FVa inactivation by APC and protein S

A thesis submitted to Imperial College London

for the degree of

Doctor of Philosophy in the Faculty of Medicine

by

Magdalena Maria Gierula, MSc

April 2018

Centre for Haematology
Faculty of Medicine
Imperial College London
Hammersmith Hospital Campus

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ABSTRACT

Factor Va enhances thrombin generation by several orders of magnitude. Its function is controlled by activated protein C (APC)-mediated and protein S enhanced proteolysis. The aim of my thesis was to clarify the molecular mechanisms underlying FVa inactivation. For this, all three proteins were recombinantly produced and characterised. As APC-mediated FVa inactivation is phospholipid dependent, assays were optimised to investigate interactions in the presence of phospholipids.

I have shown that protein S and FVa act together to enhance APC association with phospholipid membranes. The presence of protein S is mandatory, as FVa by itself does not increase binding of APC to phospholipids. These findings strongly suggest that APC, protein S and FVa together form an inactivation complex on phospholipid surfaces.

Unlike FVa, FVIIIa does not enhance APC binding to phospholipids, indicating that FVIIIa does not form a similar complex with APC and protein S. C4BP-bound protein S did not efficiently enhance APC-phospholipid binding, indicating that C4BP interfers with complex formation. Results I obtained with protein S variants with impared APC cofactor function, and FV Nara, associated with strong APC resistance, indicate that their mutations essentially abolished their ability to assemble into the tri-molecular complex.

FV-810, with partial B-domain deletion, assembled the complex as efficiently as FVa. Protein S was required for complex enhancement by FV-810. However, results for FV-810 obtained with a FVa inactivation assay indicated that effective inactivation can be achieved in the absence of protein S. These findings suggest that there must be an alternative mechanism involved in APC cofactor function which is not mediated solely by increased binding of APC to phospholipids.

In conclusion, my findings demonstrate that FVa promotes its own APC-mediated degradation by enhancing APC binding to phospholipids together with protein S, but also suggest that more than a single mechanism controls FVa inactivation.

DECLARATION OF ORIGINALITY

I, Magdalena Maria Gierula, hereby declare that the work presented in this thesis is my own. All work and data analysis of the results were performed by myself, unless otherwise specified in the text.

Magdalena Maria Gierula

ACKNOWLEDGEMENTS

I would like to take this opportunity to thank everyone who has supported me during the course of my PhD.

First and foremost, I would like to thank my supervisors, Prof. David Lane, Dr Jim Crawley and Dr Josefin Ahnström, for all of their support and guidance throughout my project. It has been a privilege to be a part of your research team. I am grateful to Prof. David Lane and Dr Josefin Ahnström for reading my dissertation in such a short space of time and providing valuable feedback.

I am incredibly grateful to Dr Josefin Ahnström, for constant encouragement and genuine care about me, both in and out of work. Josefin, you have been a dedicated mentor whose faith in me has helped me to develop as a researcher, thank you.

I would like to express my gratitude to the current and former members of the Haemostasis and Thrombosis group. It has been a great journey that I have had the pleasure of sharing with amazing colleagues.

Isabelle, I am indebted to you for your advice and help with flow cytometry. Adrienn and Salvo, I greatly appreciate all your help in the lab. I have truly enjoyed all our scientific and non-scientific discussions. Ishani, you were a great student, thank you for your hard work on the protein C part of the project. A special thanks to Mary, Adela, Angela and Patricia for keeping me sane during the roller coaster of my PhD. Tassos, I hope that one day I will learn your attitude to life, it really is inspiring. Agata and Natalia, thank you for the warm welcome in the lab and all your support. You have made the start of my doctorate really enjoyable. Agata, no one has ever sung so beautifully for me.

Ola and Marta, thank you for all lunch and coffee breaks and being such good listeners.

I am also thankful to my family who have supported me along the way. Julia, the smile on your face has helped to keep me going. Przemek, Mum thank you for stepping into my motherhood responsibilities. Without your invaluable help, this thesis would have never been completed.

Chciałam również podziękować Całej Mojej Rodzinie, która wspierała mnie przez cały okres moich studiów. Julia, uśmiech na Twojej twarzy pomagał przetrwać najgorsze. Przemku, Mamo, dziękuję za pomoc w moich macierzyńskich obowiązkach. Bez Waszej pomocy ta praca nie powstałaby.

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ABBREVIATIONS

+Ctrl	positive control
A	absorbance
a.a.	amino acid
A2AP	α2-antiplasmin
ADP	adenosine 5-diphosphate
Ang1	angiopoetin 1
AP	activation peptide
APC	activated protein C
APC-FEGRCK	FEGRCK-active site labelled APC
APMSF	amidinophenylmethanesulfonyl fluoride hydrochloride
APS	ammonium persulfate
AT	antithrombin
BHK	baby hamster kidney
bp	base pair
BSA	bovine serum albumin
BTBSCa^{2+}	0.5% BSA in 50mM Tris, pH 7.4, 150mM NaCl and 5mM $CaCl_2$
C4BP	C4b-binding protein
CAT	calibrated automated thrombography
CCM	concentrated conditioned media
CCP	complement control protein domains
cDNA	complimentary deoxyribonucleic acid
CTI	corn trypsin inhibitor
CV	column volume
DEAE	diethylaminoethyl cellulose anion exchange
DMEM/F12	Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DOPC	1,2-Dioleoyl-*sn*-glycero-3-phosphocholine
DOPE	1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine
DOPS	1,2-Dioleoyl-*sn*-glycero-3-phosphoserine
dsDNA	double-stranded DNA
DVT	deep venous thrombosis
ε	extinction coefficient
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
EPCR	endothelial protein C receptor
ETP	endogenous thrombin potential
F	factor
FBS	foetal bovine serum
FDP	fibrin degradation products
FT	flow through
G418	Geneticin®
GAS6	growth arrest specific 6
Gla	γ-carboxylated glutamic acid
GPIa/IIa	platelet receptor glycoprotein Ia/IIa
GPIbα	platelet receptor glycoprotein Ibα
GVI	glycoprotein VI
HEK	human embryonic kidney fibroblast

