	horseradish peroxidase
H_2O_2	peroxidase
H_2SO_4	sulphuric acid
Ile	interleucin
IPTG	isopropyl-β-D-thiogalactoside
KDa	kilodalton
Ki	inhibition constant

Km	Michaelis-Menten constant
LB	lubria broth
LDL	low density lipoprotein
LMWH	low molecular weight heparin
Lp(a)	lipoprotein a
Lys	lysine
Met	methionine
M	molar
NaCl	sodium chloride
NaI	sodium iodide
NaOH	sodium hydroxide
NAP	nematode anticoagulant protein
OD	optical density
PAR	protease activated receptor
PAI-1	plasminogen activator inhibitor-1
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PEG	polyethyleneglycol
PT	prothrombin time
RF	replicative form
Ser	serine
SM	skimmed milk
SV40	simian virus 40
TF	tissue factor
TFPI	tissue factor pathway inhibitor
tPA	tissue plasminogen activator
Tris	tris(hydroxymethyl)aminomethane
TSP-1	thrombospondin-1
TT	thrombin time
Vmax	maximum velocity

Vo	initial velocity
vWF	von Willebrand factor
Xgal	5-bromo-4-chloro-3-indonyl-β-D-galactoside

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CHAPTER 1

INTRODUCTION

Blood coagulation is initiated when injured blood vessels expose blood to tissue factor (TF) in the subendothelium (fig. 2.1). Coagulation factor VII (FVII) or activated factor VII (FVIIa) present in plasma binds to TF to form the FVIIa/TF complex. This complex activates factor X which on its turn activates thrombin. Thrombin is responsible for the formation of fibrin and activation of platelets to form a thrombus.

Despite many years of research using various strategies only two anticoagulants are in widespread clinical use (Hirsh and Weitz, 1999). These are coumarins and heparins (Hirsh *et al.*, 1994; Weitz, 1997). Coumarins impair the function of the vitamin K-dependent proteins including both procoagulants (thrombin, factor Xa, factor IXa and factor VIIa) and anticoagulants (activated protein C and protein S) whereas heparin enhance the inhibition of thrombin and factor Xa by antithrombin III. The non-selective mode of inhibition of both of these anticoagulants probably accounts for their therapeutic limitations in maintaining the balance between thrombosis and haemostasis (Hirsh and Weitz, 1999).

Because it initiates the coagulation process, the FVIIa/TF complex represents a good target for developing therapeutic anti-coagulants. Tissue factor pathway inhibitor (TFPI) naturally inhibits human FVIIa/TF. TFPI is a protein containing three Kunitz domains and it inhibits the FVIIa/TF complex in a factor Xa-dependent manner (Broze *et al.*, 1988).

The elaborate nature of the FVIIa/TF complex suggests additional approaches that may impair its function (Higashi and Iwanaga, 1998). FVII is a vitamin K dependent glycoprotein, which circulates in blood as a single chain glycoprotein of 406 amino acids (Kumar and Fair, 1993). Activation of factor

VII by factors IX and X involves the cleavage of an Arg152-Ile153 bond. The activated factor VII (FVIIa) then circulates as a two-chain glycoprotein composed of a heavy and light chain. The light chain of FVIIa consists of the N-terminus Gla-domain followed by two EGF-like domains. The heavy chain is the catalytic domain and disulphide bonds link the two chains (Eigenbrot, 2002; Persson, 2000; Banner *et al.* 1996). FVII makes extended contact to TF in the FVIIa/TF complex. The Gla-domain, the EGF-1 domain and the catalytic domain are involved in the interaction with TF.

TF is a small transmembrane cell surface receptor with an extracellular domain, a transmembrane domain and a cytoplasmic domain (Spicer *et al.*, 1987). The extracellular domain consists of two fibronectin type III domains, TF1 and TF2. The main binding site for FVIIa is located at the interface between the TF1 and TF2 domains (Banner *et al.*, 1996).

Compounds that block the association of TF with FVIIa can prevent the activation of the macromolecular substrate, FXa, and therefore inhibit coagulation. We designed a study to develop peptide inhibitors of FVIIa using phage display technology.

The major thrust these days is to develop inhibitors against factor X and thrombin. The fact that FVIIa acts higher up in the coagulation cascade than these two factors, made us speculate that less inhibitor might be needed to inhibit FVIIa. We thus decided on investigating the possibility of developing inhibitors against FVIIa. Although there are no FVIIa inhibitors commercially available as anticoagulants yet, studies on this have been reported (Dennis *et al.* 2000; DeCristofaro, 2002; Roberge *et al.* 2002; Dennis and Lazarus, 1994b).

We selected possible inhibitors of FVIIa 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). Different single phage colonies were picked and grown and then their ability to bind to and inhibit FVIIa were tested. We sequenced the FVIIa-inhibitory colonies and decided on one sequence to synthesise. A 7-mere cyclic peptide was synthesised and characterised by performing prothrombin times and thrombin times. We also tested the effect of this peptide kinetically on FVIIa-inhibition and also on platelet adhesion to endothelial cells and tissue factor. We 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 characterisation of novel FVIIa inhibitory peptides. It is therefore necessary to commence the literature review with a brief discussion on the mechanisms of blood coagulation.

2.1 Blood Coagulation

Blood coagulation in concert with platelet activation and fibrinolysis is part of the haemostatic response to injury and serves to maintain the integrity of the vascular system. It also helps to prevent excessive blood loss through platelet-fibrin formation. Blood coagulation is initiated when blood vessels are injured, exposing blood to tissue factor (TF) in the exposed subendothelium (figure 2.1). TF is produced constitutively by cells beneath the endothelium, as well as monocytes, macrophages, brain -, lung - and placental cells. Coagulation factor VII (FVII) or activated factor VII (FVIIa) present in plasma binds to tissue factor forming a FVIIa/TF complex which activates limited quantities of factor X (Xa) and factor IX (IXa). TF acts as a cofactor for FVII activation and enhances the proteolytic activity of FVIIa towards its substrates, factors IX and X (Broze, 1992; Ruf, 1998). Factor Xa in turn activates prothrombin to form thrombin (Tuddenham, 1996). Tissue factor pathway inhibitor (TFPI) almost immediately inhibits the FVIIa/TF complex after triggering the coagulation cascade (Figure 2.1). Thrombin accelerates its own production by activating platelets to provide coagulation surfaces where the prothrombinase complex, an enzyme complex activating prothrombin in plasma, assembles in a Ca^{2+}- dependent reaction. Thrombin also activates factors V and VIII to provide the cofactors for factor Xa in the prothrombinase complex, and for factor X activation by factor IXa respectively. Even when factor X activation is initiated by tissue factor, efficient propagation of factor X activation is critically dependent on the factors IXa and VIIIa enzyme complex (TENASE complex) (Ofosu *et al.* 1996). The FVIIa/TF complex is the

essential initiator of blood coagulation, due to activation of FX and FIX not being detectable in the absence of FVIIa or TF (Butenas and Mann, 2002; Butenas *et al.* 2000; Davie *et al.* 1991; Nemerson, 1986).

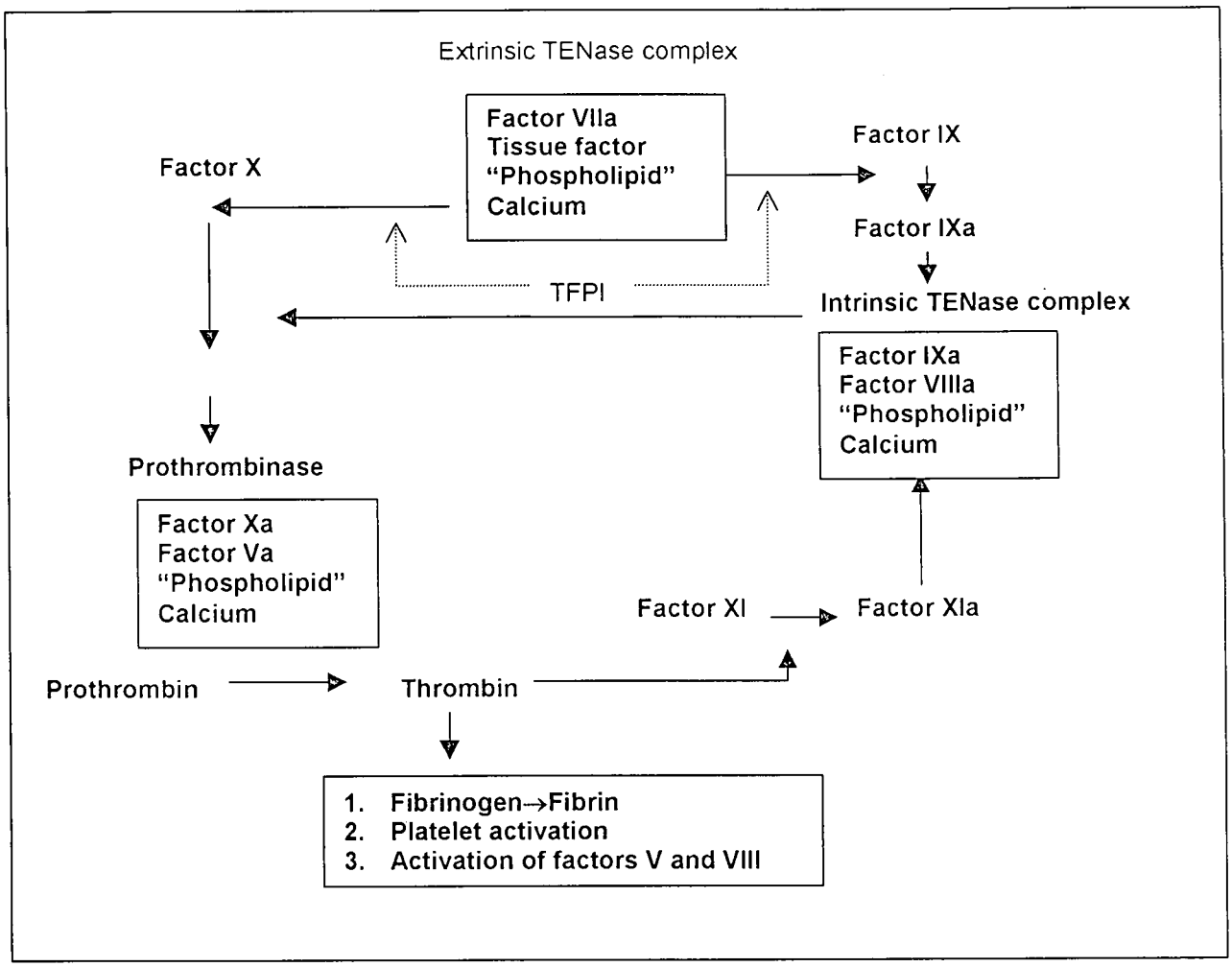

Figure 2.1 A schematic representation of the coagulation cascade. The boxes represent the components of the vitamin K-dependent complexes. The top component of each complex is the vitamin K-dependant serine protease and the second its required cofactor protein. "Phospholipid" represents the appropriate membrane surface required for precise protein assembly and is mainly supplied by platelets. Calcium ions stabilise the interactions (Broze, 1992; Meiring, 1996).

2.2 Role of platelets in thrombosis and coagulation

Since this study focuses on the characterisation of a FVIIa inhibitory peptide, studying its effect on platelet adhesion, necessitates discussion of the role of platelets in thrombosis and haemostasis.

Platelets play a major role in haemostatic plug formation following injury to blood vessels where normally they do not adhere. Several components of the subendothelium are exposed with vascular injury. They are fibrillar and non-fibrillar collagens, elastin, proteoglycans, laminin, thrombospondin, fibronectin, vWF and TF. Platelets are localised to the injury site and adhere directly to the exposed collagen through their GpIa/IIa receptor, and indirectly via circulating von Willebrand factor (vWF). Von Willebrand Factor (vWF) circulating in the blood binds to the exposed collagen following damage to the endothelium. The vWF subsequently undergoes a conformational change whereafter platelets can bind to it through their membrane glycoprotein Ib/V/IX receptor (GpIb/V/IX). vWF thus forms a bridge between the exposed collagen and the receptor.

The tethering of platelets to vWF slows down the movement of the platelets. These tethered cells roll on the damaged surface and eventually the platelets become adhered through binding of other receptors. The binding of vWF to platelets activates the platelets, resulting in a conformational change in platelet membrane glycoprotein, GpIIb/IIIa. The conformational change in GpIIb/IIIa enables fibrinogen and vWF to bind to it. The fibrinogen and vWF can then bind to adjacent platelets and this is called platelet aggregation (Jackson *et al.* 2000; George, 2000; Ofosu, 2002).

The activated platelet also releases the contents of its α- and dense granules. These contents help to reinforce platelet activation. The dense granules release ADP and calcium. ADP is responsible for aggregation of platelets, and platelets have transmembrane receptors for ADP. Fibrinogen, factor XI, factor IX, factor V, factor XIII and vWF are also secreted by the activated

platelets as well as other adhesive proteins such as fibronectin, thrombospondin and vitronectin (Ofosu, 2002; George, 2000; Gachet, 2001).

Activated platelets generate thrombin on their surface and the thrombin binds to transmembrane receptors on the platelets. This leads to further platelet activation and also aggregation. Thrombin is described as the most potent activator of platelets and the principle receptor for thrombin is PAR-1 (Ofosu, 2002). Thrombin also activates fibrinogen to fibrin polymers, which stabilises the haemostatic plug. Activated platelets provide a negatively charged surface for the coagulation enzyme complexes of the coagulation cascade. Calcium released from the granules of activated platelets is thus also involved in these platelet surface coagulation reactions (Ofosu, 2002).

TF is also present on microparticles in circulating blood and in thrombi. This circulating TF is potentially thrombogenic because thrombus formation was reduced by the addition of a TF inhibitor to native human blood (Rauch and Nemerson, 2000). Polymorphonuclear leukocytes and monocytes contain TF-positive microparticles. These microparticles adhere to platelet thrombi. Leukocytes and monocytes can transfer these TF microparticles to platelets via an interaction between the cell adhesion molecule CD15 on the leukocytes and monocytes and P-selectin on platelets. P-selectin is an α-granule-derived adhesion molecule present on activated platelets and is also known as CD62P (Østerud, 2001; Giesen *et al.* 1999; Rauch *et al.* 2000; Rauch and Nemerson, 2000). Under normal conditions, most of the TF activity on the lymphocyte and monocyte cell membrane is latent or encrypted, implying that the TF binds to FVIIa, but is not capable to initiate coagulation. Platelets play an important role in the decryption of monocyte TF through the interaction between CD15 and P-selectin. A mixture of leukocytes and platelets generates more TF activity than either of these cells alone (Østerud, 2001; Giesen and Nemerson, 2000). The transfer of TF microparticles to platelets thus results in platelets being more capable of triggering and also propagating thrombus growth.

2.3 Factor VII

Factor VII (FVII) was first purified in 1975 as a single chain inactive form (Radcliffe and Nemerson, 1975). The single chain FVII is cleaved by FXa and thrombin into a double chain active form (FVIIa) (Radcliffe and Nemerson, 1975). The total isolation of human FVII was also reported in 1981 (Bajaj *et al.* 1981; Kisiel and McMullen, 1981).

The essential role of FVII and activated FVII (FVIIa) in plasma is to bind to tissue factor forming the factor VIIa/TF complex. Activated FVII present in plasma fails to show appreciable enzyme activity until it is brought into contact with TF (Banner, 1997). FVII is a vitamin K-dependent glycoprotein and circulates in the blood as a zymogen at a concentration of about 10nM. Approximately 1% of FVII is present in the activated form (FVIIa) (Örning *et al.* 1997). Of all the coagulation factors FVII has the shortest lifetime, which is about 4 to 5 hours. The activated form of FVII (FVIIa) circulates for about 2½ hours (Nemerson, 1988).

2.3.1 Genetics and Structure

The FVII gene has 9 exons on the long arm of chromosome 13, and is located adjacent to the FX gene (Hutton *et al.* 1999). FVII is synthesised and secreted by the liver and circulates as a single chain zymogen of 406 amino acid residue (Kumar and Fair, 1993). It is synthesised with a pre-pro-leader sequence of 38 amino acids (see figure 2.2). For the mature protein to be produced, an arginyl-alanine bond of the pre-pro-leader sequence must be cleaved (Hagen *et al.* 1986; Tuddenham and Cooper, 1994).

The mature protein consists of an amino terminus γ-carboxy glutamic acid rich (Gla) domain, a short signal peptide, two epidermal growth factor-like (EGF) domains, a short activation peptide and a catalytic carboxyl serine protease domain (see figure 2.2) (Banner *et al.* 1996; Hutton *et al.* 1999). This zymogen is post-translationally modified to produce 10 Gla residues (Chang

et al. 1995). The Gla-domain extends from residue 1 to 35. It mediates calcium ion binding, which induces a conformational change leading to the expression of membrane and cofactor binding properties. Calcium ions are required for the stabilisation of the FVII/TF complex. Residues 37 to 46 form the short signal peptide, and it consists of an amphipathic helix region, followed by the two EGF-domains. The EGF domains mediate protein-protein interactions and are necessary for binding to TF. The short activation peptide following the EGF-domains is a connecting region containing an arginyl-isoleucine cleavage site for FXa at residue 152/153. Finally, the catalytic carboxyl serine protease domain is homologous to trypsin and contains a catalytic triad consisting of residues His-193, Asp-242, and Ser-344 (Higashi and Iwanaga, 1998; Broze, 1992; Hagen *et al.* 1986; Tuddenham and Cooper, 1994). This serine protease domain has two α-helices, and the ends of these two helices form a concave surface covering a part of the amino-terminus of TF. In the first helix Arg276 is the main contact residue and the contact residues are Met306 and Asp309 in the second helix. The Asp309 residue precedes the Cys310-Cys329 loop (Ruf, 1998; Banner *et al.* 1996).

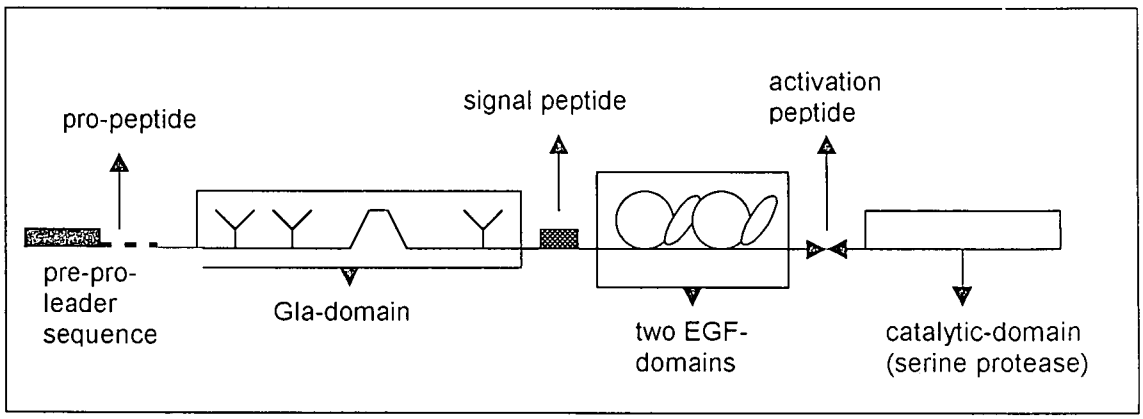

Figure 2.2 Schematic presentation of the domain structures of FVII. EGF=epidermal growth factor; Gla=γ-carboxy glutamic acid (Hutton *et al.* 1999)

Both factors Xa and IXa activate FVII. FXa, however, is approximately 800 times more efficient than FIXa. Ca^{2+} and phospholipids are essential for the

activation (Masys *et al.* 1982). Thrombin or FXIIa can also activate FVII in the absence of cofactors (Broze and Majerus, 1981).

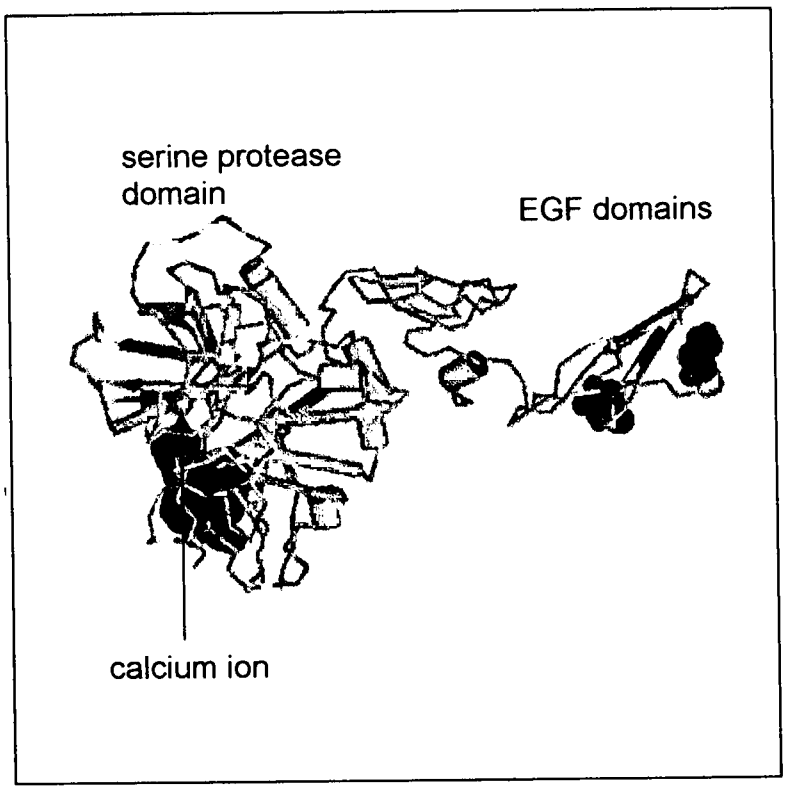

Figure 2.3 Secondary structure of Gla-domainless human FVII. The purple represents the two EGF-domains and the yellow represents the serine protease domain. The green represents a calcium ion (Pike *et al.* 1999)

FXa activates FVII by splicing the peptide bond between Arg 152 and Ile 153 of the activation peptide. This results in the formation of a light chain of 152 residues and a heavy chain of 254 residues (Eigenbrot, 2002; Banner *et al.* 1996). The light chain consists of the amino-terminus Gla-domain followed by two EGF domains. The heavy chain contains the catalytic domain, which has a core structure common to all serine proteases of the thrombin/trypsin family. It is an arginine specific serine protease, therefore it cuts its substrates proximal to an arginine residue (Banner, 1997; Banner *et al.* 1996). The heavy chain is linked to the light chain via a disulphide bridge between Cys-135 and Cys-262 (Higashi and Iwanaga, 1998). Three different binding regions are

contained within the catalytic domain of FVIIa. These three binding regions are: a TF binding region, active site binding region, and a macromolecular substrate binding region, all three these regions being important for proteolytic activity (Persson, 2000; Ruf and Dickinson, 1998). Activation of the trypsin-type serine proteases like FVIIa results in formation of a salt bridge between the amino-terminus α-amino group and the β-carboxyl group of the aspartic acid residue adjacent to the active serine. This bridge is essential for the catalytic activity since it stabilises the active conformational states of the protease domain. In FVIIa this salt bridge is formed between the amino-terminus α-amino group of Ile-153 and the β-carboxyl group of Asp-343. It is important to note that this bridge is not completely formed in FVIIa unless it is bound to TF (Higashi and Iwanaga, 1998).

Mutagenesis showed that trace amounts of FVIIa could activate FVII bound to TF. This auto-activation might be more important for the initial initiation of blood coagulation than previously considered (Nakagaki *et al.* 1991; Neuenschwander and Morrissey, 1992; Thomas, 1947). The conformational change that FVII undergoes when it binds to TF makes it susceptible to activation by trace amounts of FVIIa as well as other proteases (Nakagaki *et al.* 1991). ATIII had no apparent effect on activation of FVII by FVIIa, while it could completely block FXa dependent activation of FVII under the same conditions. FVIIa subjected to auto-activation could generate FXa in the same way as otherwise activated FVIIa (Yamamoto *et al.* 1992).

FVIIa can trigger signalling events in cells via the protease activated G protein-coupled receptor-2 (PAR2) only in the presence of TF. The other PAR's, PAR1, PAR3, and PAR4 are all activated by thrombin. The factor VIIa/TF generated FXa can also cleave PAR2 to trigger transmembrane signalling (Camerer *et al.* 2000; Riewald and Ruf, 2001).

2.4 Tissue factor

Tissue factor (TF) is a small transmembrane cell surface receptor that triggers the coagulation cascade. It does not require proteolytic modification to fully express its activity, thereby making it the primary initiator of coagulation (Edgington *et al.* 1991; Tuddenham and Cooper, 1994). Cells normally in contact with plasma - the blood cells and endothelium - do not express TF without activation. TF is present in brain, lung and placenta and in the media and adventitia of blood vessels. It is also found in the bronchial mucosa and alveolar epithelial in the lung (Ruf and Edgington, 1994). Normally, monocytes and endothelial cells do not express TF, but these cells are stimulated to express tissue factor on their surfaces by endotoxin, interleukin-1, tumour necrosis factor, cytokines and platelets. In the cellular immune response, monocytes express TF after stimulation by T-helper cells. Monocytes also express TF *in vivo* in certain pathological conditions associated with intravascular coagulation and thrombosis such as meningococcal infection and peritonitis (Broze, 1992; McVey, 1994; Hutton *et al.* 1999; Ruf and Edgington, 1994; Miller *et al.* 1981; Østerud and Flaegstad, 1983; Bajaj *et al.* 2001). TF is expressed by vascular adventitial cells, neuroglia, vascular smooth muscle and epidermal cells (McVey, 1994) TF, therefore forming a protective envelope around blood vessels and organs in order to initiate coagulation as soon as is necessary (Morrisey, 2001).

2.4.1 Genetics and structure

The gene that encodes TF has six exons and is located on the short arm of chromosome 1 (Hutton *et al.* 1999; Edgington *et al.* 1991). The mature protein consists of 263 residues and is preceded by a 32-residue signal peptide (Fisher *et al.* 1987; Spicer *et al.* 1987). The TF molecule has an extracellular domain, a transmembrane domain, and a cytoplasmic domain (see figure 2.4) (Banner *et al.* 1996; Spicer *et al.* 1987). The extracellular domain consists of two fibronectin type III domains, TF1 and TF2 connected end to end to form an angle of about 120° (Morrissey *et al.* 1997). Both TF1

and TF2 domains consist of 219 amino acids. The main binding site for FVIIa is located at the interface between the TF1 and TF2 domains (Banner *et al.* 1996). The transmembrane domain consists of a 23-residue domain and the cytoplasmic domain of a 21-residue domain. This domain is crucial for the anchoring of TF to the membrane and therefore localises the catalytic initiation of coagulation (Ruf *et al.* 1991).

Two adjacent lysine residues in TF, Lys^{165} and Lys^{166}, are important for FX activation (Kelley *et al.* 1997). These two residues interact directly with the Gla-domain of FX and are also required for the accelerated inhibition of the FVIIa/TF complex by TFPI mediated by FXa (Huang *et al.* 1996; Roy *et al.* 1991; Rao and Ruf, 1995). The structure of the FVIIa/TF complex will be discussed in more detail in the next section. TF accelerates the activation of FVII by FXa, which makes it a bifunctional coagulation cofactor by enhancing the activity of FVII as a cofactor and as a substrate. TF is thus required for activation of FX by FVIIa and the acceleration of FVII activation by FXa (Nemerson and Repke, 1985). The primary function of TF is however to anchor the complex to the membrane surface by its transmembrane domain (Krishnaswamy, 1992).

Tissue factor therefore plays a key role in the initiation of blood coagulation during physiological haemostasis, but it may also be responsible for thrombotic disorders. It is also involved in processes other than coagulation, such as intracellular signalling, metastasis, angiogenesis and inflammation. Tissue factor plays an important role in arteriosclerosis and arterial and venous thrombosis. TF is also the dominant procoagulant in many tumour cells (Pendurthi, 2002; Kirchhofer, 2001; Semeraro and Colucci, 1997).

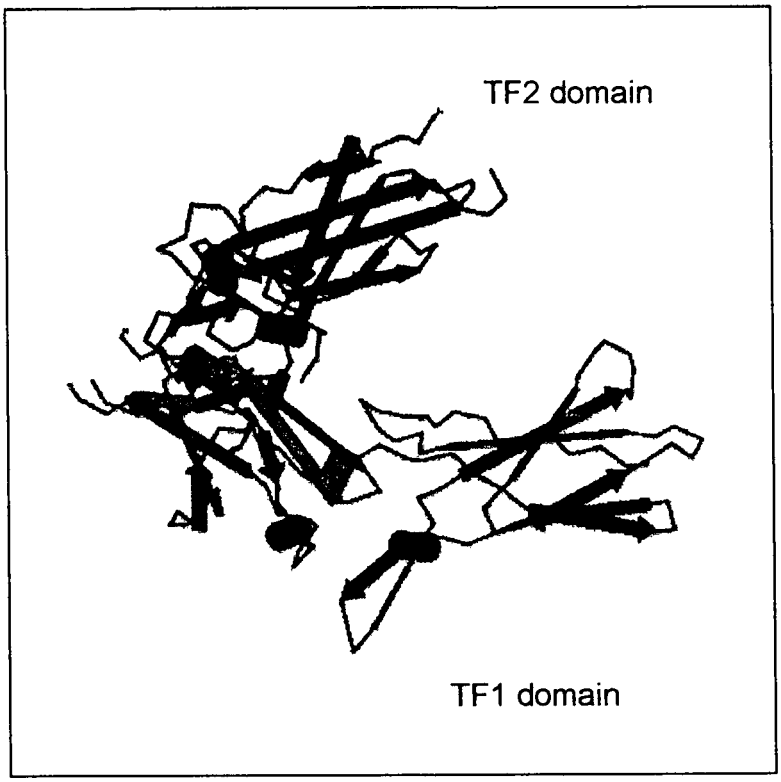

Figure 2.4 Secondary structure of the extracellular domain of human tissue factor. The red represents the TF2 domain and the TF1 domain is represented by the purple (Huang *et al.* 1998)

2.5. Factor VIIa/TF complex

TF and FVIIa form a 1:1 complex where TF acts as a cell-surface receptor for FVIIa (Nemerson, 1988). FVIIa can only reach its full catalytic potential when it is in complex with TF in the presence of calcium ions (Bom and Bertina, 1990; Banner *et al.* 1996). Free FVIIa does not recognise factors IX and X. The binding of TF to FVIIa enhances the enzymatic activity of FVIIa several thousand-fold (Broze, 1992). When bound to TF, FVIIa undergoes a conformational change, creating recognition sites for factors X and IX (Nemerson, 1988). It could be said that FVIIa on its own is "zymogen-like"; and when it is in complex with TF it becomes more "active-enzyme-like" (Banner, 1997). It was thus proposed that FVIIa exists in equilibrium and the binding to TF leads to a shift in this equilibrium towards the active state

(Pendurthi, 2002; Higashi and Iwanaga, 1998). TF binds to FVIIa with a high affinity (binding constant (Kb) of about 3nM) and complex formation is thus very rapid within a wound site (Tuddenham, 1996). Three characteristics are responsible for the dramatic enhancement of the catalytic function of FVIIa. These are a) facilitation of interaction with FX by localising the reaction to the phospholipid surface, b) allosteric activation of FVIIa, and c) the alignment of specific regions of FVIIa and TF to form a surface for recognition of its substrate, FX (Ruf, 1998). This will be discussed in some detail hereafter.

FVIIa adopts a stretched-out conformation along the elongated TF in the FVIIa/TF complex (Ruf, 1998) (See figure 2.5). Two modules on the light chain of FVIIa make distinct contact with TF. This is the amino-terminus Gla-domain of FVIIa interacting with the carboxy-terminus of TF through hydrophobic contacts because deletion of the Gla domain of FVIIa results in a decreased affinity for TF, and the Gla-domain is essential for calcium ions, which in turn is essential for TF binding (Edgington *et al.* 1997; Banner *et al.* 1996; Ruf, 1998; Higashi *et al.* 1996; Nemerson, 1988). The other module on the light chain of FVIIa in the EGF1 domain packs into a cleft formed in the TF molecule where the amino- and carboxy-terminals collide. This contact area is the largest in the complex and accounts for more than 50% of the free energy of complex formation (Edgington *et al.* 1997; Ruf, 1998). Arg79, Ile69, and Phe-71 in the EGF-1 domain are primarily involved in the high affinity binding to TF. A recent study showed the role of the EGF-1 domain of FVIIa in the conformational change of the active site, following activation of FVII forming an allosteric linkage between EGF-1 and the active site (Leonard *et al.* 2000).

The catalytic (serine protease) domain of FVIIa and its tightly associated EGF-2 domain forms a continuous interface with the amino-terminus module of TF, and this contact is responsible for the allosteric activation of FVIIa's catalytic function (Edgington *et al.* 1997; Ruf, 1998). The catalytic (serine protease) domain has a low affinity calcium-binding site that is also necessary for interaction with TF (Wildgoose *et al.* 1993). Arg304 in the catalytic domain is

implicated in the binding of FVIIa to TF, while Arg290 and Lys341 appear to be critical for proteolytic function and substrate specificity (Ruf, 1994; Neuenschwander and Morrissey, 1995). The protease domain of FVII and FVIIa docks similarly with TF and the structures of the FVIIa/TF and FVII/TF complexes thus are similar (Dickinson and Ruf, 1997).

The interaction between TF and FVIIa forms a salt bridge between Ile-153 and Asp-343 of FVIIa, and is part of the acceleration of the catalytic activity of FVIIa by TF. The formation of this salt bridge can also protect the α-amino group of FVIIa from carbamylation (Owenius, 2001; Higashi *et al.* 1994; Higashi and Iwanaga, 1998).

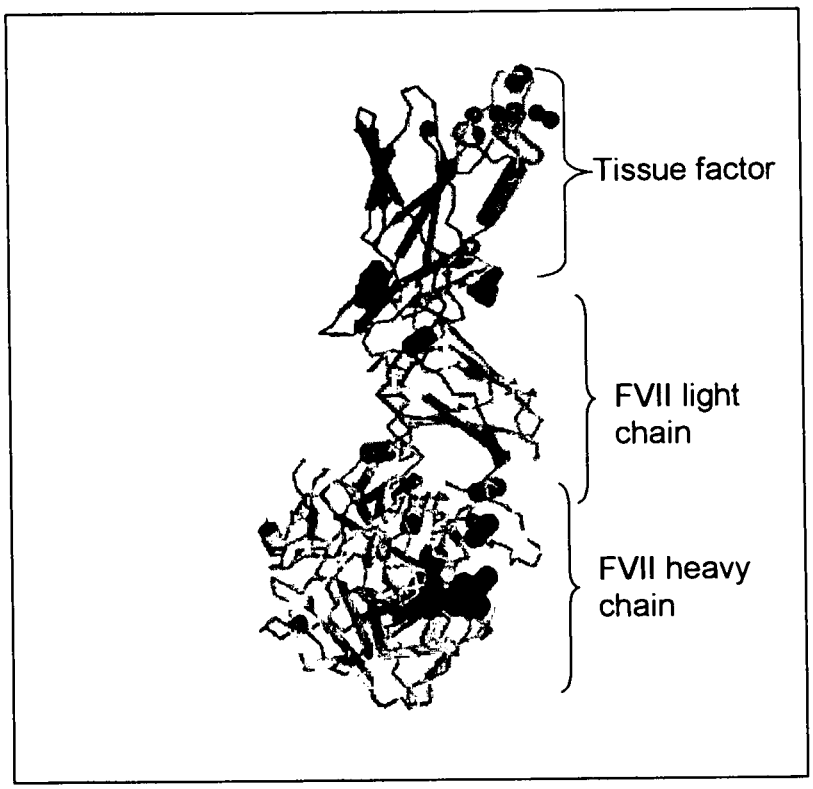

Figure 2.5 Secondary structure of the complex of active site inhibited human FVIIa with human recombinant soluble tissue factor. The light purple represents the light chain of FVIIa, the yellow represents the heavy chain of FVIIa. The blue and darker purple represents TF (Banner *et al.* 1996)

The importance of the presence of Ca^{++} and phospholipids in the FVIIa/TF complex is that it is responsible for a 15 million-fold increase in the catalytic efficiency of FX activation (Bom and Bertina, 1990). TF can however form a complex with FVIIa on membranes that only contain neutral phospholipids, but when acidic phospholipids are also present, the function of the complex is dramatically enhanced (Mann *et al.* 1990).

One mechanism, by which TF regulates the activity of FVIIa, is to properly align the active sites of the enzymes above the membrane surface and therefore phospholipid surfaces. The catalytic activity of FVIIa in complex with TF is enhanced by reducing the conformational mobility, thus a loss of rotational and transitional freedoms (McCallum *et al.* 1996; Banner, 1997; Higashi *et al.* 1994; Higashi and Iwanaga, 1998). The reactivity of the active site of FVIIa is enhanced by reorientation of the amino-terminus, a conformational change in the catalytic triad to facilitate hydrolysis of the ester substrate (Rapaport and Rao, 1995; Higashi *et al.* 1992).

Another mechanism by which TF regulates the activity of FVIIa, is by initiating allosteric cross talk between the three regions in the catalytic domain of FVIIa in the FVIIa/TF complex (Ruf and Dickinson, 1998). A short α-helix is situated at residues 307-312 in the substrate-binding region of the catalytic domain of FVIIa and this helix is distorted in free FVIIa. Free FVIIa contains a Met-residue in the 306 position. This differs from the other serine proteases. This Met-306-residue causes an unstable helix but stabilises after binding to TF, thus preventing the expression of FVIIa activity in the absence of TF. Stabilising by TF thus optimises substrate binding to the altered FVIIa (Kemball-Cook *et al.* 1999; Pike *et al.* 1999). This helix is attached to the protein body through a Cys310-Cys329 disulphide bridge (Pike *et al.* 1999).

Furthermore, the second β-strand in the B2-barrel of the FVII zymogen shows a different registration with the β-strand in the A2-barrel. In the FVIIa/TF complex however, this β-strand (B2-barrel) has shifted three residues toward the carboxy-terminus. This shift results in a shorter activation domain loop as

in the FVIIa enzyme (Eigenbrot *et al.* 2001; Eigenbrot and Kirchhofer, 2002). This is another mechanism by which TF regulates the activity of FVIIa.

Studies on mouse models have proved that inactivation of the TF gene and therefore total TF deficiency, resulted in lethality at embryonic stage. This appeared to be due to a defect in the vascular integrity of the yolk sac. However, FVII deficiency was proved not to be lethal to the embryo, but the neonates with FVII deficiency died from haemorrhage within days after their birth (Rosen *et al.* 1997; Chan, 2001; Mackman, 2001; Aasrum and Prydz, 2002). It is also known that mice expressing a mutant form of TFPI, in which the first Kunitz domain is deleted, die between embryonic day and birth. The few mice that were born with this deficiency died shortly after birth. Interestingly in this study they showed that diminishing FVII activity in these mice resulted in rescuing them from intrauterine death. They developed normally *in utero* and survived birth. However, postnatal they also died from bleeding events (Chan *et al.* 1999; Chan, 2001).

2.6 Inhibitors of the factor VIIa/TF complex

2.6.1 Tissue factor pathway inhibitor (TFPI)

TFPI is the natural inhibitor of factor Xa and the factor VIIa/TF complex. It also inhibits the FVIIa/TF complex in a FXa dependent manner by producing a feedback inhibition (Wun *et al.* 1988; Broze *et al.* 1988; Broze, 1995; Broze and Miletich, 1987a; Broze and Miletich, 1987b; Broze, 1987).

As early as 1947 TFPI was found in serum, inhibiting coagulation in the presence of Ca^{2+} (Thomas, 1947; Schneider, 1947). This inhibitor of the FVII/TF complex was recognised in 1957 (Hjort, 1957). TFPI inhibits the enzymatic activity of the FVIIa/TF complex and not just the activity of TF alone (Rao and Rapaport, 1987). It was cloned in 1988 (Wun *et al.* 1988).

TFPI is composed of three Kunitz type domains (see figure 2.6), which are intervened by linker regions. The linker regions are less structured than the domains, but is important because the isolated Kunitz domains are less potent inhibitors compared to the intact full-length TFPI. TFPI has an acidic amino-terminus region and a basic carboxy-terminal region (see figure 2.5) (Broze, 1995; Wun *et al.* 1988; Bajaj *et al.* 2001).

TFPI is synthesised mainly by the endothelial cells under normal conditions (Bajaj *et al.* 2001). It is present in three pools in blood. About three percent of the circulating TFPI is carried in platelets, and platelets release the TFPI after stimulation by thrombin (Broze, 1992). Ten percent of the TFPI circulates in plasma in association with lipoproteins. A small amount of full-length TFPI circulates in free form. The major form of TFPI in association with lipoproteins is associated with low-density lipoproteins (LDL) and has a molecular weight of 34kDa. This form of TFPI lacks the distal portion of the full-length TFPI. This distal portion includes the third Kunitz domain and this form of TFPI is carboxy-terminally truncated. TFPI associated with high-density lipoproteins has a molecular weight of 41 kDA and is also carboxy-terminally truncated.

The carboxy-terminally-truncated form of TFPI is not as efficient an anticoagulant as full-length TFPI. The highest percentage of TFPI, 80-85%, is the full-length TFPI and is associated with the endothelial cell surface by binding to glycosaminoglycan (Broze, 1995; Bajaj *et al.* 2001; Broze *et al.* 1994). TFPI binds specifically and saturably to thrombospondin-1 (TSP-1), a protein in the α-granules of platelets. TSP-1 is secreted from activated platelets at sites of vascular injury. The binding between TFPI and TSP-1 thus causes TFPI to efficiently down-regulate coagulation at vascular injury sites (Mast *et al.* 2000).

It was shown that lipoprotein a (Lp(a)) binds to TFPI and this binding inactivates TFPI. Lp(a) is a complex of low-density lipoprotein (LDL) and apolipoprotein a (apo(a)). The apo(a) portion is most likely involved in TFPI binding (Caplice *et al.* 2001).

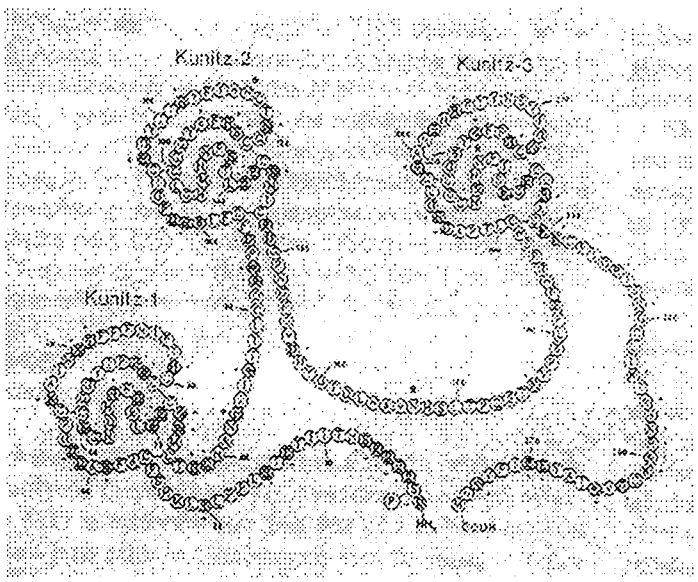

Figure 2.6 Structure of TFPI with the three Kunitz-type domains (Broze, 1995).

The first Kunitz domain binds to the active site of FVIIa. The second Kunitz domain is responsible for the inhibition of FXa, although other parts are also involved in the interaction with FXa. The carboxy-terminus is required for optimal inhibition of FXa (Wesselschmidt *et al.* 1992). The function of the third Kunitz domain is still unknown. The full-length TFPI had a tenfold higher rate constant for binding FXa than variants with a truncated carboxy-terminus (Lindhout *et al.* 1995). TFPI forms a 1:1 complex with the active site of FXa and it does not need Ca^{2+} (see figure 2.7) (Broze, 1992; Rapaport, 1991). Inhibition of the FVIIa/TF complex involves the formation of a quaternary complex (see figure 2.7). This complex contains FXa-TFPI-TF/FVIIa and its formation requires Ca^{2+} (Rapaport, 1991). The TF and FVIIa can be released from the quaternary complex by decalcification, this thus being a reversible action (Rapaport, 1991; Broze, 1995; Novotny, 1994; Higashi and Iwanaga, 1998; Bajaj *et al.* 2001).

TFPI is a slow, tight binding, competitive and reversible inhibitor, and inhibits as follows:

$$Xa + TFPI \underset{k_2}{\overset{k_1}{\Leftrightarrow}} Xa/TFPI \underset{k_4}{\overset{k_3}{\Leftrightarrow}} Xa/TFPI^*$$

Factor Xa/TFPI is the final complex and the inhibition constant (Ki) value of Xa/TFPI is 30nM (Bajaj *et al.* 2001).

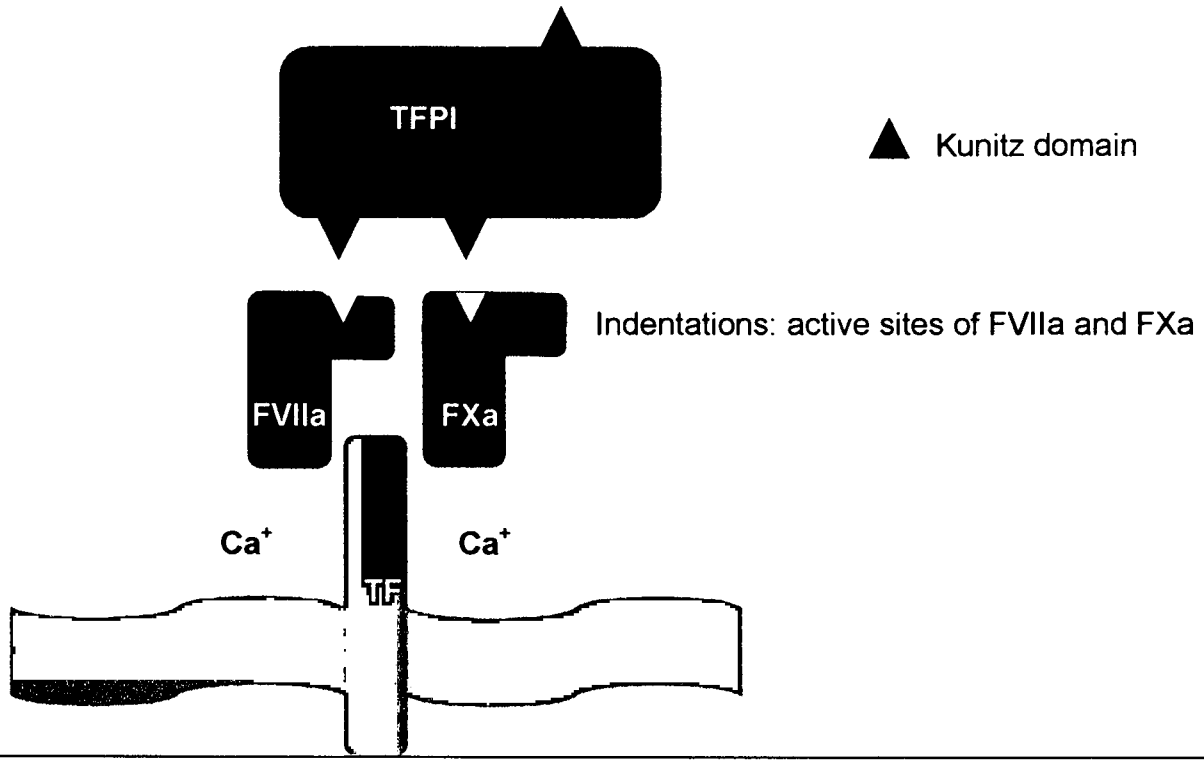

Figure 2.7 Schematic presentation of the proposed mechanism for complex formation of the FVIIa/TF/FXa/TFPI complex (Girard *et al.* 1989)

2.6.2 Antithrombin III (ATIII)

Antithrombin III is the natural inhibitor of thrombin. Although antithrombin III is not an inhibitor of FVIIa, the interaction of FVIIa with TF, however, enhances its susceptibility to inhibition by ATIII (McVey, 1994). This inhibition, in turn, is enhanced by heparin and is irreversible (Broze *et al.* 1993). The inhibition of the FVII/TF complex by AT III involves accelerated dissociation of FVIIa from the FVIIa/TF complex due to destabilisation of the salt bridge in FVIIa when ATIII binds to it. The circulating FVIIa-ATIII complexes are then unable to bind to new cell surface TF receptors (Higashi and Iwanaga, 1998; Rao *et al.*

1995). However, in the presence of sufficient amounts of FVIIa, ATIII is not an efficient inhibitor (Higashi and Iwanaga, 1998).

2.6.3 Nematode anticoagulant proteins

The nematode anticoagulant proteins (NAPs) derived from the hematophagous nematode *Ancylostoma caninum* can be used as inhibitors of blood coagulation. Their use has been based on the observation that infection by human hookworm can result in significant blood loss. NAP5 and NAP6 inhibit FXa by binding to its active site. NAPc2, on the other hand, binds to an exosite, distinct from the active site of FXa, and this binary complex inhibits the TF/FVIIa complex (Johnson and Hung, 1998; Duggan *et al.* 1999; Bergum *et al.* 2001; Stanssens *et al.* 1996). NAPc2 is functionally similar to TFPI, but differs in that it binds to an exosite on FXa (Johnson and Hung, 1998).

2.6.4 Active site inhibited factor VIIa

It is interestingly to note that by blocking the active site of FVIIa its affinity for TF is enhanced. This was done by the incorporation of a small peptide inhibitor (Phe-Phe-Arg chloromethylketone) into the active site. The reason for this could be that the incorporation of the inhibitor stabilises the protease domain and increases the number of residues in contact with TF (Sorensen *et al.* 1997). There are definite differences between active site inhibited FVIIa and FVIIa in their recycling and intracellular compartmentalisation. A fraction of both recycles back to the cell surface, but the percentage of recycled active site inhibited FVIIa is much higher than FVIIa. This means that more FVIIa are accumulated intracellularly (Iakhiaev *et al.* 2001).

Inactivated active site FVIIa was used in studies of thrombus formation on immobilised TF in a perfusion chamber. The results showed that inactivated active site FVIIa did inhibit thrombus formation and the inhibition was dose-dependent. It also showed that the antithrombotic efficacy of the inactivated

active site FVIIa depended on shear rate, the efficacy was best at a shear rate of 650 s^{-1} and represents arterial blood flow (Ørvim *et al.* 1997).

Inhibition by active site inhibited FVIIa was capable of eliminating vascular thrombus formation at sites of mechanical vascular injury in baboons and rats, without having an effect on the bleeding time (Harker *et al.* 1996; Soderstrom *et al.* 2001).

The antithrombotic effect of active site inhibited FVIIa was also tested in a rabbit model and it was shown that the effect was prolonged and even persisted after the active site inhibited FVIIa plasma levels were almost baseline (Golino *et al.* 1998).

Studies in rats showed that the anti-thrombotic effect of active site inhibited FVIIa can be totally reversed by administration of an equal dose of recombinant FVIIa (Ghrib *et al.* 2001).

2.6.5 Peptide inhibitors

Kunitz domain variants displayed on the surface of filamentous phage was used to select potent active-site inhibitors of the FVIIa/TF complex. The selection was directed against FVIIa bound to TF. The inhibitor found differed from TFPI and ATIII and was named TF71-C. This peptide inhibited the FVIIa/TF complex with a K_i of 1.9nM. This K_i represented an increase in binding affinity of more than 150-fold compared to TFPI (Dennis and Lazarus, 1994a; Dennis and Lazarus, 1994b).