HiM	high molecular weights marker
HMWK	high molecular weight kininogen
HRP	horseradish peroxidise
Hyn	β-hydroxyasparagine
IL6	Interleukin 6
ITS	insulin, transferrin and sodium selenite
k_{cat}	turnover number
K_d	dissociation constant
kDa	kilodaltons
K_m	Michaelis-Menten constant
LB	Bertani medium
LG	laminin G-type
LPS	Lipopolysaccharide
mA	milli absorbance
mAU	milli absorbance unit
MEM	minimum essential media
MFI	mean fluorescence intensity
mRNA	messanger ribonucleic acid
OPD	o-phenylenediamine dihydrochloride
PAI-1	plasminogen activator inhibitor -1
PAI-2	plasminogen activator inhibitor-2
PAR1	protease-activated receptors 1
PAR4	protease-activated receptors 4
PBS	phosphate buffered saline
PC	phosphatidylcholine
PCR	polymerase chain reaction
PE	phosphatidylethanolamine
PEI	linear polyethylenimine
PI	phosphatidylinositol
pp	plasma purified
PS	phosphatidylserine
PolyP	polyphosphate
QFF	Q sepharose fast flow
r	recombinant
Reg	regeneration fraction
RT	room temperature
S1P	sphingosine 1 phosphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SHBG	sex hormone binding globulin
SP	serine protease
SP FF	SP Sepharose Fast Flow
Sphk1	sphingosine kinase 1
SphM	sphingomyelin
SPR	surface plasmon resonance
TAFI	thrombin activated fibrinolysis inhibitor
TAM	familie of tyrosine kinases receptors, namely Tyro3, Axl, Mer
TBS	20mM Tris-HCl pH 7.4, 150mM NaCl
TBSCa2+	20mM Tris-HCl pH 7.4, 150mM NaCl, 5mM $CaCl_2$ buffer
$TBSTCa^{2+}$	20mM Tris, pH 7.4, 150mM NaCl, 5mM $CaCl_2$ and 0.1% Tween 20
TCA	trichloroacetic acid
TEMED	tetramethylethylenediamine

TF	tissue factor
TFF	tangential flow filtration
TFPI	tissue factor pathway inhibitor
Th	thrombin
TLR	toll-like receptors
TM	thrombomodulin
TNFα	tumor necrosis factor α
tPA	tissue plasminogen activator
Tris	tris(hydroxymethyl)aminomethane
TSR	thrombin sensitive region
TXA_2	thromboxane A2
uPA	urokinase plasminogen activator
VWF	von Willebrand factor
WT	wild-type

Common abbreviations not spelled out in the text

°C	Celcius degrees
g	gram
L	liter
m	meter
M	molar
mol	mole
SD	standard deviation
V	volt
%	percentage

Power prefix abbreviations

m	milli
µ	micro
n	nano
p	pico

Amino acid abbreviations

Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

1 INTRODUCTION

1.1 Haemostasis

1.1.1 Overview of Haemostasis

Haemostasis is a natural mechanism with a main function the prevention of bleeding. It also acts to keep the blood in a fluid state. Despite the importance of this process, our knowledge and understanding of all mechanisms involved in haemostasis is incomplete. In the next few sections, I will outline our current understanding of haemostasis.

Haemostasis involves several processes that for convenience can be divided into primary, secondary haemostasis and fibrinolysis. Primary haemostasis involves vasoconstriction of blood vessels and formation of an unstable platelet plug, whereas secondary haemostasis describes the coagulation of blood. Both processes are coordinated to limit blood loss from injury. Fibrinolysis refers to the break-down of the blood clot and the subsequent vessel repair mechanisms.[1-3]

1.1.1.1 Primary haemostasis

The initial step after vascular injury is vasoconstriction. Vasoconstriction is caused by contraction of the smooth muscle cells lining the blood vessel. It is enhanced by thromboxane A_2 (TXA_2), released from activated platelets and the injured endothelium. Vasoconstriction is thought to be an important protective mechanism for damaged small blood vessels.[4]

Platelets are the essential cellular component of primary haemostasis. Their adhesion, activation and aggregation leads to formation of an unstable platelet plug. In healthy blood vessels, platelets circulate freely near the vessel wall, but do not interact with the endothelial cell surface.[5,6] The endothelium expresses molecules such as nitric oxide, prostacyclin and endothelial ectonucleotidase which prevent activation of platelets.[7] However, upon disruption of the endothelium, the subendothelial matrix, containing collagen, von Willbrand factor (VWF), laminin and fibronectin, becomes exposed to the circulating blood. Two of these proteins, collagen and VWF, are major platelets ligands. VWF is a large, multimeric glycoprotein, which is not only present in subendothelial tissue, but is also stored in endothelial cells, circulates in plasma and is stored in platelet α-granules. Upon vascular

injury, circulating VWF binds to exposed collagen. The platelets adhere to immobilized VWF through a direct interaction between its A1 domain and the platelet receptor glycoprotein Ibα (GPIbα).[6] The GPIbα-VWF complex has also been found to enhance exposure of phosphatidylserine, which is important for thrombin formation (see section 1.1.3).[8] The interaction between the A1 domain of VWF and the platelet receptor GPIbα is essential for haemostasis, and deficiency of either VWF or GPIbα are associated with bleeding disorders known as von Willebrand disease and Bernard-Soulier syndrome, respectively.[6,9,10]

There are other glycoprotein receptors present on platelets, which upon activation bind to adhesive proteins such as collagen, elastin and laminin.[8] One of these is the GPIa/IIa receptor which facilitates direct binding of platelets to collagen. The platelets are also captured by collagen through an interaction mediated by glycoprotein VI (GVI). Collagen is potent platelet activator and its exposure initiates thrombus formation. Upon interaction with collagen, platelets become activated. There are several distinct intracellular signalling activation pathways. Adenosine 5-diphosphate (ADP), serotonin, thromboxane A_2 (TXA_2) and thrombin all are able to activate platelets.[2,11] Once activated, platelets undergoes shape changes and the contents of their α and dense granules are relased. These relased components include ADP, VWF, fibrinogen and serotonin. Release of stored VWF leads to adhesion of additional platelets. Released fibrinogen causes aggregation of platelets by binding and bridging to GPIIb/IIIa receptors, the most abundant platelet integrin. TXA_2, which is produced by activated platelets, is an efficient activator, but can also induce smooth muscle cell contraction, leading to vasoconstriction of the vessel.[6] ADP also efficiently activates platelets through two receptors, P2Y1 and P2Y12. ADP together with TXA_2 greatly enhances platelet aggregation. Activated platelets undergo degranulation, which triggers a positive feedback loop. More platelets are recruited to the site of injury and platelet-platelet interactions occur through fibrinogen bridging.[7] Accumulated platelets form an unstable primary plug, that then has to be strengthened and stabilised by fibrin generated through the coagulation pathways.

1.1.1.2 Secondary haemostasis

Secondary haemostasis is the formation of insoluble, cross-linked fibrin, which acts to stabilise the primary platelet plug. Like primary haemostasis, secondary haemostasis is initiated upon disruption of endothelium, when tissue factor (TF) becomes exposed to the

circulating blood. TF triggers the coagulation cascade through the activation of coagulation factors resulting in formation of thrombin, which converts soluble fibrinogen to insoluble fibrin.

1.1.1.3 Coagulation cascade

A waterfall/cascade model of coagulation, still commonly used in lectures and in text books, was introduced in 1964 by two research groups.[12,13] This model is based upon the coagulation cascade being triggered either by a vascular injury (extrinsic pathway) or anionic artificial structures (intrinsic pathway) (Figure 1.1).