Peptide inhibitors of FVIIa have been selected by using the technique of phage display. Naive polyvalent phage libraries of 20-residues were used. One selected inhibitor, E-76, is an 18-residue peptide that binds to a distinct and functionally relevant exosite on the TF/FVIIa complex. E-76 binds to the 140s loop of FVIIa. This loop stretches from residues 142-153, and its binding causes a conformational change in the 140s loop. It is a potent inhibitor of FX

activation with a median inhibitory concentration of 1nM. Although E-76 does not bind to the active site of FVIIa, it was shown to inhibit amidolytic activity towards a chromogenic substrate and the inhibition is TF-dependent. Kinetic analysis indicated that E-76 is a non-competitive inhibitor of FX, reducing the maximum reaction velocity (V_{max}), but showing no effect on the Michaelis constant (K_m) (Dennis *et al.* 2000; DeCristofaro, 2002).

Another inhibitor A-183 is a peptide that also binds to an exosite of the protease domain of FVIIa. It binds to the 60s loop. This peptide is a partial mixed-type inhibitor of FX activation with a K_i of 200pM (DeCristofaro, 2002; Dennis *et al.* 2001; Roberge *et al.* 2001).

These two peptides, E-76 and A-183, were linked to create a bifunctional peptide. Compared to their individual inhibition activity, the combined peptides showed stronger inhibition of TF-dependant coagulation. A combination of the two peptides in a 1:1 molar ratio showed complete inhibition of FX activation as compared to 78% and 92% of the two peptides separately (Roberge *et al.* 2002).

Another potent inhibitor was developed against loop I (91-102) of the second EGF-domain of FVIIa. This is a cyclic peptide that disrupts the essential interaction between the second EGF-domain and the catalytic domain of FVIIa. This peptide was named PN7051 and is a dose-dependent inhibitor of FX activation by factor VIIa/TF complex with an IC_{50} value of 10μM. The inhibition of this peptide on thrombus formation was also tested in an *ex vivo* experiment with a perfusion chamber. The peptide showed inhibition of fibrin deposition, platelet/fibrin adhesion, platelet/thrombus volume, and thrombin activation. The overall IC_{50} in this case was 0.5 mM. This peptide thus proves to be capable of inhibiting complete thrombus formation at sufficiently high concentrations (Örning *et al.* 1997; Örning *et al.* 2002).

2.7 Phage display

2.7.1 Introduction

The technique of phage display is used in this study, and for purposes of clarity I will discuss this technique in some detail.

Phage display technology describes an *in vitro* selection technique (biopanning) in which a peptide or protein is genetically fused to a coat protein of a bacteriophage. This results in the display of the fused peptide or protein on the exterior of the phage, while the DNA encoding the fusion resides in the virion (Smith, 1985). The technique allows for large numbers of phage clones greater than 10^9 different displayed sequences to be screened, this being the major advantage over other methods (Smith, 1985). The selection technique, biopanning, is carried out by incubating the pool of phage-displayed variants with the target protein to select phage clones that bind or inhibit the target protein. Phage display is therefore a very powerful technique since it links peptide or protein display with genetic information, i.e. the displayed peptide or protein allows rapid selection and the genetic information allows reliable amplification of the phages.

Phage display technology is used for a variety of purposes, which include mapping of epitopes, identification of antagonists and agonists for target molecules, engineering of human antibodies, optimising of antibody specificities, and creation of novel binding activities (Kay and Hoess, 1996). A number of applications in which phage display has been used, have been described including epitope mapping (Scott and Smith, 1990), mapping protein-protein contacts (Hong and Boulanger, 1995), and the identification of peptide mimics of non-peptide ligands (Devlin *et al.* 1990). Peptides have been identified by either panning against an immobilised purified receptor or against intact cells (Doorbar and Winter, 1994). Larger proteins such as antibodies (Barbas *et al.* 1991), hormones (Lowman *et al.* 1991), protease inhibitors (Roberts *et al.* 1992) and DNA binding proteins (Soumillion *et al.*

1994) have been displayed on phages and resulted in isolation of variants with altered specificity or affinity.

2.7.2 Structure and genome of the filamentous phage

The filamentous phages M13 and fd are mostly used for phage display, mainly because they are not lysogenic towards their hosts (Messing, 1983). The M13, fd, and f1 phages are all part of the Ff class of filamentous phages (Webster, 2001). The phages have a filamentous shape containing a single stranded closed circular molecule of DNA (Arza and Félez, 1998).

M13-filamentous and fd-phages infect *Escherichia coli* (*E.coli)* containing the F-conjugate plasmid, as it codes for the extracellular F-pili which serves as receptors for the phage (Rodi and Makowski, 1999). M13 is the best-studied member of this class and therefore I will concentrate on this phage type in further discussion (Kornberg, 1980). M13 phage is approximately 6.5 nm in diameter and 930 nm in length. The length is dependent on the length of the enclosed genome (Rodi and Makowski, 1999; Webster, 2001). The genome of the M13 phage is a single strand covalently closed DNA molecule, which consists of about 6400 nucleotides. A flexible protein cylinder encases the DNA molecule (Webster, 2001). This DNA molecule codes for 11 genes. Genes III, VI, VII, and IX code for the minor coat proteins, while gene VIII codes for the major structural protein (Webster, 2001) (see figure 2.7). Genes I, IV, and XI code for the assembly of the phage and genes II and X for DNA replication (Webster, 2001).

Coat protein gpIII, a 406 amino acid polypeptide, is located at the proximal end of the virion. Four to five copies are present. (Figure 2.7) This protein is necessary for the infection of bacterial cells (Smith, 1985; Makowski, 1994). In phage display technology, the amino-terminus of gpIII is used for the fusion of short peptides. All of the 5 copies of gpIII can be fused to short peptides; therefore these peptides are present at low valency, 1-5 copies per virion (Wilson and Finlay, 1998; New England Biolabs, 2000).

Coat protein VI is also located at the proximal end of the virion. Four to five copies of minor coat protein VI are present (Wilson and Finlay, 1998). This coat protein consists of 113 amino acids and in this case the carboxy-terminus is exposed (Makowski, 1994). Gp VI is required for the attachment of gpIII to the phage.

The major coat protein VIII (gpVIII) forms a thick flexible cylinder around the single-stranded viral genome and there are ± 2800 copies present (see figure 2.7) (Makowski, 1994; Webster, 1996). It is a 50 amino acid protein and about 10% of the 2800 copies can be fused to short peptides at the amino-terminus (Makowski, 1994; New England Biolabs, 2000). The display of the peptides or proteins in M13 can have different valencies of copies. This valency is dependent on the site of display and type of vector (Armstrong *et al.* 1996).

Minor coat proteins, gp VII and gp IX, are situated on the distal end of the virion. Four to five copies of each of gpVII (a 33 residue protein) and gpIX (a 32 residue protein) are present (Webster, 2001). These proteins are responsible for the maintenance of the phage stability and have not yet been employed for phage display (Rodi and Makowski, 1999; Wilson and Finlay, 1998).

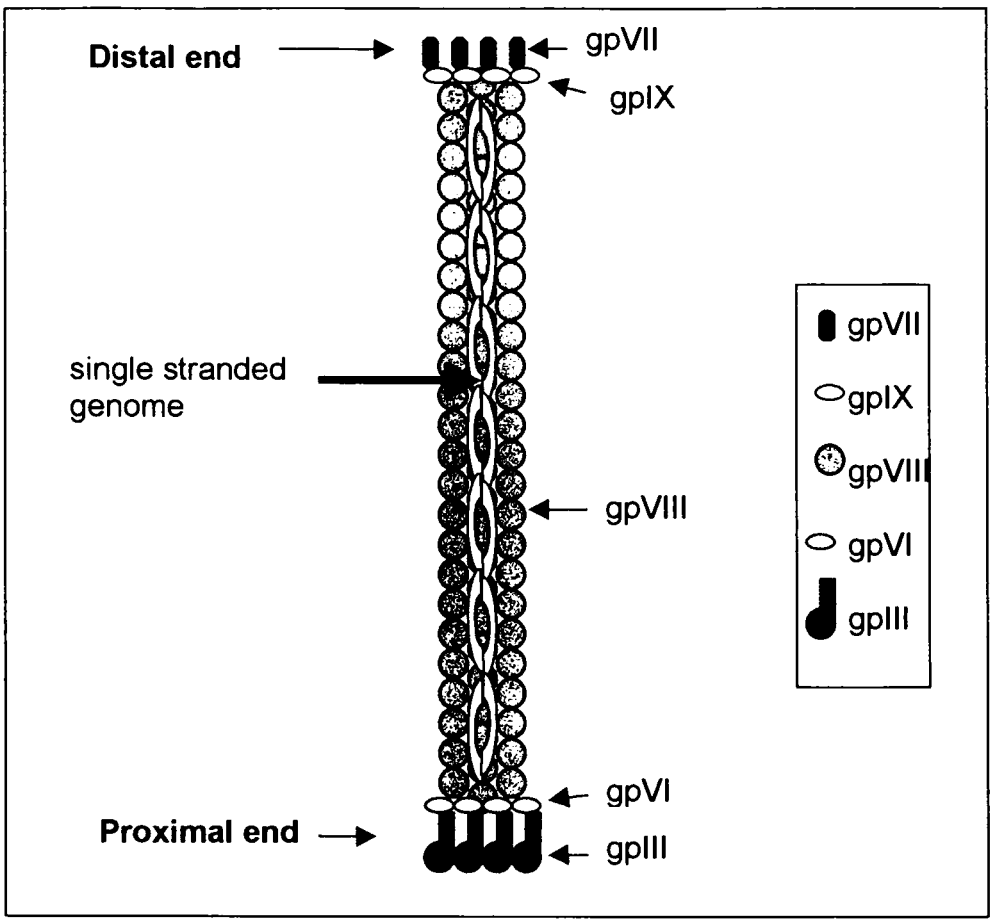

Figure 2.8 Filamentous phage structure. The single-stranded circular genome is surrounded by gpVIII. (Wilson and Finlay, 1998)

2.7.3 Life cycle of M13 phage

The life cycle of the M13 phage starts by infecting *E.coli.* cells. This is done by binding of the amino-terminus of gpIII to the F-pilus of the *E.coli.* cells. The phage then penetrates the F-pilus of the *E.coli.* cell and in this process gpVIII is stripped off in the cell membrane. The minor coat protein, gpIII, remains attached and guides the phage in the infection process. The replicating enzymes of the host cell convert the single-stranded DNA of the phage into a double-stranded circular form (Figure 2.9), the so-called replicative form (RF form). No viral product is synthesised during this phase. The viral DNA is

always indicated as the (+) strand. The complementary strand, the (-) strand, contains the coding information.

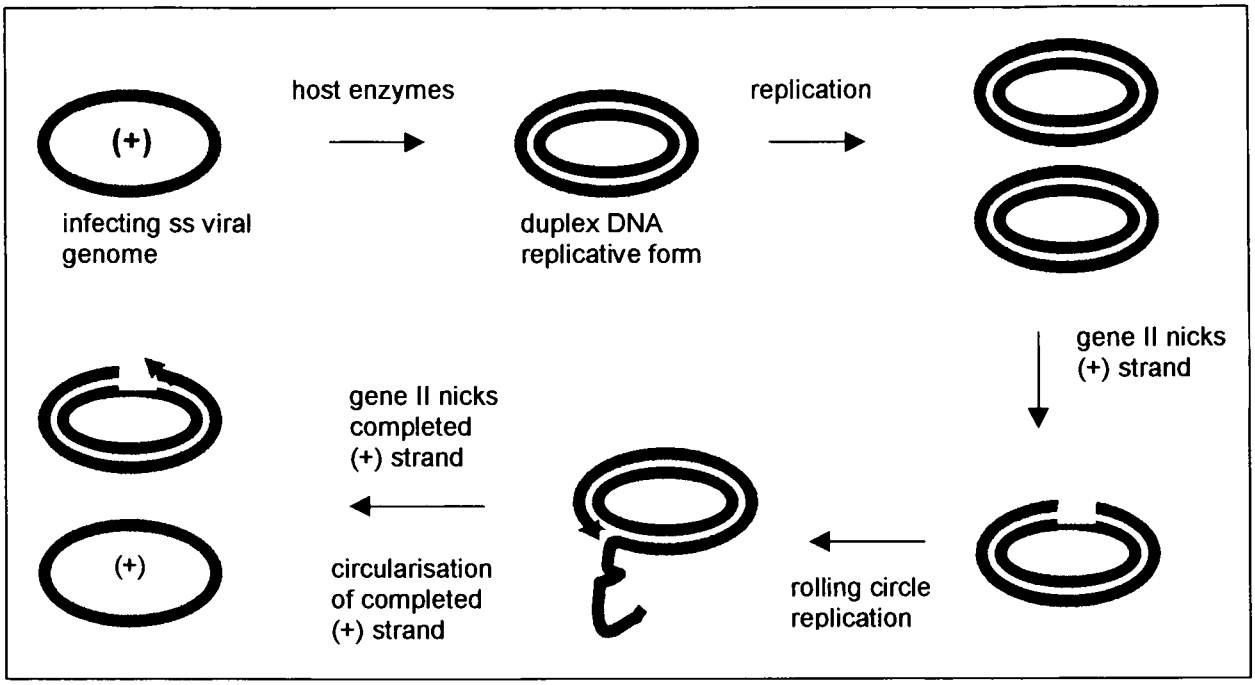

Figure 2.9 Life cycle of M13 phage (Blaber, 1998)

The next phase of the life cycle is the replication phase where transcription starts and it always occurs in the same direction. Two terminators for transcription are located at the ends of genes IV and VIII. Transcription occurs in a cascade form. Gene VIII is transcribed frequently, whereas gene III is transcribed in small amounts (Messing, 1983; Blaber, 1998).

In the next phase, the gene II product is synthesised after completion of the synthesis of the complementary strand. This protein is responsible for introducing a "nick" in the (+) strand and therefore initiating the (+) strand synthesis. DNA polymerase is responsible for extending the (+) strand at the 3'-OH end. The complementary strand is now used as template to synthesise a new copy of the (+) strand. After the (+) strand synthesis has made one trip around the (-) strand, gene II introduces another "nick" in the (+) strand. This separates the parental (+) strand from the newly synthesised (+) strand. The (-) strand continues to serve as template for the synthesis of new (+) strands.

The parental (+) strand is then circularised and can be converted again to the RF form. Gene V protein levels increase as the RF molecules accumulates in the cell. This protein binds to the (+) strand and prevents conversion of the newly synthesised single (+) strands to the RF form. This leads to the build-up of circular single stranded (+) DNA, the M13 genome. This complex of single stranded (+) DNA and gene V protein now moves to the membrane where the gene V protein is replaced and gpVIII covers the phage DNA again as it is being extruded out. The release of the phage does not require the F-pilus (Messing, 1983; Blaber, 1998).

2.7.4 Phage display of peptides and proteins

The gpIII coat protein is most commonly used for phage display. The M13 phage vector is modified and carries the lacZ gene. When the lacZ gene is present, phage plaques appear blue when grown on agar plates containing bromo-4-chloro-3-indoyl-β-D-galactoside (XGal). The presence of white plaques suggests contamination. The lac Z gene is used to distinguish phages foreign inserts from those without inserts (Armstrong *et al.* 1996).

Foreign peptides are fused to the chosen structural coat protein, mostly gpIII of the phage, by inserting the corresponding DNA sequence into the gene coding for the coat protein. The expression of the fusion protein and the subsequent incorporation into the mature phage particle will result in the display of the foreign peptide on the surface of the phage (see figure 2.9). Phage libraries are constructed by inserting random oligonucleotide sequences in the phage genome. This results in the display of random peptides on the phage surface and therefore allows selection of peptides with specific affinities or activities (Makowski, 1994) (Figure 2.8). The most common type of phage libraries are random peptide libraries. In these libraries the DNA inserts are derived from degenerate oligonucleotides with a NNK sequence where N is an equal mixture of A, G, C and T. K is an equal mixture of G and T. The degenerate oligonucleotides are synthesised chemically by adding mixtures of nucleotides. Each NNK is therefore a

mixture of 32 triplets that include codons for all 20 natural amino acids (Smith and Petrenko, 1997).

The M13 phages are used for the display of not only foreign peptides, but also proteins (Makowski, 1994). Phage display has made engineering of protein properties possible, without a detailed knowledge of the structure-function relationship (Katz, 1997). Phage display is also being used to identify highly active substrates by including a "tether" sequence recognised by monoclonal antibodies (Smith *et al.* 1995).

2.7.5 Phage display systems

The different phage display systems can be classified according to the arrangement of their coat protein genes. The display sites, which are most commonly used, are within genes III and VIII, however gene VI has also been used for display (Armstrong *et al.* 1996).

The phage display systems are differentiated on the basis of the coat protein used for display, whether fusion can be to all copies or only a fraction, and whether the fusion is encoded on the phage genome or on a separate genome. This classification was made by George Smith (1993). The valency of the display can vary between one and thousands of copies and is dependent on the vector type.

The type 3 and type 8 vectors are the simplest phage display vectors. The basic functions of these vectors have not been changed with the display of peptides/proteins. A single genome bears a single gene III in type 3 vectors and this single genome accepts foreign DNA inserts and therefore encodes a single type of pIII molecule. The same applies for type 8 vectors, however short peptides and a variety of proteins can be displayed at the N-terminus of pIII, but pVIII can display only short peptides. This is called multivalent display, because the phage contains multiple copies of the inserted peptide/protein and is used for the selection of phage with relatively low target

affinity. One limitation of multivalent display is that only small peptides can be displayed because larger inserts can interfere with the function of the coat protein. This leads to a poorly infecting phage (Lowman *et al.* 1991; Allen *et al.* 1995, Armstrong *et al.*, 1996; Phizicky and Fields, 1995).
Some of the sequences are not displayed well enough on the type 3 and 8 systems which leads to the development of two other systems, types 33 and 88 (Smith and Petrenko, 1997).

In these vector types, 33 and 88, the genome bears two different copies of gene III or VIII and this encodes two different types of coat proteins. Only one of these gpVIII or III is recombinant and bears the DNA insert, the other coat protein is the wild-type.

In another vector type, 3+3 and 8+8, there are also two different copies of the genes, but in this case the two genes are on different genomes. The wild-type version is on a phage and the recombinant version is on a phagemid. The wild-type version is also called a helper phage. In these types usually only a single copy of the peptide-bearing protein is displayed, thus both the helper phage and the phagemid will have only one copy of the peptide. A major advantage of monovalent display is that apart from the mutant coat protein provided by the phagemid, the helper phage then supplies a large excess of the wild-type coat protein, resulting in phages with good infectivity (Smith, 1997; Armstrong *et al.* 1996). In monovalent phage display, where only one copy of the inserted peptide/protein is displayed on the phage, the conditions can be designed for the selection of high-affinity interactions. The different display systems are shown in figure 2.10 (Lowman *et al.* 1991; Allen *et al.* 1995, Armstrong *et al.*, 1996; Phizicky and Fields, 1995).

Foreign peptides have also been fused to pVI. This fusion however, takes place at the C-terminus 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; Amery *et al.* 2001). Jespers *et al.* (1995) and Hufton *et al.* (1999) found that pVI is suitable for the cDNA expression. There are also the different types of pVI display. The types are type 6, 66 and 6+6 (Smith and Petrenko, 1997).

Proteins are also displayed on λ phage. Peptides or proteins are fused to the amino terminus of the D protein (11kDa) of the λ capsid. The fusions then assemble onto the viral capsid. The λ phage display system has the advantage of efficient construction and maintenance of very large libraries. A unique feature of the λ system is that the displayed peptides/proteins does not need to be secreted across the membrane because the virus assembles intracellularly prior to the release of particles (Maruyama *et al.* 1994; Sternberg and Hoess, 1995).

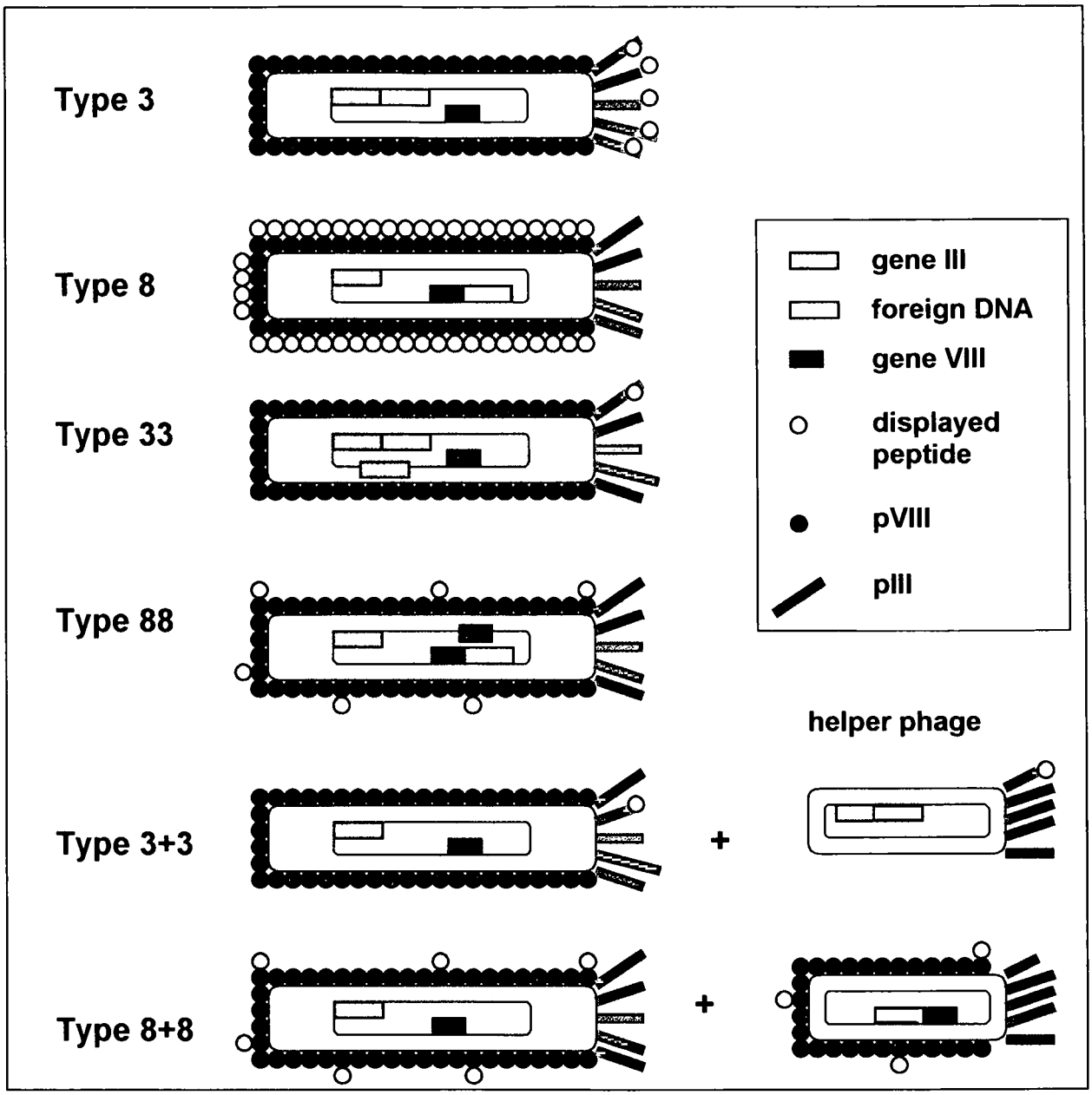

Figure 2.10 Types of phage display systems (Smith and Petrenko, 1997)

2.7.6 Applications of phage display

In the field of thrombosis and haemostasis, phage display 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 $\alpha_{IIb}\beta_3$ 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 characterise the peptide binding specificity of the $\alpha_5\beta_1$ integrin, the fibronectin receptor on platelets (Koivunen *et al.* 1994). Furthermore a phage display library of plasminogen activator inhibitor (PAI-I) mutants was used to determine the interactive sites between PAI-I and thrombin, and also between PAI-I and the tissue-type plasminogen activator (tPA) variable region 1 (Van Meijer *et al.* 1996). Three APPI Kunitz domain libraries were used to select potent active-site inhibitors of human FVIIa/TF complex as well as competitive phage selection on the same three libraries. The selection conditions were altered in this competitive phage selection technique. FXIa, plasma kallikrein, and plasmin was included in the selection, and Kunitz domain phage that specifically binds to immobilised TF/FVIIa complex were selected and enriched, and the phage that binds to the other proteases were eliminated (Dennis and Lazarus, 1994a; Dennis and Lazarus, 1994b).

A phage-epitope library was used to identify a ligand peptide mimicking the conformation dependent epitope for a monoclonal antibody (mAB 5.5). This antibody is directed against the ligand-binding site of the nicotinic acetylcholine receptor (Balass *et al.* 1993). Phage display was also used to localise the epitopes of monoclonal antibodies that were raised against human pro-enkephalin (Bottger *et al.* 1995). Epitopes were also localised in polyclonal antibodies (Dybwad *et al.* 1995). Winthrop *et al.* (2000) describes gene libraries, which can be used for antibody-based binding peptide modules.

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 localise 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

Phage display selection has also been performed against whole cells such as blood leukocytes, CHO cells to isolate antibodies against antigens on the cells (De Kruif *et al.* 1995; Hoogenboom *et al.* 1999). and insect cells. Successful selection of peptides using phage display against mouse melanoma cells has also been described (Szardenings, 1997). Pasqualini and Ruoslahti (1996) reported on the approach of organ-selective targeting based on *in vivo* screening of random peptide sequences. A new phage display technology has also been described, this is phage selection using ligand identification via expression (LIVE). This technique combines phage biology with functional selection of altered cell function through gene transfer and could be a powerful tool for identifying natural ligands (Larocca and Baird, 2001). Finally, phage display is now a well-established tool for research (Cortese *et al.* 1995) and it shows potential in the discovery of new drugs as well as the development of new vaccines (Arza and Félez, 1998; Wittrup, 1999; Sidhu, 2000).