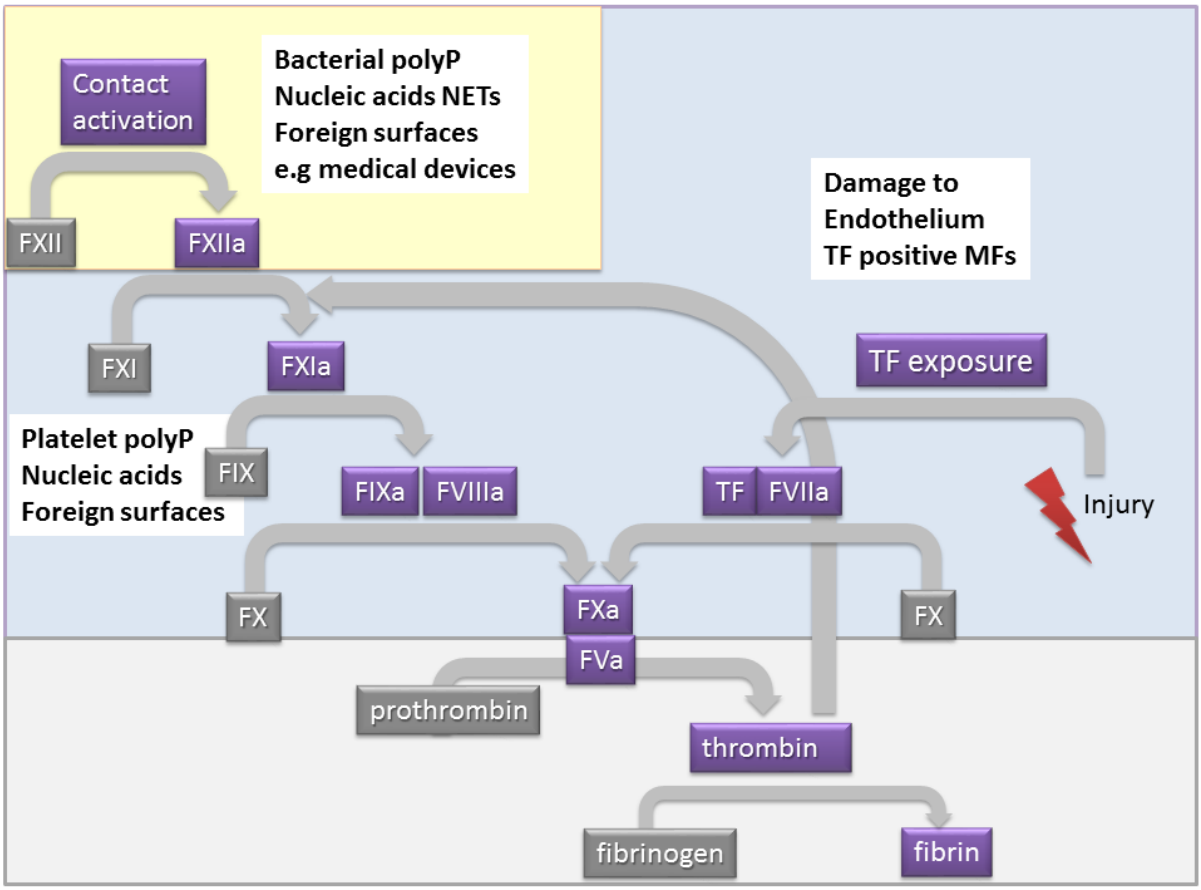


Figure 1.1. Waterfall/cascade model of blood coagulation.
The blood coagulation can be initiated by the intrinsic or the extrinsic pathway. The cascade of reactions leads to formation of thrombin which subsequently converts fibrinogen to fibrin and stabilises the clot. This simplified model of coagulation is based upon early influencial publications.[12,13]

The extrinsic pathway is initiated by TF once it is exposed to the blood after a vessel injury. Partially activated coagulation factor (F)VII (FVIIa), which is circulating in the blood, forms an extrinsic tenase complex together with tissue factor (TF) and this converts FX to its active

form, FXa. In the intrinsic pathway, FXII is activated by contact with anionic surfaces into FXIIa. FXIIa then activates FXI in the presence of its cofactor, high molecular weight kininogen (HMWK). Subsequently, FXIa activates FIX into FIXa. FIXa together with FVIIIa form the intrinsic tenase complex, which then activates FX to FXa in the common pathway. FXa, which can be generated by both pathways, forms the prothrombinase complex with its cofactor FVa and prothrombin. This complex rapidly converts prothrombin to thrombin. A small amount of generated thrombin disosiates FVIII from VWF, activates platelets and coagulation factors FV, FVIII, FXI which leads to acceleration of thrombin formation.[14] Subsequently, thrombin generates fibrin that stabilises the clot and a solid thrombus is formed.

Our understanding of the mechanisms involved in haemostasis is constantly evolving. The cascade model of coagulation, while an important conceptual advance, could not address the questions arising from clinical practice. For example, it was not clear why patients with haemophilia A and B (deficiency of FVIII and FIX, respectively) bleed if there is an alternative pattern of FX activation.[15] The surface dependent model of haemostasis, addressing some of unresolved issues, was introduced in 1992 by Mann.[16] The cell-based model of coagulation emphasises the function of cells in providing an active surface upon which activated coagulation factors could be assembled. A developed cell model of blood coagulation includes three phases: initiation, amplification and propagation (Figure 1.2).[17]

1.1.1.3.1 Initiation phase

The initiation phase is triggered upon injury and disruption of the endothelium, resulting in exposure of TF to the blood stream. TF is a 47kDa transmembrane protein, which is expressed mainly on the surface of smooth muscle cells and adventitia cells, which are not exposed to the blood stream under normal physiological conditions. Upon vessel injury TF becomes exposed to blood and is able to bind circulating FVIIa. FVII is the precursor form (zymogen) of a serine protease, FVIIa. In contrast to other coagulation factors, it circulates in plasma in a partially active form, as well as its zymogen. The molecular mechanisms of FVII activation are complex yet to be completely clarified. However, in addition to TF, FIXa, FXa, FXIIa thrombin and plasmin are all believed to activate FVII.[18] It has also been suggested that FVII is able to activate itself.

Once bound to TF, FVIIa becomes fully active and activates FIX and FX to their active forms, FIXa and FXa, respectively. During this initiation phase, FXa only generates small amounts of thrombin. This is due to rapid inhibition of the TF/FVIIa/FXa complex by the specific inhibitor tissue factor pathway inhibitor (TFPI; see further details in section 1.1.2). Also another coagulation inhibitor, antithrombin, down-regulates the generation of thrombin by inhibiting FXa and thrombin (see Figure 1.2). However, the small amounts of thrombin generated is enough to move the coagulation cascade into the amplification phase.