Sometimes the recombinant libraries have a poor display of some peptides or poor production of phage clones displaying peptides. Other disadvantages are the fact that some of the binding peptides could be missed because of overpanning. Phage display also has the disadvantage that it is not suitable

for the selection of protein that requires posttranslation modification (Scott, 2000; Silverman, 2000).

Another method that can be used to identify protein-protein interactions is the yeast two-hybrid system. This method, however, also has its limitations. Proteins that cannot fold correctly in the cytoplasm may not be suitable for use in this system while it has been described that proteins can fold correctly on phage. This occurs when phage is expressed in *E.coli* that are severely defective in disulphide bond formation. Interactions such as glycosylation and disulfide bond formation might not occur in the two-hybrid system because the proteins that are generated are targeted to the nucleus. It was also found that some hybrid genes could be harmful or lethal when they are expressed in yeast (Allen *et al.* 1995; Bardwell *et al.* 1991). In 2000 a bacterial two-hybrid selection system was described for the studying of protein-DNA and protein-protein interactions. This system has advantages over the hybrid system in that it can analyse libraries larger than 10^8 in size and it also has a faster growth rate (Joung *et al.* 2000).

CHAPTER 3

MATERIALS AND METHODS

American Diagnostica Incorporated, Greenwich, CT, USA kindly supplied the recombinant human FVIIa (cat # 407rec/N), tissue factor, murine monoclonal antibody against human FVIIa and the chromogenic substrate, Spectrozyme FVII as part of our collaboration agreement with them. General laboratory reagents were of analytical grade and purchased from various suppliers as outlined in the text.

3.1 Phage display

3.1.1 Phage Display Peptide Libraries

We used two peptide libraries, a cyclic 7-mere Phage Display Peptide Library Kit (Ph.D.-C7C cat. # 8120) and a linear 12-mere Phage Display Peptide Library Kit (Ph.D.-12 cat. # 8110). The kits were purchased from New England Biolabs, Beverly, MA, USA. Both libraries are gpIII fusions with 5 copies of the fused peptide per phage. The cloning vector for both is M13KE (New England Biolabs, 2000).

The Ph.D.-12 peptide library kit includes a combination library of random 12-mere peptides fused to the minor coat protein, gpIII of M13 phages. The 12-mere peptides are expressed at the amino-terminus of gpIII. This 12-mere library contains approximately 2.7×10^9 electroporated sequences. These sequences were amplified once to yield about 55 copies of each sequence in 10μl of the phage library (New England Biolabs, 1998b).

The Ph.D.-C7C peptide library kit includes random 7-mere peptide sequences, which are flanked by a pair of cysteine residues. This cyclic 7-

mere library is therefore also a combination library of random 7-mere peptides fused to gpIII. The cysteine residues will spontaneously form a disulphide cross-link under nonreducing conditions. This will result in the display of cyclic peptides, in contrast to the linear peptides displayed in the 12-mere Ph.D.-12 library. The cyclic peptides are also displayed at the amino-terminus of gpIII. This library contains approximately 3.7 x 10^9 electroporated sequences. These were also amplified once to yield about 50 copies of each sequence in 10μl of the phage library. An advantage of the cyclic library is that binding is more tightly than in the linear library. It was also shown that the cyclic libraries show more stringent sequence specificity than the linear ones (New England Biolabs, 1998a; McLafferty *et al.*, 1993).

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 Biotinylation of FVIIa

FVIIa was biotinylated with sulfo-NHS-LC-biotin (74 μg biotin/100 μg FVIIa) (Pierce, Illinois, USA) for 2 hours at 4°C. To separate the biotinylated factor VIIa from the free biotin, the solution was run through a PD-10 sephadex column (Whitehead Scientific, RSA). Twenty fractions of 500 μl each were collected and the protein concentration of each fraction measured at 280 nm. The peak protein fractions were pooled and the protein concentration determined by using the Biorad method (Sambrook and Russell, 2001).

The percentage biotinylation was measured with a colorimetric assay based on displacement of the dye HABA [2-(4'-hydroxyazobenzene)benzoic acid] according to the instructions of the manufacturer (Pierce, Illinois, USA cat. # 28010).

3.1.3 Biopanning

We used two methods of biopanning to select FVIIa-binding phages (biotinylated and direct method). In the first method we incubated 2.10^{11} phages of each library with 10 μg of biotinylated FVIIa. By using streptavidine magnetic beads, we could separate the FVIIa-binding phages from the unbound phages. In the second method we coated 20 μg FVIIa to the inside of an immune-tube.

The first method (biotinylation method) was done as follows:
Streptavidine magnetic beads (1.5 mg; Roche Molecular Biochemicals, Mannheim, Germany) were first washed twice with 200 μl PBS. The beads were blocked with 500 μl of 2% Skimmed milk (SM) (DIFCO Laboratories, Detroit, USA) in PBS for one hour at room temperature (tube 1). After blocking, 170 μl of this mixture was removed and placed in another tube to which 2.10^{11} phages of each phage library (C7C or L12) were added in a final concentration of 2% SM and incubated for an hour at room temperature while rotating. Biotinylated FVIIa (10 μg) and SM were added to the other tube in a final concentration of 2% SM and incubated at room temperature for 30 minutes. The phage solution was then put on the magnet and the phages that did not bind to the magnetic beads were added to the tube with the biotinylated FVIIa and rotated overnight at 4°C. In this way the non-specific binding phages that bind only to the magnetic beads were removed. The next day, the phage and FVIIa solution were put on the magnet and the phages that bind to FVIIa will stay bound to the beads while the non-binding phages were removed and kept as the "INPUT" phages. The beads were washed 10 times with 0.1% Tween-20 in PBS and the FVIIa-binding phages were eluted non-specifically with 0.5 μl of 0.2 M glycine (pH2) solution (a high acid solution) for 15 minutes at room temperature. The tube was placed on the magnet and the supernatant was added to another tube containing 125 μl of Tris (pH 8) to neutralise the low pH. These phages we called the "OUTPUT" phages, they were the phages that bound to FVIIa.

In the second method, we coated 20 μg of FVIIa to the inside of a Maxisorb immune-tube (Nunc, IL, USA) by adding a solution of FVIIa in PBS (20 μg FVIIa/1 ml PBS) to the immune-tube and rotated it for 2 hours after which we incubated it overnight at 4°C. The next day we removed the non-binding phages ("INPUT" phages) and eluted the FVIIa-binding phages from the tube non-specifically also with 0.5 μl of 0.2 M glycine (pH 2) for 15 minutes. To neutralise the low pH, we also added 125 μl Tris (pH 8) These are the "OUTPUT" phages.

Simultaneously, a pre-culture of ER2537 *E.coli* cells was diluted 1:100 in 40 ml Lubria Broth-medium (LB-medium, DIFCO Laboratories, Detroit, USA) and incubated at 37°C for about 2 hours until the *E.coli* cells were in the log-phase of growth, i.e. an OD_{600} of between 0.6 and 1.

We made dilutions of the "INPUT" and "OUTPUT" phages and plated it out on Lubria Broth/ isopropyl-β-D-thiogalactoside/5-bromo-4-chloro-3-indolyl-β-D-galactoside plates (LB/IPTG/XGal plates). This was done by diluting ten μl of the "INPUT" and "OUTPUT" phages in LB-medium. The "INPUT" phages were diluted up to 10^{-9} and the "OUTPUT" phages to 10^{-4}. Two hundred μl of the log-phase *E.coli* cells were added to 10 μl of each of the last 4 dilutions of "INPUT" and "OUTPUT" phages and plated out on LB/IPTG/XGal plates which were incubated at 37°C overnight. LB/IPTG/XGal plates were used to ensure phage colonies that contain the random DNA inserts, were picked (in the next section) because phages with inserts contains the lac Z gene and such colonies colour blue in the presence of XGal.

The "OUTPUT" phages were amplified by infecting 40 ml of log-phase *E. coli* cells and incubating it overnight at 37°C. The next day the infected cell cultures were centrifuged at 23 000 g for 15 minutes at 4°C to remove the *E.coli*. The phages in the supernatant were precipitated on 20% Polyethyleneglycol/sodium chloride (PEG/NaCl, PEG, 0.03 M, NaCl, 2.5 M) for two hours. Following centrifugation, the phage pellet was dissolved in 1 ml

PBS and precipitated again on 20% PEG/NaCl. The amplified phages were finally dissolved in 0.5 ml PBS and the phage concentration determined by reading the OD at 260 nm and calculating the phage concentration (phages/ml) as follows:

$$\text{Amount phages/ml} = OD_{260} \times \text{dilution} \times \text{constant}$$

where the constant is $2.214.10^{11}$ since the OD_{260} of $2.214.10^{11}$ phages is equal to 1. We used 2.10^{11} of the purified phages for the next round of selection. Three rounds of selection were done all together. During the second and third selection rounds, we eluted the FVIIa binding phages specifically with 10 μg of Murine antihuman FVIIa, a monoclonal antibody against human FVII/FVIIa (ADI, Greenwich, CT, USA). This antibody binds to the catalytic site of FVIIa and blocks the assembly of the FVIIa/TF complex and it also blocks FVIIa mediated plasma coagulation.

3.1.4 Global Elisa

A global ELISA was performed on the amplified phages of each round of panning. The initial phage concentration on the ELISA was $5x10^{10}$ phages and these phages were then diluted 1:2 until a concentration of $7.81x10^{8}$.

Half of an ELISA plate (48 wells) was coated overnight with 20 μg/ml FVIIa at 4°C. The plate was blocked with 4% SM in PBS for 2 hours at room temperature and washed with 3 times 0.1% Tween-20 in PBS. 5.10^{10} phages of each round was added 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 7 times 1:2 into the other wells of the columns. No phages were added to the last well of each column. These wells served as a negative control. We added the same amount of phages to the non-coated wells as to the coated wells to make sure that we have not selected plastic-binding phages instead of FVIIa-binding phages. After incubation for 2 hours at room temperature, the plate was washed 9 times with 0.1% Tween-20. A horseradish peroxidase conjugated anti-M13 phage antibody (Amersham Pharmacia Biotech, NJ, USA), was added and incubated for 1 hour. The

substrate, orto-phenyldiamine-dihydrochloride (OPD) and peroxidase (H_2O_2) was added after 12 wash steps and incubated at room temperature for 10 minutes to develop a colour reaction. The reaction was stopped with 1 M H_2SO_4. The optical density was read at 490 nm on an EL312e Microplate Bio-Kinetics reader (Bio-tek instruments, Vermont, USA).

The global ELISA indicated that we have selected the highest concentration of FVIIa-binding phages in round 3 since the OD_{490} of the round 3 phages was the highest. We thus grew single phage colonies of the third selection round to search for displayed peptides that bind to and inhibit FVIIa.

3.1.5 Growing and amplification of single colonies

We picked one hundred and fourty-four (3 times forty-eight) colonies of each library from the IPTG/XGal plates of the third selection round. They were grown in 2 ml of a 1:50 diluted pre-culture of *E.coli* cells in LB-medium in a culture tube overnight at 37°C. Half of each culture was centrifuged and the supernatants containing the amplified phages were used for an ELISA to determine which colonies bind to FVIIa (binding ELISA). A cell stock was prepared with the other half of the cultures. It was mixed with glycerol (35%) and frozen for later use.

3.1.6 ELISA to identify FVIIa binding phage colonies (Binding ELISA of single colonies)

In order to distinguish between actual FVIIa binders and "plastic" binders, each colony was tested in a FVIIa-coated and non-coated well. Half of an ELISA plate (48 wells) was coated overnight with 20 μg/ml FVIIa at 4°C. The plate was blocked with 4% SM in PBS for 2 hours at room temperature and washed 3 times with 0.1% Tween-20 in PBS. 100 μl of the supernatant of each grown colony of round 3 was added to a coated and non-coated well of the ELISA plate in a final concentration of 2% SM, incubated for 2 hours at room temperature and then washed 9 times with 0.1% Tween-20. A

horseradish peroxidase conjugated anti-M13 phage antibody (Amersham Pharmacia Biotech, NJ, USA), was added and incubated for 1 hour. The substrate, orto-phenyldiamine-dihydrochloride (OPD) and peroxidase (H_2O_2) was added after 12 wash steps and incubated at room temperature for 10 minutes to develop a colour reaction. The reaction was stopped with 1 M H_2SO_4. The optical density was read at 490 nm on an EL312e Microplate Bio-Kinetics reader (Bio-tek instruments, Vermont, USA). Wells with the most intense colour indicate either the strongest binding phages or a high concentration of weak FVIIa-binding phages.

Since we only found 6 colonies of each library that had high colour intensity, we decided to grow all of them up from the cell stock and tested them for concentration dependant binding to FVIIa in a dilution ELISA.

3.1.6.1 Dilution ELISA

We diluted (1:2 dilution) the 12 colonies (6 of each library) that was grown up and tested them for concentration dependent binding to FVIIa. The highest phage concentration was $2.5.10^{10}$ and we diluted it 1:2 as in the global ELISA. Each colony was also diluted on non-coated wells to distinguish between FVIIa-binding phages and non-specific plastic binding phages. The rest of the ELISA was done the same way as the global ELISA. We repeated this ELISA three times.

3.1.6.2 Inhibition ELISA

This ELISA was performed do determine whether TF was able to prevent the 12 strongest binding colonies from binding to FVIIa. Different concentrations of TF were added to FVIIa coated wells. American Diagnostica Inc. (Greenwich, CT, USA) kindly supplied TF. After incubation for 15 minutes, 5.10^{10} phages in 2% SM (off all 12 colonies) were added to each well of a column and incubated for 2 hours. Bound phages were detected after 1 hour of incubation with the anti-M13 antibody, visualisation done with OPD and

H_2O_2 and absorbency measured at 490 nm. Only one colony from the cyclic 7-mere library showed inhibition and we repeated the inhibition ELISA with this colony three times.

3.1.7 Prothrombin time (PT)

The effect of a dilution series of phage colonies on the PT was determined. Phages of the 12 strongest binding colonies (6 from each library) were added to human plasma. For the control, PBS was added to the plasma. A negative control was also performed where a non-binding phage was added to the plasma. To prepare platelet poor plasma 5 ml of citrated blood was centrifuged at 2000 g for 10 minutes and the plasma aspirated. The PT was determined by incubating 100 µl of plasma with 50µl of different concentrations of phages for 10 minutes, using PBS as control. Two hundred µl of Dade Innovin (Dade Behring, Marburg, Germany) was added and the clotting time was measured on the STart®4coagulation timer (Diagnostica Stago, Asnieres, France). Only one colony of each library showed lengthening in PT. We then repeated the PT three times with each of these 2 colonies.

3.1.8 Sequencing of FVIIa-inhibiting phages

3.1.8.1 DNA preparation

We sequenced both colonies that showed lengthening of the PT.

DNA was prepared to sequence the phages. Briefly, 100 µl of the amplified phages were diluted in PBS and precipitated on 20% PEG/NaCl for one hour. The phages were then centrifuged for 10 minutes at 18000 g and the pellet dissolved in 100 µl of a sodium iodide buffer (10 mM Tris, 1 mM EDTA, 4 M NaI), incubated for 10 minutes at room temperature and centrifuged again for 10 minutes. The pellet was washed with 70% ethanol and left to dry. The DNA was rehydrated in 30 µl of distilled H_2O.

The DNA concentration was determined by preparing a 10x and 20x dilution and reading the OD at 260 nm. The DNA concentration was calculated by a standard method (Sambrook and Russell, 2001).

3.1.8.2 Polymerase chain reaction (PCR)

We used the DYEnamic ET Terminator Cycle Sequencing Premix Kit (Amersham Pharmacia Biotec Inc, NJ, USA.) for sequencing reactions. A PCR reaction mixture was prepared by using 8 μl premix reagent, 0.5 μl primer (5 pmol, 5'-CCC TCA TAG TTA GCG TAA CG-3') and 2 μg DNA from the inhibiting phages. The final volume of the reaction was 25 μl. The PCR was performed on the Gene Amp PCR System (Applied Biosystems, CA, USA). One cycle was performed at 94°C for 1 minute. Twenty-five cycles were performed at, 94°C for 30 seconds, 45°C for 30 seconds and 60°C for 4 minutes.

After the PCR, the DNA was extracted by adding 2 μl of 3 M sodium acetate and 76 μl 100% ethanol to the PCR product and incubated at 4°C for 15 minutes. It was then centrifuged for 15 minutes, the pellet washed with 70% ethanol and dried in a vacuum drier for 2 minutes at medium heat. Template suppression reagent (TSR, 30 μl) (Applied Biosystems, CA, USA) was added to the dried DNA. The DNA was then denatured at 95°C for 1 minute. Sequencing was performed on the ABI Prism 310 Genetic Analyser (Applied Biosystems, CA, USA). Interestingly the sequence from the cyclic 7-mere phage fitted exactly into the middle of the sequence from the linear 12-mere phage. We compared these sequences to the sequences of TF, FX and TFPI and could not find any comparison. Unfortunately according to a contract of agreement with the company ADI, who supplied the reagents for this study, it will not be possible to disclose the peptide sequence. A cyclic heptapeptide with a sequence similar to the peptide sequence displayed on the phage colony form the cyclic library was synthesised. American Diagnostica Inc. (ADI, Greenwich, CT, USA) kindly supplied the peptide. The purity of the peptide was higher than 80%.

3.2 Tests performed on the synthesised peptide

3.2.1 Prothrombin times and thrombin times

The effect of the peptide on PT and thrombin time (TT) was measured in normal pooled plasma. PT was measured as described (see 3.1.7). Final peptide concentrations in the PT reaction were 1.17 mM, 0.58 mM, and 0.3 mM. As a control, we added PBS to plasma. For a negative control we added a peptide that does not bind to FVIIa.

TT was measured by incubating 100 µl of plasma with the same peptide concentrations as for the PT, for 10 minutes. 50 µl of Dade thrombin reagent (Dade Behring, Marburg, Germany) was added and the clotting time was measured on the STart®4coagulation timer (Diagnostica Stago, Asnieres, France). Again we used PBS as a control and a peptide that does not bind to FVIIa as a negative control. We repeated the PT and TT three times.

3.2.2 Perfusion studies with endothelial cells

The antithrombotic effect of the peptide was tested in perfusion studies in the laboratory of Prof. Jolan Harsfalvi from the University of Debrecen, Hungary. The parallel flow chamber, described by Sakaríassen in 1983, was used (Sakaríassen *et al.* 1983). Thermanox Plastic cover slips (Nunc, IL, USA) were coated with human microvascular endothelial cells (HMEC-1) as follows: The cover slips were sterilised in 80% ethanol. They were then dipped into sterile 1% gelatine followed by dipping into 0.5% gluteraldehide. The cover slips were then left to dry before coated with the endothelial cell suspension. We coated cover slips with simian virus 40 (SV40)-immortalised human microvascular endothelial cells (HMEC-1), a generous gift from E.W. Ades and T.J. Lawley from the Centres for Disease Control and Prevention and Emory University School of Medicine, Atlanta, GA. The cells were cultured as described by Ades *et al.*(1992) with slight modification. MCDB131 culture medium (Sigma Chemical Co, St Louis, MO, USA) was supplemented with

15% heat denaturated human serum and 20 mg/ml gentamicine. The medium was substituted every 2 days under sterile conditions. The cells were detached from the flask surface by treatment with a mixture of 0.125% (w/v)-Trypsin and 0.01% (w/v)-EDTA. After the treatment with trypsin, 10^5 cells in 1 ml culture medium were added to the culture wells containing the cover slips and incubated at 37°C with 5% CO_2. The medium was substituted after 4 hours and when the cover slips were completely covered, they were treated with 1 mmol/L ammonia solution to expose the extracellular matrix (ECM) of the endothelial cells. The ammonia solution damages the endothelial cell to express TF among other proteins on the surface of the cover slips. The cover slips were washed with PBS and stored at -4°C.

Ten ml of blood from normal humans was collected in 200 U/ml low-molecular weight heparin (Clexane, Rhône-Poulenc Rorer, France). The different final concentrations of the peptide in whole blood were 2.34 μM, 4.7 μM, and 9.35 μM. The blood was incubated at 37°C for 10 minutes before starting the experiment. The chamber was first rinsed with HEPES buffer (0.01M HEPES, 0.15M NaCl, pH 7.35). The cover slips were placed in the perfusion chamber perpendicularly to blood flow. Constant blood flow was obtained and maintained with a peristaltic pump, and the blood was recirculated through the chamber. The effect of the peptides on platelet adhesion onto the cover slips was studied at 37°C at shear rates of 1000^{-s} and 200^{-s}. Blood flow was maintained for 5 minutes followed by rinsing the cover slips with HEPES buffer. The cover slips were removed from the chamber, rinsed in HEPES buffer and fixed in methanol. They were stained in May-Grünwald (diluted 1:1 in Sörensen buffer) for 10 minutes and in Giemsa stain (diluted 1:5 in Sörensen buffer) for 30 minutes and finally washed in Sörensen buffer (32 mM KH_2PO_4 and 40 mM Na_2HPO_4).

Thirty fields per cover slip were analysed. The percentage coverage on each field was determined with a Zeiss light microscope connected to a computerised image analyser (Virginia, MTAKFKI, Budapest).

The peptide was also tested for inhibition of platelet adhesion to collagen and TF.

3.2.3 Perfusion studies with collagen

The Thermanox cover slips were coated with 100 μg/ml Horm Collagen reagent (Nycomed, Oslo) for one hour at room temperature and left to dry before use. Perfusion studies were performed as described in 3.2.2. We used the same peptide concentrations and shear rates as in 3.2.2.

3.2.4 Perfusion studies with TF

Twenty ml of blood from normal humans was collected in 200 U/ml low-molecular weight heparin (Clexane, Rhône-Poulenc Rorer, Port Elizabeth, South Africa). We used Innovin (Dade Behring, Marburg, Germany) as a source of tissue factor. The Thermanox cover slips were cleaned for 30 minutes in 5 parts of ethanol (98% pure) and 1 part of 10 mM NaOH and rinsed with ethanol. They were then coated with 100μl of Innovin and dried for 45 minutes before use.

The same method of perfusion with platelet aggregates as in HMEC-1 covered slips were used as described in 3.2.2. Five fields per coverslip were analysed. A photo of each field was taken with a Zeiss microscope connected to an Olympus digital camera and the percentage coverage was determined with an image analysing program. We used final peptide concentrations of 29.2 μM and 58.5 μM at shear rates of 200 s^{-1}, 650 s^{-1} and 1300 s^{-1}. Since the inhibition effect was more pronounced at a shear of 1300 s^{-1}, we did the perfusion experiment at this shear with a higher peptide concentration of 117 μM as well.

3.2.5 Kinetic assay

The chromogenic substrate, Spectrozyme FVIIa, that was used for the kinetic studies were kindly supplied by American Diagnostica.

Chromogenic substrate hydrolysis was detected using the EL312e Microplate Bio-Kinetics reader (Bio-tek instruments, Vermont, USA) equipped with kinetic module software. Chromogenic substrate hydrolysis was followed for 40 minutes at 405 nm. Every 5 minutes a reading was taken. The first kinetic assays were done with different FVIIa concentrations (800 nM, 400 nM, 200 nM, 100 nM and 50 nM) to determine the optimal FVIIa concentration to use in further assays. The optimal FVIIa concentration was 400 nM. The next assays were done with this FVIIa concentration and different peptide concentrations (0 mM, 0.057 mM, 0.114 mM, 0.171 mM, 0.228 mM and 0.456 mM) and different substrate concentrations (0 mM, 0.5625 mM, 1.125 mM, 2.25 mM, 4.5 mM and 9 mM) were used. The total reaction volume was 200 µl. All reagents were diluted in the reaction buffer (0.05 M Tris, 0.1 M NaCl pH 8.4). We used Dade Innovin (Dade Behring, Marburg, Germany) as a source of TF. All reactions contained 100 µl of Dade Innovin (Dade Behring, Marburg, Germany). The reactions were started by the addition of different substrate concentrations to a mixture of 400 nM FVIIa and different peptide concentrations.

We determined the initial velocity (Vo-value) of each reaction by dividing the change in absorbency over the first 15 minutes by the time period (15 minutes). The reaction curves at 400 nm FVIIa are straight lines over the first 15 minutes (see figure 4.10). We plotted the mean Vo-values against the different substrate concentrations at the different peptide concentrations and calculated the maximum velocity (Vm-value) and apparent Michaelis-Menten constant (Km'-value) of each peptide concentration by using one-site binding non-linear regression (r^2>0.990). Each reaction was repeated 4 times. To determine the type of inhibition of the peptide, we plotted the Lineweaver-Burk plot (1/Vo vs 1/substrate). We determined the inhibition constant (Ki-value) by

plotting the apparent Km values against the peptide concentrations (see figure 4.13). The Ki-value is then measured at the x-axis intercept.

CHAPTER 4

RESULTS

4.1 Biotinylation of FVIIa and biopanning on biotinylated FVIIa

The biotinylation of FVIIa was successful because 14.6 molecules of biotin were bound to a molecule of FVIIa. The biotinylation was calculated according to the instructions of the manufacturer of biotin (Pierce, Illinois, USA). We however did not find any FVIIa binding phages in either of the three selection rounds as indicated by the global ELISA. Therefore we decided to continue biopanning by coating FVIIa directly to the immune-tube.

4.2 Biopanning of FVIIa coated directly in immune-tubes

The concentration of the amplified phages increased after each round of panning. The concentrations of the amplified phages were $1.99.10^{11}$ for round 1, $4.95.10^{11}$ for round 2, and $1.5.10^{12}$ for round 3 for the cyclic 7-mere library. For the linear 12-mere library the phage concentrations were $2.31.10^{11}$ for round 1, $5.22.10^{11}$ for round 2 and $6.22.10^{11}$ for round 3.

The global ELISA indicated that we have selected the highest concentration of FVIIa-binding phages in round 3 since the optical density of these phages was the highest. We therefore decided to use the phages from round 3 for binding studies.