1.1.1.3.2 Amplification phase

Platelets adhered to the site of injury play a pivotal role in blood coagulation. Platelets are activated by collagen and other agents (see above), but can also be activated by thrombin generated during the initiation phase through specific protease-activated receptors 1 and 4 (PAR1 and PAR4). Activated platelets provide a procoagulant membrane surffface upon which coagulation factors can accumulate and where further coagulation is facilitated. Upon activation, platelets release partially activated FV from their α-granules. Importantly, during this amplification phase, thrombin activates FV and FVIII, the latter released from its carrier protein VWF, into FVa and FVIIIa. FVa and FVIIIa are two important cofactors for the assembly of the prothrombinase and intrinsic tenase complexes, respectively.[15,19] A number of studies have shown that thrombin also can activate FXI.[20-23] Oliver *et al*. demonstrated that thrombin mediated activation of FXI was enhanced by activated platelets, while a study by Choi strongly suggested that this activation is greatly accelerated by polyphosphate (polyP) secreted from activated human platelets.[24,25] Given that FXIa is an efficient activator of FIX it can therefore be argued that FXI activation by thrombin is an important part of the amplification phase as the activation of FV and FVIII.

1.1.1.3.3 Propagation Phase

During the propagation phase, FIXa assembles into the intrinsic tenase complex together with its cofactor FVIIIa on the surface of activated platelets. Some FXIa, generated during the initiation phase by TF/FVIIa, but also FXIa converts FIX into its active form, FIXa. The FIXa/FVIIIa complex then activates FX to FXa. It has been established that the FIXa/FVIIIa mediated activation of FX is ~50-fold more potent than that mediated by TF/FVIIa.[18,26] The essential role of this tenase complex for efficient thrombin generation is reflected in two

bleeding disorders. Haemophilia A is caused by deficiency of FVIII and deficiency of FIX leads to haemophilia B. Both diseases are associated with spontaneous bleeding into joints, muscles and internal organs.[2] Once FXa is generated it forms the prothrombinase complex together with FVa, rapidly generating large quantities of thrombin. The presence of FVa enhances thrombin generation during the propagation phase by several orders of magnitude.[27] Thrombin converts fibrinogen into insoluble fibrin which, together with thrombin-activated FXIII, stabilises the clot.[15,18]

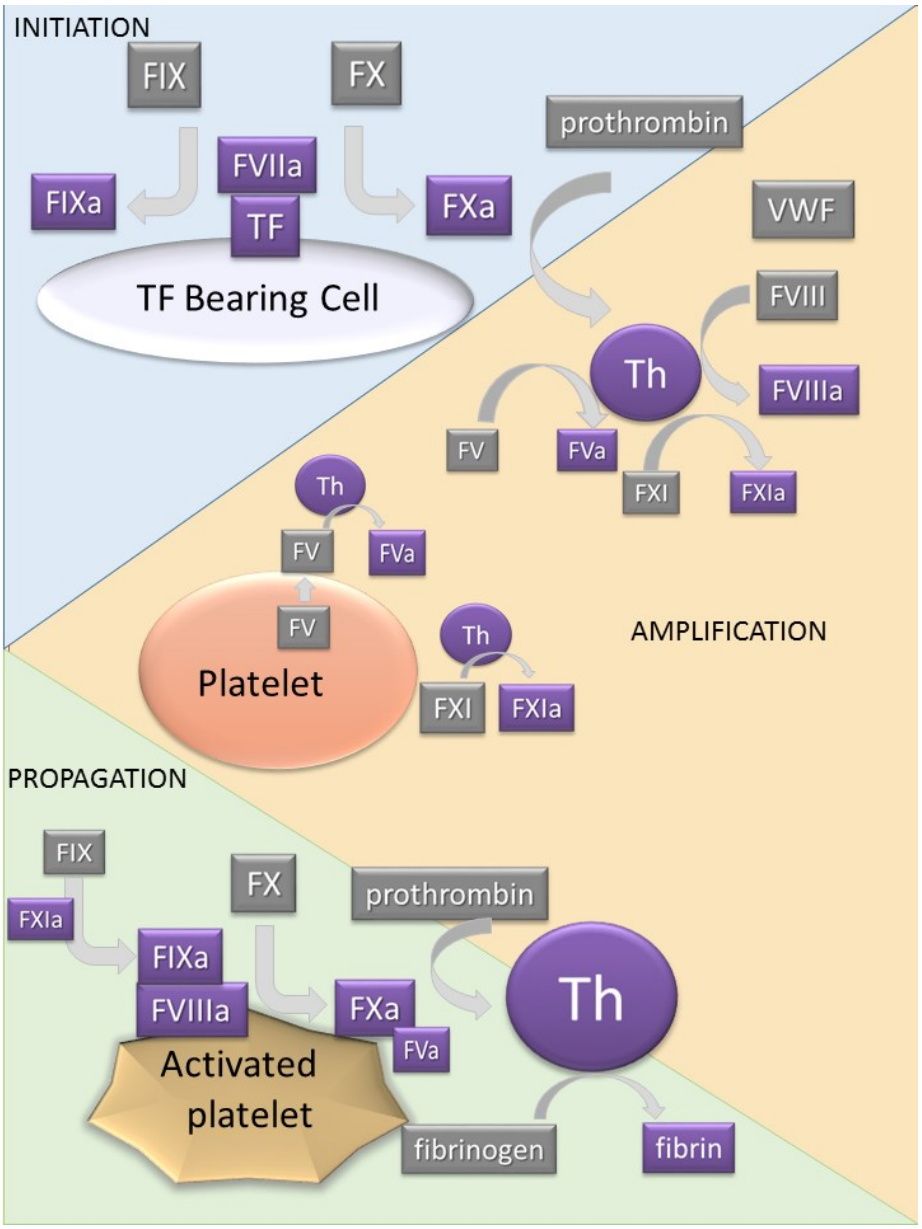


Figure 1.2. The cell model of the blood coagulation
There are three phases in the cell model of the blood coagulation: initiation, amplification and propagation. The initiation phase occurs on TF bearing cells, while the propagation phase takes place on the activated platelets.Th, thrombin. Adapted from Hoffman *et al.,*2003.[17]

1.1.1.4 Fibrinolysis

The role of the third phase of haemostasis is to degrade blood clots and thereby prevent vessel occlusion, thrombosis.[1] The fibrin clot has to be broken down and the tissue repaired. Fibrinolysis is a process responsible for the degradation of the clot. Plasmin, the main protease of this process, is produced in the liver as an inactive zymogen, plasminogen. Plasminogen is incorporated into the fibrin clot during its formation. To initiate wound healing, plasminogen is activated and plasmin starts to degrade the clot.[28] Activation of plasminogen is mediated by tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA). Fibrin enhances its own degradation by binding to both plasminogen and tPA. Plasminogen activation mediated by fibrin-bound tPA is ~500-fold more efficient than the activation in solution.[28,29] Once activated, plasmin cleaves fibrin into small fragments, called fibrin degradation products (FDP). FDP may have predictive and prognostic value as elevated levels are associated with conditions such as pulmonary embolism, DVT or cancer induced thrombosis.[30-32]