With the binding ELISA of the single colonies, we only found twelve phage colonies that bind strongly to FVIIa. Six colonies were from the linear 12-mere library and the other six from the cyclic 7-mere library. Because we had limited quantities of the very expensive FVIIa, we did not search for more binding colonies.

We performed a dilution ELISA with the twelve colonies. All the colonies of each library showed about the same binding affinity to FVIIa. To simplify the graph, we only plotted the binding affinity of one colony of each library. These results can be seen in figure 4.1. The OD_{490} increased with increasing phage concentrations. These two colonies thus bind concentration dependently to FVIIa. We repeated this ELISA three times. The colonies of the cyclic 7-mere library bind with a slightly higher affinity than the colonies of the linear 12-mere library.

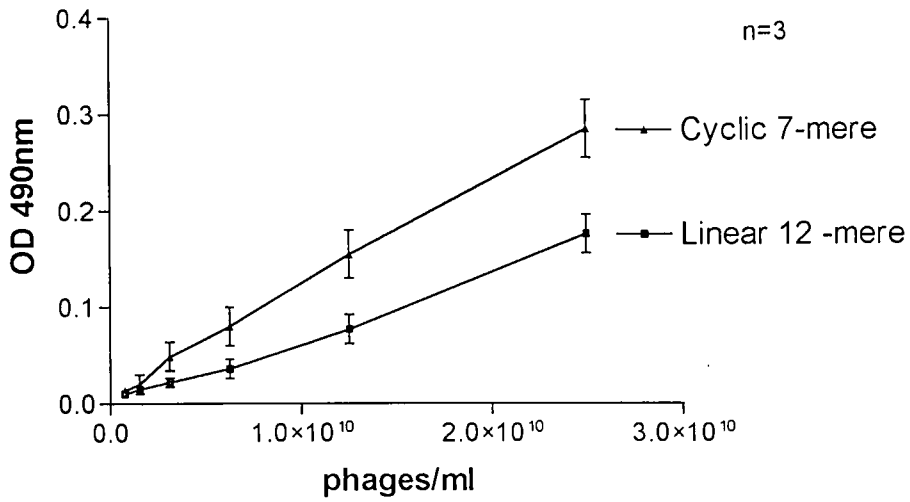

Figure 4.1 Dilution ELISA of both the cyclic 7-mere and linear 12-mere colonies. The OD_{490} increased with increasing concentration of phages. The cyclic 7-mere colony binds stronger to FVIIa than the linear 12-mere one (n=3).

An inhibition ELISA was also performed to determine if TF was able to prevent the twelve colonies from binding to FVIIa. No inhibition effect was seen with the linear colonies. Only one colony (colony 38) of the cyclic library showed a modest inhibition. This is shown in figure 4.2.

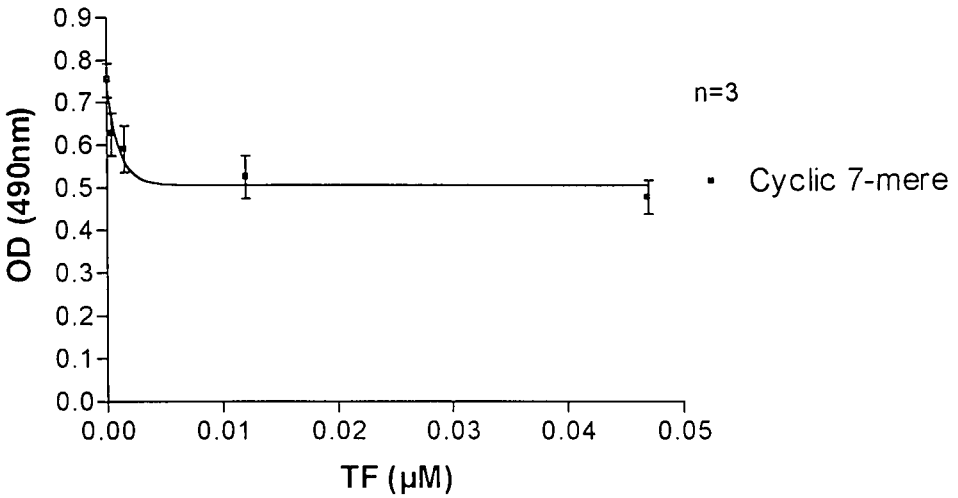

Figure 4.2 Inhibition ELISA of the cyclic 7-mere colony (colony 38) at different TF concentrations. TF concentrations from 0 to 0.05 µM were added to FVIIa coated wells and incubated for 15 minutes. 5.10^{10} phages of the cyclic 7-mere colony were added to the wells and incubated for 2 hours where after the FVIIa bound phages were detected using an anti-phage antibody (n=3).

We also performed PT's on different concentrations of the 6 linear 12-mere colonies and the 6 cyclic 7-mere colonies that showed binding in the binding ELISA of single colonies. Only two colonies (one from the cyclic 7-mere library and one from the linear 12-mere library) showed lengthening of the PT. The colony from the cyclic library that lengthened the PT is the same colony that showed inhibition in the inhibition ELISA. This was colony 38. Again we only show the graphs of the two phage colonies that lengthened the PT. Figure 4.3 shows the graph of the lengthening in the PT of the linear 12-mere colony and figure 4.4 that of the cyclic 7-mere colony. For the control, PBS was added to the plasma. A negative control was also performed where a non-binding phage was added to the plasma.

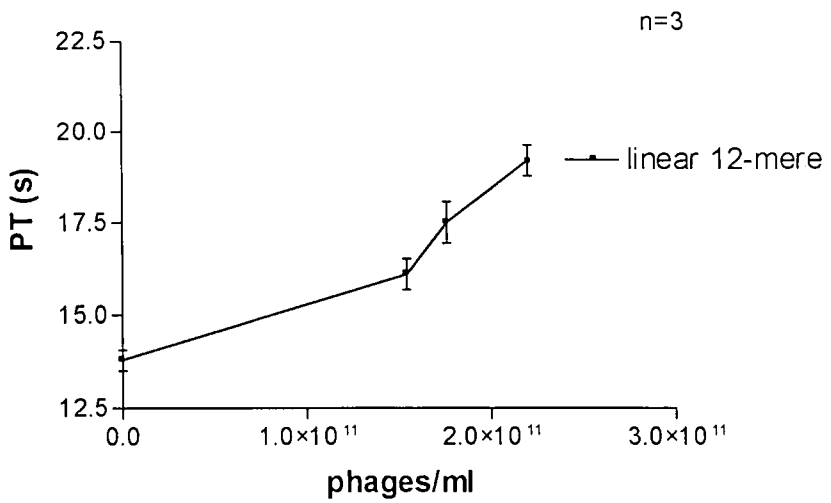

Figure 4.3 PT's with increasing concentrations of the linear 12-mere colony. Increasing concentrations of this phage colony were incubated for 10 minutes with human plasma before adding the PT reagent (Dade Innovin). This phage colony lengthened the PT concentration dependently. At the highest phage concentration ($2.3.10^{11}$ phages) the PT was prolonged with 5.4 seconds. (n=3)

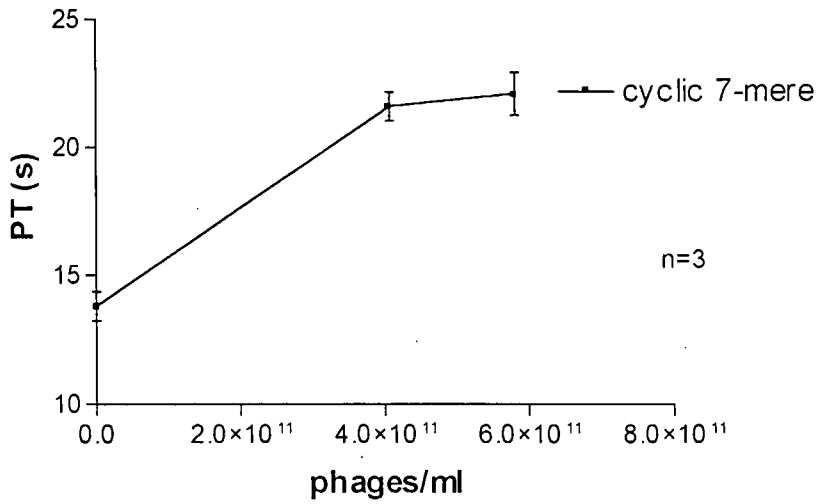

Figure 4.4 PT's with increasing concentrations of the cyclic 7-mere colony. Increasing concentrations of this phage colony were incubated for 10 minutes with human plasma before adding the PT reagent (Dade Innovin). The PT was prolonged with 8.3 seconds at the highest phage colony concentration

($5.8.10^{11}$ phages). This phage colony also lengthened the PT concentration dependently. (n=3)

4.3 Sequences of the phage colonies

We sequenced both the phage colonies (one from the cyclic 7-mere and one from the linear 12-mere library) that showed lengthening in PT. Interestingly, the sequence of the colony from the cyclic 7-mere library fitted exactly into the middle of the sequence of the colony from the 12-mere library. We compared these sequences to the sequences of TF, FX and TFPI and could not find any comparison. Unfortunately, it is not possible to disclose the sequence because of our agreement with American Diagnostics Incorporated (ADI). Because the cyclic colony binds stronger to FVIIa we decided to synthesise a cyclic peptide with the sequence similar to the peptide sequence displayed on this colony. Cyclic peptides are also more stabile than linear ones and more resistant to proteolysis (Pearce, 2001).

4.4 Effect of peptide on prothrombin times and thrombin times

PT's were done on human plasma. High concentrations of the peptide were needed to prolong the PT. The final peptide concentrations were 1.17 mM, 0.58 mM, and 0.3 mM. Figure 4.5 shows the prolongation of the PT with human plasma. The PT was prolonged by 30 seconds at the highest final peptide concentration of 1.17 mM. We repeated the experiment three times. As a control, we added PBS to plasma. For a negative control we added a peptide that does not bind to FVIIa.

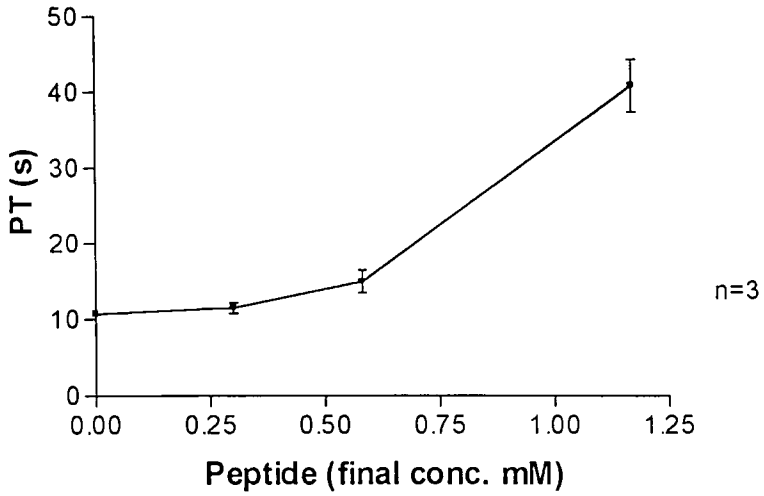

Figure 4.5 Prolongation of PT in human plasma. Different peptide concentrations were added to normal pooled human plasma and incubated for 10 minutes before the PT reagent (Dade Innovin) was added. At the highest peptide concentration, 1.17 mM, the PT was prolonged with 30 seconds. (n=3)

The TT also showed prolongation at high concentrations of the peptide. Prolongation of the TT in human plasma, with the same peptide concentrations as the PT's, are shown in figure 4.6.

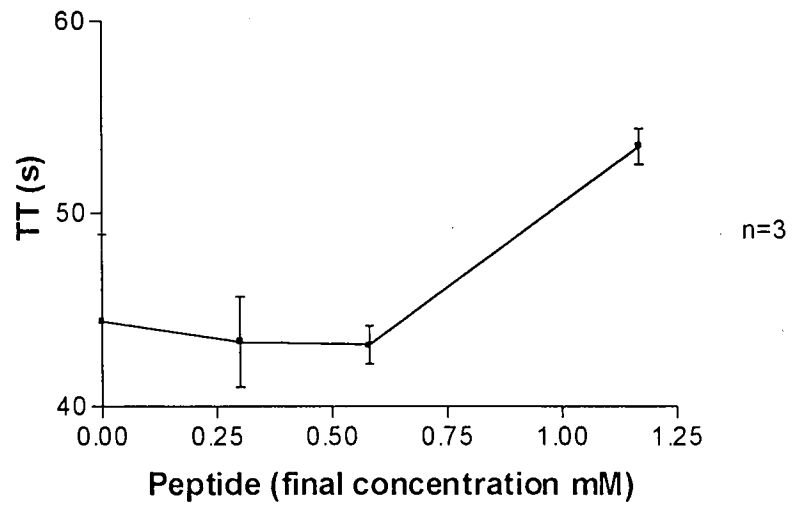

Figure 4.6 Prolongation of TT in human plasma. Different peptide concentrations were incubated with normal pooled human plasma for 10

minutes before the TT reagent (Dade thrombin) was added. The TT is prolonged at the highest peptide concentration with 9.1 seconds. (n=3)

4.5 Perfusion studies with endothelial cells

We used different peptide concentrations (2.34 μM, 4.7 μM and 9.35 μM) to study the effect of this peptide on platelet adhesion to human microvascular endothelial type 1 cells (HMEC-1 cells). We performed each peptide concentration in triplicate and table 4.1 shows the mean values and the standard deviation of the percentage of coverage. We used a low and a high shear (200 s^{-1} and 1000 s^{-1}) that simulate conditions for venous and arterial blood flow.

	Percentage coverage at a shear rate 200 s^{-1}	**Percentage coverage at a shear rate 1000 s^{-1}**
Control	17.9±0.96	11.6±0.64
2.34 μM	14.5±1.40	9.0±1.29
4.7 μM	8.1±0.20	6.1±2.24
9.35 μM	8.3±0.55	3.0±0.60

Table 4.1 Percentage of coverage on cover slips at a shear rate of 200 s^{-1} and 1000 s^{-1} (n=3).

The percentage coverage at both shears decreased with increasing peptide concentrations. Photo's of platelet adhesion (50x enlarged) at both shear rates can be seen in figure 4.7. The platelet adhesion of 9.35 μM and 4.7 μM peptide at 200 s^{-1} are the same. I therefore show the effect of only the 4.7 μM peptide at 200 s^{-1}.

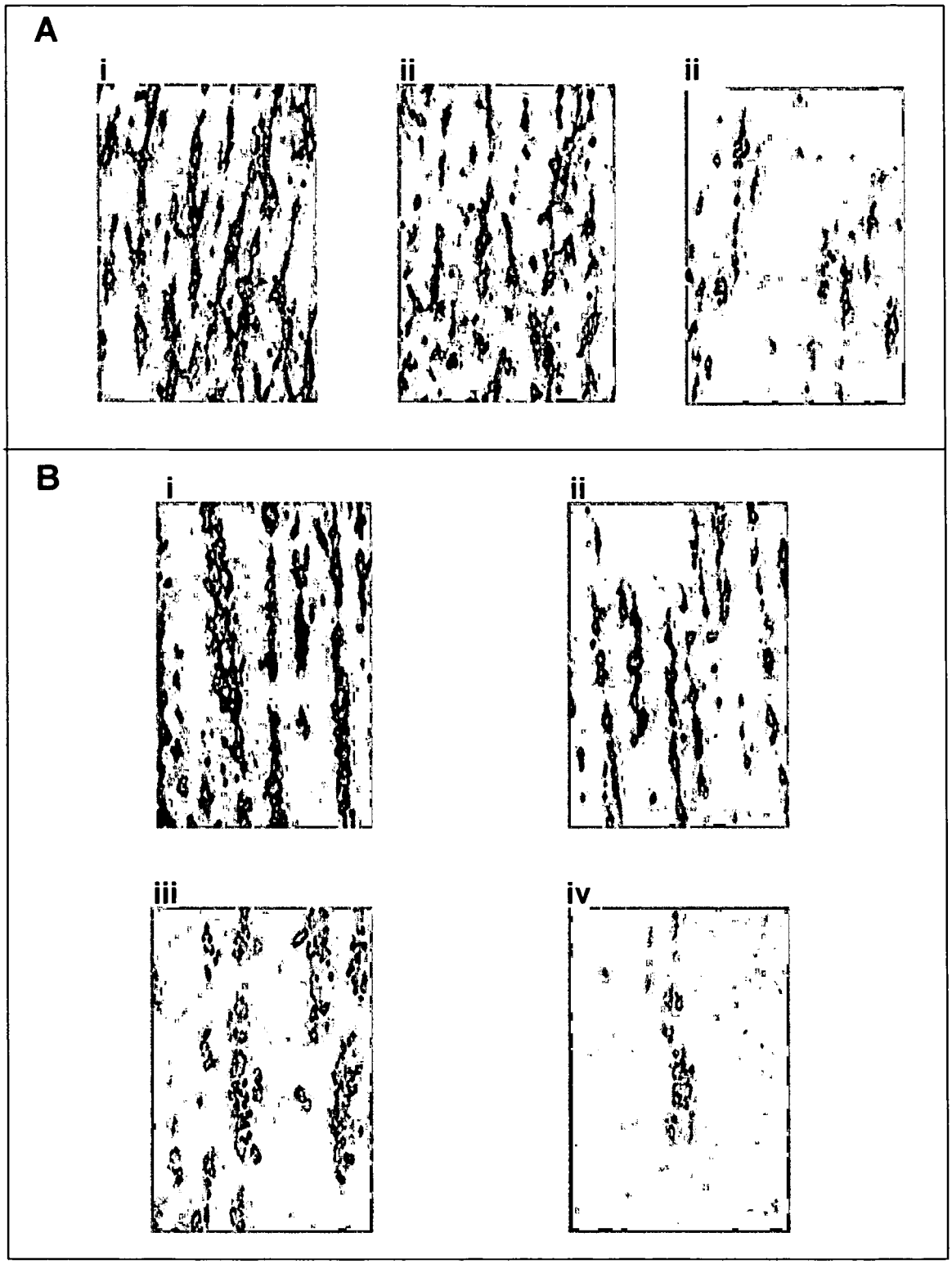

Figure 4.7 Platelet adhesion to HMEC-1 cells at shear rates (A) 200 s^{-1} and (B) 1000 s^{-1}.

A: (i) Control, (ii) 2.34 μM peptide, (iii) 4.7 μM peptide

B: (i) Control, (ii) 2.34 μM, (iii) 4.7 μM and (iv) 9.35 μM peptide.

4.6 Perfusion studies with collagen

We also did perfusion studies to determine the effect of different concentrations of the peptide on platelet adhesion to collagen. The peptide however does not have any influence on platelet adhesion to collagen at concentrations of 2.34 μM, 4.7 μM and 9.35 μM peptide. These results are not shown.

4.7 Perfusion studies with TF

By coating the coverslips with Innovin (Dade Behring, Marburg, Germany), we could study the effect of this peptide on platelet adhesion to TF because Innovin is a source of TF. We performed each peptide concentration only in duplicate because of limitations of the amount of peptide. Three different shear rates (200 s^{-1}, 650 s^{-1} and 1300 s^{-1}) were used. The mean values of platelet coverage at shears of 200 s^{-1}, 650 s^{-1}, and 1300 s^{-1}, are summarised in table 4.2. The photos (100x enlarged) of the platelet adhesion at 200 s^{-1} and 650 s^{-1} shear rates are shown by figure 4.8 and that of the 1300 s^{-1} shear rate are shown by figure 4.9.

	Percentage coverage at a shear rate 200 s^{-1}	**Percentage coverage at a shear rate 650 s^{-1}**	**Percentage coverage at a shear rate 1300 s^{-1}**
Control	6.1	11.4	22
29.2 μM	No effect	No effect	10.4
58.5 μM	4.1	4.7	2.9
117 μM	Not done	Not done	3.3

Table 4.2 Percentage of coverage on TF-coated coverslips at shears 200 s^{-1}, 650 s^{-1} and 1300 s^{-1} (n=2).

A peptide concentration of 29.2 μM had no effect on the percentage coverage at shears 200 s^{-1} and 650 s^{-1} and those photos are not shown in figure 4.8.

This concentration however had an effect at the high shear of 1300 s^{-1}. Platelet adhesion to the TF-coated coverslips at 1300 s^{-1} was inhibited in a dose-dependent manner by the peptide. At the lower peptide concentrations the effect was not as pronounced as that at the high concentration.

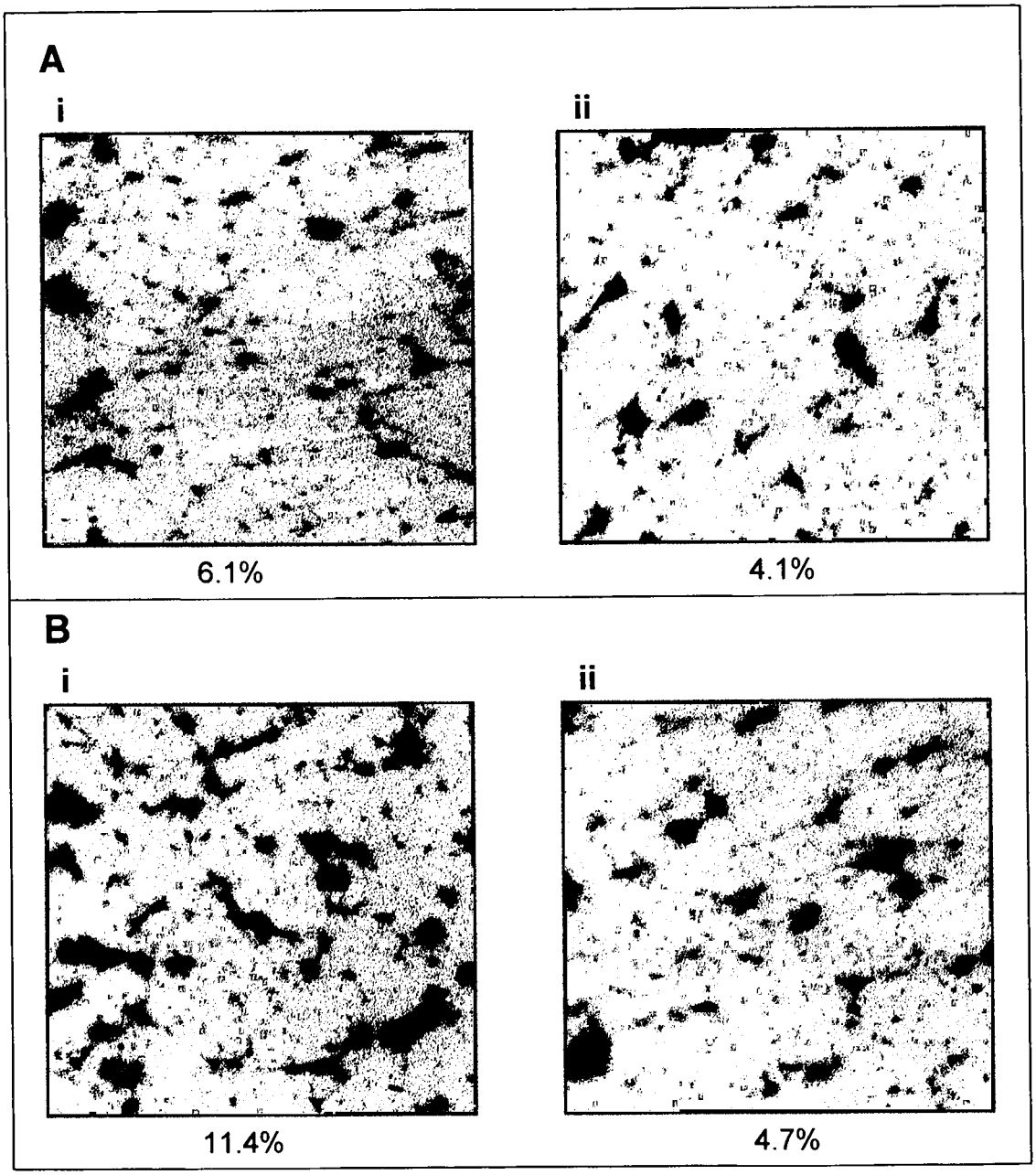

Figure 4.8 Platelet adhesion on TF coated coverslips at shear rates (A) 200 s^{-1} and (B) 650 s^{-1}. The percentage platelet coverage are shown beneath each photo

A: (i) Control, (ii) 58.5 μM peptide (200 s^{-1})

B: (i) Control, (ii) 58.5 μM peptide (650 s^{-1})

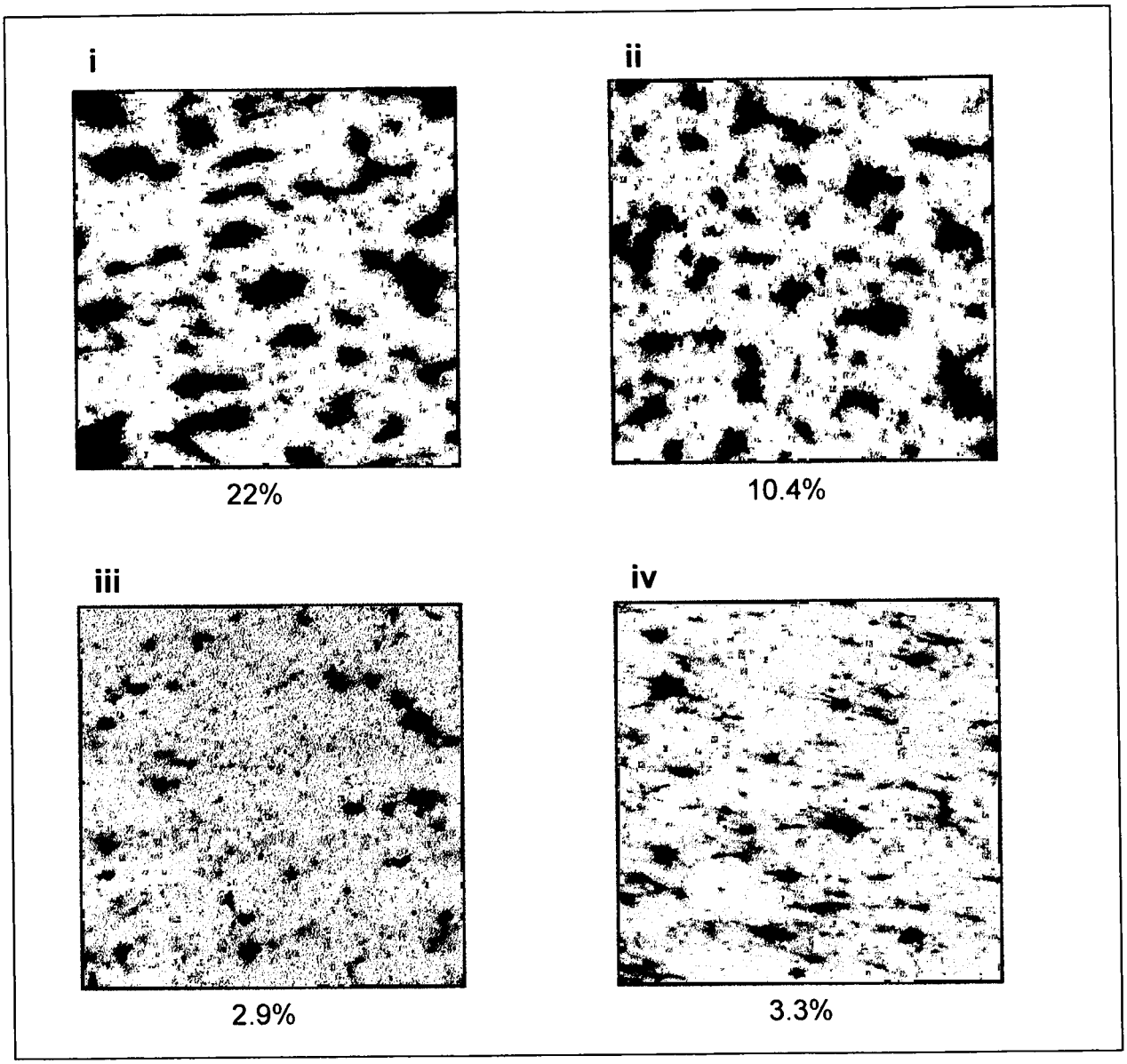

Figure 4.9 Platelet adhesion on TF coated coverslips at shear 1300 s^{-1}.
(i) Control, (ii) 29.2 μM peptide, (iii) 58.5 μM peptide, and (iv) 117 μM. The percentage coverage is shown beneath each plot

Less coverage was observed at the low shear ($200s^{-1}$) when compared to the higher shears. This was expected, since thrombi that form at low shear rates are platelet-poor and fibrin-rich (Sakariassen *et al.* 2001; Roth, 1992). The higher shears show more platelet adhesion in the control study. The peptide also has a more pronounced effect on platelet adhesion at high shear. Low peptide concentrations (29.2 μM) do not have any effect on platelet adhesion at low shears. At high shears it however has a ± 50% decrease in platelet adhesion. Concentrations of 58.5 μM peptide has an effect at all three shear rates, but is more pronounced at the high shear rate.