Fibrinolysis is tightly regulated by inhibitors of plasmin and plasminogen activator. There are three main serine protease inhibitors, plasminogen activator inhibitor-(PAI-1), plasminogen activator inhibitor-2 (PAI-2), and α2-antiplasmin (A2AP). While PAI-1 and PAI-2 inhibit plasminogen activators tPA and uPA, A2AP inactivates plasmin not bound to fibrin. Thrombin activated fibrinolysis inhibitor (TAFI), is a carboxypeptidase which cleaves off the C-terminal lysine and arginine residues on fibrin, and, by doing so, removes plasminogen binding sites. It is therefore also an inhibitor of fibrinolysis.[28]

1.1.2 Anticoagulant pathways

While inherited diseases with impaired haemostasis causing a bleeding phenotype (such as haemophilia A and B) are rare, excessive clotting, thrombosis is common, particularly deep venous thrombosis (DVT). Blood coagulation is closely regulated during its different phases to prevent excessive thrombin formation and blood clotting. There are three main anticoagulant pathways, the tissue factor pathway inhibitor (TFPI), activated protein C (APC) and antithrombin pathways, see Figure 1.3.

TFPI is an important inhibitor of the initiation step of coagulation. TFPI inhibits activation of FX and FIX through inhibition of the TF/VIIa complex, the activity of FXa directly and the formation of thrombin from the prothrombinase complex.[33-35] TFPI is a protease inhibitor comprising of a N-terminal tail, three Kunitz-domains, Kunitz domains 1, 2 and 3, and a C-terminal tail. The second Kunitz domain directly inhibits FXa, the first Kunitz domain binds to the TF/FVIIa complex and blocks further activation of FX and FIX.[34,35] Protein S and various molecular forms of FV have been found to function as cofactors for TFPI and enhance its anticoagulant activities.[36-39] Protein S and FV have been shown to interact with the third Kunitz domain and the basic C-terminus of TFPI, respectively,[40,41] See sections 1.2.3.3 and 1.4.2.1. for further details.

APC plays an important role in the down-regulation of thrombin generation. This serine protease inactivates FVIIIa and FVa, the cofactors of the intrinsic tenase and prothrombinase complex, respectively.[42-45] APC-mediated proteolysis is calcium and phospholipid dependent. Protein S serves as a cofactor for both APC-catalysed inactivations.[46-48] FV acts as a synergistic cofactor, together with protein S, during FVIIIa inactivation.[49] APC-mediated inactivation of FVa is the main subject of this thesis and will be described in further detail in section 1.3.3.1.1 below.

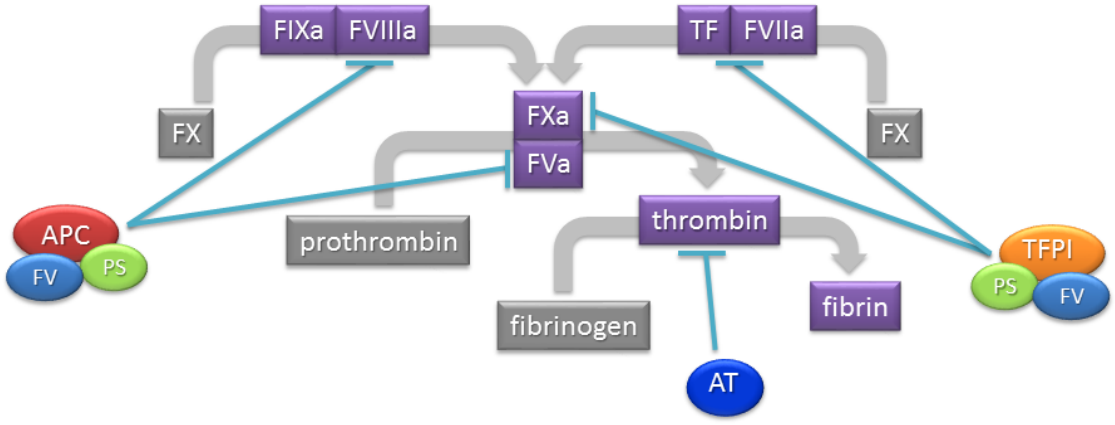


Figure 1.3. Regulation of the blood coagulation by the anticoagulant pathways.
There are three main anticoagulant pathways regulating the blood coagulation, the TFPI, APC and antithrombin pathways. TFPI inhibits FXa and TF/FVIIa. APC inactivates FVa and FVIIIa while AT inhibits thrombin (illustrated) and FVIIa, FIXa, FXa, FXIIa (not illustrated).

Antithrombin is a serine protease inhibitor which inhibits most of the activated coagulation factors that are serineproteases. Antithrombin inhibits thrombin, FIXa, FXa, FXIa, FXIIa and

FVIIa (in the presence of heparin). The role of antithrombin is to "mop up" any active coagulation factors which have "escaped" the site of injury. Similarly to TFPI and APC, antithrombin on its own is an inefficient anticoagulant. Heparin and endogenous heparan sulphates bind to antithrombin through a specific pentasaccharide sequence and stimulate its activity by up to 1000-fold.[50,51]

1.1.3 The importance of membranes

Membrane/protein interactions play a crucial role in blood coagulation, as many of reactions are dependent upon binding to negatively charged phospholipid membranes. The cell model of thrombin generation illustrates the importance of phospholipid membranes. Blood clotting complexes, as well as anticoagulant complexes, assemble on phospholipid surfaces provided by activated endothelial cells or activated platelets.[52] Binding of coagulation factors to the membrane results in an increase of their local concentration, thereby enhancing their binding into active complexes. Phospholipid binding can also lead to conformational changes of the proteins to obtain required orientation for proteolytic activation to occur. Protein/membrane interactions often increases velocity of enzymatic reactions with effects on the Michaelis-Menten constant (K_m) and the turnover number k_{cat}.[53]