4.8 Kinetic assay

The first step in the kinetic assay was to determine the optimal FVIIa concentration. This was determined at 400 nM. We then determined the Vo-values of the reaction at different peptide and different substrate concentrations. Figure 4.10 shows the change in optical density over time of the control (0 mM peptide) at different substrate concentrations with 400 nM FVIIa. We included this figure to show that the reaction curves are straight lines over the first 15 minutes, since we calculated the Vo-values as the difference in optical density over the first 15 minutes over this time period.

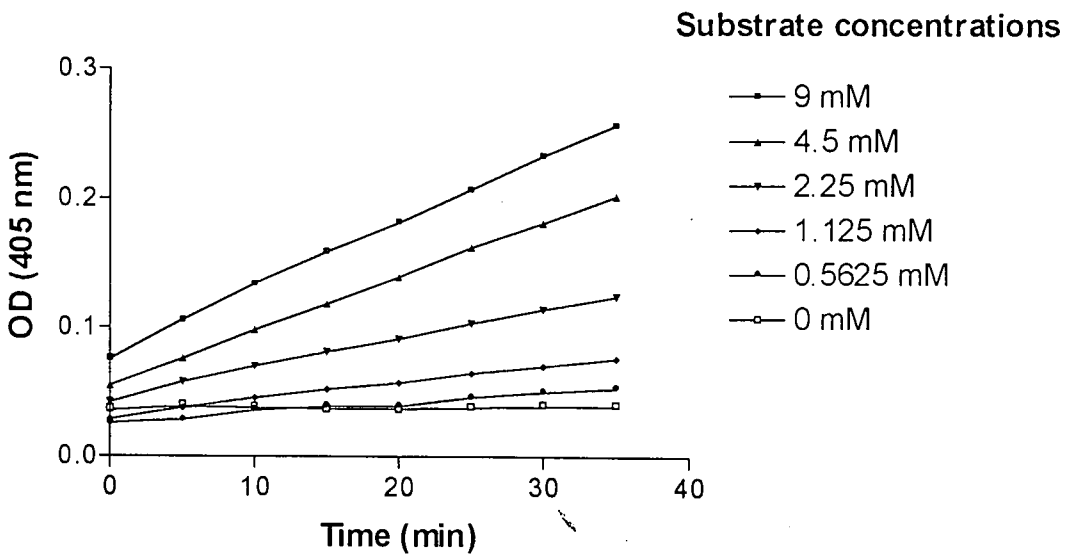

Figure 4.10 Different substrate concentrations at 0 mM peptide.

Figure 4.11 shows the Michaelis-Menten kinetics of the inhibition of FVIIa by the different peptide concentrations.

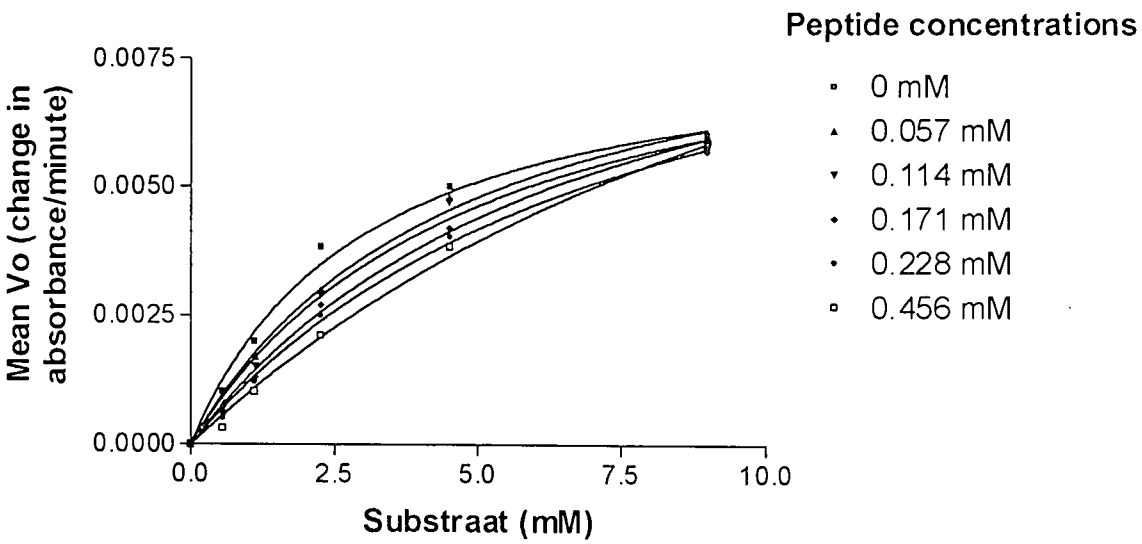

Figure 4.11 Michaelis-Menten kinetics of the inhibition of FVIIa by the peptide (n=4).

According to figure 4.11 the Vm-values of the different peptide (inhibitor) concentrations are unchanged but the Km-values increased with increasing peptide concentrations. This could indicate that the peptide is a competitive inhibitor. To confirm this, we plotted the 1/Vo values against the 1/substrate concentration (figure 4.12). This Lineweaver-Burk plot shows that the inhibition of the peptide is competitive because the slope of the graphs increased with increasing peptide (inhibitor) concentrations.

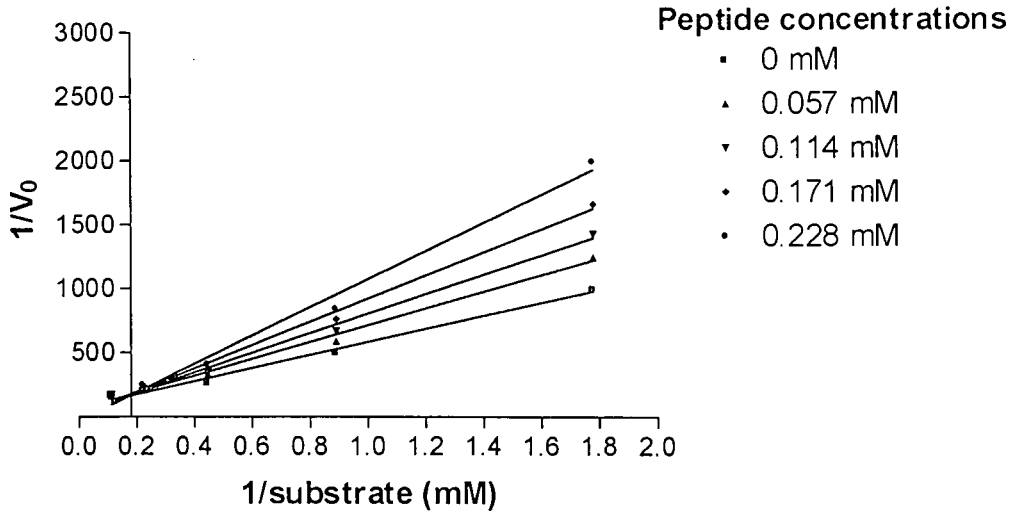

Figure 4.12 Linewaever-Burk plot showing the effect of competitive inhibition.

To determine the inhibition constant (Ki) we plotted the apparent Km (Km') values against the peptide concentrations in figure 4.13. We could fit a straight line to the graph and determine the Ki value as the x-axis intercept at 0.1232mM.

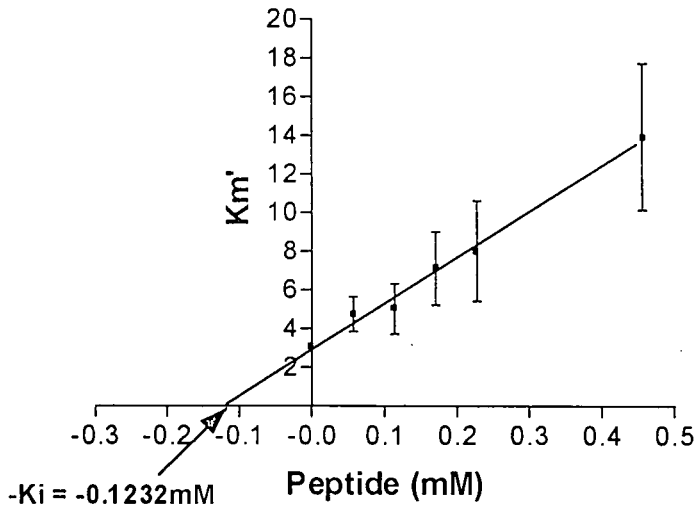

Figure 4.13 The Km value plotted against the peptide concentrations. From this graph the Ki could be determined by measuring the X-intercept of a linear regression. (n=4)

CHAPTER 5

DISCUSSION

The importance of the FVIIa/TF complex for the initiation of not only hemostasis, but also of thrombosis is now generally accepted (Davie *et al.*, 1991). Several studies have shown the impact of this binary complex on arterial thrombus formation (Barstad *et al*, 1995; Ørvim *et al*, 1994). Recently it was confirmed by selective inhibition of the FVIIa/TF complex by active site inactivated rFVIIa (Ørvim *et al*, 1997). In one study blockade of coagulation at the level of the FVIIa/TF complex provided full antithrombotic protection without abnormal bleeding (Harker *et al.*, 1996). Furthermore, although factor X and thrombin are the most popular candidates against which inhibitors are selected, one can argue that less inhibitor might be needed to inhibit FVIIa, since it is higher up in the coagulation cascade and is not responsible for its own generation in the same magnitude than thrombin and FXa (Ofosu *et al.* 1996).

We selected peptide inhibitors to FVIIa, because the advantage of smaller peptides is that they are non-immunogenic. Markwardt (1990) showed that small peptide inhibitors to thrombin are non-immunogenic and non-toxic.

We used the technique of phage display to select peptide inhibitors to FVIIa. Phage display describes a selection technique in which a peptide/protein is expressed as a fusion with a coat protein of a bacteriophage, resulting in the display of the fused protein on the surface of the virion, while the DNA encoding the fusion resides within the virion. Therefore phage display technology 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. This technique also allows for large numbers of phage clones ($>10^9$ different sequences) to be screened which make it a very powerful technique (Smith,

1985). It has advantages over other methods for studying protein-protein interactions in the fact that it allows proteins to fold correctly on the phage surface (Allen *et al.*, 1995; Bardwell *et al.*, 1991).

We started the phage display selection process by using biotinylated FVIIa. The ability to use soluble antigen for selections imparts a greater degree of control over the selection process (Smith *et al.*, 1995). By using biotinylated antigen, soluble selections can be achieved. Phages are allowed to bind the biotinylated antigen and are recovered using streptavidine-coated magnetic beads. Although the biotinylation of FVIIa was successful, we could not find any FVIIa-binding phages. It could be that FVIIa became inactive after biotinylation. We therefore decided to continue biopanning in immune-tubes. To achieve this, we coated the immune-tube with FVIIa and incubated the phage libraries with it. We used a murine monoclonal antibody against human FVIIa to elute the FVIIa-binding phages specifically from the immune-tube in the last two rounds of selection. This antibody binds to the active site of FVIIa and blocks the assembly of the FVIIa/TF complex. It also blocks FVIIa-mediated plasma coagulation. Since this antibody replaced the phages from FVIIa, it means that the selected phages bind to the catalytic domain of FVIIa, because this antibody inhibits FVIIa in a two-stage chromogenic assay involving the chromogenic substrate for FXa, spectrozyme Xa (Clarke *et al.*, 1992). We did three selection rounds and found that the twelve strongest FVIIa binding phage clones included six from the cyclic 7-mere library and six from the linear 12-mere library. From the 12 strong-binding colonies, only 2 (one from each library) lengthened the PT in a concentration dependent manner (fig. 4.3 and 4.4). Interestingly, the sequence from the cyclic 7-mere library fitted exactly into the middle of the sequence from the linear 12-mere library. No similarities were found between either of the cyclic and linear sequences and those of TF, FX or TFPI. After the three rounds of enrichment (biopanning), 12 phage colonies were tested for binding to FVIIa. The phage colonies from the cyclic heptapeptide library bind with a higher affinity than the linear 12-mere sequence (figure 4.1). This is not surprising since it is known that cyclic peptides bind more tightly to their ligands than the same linear

sequences due to improved binding entopy (Das and Meirovitch, 2001). Furthermore, TF concentrations from 0 to 0.05 μM prevented the binding of one of the colonies from the cyclic peptide library to FVIIa. This suggests that this cyclic heptapeptide sequence bind to the same binding site as TF to FVIIa. The effect is nevertheless modest, since TF does not prevent the phages completely from binding to FVIIa (figure 4.2). One phage colony from each library lengthened the prothrombin time (PT) in a dose-dependent manner (see figures 4.3 and 4.4). Unfortunately only limited numbers of phages can be used in any assay because of the inherent size restrictions of the phages. In view of this, no further results on inhibition could be obtained by using the phages.

A cyclic peptide with the corresponding sequence as the peptide sequence displayed on the cyclic 7-mere colony was synthesised. We cannot disclose the sequence of the peptide since we have a secrecy contract with American Diagnostica Incorporated (Greenwich, USA). There were two reasons why we selected the cyclic peptide. First, it binds stronger to FVIIa (see figure 4.1), and second, cyclic peptides are more stable than those with linear sequences (Fabiola *et al.*, 2001). Furthermore, cyclic peptides are more resistant to proteolysis (Pearce, 2001).

We characterised the peptide by studying its effect on platelet adhesion to human vascular endothelial cell matrix (HMEC-1) in a parallel flow chamber, under both arterial and venous shear stress conditions (shear rates of 1000 s^{-1} and 200 s^{-1} respectively). It is interesting to note that the percentage coverage in the control study was higher at venous flow (shear rate of 200s^{-1}) than at arterial flow (shear rate of 1000 s^{-1}). This is because minute levels of TF are expressed on the endothelium which is a major initiator of venous thrombosis (Salzman and Hirsh, 1993). The peptide inhibits platelet adhesion in a dose-dependent manner at both shear stress conditions. The inhibiting effect was more pronounced at arterial flow conditions (1000 s^{-1}), because the highest peptide concentration (9.35 μM) inhibited coverage by approximately 74%. At the low shear rate (200 s^{-1}) inhibition was approximately 54% at the

same peptide concentration (see table 4.1 and figure 4.7A). We cannot explain this. We can however argue that the inhibition is more effective at arterial flow conditions is because platelets are more involved in thrombi formed at arterial shear whereas fibrin is more involved in thrombi formed at venous shear (Sakaríassen *et al.*, 2001; Roth, 1992). This was also observed in the perfusion studies where TF (Innovin)-coated coverslips were used. In this case the control studies indicate that platelets adhere much more to the TF-coated coverslips at high shear rates (1300 s^{-1}), where the percentage coverage was 22% (see table 4.2 and figure 4.9). At shear rates of 650 s^{-1} and 200 s^{-1}, percentage coverage were 11.4% and 6% respectively. It is also interesting that the fibrin network was highly visible at low shear rates (200 s^{-1}) (see table 4.2 and figure 4.8). It is known that TF in high concentrations emerges as a potent inducer of both arterial and venous thrombosis. TF is present in relatively high concentrations on the cover slips. We can explain the higher platelet coverage at high shears to the fact that platelets are more involved in arterial thrombogenesis (Sakaríassen *et al.*, 2001)

This cyclic peptide affected platelet adhesion to the TF-coated coverslips a high shear rate of 1300 s^{-1} in a dose-dependent manner. We did not observe a concentration-dependent effect at medium and low shears of 650 s^{-1} and 200 s^{-1} respectively because the percentage coverage was too low to observe inhibitory effects. The peptide at a concentration of 56.5 μM however inhibited platelet adhesion by 58% at 650 s^{-1}. The effect at 200 s^{-1} could again not be detected since the percentage coverage was too low. PN7051 also inhibits platelet adhesion to immobilised TF in a parallel-plate perfusion chamber at a shear rate of 650 s^{-1}. Fifty percent inhibition was observed at 500 μM. Our peptide inhibited platelet adhesion by 58% at 58.5 μM at the same shear, i.e. it seems to have a stronger effect on platelet adhesion and thrombus formation than PN7051 (Örning *et al.*, 2002).

Furthermore, this peptide had no effect on collagen-coated coverslips in the perfusion studies. It is known that other coagulation inhibitors such as hirudin and PPACK also had no effect on platelet adhesion to collagen (Harker *et al.*,

1996). A possible explanation could be that there is no TF involved on the collagen surface and coagulation is not initiated by the FVIIa/TF complex, but platelets are activated by vWF.

Thus, we can summarise the effect of the peptide on platelet adhesion at different shears on the different matrixes in the perfusion studies, as follows. The peptide only has an effect on matrixes where TF is present, because there was no effect on the collagen matrix. The amount of TF on the matrixes also has an influence on the platelet adhesion at different shears, since at minute levels of TF such as the HMEC-1 matrix, the platelet coverage is higher at venous flow conditions. While at high TF concentrations such as the TF matrix, the platelet coverage is much higher at arterial flow conditions. At venous flow conditions, very low platelet coverage occurs on the TF matrix and therefore we cannot compare the effect of the peptide on the TF matrix at different shears. We can however compare the effect of the peptide at different shears on the HMEC-1 matrix. Here it is clear that the peptide has a stronger inhibition effect at arterial conditions than at venous conditions, especially at high peptide concentrations, although the platelet coverage is better at venous conditions. We have no explanation for this.

It is important to note that all the perfusion studies were performed in heparanised blood. It is known that LMWH does not block all coagulant activity, but allow some thrombin formation and thus some fibrin deposition on the surface (Sakarissen *et al.*, 2001). Fibrin networks can be clearly observed on the TF matrix at low shear rates.

The kinetic data indicates that this peptide is a competitive inhibitor of FVIIa. Michaelis-Menten kinetics shows that the Vm-values stays the same with increasing peptide concentrations, but the Km-values increased. Furthermore the 1/Vo versus 1/substrate curves (Lineweaver-Burk plots) of different peptide concentrations cross-section on the y-axis, which is also an indication of competitive inhibition (see figures 4.11 and 4.12). This is because the Lineweaver-Burk equation in the presence of a competitive inhibitor is,

1/Vo = Km'/Vm * 1/substrate concentration + 1/Vm, and the slope of the plots at different peptide concentrations increase with increasing peptide concentrations. It is important to note that a competitive inhibitor does not necessarily binds to the same binding site as the substrate, but can also bind to a different binding site on the enzyme in such a way that the binding of either one causes a conformational change in the enzyme which prevent s the other from binding. This inhibition gives competitive kinetics (Dixon and Webb, 1979).

The chromogenic substrate we used in the kinetic studies contains an arginine sequence and binds to the catalytic site of FVIIa. Since FVIIa is a serine protease, it cleaves the substrate at a site adjacent to an arginine residue. The peptide does not contain an arginine residue and therefore we can accept that the peptide does not bind to the same binding site as the substrate. The peptide does however prevent the splicing of the colour reagent of the chromogenic substrate. Therefore the binding of the inhibitor to FVIIa may cause a conformation change to prevent the chromogenic substrate from being spliced. Since we selected phages that binds to FVIIa and eluted them with an antibody that binds to the catalytic site of FVIIa and prevents the formation of the FVIIa/TF complex, we can argue that this peptide also binds to the catalytic site and also prevent the binding of TF to FVIIa. We know that the catalytic domain (serine protease domain) contains three different binding regions which are a TF binding region, active site binding region and a macromolecular substrate binding region and all three of these regions are involved in the proteolytic activity (Persson, 2000). Since free FVIIa does not recognise the substrate, factors IX and X, the binding of TF to FVIIa is necessary for the binding of the substrate (Broze, 1992). We can thus argue that this peptide binds to FVIIa in such a way that it prevents the formation of the FVIIa/TF complex and therefore FVIIa does nor recognise the substrate and does not splice the colour reagent of the chromogenic substrate. The Ki-value is determined at 0.1232 mM. Our peptide is not a strong inhibitor of FVIIa, when the Ki-value is compared to that of other FVIIa inhibitors. For example, TFPI inhibits the activation of FXa by a Ki of 30 nM. Similarly,

Kunitz domain variants of TFPI inhibit the FVIIa/TF complex with a Ki of 1.9 nM (Dennis and Lazarus, 1994a; Dennis and Lazarus, 1994b). Another peptide inhibitor, A-183, that binds to the exosite of the protease domain of FVIIa, inhibits FX activation with a Ki of 200 pM (De Cristofaro, 2002; Dennis *et al.*, 2001; Roberge *et al.*, 2001).

One must take into account that we used Innovin (Dade Behring, Marburg, Germany) as a source of TF in the kinetic studies. It is now know that the Innovin contains only a small percentage of TF. This may explain the large difference in Ki-values from other studies where they used recombinant TF.

This cyclic heptapeptide lengthens the PT dose-dependently. Comparable to other inhibitors of TF-dependent coagulation a peptide concentration of 1.17 mM lengthened the PT 3-fold. PN7051, another cyclic inhibitor of the FVIIa/TF complex that represents loop I of the second EGF-like domain of FVII, also prolongs the clotting time dose-dependently with an IC_{50} of 1.3 mM (Örning *et al.*, 2002). The reason for the lengthening in PT may be the binding of the peptide to FVIIa and preventing the FVIIa/TF complex from forming, which prevents the activation of FXa. Our peptide also lengthens the TT but only at concentrations in excess of 0.58 mM, indicating that it may also inhibit FXa function. Moreover, the lengthening in TT is not as pronounced as the lengthening in PT. At the highest concentration of 1.17 mM, the lengthening in TT was 9.1 seconds (±0.25-fold). It may be so because determination of the TT is not TF-dependent. This difference in the effect on PT and TT can again be compared to that of the cyclic inhibitor, PN7051, which inhibits the prothrombinase complex with an IC_{50} of 0.5 mM and TF-dependent FX activation with an IC_{50} of 20 μM using the same concentrations of FVIIa and FX (Örning *et al.*, 2002). It is of interest to note that peptide concentrations higher than 500 μM lengthened the PT and TT. In a study where the effect of two distinct peptide exosite inhibitors of FVIIa on the PT was determined, the lengthening in PT started at concentrations higher than 100 nM. Our peptide is therefore not as potent as exosite inhibitors of FVIIa (Roberge *et al.*, 2001).

The inhibition of our peptide on thrombin formation may be explained as follows. This peptide prevents the formation of the FVIIa/TF complex and thus prevents the activation of FX since the formation of the FVIIa/TF complex is necessary to activate FX. We can therefore argue that the peptide inhibits thrombin formation.

To summarise, one can argue that our peptide acts on three levels of the coagulation process to inhibit thrombus formation by preventing the formation of the FVIIa/TF complex and thereby preventing the activation of FX. First, at vessel wall injury where TF within the vessel wall is exposed to FVIIa in the circulating blood (Broze, 1992). Second, on leukocytes containing circulating TF, which is transferred to the platelet thrombus (Rauch *et al.*, 2000). This is the so-called blood-borne TF (Rauch and Nemerson, 2000). Third, on platelet surfaces where the extrinsic and intrinsic tenase complexes are formed (Mann *et al.*, 1990).

The existence of blood-borne TF as described by Rauch and Nemerson (2000) provides another mechanism by which inhibitors of FVIIa, or the FVIIa/TF complex, such as our peptide prevents thrombosis. They described thrombogenic TF on leukocyte-derived microparticles and their incorporation into spontaneous human thrombi. Polymorphonuclear leukocytes and monocytes transfer the TF-positive microparticles to platelets, thereby making them capable of triggering and propagating thrombosis. This is done by the interaction of leukocytes via their CD15 receptor on the cell membrane with CD62P (P-selectin) on the platelet membrane. P-selectin is an α-granule-derived activation dependent adhesion molecule found on platelets. Platelets therefore become TF-positive after exposure to the TF-positive microparticles. The activation of the coagulation factors IX and X occurs therefore very close to platelet surfaces and thereby favouring the formation of the intrinsic and extrinsic tenase complexes on their highly procoagulant surfaces. Since our peptide is able to prevent FX activation by preventing the formation of the

FVIIa/TF complex, one can argue that it can also act on platelets to inhibit formation of platelet-rich thrombi.

Shortfalls

In a study of this nature where highly expensive products are used, it is necessary to be economical. One aspect of the study that was affected is the kinetic studies. We unfortunately did not have enough peptide or FVIIa to repeat the reactions more than 4 times. This can explain the large standard deviation of the apparent Km-values in figure 4.13 of the kinetic studies. Another explanation for the large Km'-values at high peptide concentrations can be the impurities of the peptide since it maybe not 100% pure, but at least more than 80%. Another aspect is the perfusion studies with Innovin-coated coverslips. These studies could only be done twice at low shears and high shears.

Future studies

The kinetic studies indicate that this peptide is a competitive inhibitor of FVIIa that prevents the binding of TF to FVIIa. It would be interesting to see by X-ray cristallography where exactly this peptide binds to FVIIa. Since we could not determine the effect of this peptide on platelet adhesion to a TF-matrix at low shears, we would like to investigate the effect of this peptide on platelets more intense. By using flow cytometry, we will be able to determine if this peptide has an inhibitory effect on platelets stimulated with ionophore or thrombin. We will measure P-selectin, CD63 (a lisosomal protein that indicates the platelet release reaction), PAC-1 (GpIIb/IIIa expression on the platelet membrane) and TF expression. We will also be able to determine whether this peptide prevents platelet activation. It would further be interesting to determine the *in vivo* effect of this peptide in animal models.

Finally, I hope that the results discussed in this thesis have convinced the reader that the FVIIa/TF complex is of utmost importance not only for maintaining normal hemostasis, but also by playing a crucial role in the development of arterial thrombosis, still the leading cause of death in our Western society. We also hope that the results showing inhibition of FVIIa may suggest a novel therapeutic approach to prevent thrombosis in patients and may prove to be effective also in disorders associated with increased blood TF. I believe that the study of FVIIa and its inhibitors will remain important research topics in years to come and that it may result in important social benefits.

CHAPTER 6

ABSTRACT

The importance of FVIIa and the FVIIa/TF complex for the initiation of not only hemostasis but also thrombosis is now generally accepted. It was shown that the blockade of coagulation at the level of FVIIa provided full antithrombotic protection without abnormal bleeding (Harker *et al*, 1996), therefore FVIIa is a suitable candidate for the development of novel antithrombotics.

We selected inhibitors to FVIIa using the technique of phage display. A repeated selection of phages from a cyclic heptapeptide and a linear 12-mere phage display library resulted in the enrichment of phages that bind to human FVIIa. We selected twelve colonies (6 from each library) that showed the strongest binding to FVIIa. The colonies from the cyclic 7-mere library showed a higher affinity binding for FVIIa than the colonies from the linear 12-mere library. TF also prevents the binding of one of the cyclic colonies to FVIIa. This colony as well as one colony from the linear library showed lengthening of the prothrombin time (PT) as well as the thrombin time (TT) in a dose-dependent manner.

A cyclic heptapeptide was synthesised with the corresponding sequence as the sequence displayed on the cyclic 7-mere colony. The peptide showed lengthening of the PT and TT in a dose-dependent manner with a more pronounced effect on the PT than the TT. We also studied the effect of this peptide on platelet adhesion on human vascular endothelial cell matrix, collagen and TF under both venous and arterial shear stresses. The peptide inhibits platelet adhesion to HMEC-1 under both shear stresses. The effect on arterial shear is however more pronounced. It does not inhibit platelet adhesion to collagen, but has a dose-dependent inhibitory effect on platelet adhesion TF at arterial shear. Kinetic analysis of the peptide showed that this peptide is a competitive inhibitor of FVIIa by altering the Km-values but not the

Vmax-values. The Lineweaver-Burk plot also indicates a competitive inhibition, because the slope of the graphs increased with increasing inhibitor concentrations. The Ki-value was determined at 0.1232 mM.

In summary, this peptide inhibits thrombus formation by preventing FVIIa from binding to TF and therefore preventing the activation of FX by the FVIIa/TF complex. This study suggests that inhibitors to FVIIa provide a novel therapeutic approach to prevent thrombosis.

ABSTRAK

Dit word nou algemeen aanvaar dat faktor VIIa en die faktor VIIa/Weefselfaktor (FVIIa/WF) kompleks nie alleen 'n baie belangrike rol in hemostase speel nie, maar ook in trombose. Deur die stollingskaskade op die vlak van FVIIa te blokkeer, lei tot volledige inhibisie van trombose sonder die moontlikheid van 'n bloedingsneiging (Harker *et al*, 1996). FVIIa is dus 'n geskikte kandidaat waarteen nuwe anti-trombotiese middels ontwikkel kan word.

Die tegnologie van peptiedblootlegging op fage is gebruik om inhibitore teen FVIIa te selekteer. 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 bind aan FVIIa. Ons het twaalf kolonies wat sterk aan FVIIa bind geselekteer (6 van elke biblioteek). Die sikliese kolonies het met hoër affiniteit aan FVIIa gebind. Ons het ook gevind dat WF die binding van een van die sikliese kolonies aan FVIIa verhoed. Hierdie kolonie asook een van die lineêre biblioteek het dosis-afhanklike verlenging getoon met die protrombien tyd (PT) en die trombien tyd (TT).

Ons het 'n sikliese hepta-peptied identies aan die peptied wat blootgelê word op die sikliese faag kolonie, laat sintetiseer. Die peptied verleng die PT en TT. Die effek was dosis-afhanklik en was meer uitgesproke in die PT as in die TT. Ons het ook die effek van die peptied op plaatjie adhesie gaan ondersoek. Ons het verskillende oppervlaktes gebruik, nl. menslike vaskulêre endoteelsel-matriks, kollageen en WF. Daar is van beide veneuse en arteriële skuifkragte gebruik gemaak. Die peptied inhibeer plaatjie adhesie aan die menslike vaskulêre endoteel. Die effek was meer uitgesproke in die geval van arteriële vloei. Die peptied het egter geen effek getoon op plaatjie-adhesie aan kollageen nie. Daar was wel 'n dosis-afhanklike effek op plaatjie adhesie aan WF in arteriële vloei. Ons het ook kinetiese analises van die peptied gedoen. Die peptied is 'n kompeterende inhibeerder van FVIIa. Die

Km-waardes varieer met verskillende peptiedkonsentrasies, maar nie die Vmaks-waardes nie. Die Ki waarde is 0.1232 mM.

Ter opsomming kan ons sê dat hierdie peptied trombusvorming inhibeer deur te verhoed dat FVIIa aan WF bind en daardeur die aktivering van faktor X deur die FVIIa/WF kompleks verhoed. Hierdie studie dui daarop dat die ontwikkeling van inhibitore teen FVIIa 'n nuwe benadering is om trombose te voorkom.

REFERENCES

Aasrum, M and Prydz,H. (2002) Gene targeting of tissue factor, factor X, and factor VII in mice: their involvementin embryonic development. *Biochemistry (Mosc)* **67**, 25-32.

Allen, J.B., Walberg, MW., Edwards, M.C. and Elledge, S.J. (1995) Finding prospective partners in the library: the two-hybrid system and phage display find a match. *Trends in Biotechnology* **20**, 511-516.

Amery, L.. (2001) Identification of a novel human peroxisomal 2,4-dienoyl-CoA reductase related protein using the M13 phage protein VI phage display technology. *Comb Chem High Throughput Screen* **4**, 545-552.

Armstrong, N., Adey, N.B., McConnell, S.J. and Kay, B.K. (1996) Vectors for phage display. In: Kay, B.K., Winter, J. and McCafferty, J., (Eds.) *Phage display of peptides and proteins*, 1st ed. pp. 35-53. San Diego: Academic Press Inc.]

Arza, B. and Félez, J. (1998) The emerging impact of phage display technology in thrombosis and haemostasis. *Thrombosis and Haemostasis* **80**, 354-365.

Bajaj, M.S., Birktoft, J.J., Steer, S.A. and Bajaj, S.P. (2001) Structure and biology of tissue factor pathway inhibitor. *Thrombosis and Haemostasis* **86**, 959-972.

Bajaj, S.P., Rapaport, S.I. and Brown, S.F. (1981) Isolation and characterization of human factor VII. *The Journal of Biological Chemistry* **256**, 253-259.

Balass, M., Heldman, Y., Cabilly, S., Givol, D., Katchalski-Katzir, E. and Fuchs, S. (1993) Identification of a hexapeptide that mimics a conformation-dependent binding site of acetylcholine receptor by use of a phage-epitope library. *Proceedings of the National Academy of Sciences* **90**, 10638-10642.

Banner, D.W. (1997) The factor VIIa/tissue factor complex. *Thrombosis and Haemostasis* **78**, 512-515.

Banner, D.W., D'Arcy, A., Chène, C., Winkler, F.K., Guha, A., Konigsberg, W.H., Nemerson, Y. and Kirchhofer, D. (1996) The crystal structure of the complex of blood coagulation factor VIIa with soluble tissue factor. *Nature* **380**, 41-46.

Barbas, C.F., Bain, J.D., Hoekstra, D.M. and Lerner, R.A. (1991) Assembly of combinatorial antibody libraries on phage surfaces: the gene III site. *Proceedings of the National Academy of Sciences* **88**, 7978-7982.

Bardwell, J.C., McGovern, K. and Beckwith, J. (1991) Identification of a protein required for disulfide bond formation. *Cell* **67**, 581-589.

Barstad, R.M., Hamers, M.J.A.G., Kierulf, P. and Sakaríassen, K.S. (1995) Procoagulant human monocytes mediate tissue factor/factor VIIa-dependent platelet-thrombus formation when exposed to flowing nonanticoagulated human blood. *Arteriosclerosis, Thrombosis, and Vascular Biology* **15**, 11-16.

Bergum, P.W., Cruikshank, A., Maki, S.L., Kelly, C.R., Ruf, W. and Vlasuk, G.P. (2001) Role of zymogen and activated factor X as scaffolds for the inhibition of the blood coagulation factor VIIa-tissue factor complex by recombinant nematode anticoagulant protein c2. *The Journal of Biological Chemistry* **276**, 10063-10071.

Blaber, M. (1998) M13 Phage. Anonymous

Bom, V.J.J. and Bertina, R.M. (1990) The contributions of Ca^{2+}, phospholipids and tissue-factor apoprotein to the activation of human blood-coagulation factor X by activated factor VII. *Biochemical Journal* **265**, 327-336.

Bonnefoy, A., Harsfalvi, J., Pflieger, G., Fauvel-Lafève, F. and Legrand, C. (2001) The subendothelium of the HMEC-1 cell line supports thrombus formation in the absence of Von Willebrand factor and collagen types I, III and VI. *Thrombosis and Haemostasis* **85**, 552-559.

Bottger, V., Bottger, A., Lane, E.B. and Spruce, B.A. (1995) Comprehensive epitope analysis of monoclonal anti-proenkephalin antibodies using phage display libraries and synthetic peptides: revelation of antibody specificities caused by somatic mutations in the variable region genes. *Journal of Molecular Biology* **247**, 932-946.

Broze, G.J. (1987) The tissue factor inhibitor is also a FXa inhibitor. *Clinical Research* **35**,

Broze, G.J. (1992) The role of tissue factor pathway inhibitor in a revised coagulation cascade. *Seminars in Hematology* **29**, 159-169.

Broze, G.J. (1992) Why do hemophiliacs bleed? *Hospital Practice* 71-86.

Broze, G.J. (1995) Tissue factor pathway inhibitor. *Thrombosis and Haemostasis* **74**, 90-93.

Broze, G.J., Lange, G.W., Duffin, K.L. and MacPhail, L. (1994) Heterogeneity of plasma tissue factor pathway inhibitor. *Blood Coagulation and Fibrinolysis* **5**, 551-559.

Broze, G.J., Likert, K. and Higuchi, D. (1993) Inhibition of factor VIIa/tissue factor by antithrombin III and tissue factor pathway inhibitor. *Blood* **82**, 1679-1680.

Broze, G.J., Girard, T.J. and Novotny, W.F. (1990) Regulation of coagulation by a multivalent Kunitz-type inhibitor. *Biochemistry* **29**, 7539-7546.

Broze, G.J. and Majerus, P.W. (1981) Human factor VII. *Methods in Enzymology* **80**, 228-237.

Broze, G.J. and Miletich, J.P. (1987a) Characterization of the inhibition of tissue factor in serum. *Blood* **69**, 150-155.

Broze, G.J. and Miletich, J.P. (1987b) Isolation of the tissue factor inhibitor produced by HepG2 hepatoma cells. *Proceedings of the National Academy of Sciences* **84**, 1886-1890.

Broze, G.J., Warren, L.A., Novotny, W.F., Higuchi, D., Girard, J.J. and Miletich, J.P. (1988) The lipoprotein-associated coagulation inhibitor that inhibits the factor VII-tissue factor complex also inhibits factor Xa: insight into its possible mechanism of action. *Blood* **71**, 335-343.

Butenas, S. and Mann, K.G. (2002) Blood coagulation. *Biochemistry (Mosc)* **67**, 3-12.

Butenas, S., van't Veer, C., Cawthern, K., Brummel, K.E. and Mann, K.G. (2000) Models of blood coagulation. *Blood Coagulation and Fibrinolysis* **11**, 9-13.

Camerer, E., Huang, W. and Coughlin, S.R. (2000) Tissue factor- and factor X-dependent activation of protease-activated receptor 2 by factor VIIa. *Proceedings of the National Academy of Sciences* **97**, 5255-5260.

Caplice, N.M., Panetta, C., Peterson, T.E., Kleppe, L.S., Mueske, C.S., Kostner, G.M., Broze, G.J. and Simari, R.D. (2001) Lipoprotein (a) binds and inactivates tissue factor pathway inhibitor: a novel link between lipoproteins and thrombosis. *Blood* **98**, 2980-2987.

Chan, J.C., et al. (1999) Factor VII deficiency rescues the intrauterine lethality in mice associated with a tissue factor pathway inhibitor deficit. *Journal of Clinical Investigation* **103**, 475-482.

Chan, J.C., (2001) Gene targeting in hemostasis. Factor VII. *Frontiers in Bioscience* **6**, 98-104.

Chan, J.C., (2001) Gene targeting in hemostasis. Tissue factor pathway inhibitor. *Frontiers in Bioscience* **6**, 216-221.

Chang, J., Stafford, D.W. and Straight, D.L. (1995) The roles of factor VII's structural domains in tissue factor binding. *Biochemistry* **34**, 12227-12232.

Clarke, B.J., Ofosu, F.A., Sridhara, S., Bona, R.D., Rickles, F.R., Blajchman, M.A. (1992) The first epidermal growth factor domain of human coagulation factor VII is essential for binding with tissue factor. *FEBS letters* **298**, 206-210.

Cortese, R., Monaci, P., Nicosia, A., Luzzago, A., Felici, F., Galfré, G., Pessi, A., Tramontano, A. and Sollazzo, M. (1995) Identification of biologically active peptides using random libraries displayed on phage. *Current Opinion in Biotechnology* **6**, 73-80.

Das, B., Meirovitch, H. (2001) Optimisation of solvation models for predicting the structure of surface loops in proteins. *Proteins* **43**, 303-314.

Davie, E.W., Fujikawa, K. and Kisiel, W. (1991) The coagulation cascade: initiation, maintenance, and regulation. *Biochemistry* **30**, 10363-10370.

DeCristofaro, R. (2002) Conformational transitions in factor VIIa: can we stabilize the inactive form of the enzyme? *Thrombosis and Haemostasis* **87**, 4-6.

De Kruif, J. Terstappen, L. Boel, E. and Logtenberg, T. (1995) Rapid selection of cell subpopulation-specific human monoclonal antibodies from a synthetic phage antibody library. *PNAS* **92**, 3938-3942.

Dennis, M.S., Eigenbrot, C. and Skelton, N.J. (2000) Peptide exosite inhibitors of factor VIIa as anticoagulants. *Nature* **404**, 465-470.

Dennis, M.S. and Lazarus, R.A. (1994a) Kunitz domain inhibitors of tissue factor-factor VIIa. I. Potent inhibitors selected from libraries by phage display. *The Journal of Biological Chemistry* **269**, 22129-22136.

Dennis, M.S. and Lazarus, R.A. (1994b) Kunitz domain inhibitors of tissue factor-factor VIIa. II. Potent and specific inhibitors by competitive phage selection. *The Journal of Biological Chemistry* **269**, 22137-22144.

Dennis, M.S., Roberge, M., Quan, C. and Lazarus, R.A. (2001) Selection and characterization of a new class of peptide exosite inhibitors of coagulation factor VIIa. *Biochemistry* **40** , 9513-9521.

Devlin, J.J., Panganiban, L.C. and Devlin, P.E. (1990) Random peptide libraries: a source of specific binding molecules. *Science* **249**, 404-406.

Dickinson, C.D. and Ruf, W. (1997) Active site modification of factor VIIa affects interactions of the protease domain with tissue factor. *The Journal of Biological Chemistry* **272**, 19875-19879.

Dixon, M. and Webb, E.C. (1979) Enzyme inhibition and activation. In: Dixon, M., Webb, E.C., Thorne, C.J.R. and Tipton, K.F. (Eds.) *Enzymes*, pp 332-467. London: Longman Group Ltd.

Doorbar, J. and Winter, G. (1994) Isolation of a peptide antagonist to the thrombin receptor using phage display. *Journal of Molecular Biology* **244**, 361-369.

Duggan, B.M., Dyson, H.J. and Wright, P.E. (1999) Inherent flexibility in a potent inhibitor of blood coagulation, recombinant nematode anticoagulant protein c2. *European Journal of Biochemistry* **265**, 539-548.

Dybwad, A., Bogen, B., Natvig, J.B., Forre, O. and Sioud, M. (1995) Peptide phage libraries can be an efficient tool for identifying antibody ligands for polyclonal antisera. *Clinical Experimental Immunology* **102**, 438-442.

Edgington, T.S., Dickinson, C.D. and Ruf, W. (1997) The structural basis of function of the TF.FVIa complex in the cellular initiation of coagulation. *Thrombosis and Haemostasis* **78** , 401-405.

Edgington, T.S., Mackman, N., Brand, K. and Ruf, W. (1991) The structural biology of expression and function of tissue factor. *Thrombosis and Haemostasis* **66**, 67-79.

Eigenbrot, C. (2002) Structure, function and activation of coagulation factor VII. *Current. Protein and Peptide Sciences* **3**, 287-299.

Eigenbrot, C. and Kirchhofer, D. (2002) New insights into how tissue factor allosterically regulates factor VIIa. *TCM* **12**, 19-26.

Eigenbrot, C., Kirchhofer, D., Dennis, M.S., Santell, L., Lazarus, R.A., Stamos, J. and Ultsch, M.H. (2001) The factor VII zymogen structure reveals reregistration of beta strands during activation. *Structure* **9**, 627-636.

Fabiola, G.F., Bobde, V., Damodharan, L., Pattabhi, V., Durani, S. (2001) Conformational preferences of heterochiral peptides. Crystal structures of heterochiral peptides Boc-(D)Val-(D)Ala-Leu-Ala-OMe and Boc-Val-Ala-Leu-(D)Ala-OMe-enhanced stability of beta-sheet through C-H-O hydrogen bonds. *Journal of Biomolecular Structure Dynamics* **18**, 579-594.

Fisher, K.L., Gorman, C.M., Vehar, G.A., O'Brien, D.P. and Lawn, R.M. (1987) Cloning and expression of human tissue factor cDNA. *Thrombosis Research* **48**, 89-99.

Gachet, C. (2001) ADP receptors of platelets and their inhibition. *Thrombosis and Haemostasis* **86**, 222-232.

George, J.N. (2000) Platelets. *The Lancet* **355**, 1531-1539.

Ghrib, F., Leger, P., Ezban, M., Kristensen, A., Cambus, J. and Boneu, B. (2001) Anti-thrombotic and haemorrhagic effects of active site-inhibited factor VIIa in rats. *British Journal of Haematology* **112**, 506-512.

Giesen, P.L.A. and Nemerson, Y. (2000) Tissue factor on the loose. *Seminars in Thrombosis and Hemostasis* **26**, 379-384.

Giesen, P.L.A., Rauch, U., Bohrmann, B., Kling, D., Fallon, J.T., Badimon, J.J., Himber, J., Riederer, M.A. and Nemerson, Y. (1999) Blood-borne tissue factor: another viem of thrombosis. *Proceedings of the National Academy of Sciences* **96**, 2311-2315.

Girard, T.J., Warren, L.A., Novotny, W.F., Likert, K., Brown, S.G., Miletich, J.P. and Broze, G.J. (1989) Functional significance of the Kunitz-type inhibitory domains of lipoprotein-associated coagulation inhibitor. *Nature* **338**, 518-520.

Golino, P., Ragni, M., Cirillo, P., D'Andrea, D., Scognamiglio, A., Ravera, A., Buono, C., Ezban, M., Corcione, N., Vigorito, F., Condorelli, M. and Chiariello, M. (1998) Antithrombotic effects of recombinant human, active site-blocked factor VIIa in a rabbit model of recurrent arterial thrombosis. *Circulation Research* **82**, 39-46.

Goodson, R.J., Doyle, M.V., Kaufman, S.E. and Rosenberg, S. (1994) High-affinity urokinase receptor antagonists identified with bacteriophage peptide display. *Proceedings of the National Academy of Sciences* **91**, 7129-7133.

Hagen, F.S., Gray, C.L. and O'Hara, P. (1986) Characterization of a cDNA coding for human factor VII. *Proceedings of the National Academy of Sciences* **83**, 2412-2416.

Hanson, S.R. and Kotzé, H.F. (1986) Radionuclide Labelled Cellular Blood Elements In: Du P Heyns, A., (Ed) *Applications in atheroclerosis and thrombosis*, pp. 69-75. Tygerberg: MRC Press.

Harker, L.A., Hanson, S.R., Wilcox, J.N. and Kelly, A.B. (1996) Antithrombotic and antilesion benefits without hemorrhagic risks by inhibiting tissue factor pathway. *Haemostasis* **26** , 76-82.

Higashi, S. and Iwanaga, S. (1998) Molecular interaction between factor VII and tissue factor. *International Journal of Hematology* **67**, 229-241.

Higashi, S., Matsumoto, N. and Iwanaga, S. (1996) Molecular mechanism of tissue factor-mediated accelaration of factor VIIa activity. *The Journal of Biological Chemistry* **271**, 26569-26574.

Higashi, S., Nishimura, H., Aita, K. and Iwanaga, S. (1994) Identification of regions of bovine factor VII for binding to tissue factor. *The Journal of Biological Chemistry* **269**, 18891-18898.

Higashi, S., Nishimura, H., Fujii, S., Takada, K. and Iwanaga, S. (1992) Tissue factor potentiates the factor VIIa-catalyzed hydrolysis of an ester substrate. *The Journal of Biological Chemistry* **267**, 17990-17996.

Hirsh, J. and Weitz, J.I (1999) Thrombosis: New antithrombotic agents. *Lancet* **353**, 1431-1436.

Hirsh, J., Ginsberg, J.S. and Marder, V.J. (1994) In: Colman, R.W., Hirsh, J., Marder, V.J. and Salzman, E.W., (Eds) *Haemostasis and thrombosis: Basic principles and clinical practice*, pp. 1567-1583. Philadelphia: JB Lippincott.

Hjort, P.F. (1597) Intermediate reactions in the coagulation of blood with tissue thromboplastin. *The Scandinavian Journal of Clinical & Laboratory Investigation* **9** (27):1-182.

Hong, S.S. and Boulanger, P. (1995) Protein ligands of the human adenovirus type 2 outer capsid identified by biopanning of phage-displayed peptide library on separate domains of wild-type and mutant penton capsomers. *EMBO Journal* **14**, 4714-4727.

Hoogenboom, H.R., Lutgerink, J.T., et al. (1999) Selection-dominant and nonaccessible epitopes on cell-surface receptors revealed by cell-panning with a large phage antibody library. *European Journal of Biochemstry* **260**, 774-784.

Huang, M., Syed,R., Stura,E.A., et al. (1998) The mechanism of an inhibitory antibody on TF-initiated blood coagulation revealed by the crystal structures of human tissue factor, Fab 5G9 and TF.G9. *The Journal of Molecular Biology* **275**, 873-894.

Huang, Q., Neuenschwander, P.F., Rezaie, A.R. and Morrissey, J.H. (1996) Substrate recognition by tissue factor-factor VIIa. *The Journal of Biological Chemistry* **271**, 21752-21757.

Hufton, S.E., Moerkerk, P.T., Meulemans, E.V., De Bruïne, A., Arends, J. and Hoogenboom, H.R. (1999) Phage display of cDNA repertoires: the pVI display system and its applications for the selection of immunogenic ligands. *Journal of Immunological Methods* **231**, 39-51.

Hutton, R.A., Laffan, M.A. and Tuddenham, E.G.D. (1999) Normal Haemostasis. In: Hoffbrand, A.V., Lewis, S.M. and Tuddenham, E.G.D., (Eds.) *Postgraduate Haematology*, pp. 550-580. Oxford: Butterworth Heinemann.