The function of a phospholipid membrane strongly depends on its composition. Cell membranes are formed as double layers and comprise 70% of phospholipids. Phospholipid groups include phosphatidylcholine (PC), sphingomyelin (SphM), phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI). The composition of the membrane differs between outer and inner layers. The outer membrane consists mainly of PC and SphM while the inner membrane comprises of negatively charged phospholipids.[52] It was first discovered by Bevers that activated platelets can change the composition of their phospholipid membrane.[54] Phospholipids are translocated between outer and inner layers by phospholipid scramblases in a process called lipid scambling. Translocation of PS has the most explicit effect on the cell surface as this phospholipid is negatively charged at physiological pH and is not normally present in the outer layer. Exposure of PS upon platelet activation has been reported to increase from 2 to 12%.[55] Activated platelets and endothelial cells with exposed negatively charged phospholipids provide a procoagulant surface for coagulant processes. Assembly of the tenase and prothrombinase complexes is completely phospholipid

dependent.[56-59] Also APC-catalysed inactivation of FVa and FVIIIa depends on presence of anionic phospholipids.[48] However, not only PS is essential for coagulation and the regulation of coagulation to occur. PE, which constitute up to 40% of the activated cell surface, has been shown to have a pronounced effect of APC-mediated inactivation of FVa.[54] According to Smirnov *et al*. the presence of PE increases the activity of APC by 3-5 fold.[60,61] In agreement with their study, Norstrøm and colleaugues showed that addition of PE to the phospholipid membranes increased the rate of cleavage at both Arg306 and Arg506 by approximately 3-fold.[62] It has been also reported that the plasma glycolipid, glucosylceramide, enhances the affinity between APC and negatively charged phospholipid membranes by approximately 5-fold.[63]

Many phospholipid binding proteins from both the pro- and anti-coagulant pathways, such as FVII, FX, prothrombin, protein C and protein S, contain a Gla-domain. For these proteins ɣ-carboxylated glutamic acid (Gla) residues are responsible for Ca^{2+}-mediated binding to the phospholipid surface. Binding of calcium is necessary for conformational changes of the nascent Gla-domain that increase its affinity for anionic membranes.[64]

1.2 Factor V

As described above, FV plays an important role in the coagulation cascade. FV circulates in plasma as an inactive procofactor with little or no procoagulant activity.[65] Upon activation, FV exerts procoagulant properties. However, FV also plays important roles in the anticoagulant pathways. Its various functions strongly depend on proteolytic events that result in complex structural changes, described in further detail in what follows.

1.2.1 FV expression and structure

FV is a single-chain glycoprotein with a MW of 330kDa, circulating in plasma with a concentration range of 7 to 14 ug/ml (21-42nM).[66] A partially proteolysed form of FV, comprising 18-25 % of total FV, is found in α-granules of platelets.[67]

FV is mainly synthesised by hepatocytes in the liver,[68,69] but also by megakaryocytes[70]; lymphocytes have also been found to express some FV.[71] The gene encoding FV (*F5*) is located on chromosome 1, region of 1q21-25 and contains 25 exons of different sizes from 72 to 2820

bp.[72,73] FV is homologous to factor VIII and has the same domain organisation, namely A1-A2 B-A3-C1-C2. The A and C domains of FV and FVIII have 30-40% sequence homology while the B domains are not structurally similar.[74]

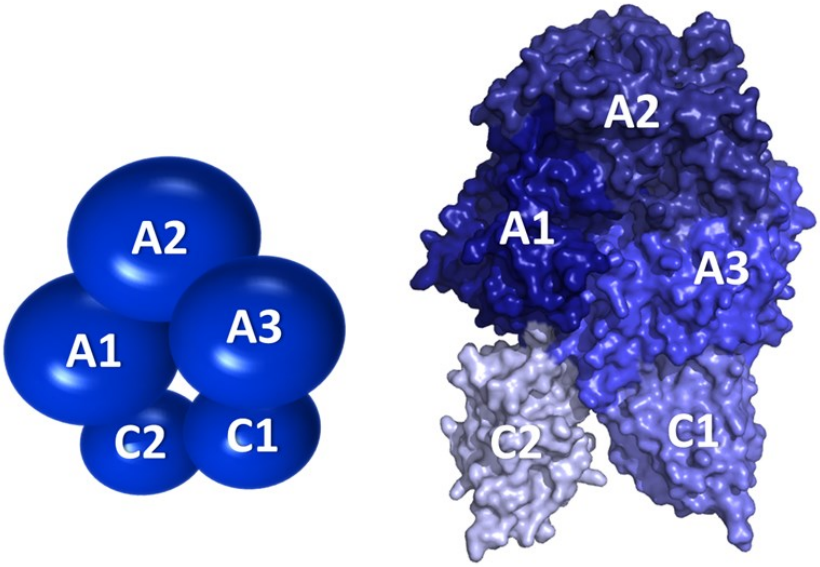


Figure 1.4. Molecular model of FVa.
FVa is composed of a heavy and a light chain which are non-covalently connected. The heavy chain of FVa consists of the A1 and A2 domains while the light chain comprises of the A3, C2 and C1 domains. The model was visualised in PyMOL. Its unpublished coordinates were kindly provided by Prof. Jim Huntington, University of Cambridge.

FV undergoes a number of post-translational modifications such as sulphation, phosphorylation and glycosylation. Sulphation is required for activation by thrombin and full cofactor function. In contrast, sulphation of FV does not have any effect on activation by FXa. The six tyrosine residues upon which sulphation occurs are at positions 696, 698, 1494, 1540, 1515 and 1565.[75,76]

FV is heavily glycosylated, in particular its B domain. Both O-linked and N-linked glycans are present. 37 potential N-linked glycosylation sites are found in the whole protein, 25 of which are localised in the B domain, nine on the heavy chain and three in the light chain.[77,78] FV is present in plasma in two glycosylation isoforms, FV1 and FV2, at a ratio of approximately 30:70 (FV1:FV2).[79] The FV1 form alone is glycosylated at Asn2181 within the C2-domain. Due to this glycosylation, FV1 has decreased affinity to phospholipid membranes, compared to FV2, and is therefore 7-fold less efficient in enhancing thrombin generation The APC cofactor activity of FV1 is also reduced (see section 1.2.3.2).[79-81]

1.2.2 FV activation

FV circulates in blood as a procofactor which has to be activated to express its cofactor function for FXa. FV can be activated by thrombin, meizothrombin (see section 1.2.3.1), factor Xa and other proteases such as Russell's viper venom protease, plasmin, calpain and cathepsin.[82] Only activation by thrombin and FXa are considered to be physiologically relevant.

Thrombin cleaves FV at three positions, Arg709, Arg 1018 and Arg1545 (Figure 1.5), leading to either complete or partial removal of the large B-domain. The resulting active form of FV (FVa) is composed of a heavy (105kDa) and a light chain (74kDa in FV1 or 71 kDa in FV2), which are non-covalently associated in circulation (Figure 1.4 and 1.5).[83-85] The presence of the B-domain maintains the inactive procofactor state of FV and the FXa cofactor activity of FVa therefore depends on efficiency and completeness of activation.[86] The FV B-domain contains an acidic (a.a. 1493-1537) and a basic (a.a. 963-1008) region, both of which have been shown to be highly conserved among species (from fish to mammals). It has been demonstrated that these regions interact with each other and thereby keep FV in an inactive state.[86] While cleavages at Arg709 and Arg1018 lead to partial cofactor function, cleavage at Arg1545 is required for complete removal of the B-domain and full activation of FV (Figure 1.5).[82,87] The first two cleavages are kinetically favoured over the Arg1545 cleavage and may occur at very low thrombin concentrations. Partially activated forms of FV have been suggested to play an important role during the initiation phase of coagulation when the thrombin concentration is low. Molecular forms of FV containing acidic region can be either released by α-granules of platelets or can be generated by FXa during initation phase of coagulation (see Figure1.5). FXa can cleave FV at the same three positions as thrombin, Arg709, Arg1018 and Arg1545. However, the order of cleavages is different compared to activation by thrombin. FXa initially cleaves FV at Arg1018 and subsequently at Arg709, followed by cleavage at Arg1545. An additional FXa cleavage site in FV was reported by Thorelli *et al*. at Arg1765, however the importance of this cleavage site in FV function is not clear.[87]

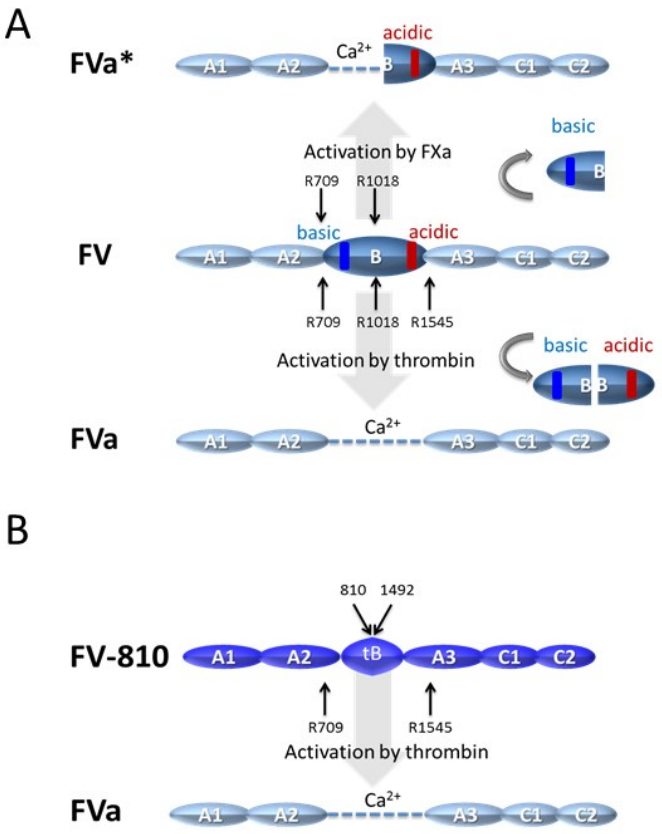


Figure 1.5. Schematic representation of FV and FV-810 activation.
(A) FV is activated by thrombin or FXa by proteolytic cleavage at positions R709, R1018 and R1545. FVa* is a partially activated FV which contains acidic region responsible for interaction with TFPIα. FVa* is generated by FXa-catalised activation of FV during initiation phase of coagulation or released from α-granules of platelets.
(B) FV-810, a B-domain truncated FV derivative containing two thrombin cleavages sites Arg709 and Arg1545. Once activated, FV-810 is a protein indistinguishable from FVa.

While the activation of FV by FXa is often overlooked, a study by Shuijt *et al*. showed that FXa dependent activation of FV may be crucial during the initiation phase of coagulation.[88] Interestingly, Wood *et al.*[40] recently demonstrated that FXa cleavage at FV Arg709 and Arg1018, leads to exposure of the FVa acidic region and thereby available for interaction with TFPI-α. This interaction, in turn, leads to inhibition of the prothrombinase complex during the initiation stages of coagulation (Figure 1.2). Together these results suggest an important role of FV activated by FXa in the initial phases of coagulation, both for its initiation and regulation. FVa activity is in turn regulated by proteolysis by APC, described in detail in sections 1.3.3.1.1.

As mentioned earlier in this thesis, the FV B-domain maintains FV in an inactive procofactor state and it has been shown that the thrombin-mediated proteolysis unmasks binding sites

for the other important members of the prothrombinase complex, i.e. prothrombin and FXa.[89,90]From these findings it was therefore suggested that the main role of the large B-domain is to sterically hinder the formation of the prothrombinase complex. This hypothesis was initially studied by Kane and colleagues who created a FV derivative, FV-810, lacking a substantial part of B domain (residues 811-1491).[91] Unlike WT FV, this variant is expressed as a single chain protein. Using this variant the authors showed that FV-810 was able to form a complex with FXa and prothrombinase as efficiently as FVa, without the need of prior activation. Importantly, due to the truncation of the highly glycosylated B-domain, FV-810 is expressed at up to 5-fold higher level than WT FV and was therefore suggested to be a suitable alternative to the use of FVa in experimental research. [91]

1.2.3 FV function

As mentioned earlier in this thesis, FV circulates in plasma as an inactive procofactor with little or no procoagulant activity.[65] However, FV has also been recognised as an important anticoagulant protein. Its procoagulant and anticoagulant functions are described in the following sections.

1.2.3.1 FXa cofactor function

For many years the only know function of FV, was its procoagulant function. As mentioned already in section 1.1.1.3, activated FV, together with FXa, assemble into the prothrombinase complex on negatively charged phospholipid membranes. This high affinity complex rapidly converts prothrombin to thrombin. The complex is several orders of magnitude more efficient than FXa on its own.[27,56,92] FVa contains binding sites for prothrombin, FXa and phospholipids and exerts its cofactor function through coordinated binding.

FVa has very high affinity towards membrane surfaces, reported to be within a range of 0.01-1nM.[74] It has been demonstrated that FVa enhances binding of FXa and prothrombin to phospholipid surfaces.[56,58,93,94] In the presence of phospholipids the affinity of the FXa/FVa interaction (see model in Figure 1.6) increases to 0.28nM, compared to 3.3nM in the absence of phospholipid membranes.[56,95] Additionally, FVa enhances the catalytic efficiency of FXa by changing the order of proteolytic cleavage of prothrombin. FXa-mediated activation of

prothrombin to thrombin involves two cleavages, at Arg271 and Arg320. Which intermediate cleavage product is formed during activation depends on the order of cleavage. Initial cleavage at Arg320 results in formation of meizothrombin while the activation starting with cleavage at Arg271 leads to the generation of prothrombin 2.