Iakhiaev, A., Pendurthi, U.R. and Rao, L.V.M. (2001) Active site blockade of factor VIIa alters its intracellular distribution. *The Journal of Biological Chemistry* **276**, 45895-45901.

Jackson, S.P., Mistry, N. and Yuan, Y. (2000) Platelets and the injured vessel wall - "Rolling into action". *TCM* **10**, 192-197.

Jespers, L.S., Messens, J.H., De Keyser, A., Eeckhout, D., et al. (1995) Surface expression and ligand-based selection of cDNAs fused to filamentous phage gene VI. *Biotechnology* **13**, 378-382.

Johnson, K. and Hung, D. (1998) Novel anticoagulants based on inhibition of the factor VIIa/tissue factor pathway. *Coronary Artery Disease* **9**, 83-87.

Joung, J.K., Ramm, E.I. and Pabo, C.O. (2000) A bacterial two-hybrid selection system for studying protein-DNA and protein-protein interactions. *PNAS* **97**, 7382-7387.

Katz, B.A. (1997) Structural and mechanistic determinants of affinity and specificity of ligands discovered or engineered by phage display. *Annual Review of Biophysics and Biomolecular Structure* **26**, 27-45.

Kay, B.K. and Hoess, R.H. (1996) Principles and applications of phage display. In: Kay, B.K., Winter, J. and McCafferty, J., (Eds.) *Phage display of peptides and proteins*, 1st ed. pp. 21-34. San Diego: Academic Press Inc.

Kelley, R.F., Refino, C.J., O'Connell, M.P., Modi, N., Sehl, P., Lowe, D., Pater, C. and Bunting, S. (1997) A soluble tissue factor mutant is a selective anticoagulant and antithrombotic agent. *Blood* **89**, 3219-3227.

Kemball-Cook, G., Johnson, D.J., Tuddenham, E.G.D. and Harlos, K. (1999) Crystal structure of active site-inhibited hukan coagulation factor VIIa (des-Gla). *Journal of Structural Biology* **127**, 213-223.

Kirchhofer, D., Eigenbrot, C., Lipari, M.T/, Maran, P., Peek, M. and Kelley, R.F. (2001) The tissue factor region that interacts with factor Xa in the activation of factor VII. *Biochemistry* **40**, 675-682.

Kisiel, W. and McMullen, B.A. (1981) Isolation and characterization of human factor VII_a. *Thrombosis Research* **22**, 375-380.

Koivunen, E., Wang, B. and Ruoslahti, E. (1994) Isolation of a highly specific ligand for the alpha 5 beta 1 integrin from a phage display library. *The Journal of Cell Biology* **124**, 373-380.

Kornberg, A. (1980) *DNA Replication*, 1 st ed. San Francisco: Freeman.

Kotzé, H.F., Lamprecht, S., Badenhorst, P.N. (1995) A four hour infusion of recombinant hirudin results in long-term inhibition of arterial-type thrombosis in baboons. *Blood* **85**, 3158-3163.

Krishnaswamy, S. (1992) The interaction of human factor VIIa with tissue factor. *The Journal of Biological Chemistry* **267**, 23696-23706.

Kumar, A. and Fair, D.S. (1993) Specific molecular interaction sites on factor VII involved in factor X activation. *European Journal of Biochemistry* **217**, 518

Larocca, D. and Baird, A. (2001) receptor-mediated gene transfer by phage display vectors: applications in functional geneomics and gene therapy. *Drug Discovery Today* **6**, 793-800.

Lener, D., Benarous, R. and Calogero, R.A. (1995) Use of a constrain phage displayed-peptide library for the isolation of peptides binding to HIV-1 nucleocapsid protein (NCp7). *FEBS Letters* **361**, 85-88.

Leonard, B.J.N., Clarke, B.J., Sridhara, S., Kelley, R., Ofosu, F.A. and Blajchman, M.A. (2000) Activation and active site occupation alter conformation in the region of the first epidermal growth factor-like domain of human factor VII. *The Journal of Biological Chemistry* **275**, 34894-34900.

Lindhout, T., Franssen, J. and Willems, G. (1995) Kinetics of the inhibition of tissue factor-factor VIIa by tissue factor pathway inhibitor. *Thrombosis and Haemostasis* **74**, 910-915.

Lowman, H.B., Bass, S.H., Simpson, N. and Well, J.A. (1991) Selecting high-affinity binding proteins by monovalent phage display. *Biochemistry* **30**, 10832-10838.

Mackman, N. (2001) Gene targeting in hemostasis. Tissue factor. *Frontiers in Bioscience* **6**, 208-215.

Makowski, L. (1994) Phage display: structure, assembly and engineering of filamentous bacteriophage M13. *Current Opinion in Structural Biology* **4**, 225-230.

Mann, K.G., Nesheim, M.E., Church, W.R., Haley, P. and Krishnaswamy, S. (1990) Surface-dependent reactions of the vitamin K-dependent enzyme complexes. *Blood* **76**, 1-16.

Markwardt, F. (1990) Hirudin and derivates as anticoagulant agents. *Thrombosis and Haemostasis* **66**, 141-152.

Maruyama, I.N., Maruyama, H.I. and Brenner, S. (1994) Lambda foo: a lambda phage vector for the expression of foreign proteins. *Proceedings of the National Academy of Sciences* **91**, 8273-8277.

Mast, A.E., Stadanlick, J.E., Lockett, J.M., Dietzen, D.J., Hasty, K.A. and Hall, C.L. (2000) Tissue factor pathway inhibitor binds to platelet thrombospondin-1. *The Journal of Biological Chemistry* **275**, 31715-31721.

Masys, D.R., Bajaj, S.P. and Rapaport, S.I. (1982) Activation of human factor VII by activated factors IX and X. *Blood* **60**, 1143-1150.

McCallum, C.D., Hapak, R.C., Neuenschwander, P.F., Morrissey, J.H. and Johnson, A.E. (1996) The location of the active site of blood coagulation factor VIIa above the membrane surface and its

reorientation upon association with tissue factor. *The Journal of Biological Chemistry* **271**, 28168-28175.

McLafferty, M.A., Kent, R.B., Ladner, R.C., and Markland, W. (1993) M13 bacteriophage displaying disulfide-constrained microproteins. *Gene* **128**, 29-36.

McVey, J.H. (1994) Tissue factor pathway. *Baillière's Clinical Haematology* **7**, 469-484.

Meiring, S.M. (1996) Plasma elimination of recombinant (r)-hirudin and the r-hirudin-thrombin complex in baboons. University of the Orange Free State.

Messing, J. (1983) New M13 vectors for cloning. *Methods in Enzymology* **101**, 20-78.

Miller, C.L., Graziano, C., Lim, R.C. and Chin, M. (1981) Generation of tissue factor by patient monocytes: correlation to thromboembolic complications. *Thrombosis and Haemostasis* **46**, 489-95.

Morrissey, J.H. (2001) Tissue factor: an enzyme cofactor and a true receptor. *Thrombosis and Haemostasis* **86**, 66-74.

Morrissey, J.H., Neuenschwander, P.F., Huang, Q., McCallum, C.D., Su, B. and Johnson, A.E. (1997) Factor VIIa-tissue factor: functional importance of protein-membrane interactions. *Thrombosis and Haemostasis* **78**, 112-116.

Nakagaki, T., Foster, D.C., Berkner, K.L. and Kisiel, W. (1991) Initiation of the extrinsic pathway of blood coagulation: evidence for the tissue factor dependent autoactivation of human coagulation factor VII. *Biochemistry* **30**, 10819-10824.

Nemerson, Y. (1986) An ordered addition, essential activation model of the tissue factor pathway of coagulation: evidence for a conformational cage. *Biochemistry* **25**, 4020-4033.

Nemerson, Y. (1988) Tissue factor and haemostasis. *Blood* **71**, 8.

Nemerson, Y. and Repke, D. (1985) Tissue factor accelerates the activation of coagulation factor VII: the role of a bifunctional coagulation cofactor. *Thrombosis Research* **40**, 351-358.

Neuenschwander, P.F. and Morrissey, J.H. (1992) Deletion of the membrane anchoring region of tissue factor abolishes autoactivation of factor VII but not cofactor function. *The Journal of Biological Chemistry* **267**, 14477-14482.

Neuenschwander, P.F. and Morrissey, J.H. (1995) Alteration of the substrate and inhibitor specificities of blood coagulation factor VIIa: importance of amino acid residue K^{192}. *Biochemistry* **34**, 8701-8707.

New England Biolabs (1998b) Ph.D.-12™ Phage display peptide library kit. Instructional manual. New England Biolabs: New England Biolabs.

New England Biolabs (1998a) Ph.D.-C7C™ Phage display peptide library kit. Instruction Manual. New England Biolabs: New England Biolabs.

New England Biolabs (2000) Phage display peptide libraries.

Novotny, W.F. (1994) Tissue factor pathway inhibitor. *Seminars in Thrombosis and Hemostasis* **20**, 101-108.

O'Brien, D.P. (1989) The molecular biology and biochemistry of tissue factor. *Baillière's Clinical Haematology* **2**, 801-820.

O'Neil, K.T., Hoess, R.H., Jackson, S.A., Ramachandran, N.S., Mousa, S.A. and DeGrado, W.F. (1992) Identification of novel peptide antagonists for GPIIb/IIIa from a conformationally constrained phage peptide library. *Proteins* **14**, 509-515.

Ofosu, F.A. (2002) The blood platelet as a model for regulating blood coagulation on cell surfaces and its consequences. *Biochemistry (Mosc)* **67**, 47-55.

Ofosu, F.A., Liu, L. and Freedman, J. (1996) Control mechanisms in thrombin generation. *Seminars in Thrombosis and Hemostasis* **22**, 303-308.

Örning, L., Fischer, P.M., Hu, C., Agner, E., Engebretsen, M., Husbyn, M., Petersen, L.B., Ørvim, U., Llinas, M. and Sakaríassen, K.S. (2002) A cyclic pentapeptide derived from the second EGF-like domain of factor VII is an inhibitor of tissue factor dependent coagulation and thrombus formation. *Thrombosis and Haemostasis* **87**, 13-21.

Örning, L., Stephens, R.W., Petersen, L.B., Hamers, M.J.A.G., Stormorken, H. and Sakaríassen, K.S. (1997) A peptide sequence from the EGF-2 like domain of FVII inhibits TF-dependent FX activation. *Thrombosis Research* **86**, 57-67.

Ørvim, U., Barstad, R.M., Örning, L., Petersen, L.B., Ezban, M., Hedner, U. and Sakaríassen, K.S. (1997) Antithrombotic efficacy of inactivated active site recombinant factor VIIa is shear dependent in human blood. *Arteriosclerosis, Thrombosis, and Vascular Biology* **17**, 3049-3056.

Ørvim, U., Roald, H.E., Stephens, R.W., Roos, N. and Sakaríassen, K.S. (1994) Tissue factor-induced coagulation triggers platelet thrombus formation as efficiently as fibrillar collagen at arterial blood flow conditions. *Arteriosclerosis and Thrombosis* **14**, 1976-1983.

Østerud, B. (2001) The role of platelets in decrypting monocyte tissue factor. *Seminars in Hematology* **38**, 2-5.

Østerud, B. and Flaegstad, T. (1983) Increased tissue thromboplastin activity in monocytes of patients with meningococcal infection: related to an unfavourable prognosis. *Thrombosis and Haemostasis* **49**, 5-7.

Owenius, R., Osterlund, M., Svensson, M., Lindgren,M. et al. (2001) Spin and fluorescent probing of the binding interface between tissue factor and factor VIIa at multiple sites. *Biophysics Journal* **81**, 2357-2369.

Pasqualini, R. and Ruoslahti, E. (1996) Organ targeting in vivo using phage display peptide libraries. *Nature* **380**, 364-366.

Pearce, J. (2001) Going round in circles to avoid proteolysis. *Trends in Biochemical Sciences* **26**, 282.

Pendurthi, U.R. and Rao, L.V.M. (2002) Factor VIIa/tissue factor-induced signaling: a link between clotting and disease. *Vitamins and Hormones* **64**, 323-355.

Persson, E. (2000) Structure of human coagulation activated factor VII. *Blood Coagulation and Fibrinolysis* **11**, 15-17.

Phizicky, E.M. and Fields, S. (1995) Protein-protein interactions: methods for detection and analysis. *Microbiological Reviews* **59**, 94-123.

Pike, A.C.W., Brzozowski, A.M., Robert, S.M., Olsen, O.H. and Persson, E. (1999) Structure of human factor VIIa and its implications for the triggering of blood coagulation. *Proceedings of the National Academy of Sciences* **96**, 8925-8930.

Radcliffe, R. and Nemerson, Y. (1975) Activation and control of factor VII by activated factor X and thrombin. *The Journal of Biological Chemistry* **250**, 388-395.

Rao, L.V.M., Nordfang, O., Hoang, A.D. and Pendurthi, U.R. (1995) Mechanism of antithrombin III inhibition of factor VIIa/tissue factor activity on cell surfaces. Comparison with tissue factor pathway inhibitor/factor Xa-induced inhibition of factor VIIa/tissue factor activity. *Blood* **85**, 121-129.

Rao, L.V.M. and Rapaport, S.I. (1987) Studies of a mechanism inhibiting the initiation of the extrinsic pathway of coagulation. *Blood* **69**, 645-651.

Rao, L.V.M. and Ruf, W. (1995) Tissue factor residues Lys165 and Lys166 are essential for rapid formation of the quaternary complex of tissue factor.VIIa with Xa.tissue factor pathway inhibitor. *Biochemistry* **34**, 10867-10871.

Rapaport, S.I. (1991) The extrinsic pathway inhibitor: a regulator of tissue factor-dependent blood coagulation. *Thrombosis and Haemostasis* **66**, 6-15.

Rapaport, S.I. and Rao, L.V.M. (1995) The tissue factor pathway: how it has become a "Prima Ballerina". *Thrombosis and Haemostasis* **74**, 7-17.

Rauch, U., Bonderman, D., Bohrmann, B., Badimon, J.J., Himber, J., Riederer, M.A. and Nemerson, Y. (2000) Transfer of tissue factor from leukocytes to platelets is mediated by CD15 and tissue factor. *Blood* **96**, 170-175.

Rauch, U. and Nemerson, Y. (2000) Tissue factor, the blood, and the arterial wall. *TCM* **10**, 139-142.

Renschler, M.F., Bhatt, R.R., Dower, W.J. and Levy, R. (1994) Synthetic peptide ligands of the antigen binding receptor induce programmed cell death in a human B-cell lymphoma. *Proceedings of the National Academy of Sciences* **91**, 3623-3627.

Ribeiro, M.J., Phillips, D.J., Benson, J.M., Evatt, B.L., Ades, E.W. and Hooper, W.C. (1995) Hemostatic properties of the SV-40 transfected human microvascular endothelial cell line (HMEC-1). A representative in vitro model for microvascular endotheliu. *Thrombosis Research* **79**, 153-161.

Riewald, M. and Ruf, W. (2001) Mechanistic coupling of protease signaling and initiation of coagulation by tissue factor. *Proceedings of the National Academy of Sciences* **98**, 7742-7747.

Roberge, M., Peek, M., Kirchhofer, D., Dennis, M.S. and Lazarus, R.A. (2002) Fusion of two distinct peptide exosite inhibitors of factor VIIa. *Biochemical Journal* **363**, 387-393.

Roberge, M., Santell, L., Dennis, M.S., Eigenbrot, C., Dwyer, M.A. and Lazarus, R.A. (2001) A novel exosite on coagulation factor VIIa and its molecular interactions with a new class of peptide inhibitors. *Biochemistry* **40**, 9522-9531.

Roberts, B.L., Markland, W., Ley, A.C., Kent, R.B., White, D.W., Guterman, S.K. *et al.* (1992) Directed evolution of a protein: selection of potent neutrophil elastase inhibitors displayed on M13 fusion phage. *Proceedings of the National Academy of Sciences* **89**, 2429-2433.

Rodi, D.J. and Makowski, L. (1999) Phage-display technology - finding a needle in a vast molecular haystack. *Current Opinion in Biotechnology* **10**, 87-93.

Rosen, E.D. *et al.* (1997) Mice lacking factor VII develop normally but suffer fatal perinatal bleeding. *Nature* **390**, 290-294.

Roth, G.J. (1992) Platelets and blood vessels. *Immunology Today* **13**, 100-105.

Roy, S., Hass, P.E., Bourell, J.H., Henzel, W.J. and Vehar, G.A. (1991) Lysine residues 165 and 166 are essential for the cofactor function of tissue factor. *The Journal of Biological Chemistry* **266**, 22063-22066.

Ruf, W. (1994) Factor VIIa residue Arg^{290} is required for efficient activation of the macromolecular substrate factor X. *Biochemistry* **33**, 11631-11636.

Ruf, W. (1998) The interaction of activated factor VII with tissue factor: insight into the mechanism of cofactor-mediated activation of activated factor VII. *Blood Coagulation and Fibrinolysis* **9**, S73-S78.

Ruf, W. and Dickinson, C.D. (1998) Allosteric regulation of the cofactor-dependent serine protease coagulation factor VIIa. *TCM* **8**, 350-356.

Ruf, W. and Edgington, T.S. (1994) Structural biology of tissue factor, the initiator of thrombogenesis in vivo. *The FASEB Journal* **8**, 385-390.

Ruf, W., Rehemtulla, A. and Edgington, T.S. (1991) Phospholipid-independent interactions required for tissue factor receptor and cofactor function. *The Journal of Biological Chemistry* **266**, 2158-2166.

Sakaríassen, K.S., Aarts, P.A.M.M., DeGroot, P., Houdijk, W.P.M. and Sixma, J.J. (1983) A perfusion chamber developed to investigate platelet interaction in flowing blood with human vessel wall cells, their extracellular matrix, and purified components. *Journal of Laboratory and Clinical Medicine* **102**, 522-535.

Sakaríassen, K.S., Hanson, S.R. and Cadroy, Y. (2001) Methods and models to evaluate shear-dependent and surface reactivity-dependent antithrombotic efficacy. *Thrombosis Research* **104**, 149-174.

Salzman, E.W. and Hirsh, J. (1993) The epidemiology, pathogenesis and natural history of venous thrombosis. In: Colman, R.W., Hirsh, J., Marder, V., Salzman, E.W., (Eds.) Hemostasis and thrombosis. Basic principles and clinical practice. pp 1275-1296. Philadelphia. J.B. Lippincott.

Sambrook, J. and Russell, D.W. (2001) *Molecular Cloning. A laboratory manual*, 3rd edn. New York: Cold Spring Harbor Laboratory Press.

Schneider, C.L. (1947) The active principle of placental toxin: thromboplastin; its inactivation in blood: antithromboplastin. *American Journal of Physiology* **149**, 123-129.

Scott, J.K. (2000) Peptide libraries. In: Barbas, C.F., Burton, D.R., Scott, J.K. and Silverman, G.J., (Eds.) Phage display: a laboratory manual, 1st ed. pp 4.1-4.13. New York: Cold Spring Harbor Laboratory Press.

Scott, J.K. and Smith, G.P. (1990) Searching for peptide ligands with an epitope library. *Science* **249**, 386-390.

Semeraro, N. and Colucci, M. (1997) Tissue factor in health and disease. *Thrombosis and Haemostasis* **78**, 759-764.

Sidhu,S.S. (2000) Phage display in pharmaceutical biotechnology. Current opinion in *Biotechnology* **11**, 610-616.

Silverman, G.J. (2000) Construction and selection from cDNA phage-display. In: Barbas, C.F., Burton, D.R., Scott, J.K. and Silverman, G.J., (Eds.) Phage display: a laboratory manual, 1st ed. pp 21.1-21.34. New York: Cold Spring Harbor Laboratory Press.

Smith, G.P. (1985) Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. *Science* **228**, 1315-1317.

Smith, G.P. (1993) Surface display and peptide libraries. *Gene* **128**, 1-2.

Smith, G.P. and Petrenko, V.A. (1997) Phage display. *Chemical Reviews* **97**, 391-410.

Smith, M.M., Shi, L. and Navre, M. (1995) Rapid identification of highly active and selective substrates for Stromelysin and Matrilysin using bacteriophage peptide display libraries. *The Journal of Biological Chemistry* **270**, 6440-6449.

Soderstrom, T., Hedner, U. and Arnljots, B. (2001) Active site-inactivated factor VIIa prevents thrombosis without increased surgical bleeding: topical and intravenous administration in a rat model of deep arterial injury. *Journal of Vascular Surgery* **33**, 1072-1079.

Sorensen, B.B., Persson, E., Freskgård, P., Kjalke, M., Ezban, M., Williams, T. and Rao, L.V.M. (1997) Incorporation of an active site inhibitor in factor VIIa alters the affinity for tissue factor. *The Journal of Biological Chemistry* **272**, 11863-11868.

Soumillion, P., Jespers, L., Boucher, M., Marchand-Brynaert, J., Winter, G. and Fastrez, J. (1994) Selection of α – lactomase on filamentous bacteriophage by catalytic activity. *Journal of Molecular Biology* **237**, 415-422.

Spicer, E.K., Horton, R., Bloem, L., Bach, R., Williams, K.R., Guha, A., Kraus, J., Lin, T., Nemerson, Y. and Konigsberg, W. (1987) Isolation of cDNA clones coding for human tissue factor: primary structure of the protein

and cDNA. *Proceedings of the National Academy of Sciences* **84**, 5148-5152.

Stanssens, P., Bergum, P.W., Gansemans, Y., Jespers, L., Laroche, Y., Huang, S., Maki, S.L., Messens, J., Lauwereys, M., Cappello, M., Hotez, P.J., Lasters, I. and Vlasuk, G.P. (1996) Anticoagulant repertoire of the hookworm *Ancylostoma caninum*. *Proceedings of the National Academy of Sciences* **93**, 2149-2154.

Sternberg, N. and Hoess, R.H. (1995) Display of peptides and proteins on the surface of bacteriophage lambda. *Proceedings of the National Academy of Sciences* **92**, 1609-1613.

Szardenings, M., Tornroth, S., et al. (1997) Phage display selection on whole cells yields a peptide specific for melanocortin receptor 1. *Journal of Biological Chemistry* **272**, 27943-27948.

Thomas, L. (1947) Studies on the intravascular thromboplastic effect of tissue suspensions in mice. *Bulletin of the Johns Hopkins Hospital* **81**, 26-42.

Tuddenham, E.G.D. (1996) The tissue factor-factor VII complex: recent advances towards elucidating the structure and function of the initiator of haemostasis. *Haemostasis* **26**, 20-24.

Tuddenham, E.G.D. and Cooper, D.N. (1994) Factor VII. In: Motulsky, A.G., Bobrow, M., Harper, P.S. and Scriver, C., (Eds.) *The Molecular Genetics of Haemostasis and its Inherited Disorders*, pp. 112-121. Oxford: Oxford University Press]

Tuddenham, E.G.D. and Cooper, D.N. (1994) Tissue factor. In: Motulsky, A.G., Bobrow, M., Harper, P.S. and Scriver, C., (Eds.) *The Molecular Genetics of Haemostasis and its Inherited Disorders*, pp. 194-203. Oxford: Oxford University Press]

Van Meijer, M., Roelofs, Y., Neels, J., Horrevoets, A.J.G., Van Zonneveld, A. and Pannekoek, H. (1996) Selective screening of a large phage display library of plasminogen activator inhibitor 1 mutants to localize interaction sites with either thrombin or the variable region 1 of tissue-type plasminogen activator. *The Journal of Biological Chemistry* **271**, 7423-7428.

Webster, R. (2001) Filamentous phage biology. In: Barbas, C.F., Burton, D.R., Scott, J.K. and Silverman, G.J., (Eds.) *Phage Display. A Laboratory Manual*, 1st ed. pp. 1.1-1.37 New York: Cold Spring Harbor Laboratory Press]

Webster, R.E. (1996) Biology of the filamentous bacteriophage. In: Kay, B.K., Winter, J. and McCafferty, J., (Eds.) *Phage display of peptides and proteins*, 1st ed. pp. 1-20. San Diego: Academic Press Inc.]

Weitz, J.I. (1997) Low molecular weight heparins. *New England Journal of Medicine* **337**, 688-698.

Wesselschmidt, R., Likert, K., Girard, T.J., Wun, T.C. and Broze, G.J. (1992) Tissue factor pathway inhibitor: the carboxy-terminus is required for optimal inhibition of factor Xa. *Blood* **79**, 2004-2010.

Wildgoose, P., Foster, D., Wiberg, F.C., Birktoft, J.J. and Petersen, L.C. (1993) Identification of a calcium binding site in the protease domain of human blood coagulation factor VII: evidence for its role in factor VII-tissue factor interaction. *Biochemistry* **32**, 114-119.

Wilson, D.R. and Finlay, B.B. (1998) Phage display: applications, innovations, and issues in phage and host biology. *Canadian Journal of Microbiology* **44**, 313-329.

Winthrop, M.D., Denardo, G.L. and Denardo, S.J. (2000) Antibody phage display applications for nuclear medicine imaging and therapy. *Quarterly Journal of Nuclear Medicine* **44**, 284-295.

Wittrup, K.D. (1999) Phage on display. *Trends in Biotechnology* **17**, 423-424.

Wright, R.M., Gram, H., Vattay, A., Byme, S., Lake, P. and Dottavio, D. (1995) Binding epitope of somatostatin defined by phage-displayed peptide libraries. *Biotechnology* **13**, 165-169.

Wun, T.C., Kretzmer, K.K., Girard, T.J., Miletich, J.P. and Broze, G.J. (1988) Cloning and characterization of a cDNA coding for the lipoprotein-associated coagulation inhibitor shows that it consists of three tandem Kunitz-type inhibitory domains. *The Journal of Biological Chemistry* **263**, 6001-6004.

Yamamoto, M., Nakagaki, T. and Kisiel, W. (1992) Tissue factor-dependent autoactivation of human blood coagulation factor VII. *The Journal of Biological Chemistry* **267**, 19089-19094.

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