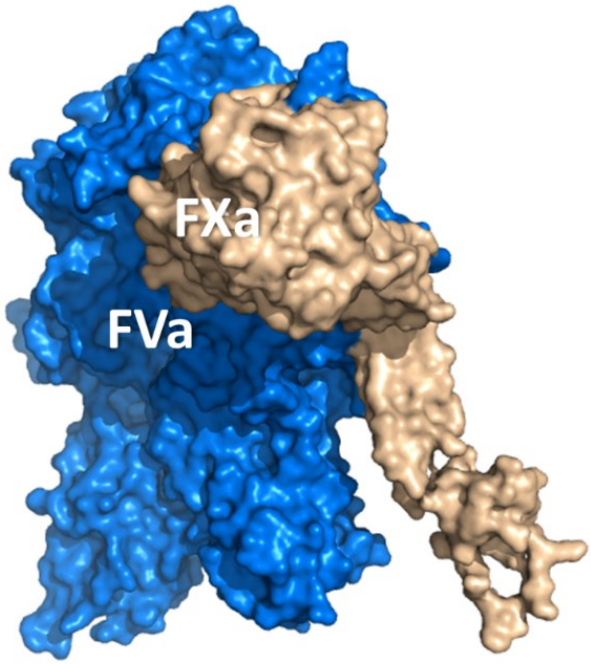


Figure 1.6. Molecular model of the prothrombinase complex.
FXa forms the prothrombinase complex with its cofactor, FVa. FXa binds to FVa with high affinity (K_d 3.3nM) which is even greater in the presence of phospholipids (K_d 0.28nM). The presence of FVa increases thrombin generation by several order of magnitude. This model of the prothrombinase complex, adapted from Lee *et al.,* is visualised using PyMOL software.[96]

In the presence of FVa, activation of prothrombin proceeds through the meizothrombin pathway at rates several order of magnitudes higher than that of the prothrombin 2 pathway, occurring in the absence of FVa.[97,98] Deficiency of FV in humans is associated with mild to severe bleeding problem.[74,99] Surprisingly, considering the importance of the procoagulant functions of FVa, this is not a lethal disease. This can be explained by its anticoagulant functions, described in detail in the following sections.

1.2.3.2 APC- cofactor function

The anticoagulant APC cofactor function of FV was identified shortly after the discovery of the APC resistance in individuals with a FV R506G mutation, commonly known as FV Leiden (see section 1.2.4).[100-104] FV acts as a synergistic APC cofactor, together with protein S, during the

inactivation of FVIIIa, enhancing the degradation by ~11-fold compared to in the absence of cofactors.[49,100,105] It has been demonstrated that the C-terminal part of the FV B-domain (the last 70 amino acids) and its association to the FV A3 domain are required for APC-cofactor function. Therefore, only partially activated FV, cleaved at Arg709 and Arg1018 can function as anticoagulant FV. After proteolysis at Arg1545, the APC-anticoagulant properties of FV are lost.[106] It has also been shown that APC-mediated cleavage at FV Arg506 stimulates its APC cofactor function.[107] Further details on the APC cofactor function of FV can be found in section 1.3.3.1.2.

1.2.3.3 TFPI- cofactor function

TFPI cofactor function is a relatively recent discovery. It has been observed that patients with severe FV deficiency have greatly reduced levels of TFPIα, suggesting that TFPIα levels are somehow regulated by FV levels. Using immunodepletion and direct binding experiments, Ducker *et al*. suggested that FV and TFPIα interact with each other and circulates as a complex with an affinity estimated to be around 13nM.[108] However, the function and importance of this interaction were not fully appreciated at the time of this discovery.

In 2001, a novel FV mutation (A2240G) was identified in a large Texas family with an inherited bleeding disorder.[109] The A2240G mutation led to a substitution of serine to glycine at position 756, localised in the region of the B-domain not required for APC or FXa cofactor activity. Since FV levels and activities in clotting assays were normal, the A2240G mutation was not recognised as a cause of disease. More than 10 years later it was revealed that the A2240G mutation leads to different splicing of FV and as a result a truncated form of FV is generated, so called FV-short.[110] FV-short lacks 702 amino acids from the B-domain, amino acids 756-1458. It was shown that FV-short forms a high affinity complex with TFPIα, much higher affinity than that with full-length FV (90pM). As a result of the increased affinity, the TFPIα concentration in plasma from individuals with the FV A2240G mutation was elevated ~10-fold, which was shown to be the cause of the bleeding disorder.[110]

Shortly after, Wood *et al*. showed that partially activated FV, cleaved at Arg709 and Arg1018 (FXa* in Figure 1.5), which still contains the acidic region of the B-domain, can enhance TFPI mediated inhibition of prothrombinase activity.[40] This was suggested to be mediated through

inhibition of the formation of the protohrombinase complex caused by a high affinity interaction between the acidic region of FV and the basic C-terminal tail in TFPIα.

Protein S is not only a cofactor for APC, but also for TFPI: it enhances TFPIα mediated inhibition of FXa by approximately 4-10 fold.[36,37] Recently our lab demonstrated that full-length FV serves as a synergistic cofactor for TFPI together with protein S during FXa inhibition.[39] Similar to its APC-cofactor function,[106] TFPI cofactor activity of FV is lost after complete activation.[39] These various cofactor roles of protein S are considered further in the sections below.

1.2.4 FV mutations

Since FV exhibits both pro- and anti-coagulant properties, mutations in the FV gene can result in thrombotic as well as bleeding disorders. Parahaemophilia is a rare bleeding disorder described by Owren, which lead to the discovery of FV.[110,111] Individuals with parahaemophilia have deficiency of FV and suffer from mild to severe bleeding symptoms. Another type of FV deficiency is its combined deficiency with FVIII. Low levels of both coagulation factors are caused by mutations in the genes LMAN1 and MCFD2, encoding proteins required for secretion of FV and FVIII.[98,99,112] Combined deficiency of FV and FVIII is associated with mild bleeding problems, occurring mainly after surgery or trauma.

The discovery of APC resistance lead to the identification of the most common mutation in FV, FV Leiden. FV Leiden is a single point mutation in the *F5* gene, resulting in substitution of Arg506 to a glutamine. FV Leiden exerts increased risk of venous thrombosis and is the most common risk factor among Caucasians (prevalence of 2-15% in Europe).[112,113] The prevalence of the FV Leiden mutation is higher in Europe than in Africa, Australia or Asia. In a pooled study from seven European centres and one centre from Brazil, FV Leiden was found in ~20% of all thromboembolism cases.[114] Individuals heterozygous for FV Leiden have 5-fold elevated risk of developing venous thrombosis while this is increased to 80-fold in individuals homozygous for the mutation.[115] Two other mutations, FV Cambridge and FV Hong Kong, with amino acid substitutions at the APC cleavage site at FV Arg306, have been identified in patients presenting thrombosis.[116,117] While the FV Cambridge mutation is an Arg306Thr substitution, the Arg306 residue is mutated to a Gly in FV Hong Kong.

Recently, a novel mutation, FV Nara (W1920R), was identified in a Japanese boy.[118] The mutation is associated with reoccurring DVT. APC mediated FV Nara inactivation was reported to be 11-fold less efficient than that of wild-type (WT) FV and reduced by 4.5 fold in comparison to FV Leiden. Unlike earlier identified FV mutations, FV Nara is the result of an amino acid substitution at position 1920, localised in the FV C1 domain, far from the APC cleavage sites. The authors suggested that Try1920 could be a potential interaction site for either APC or protein S.

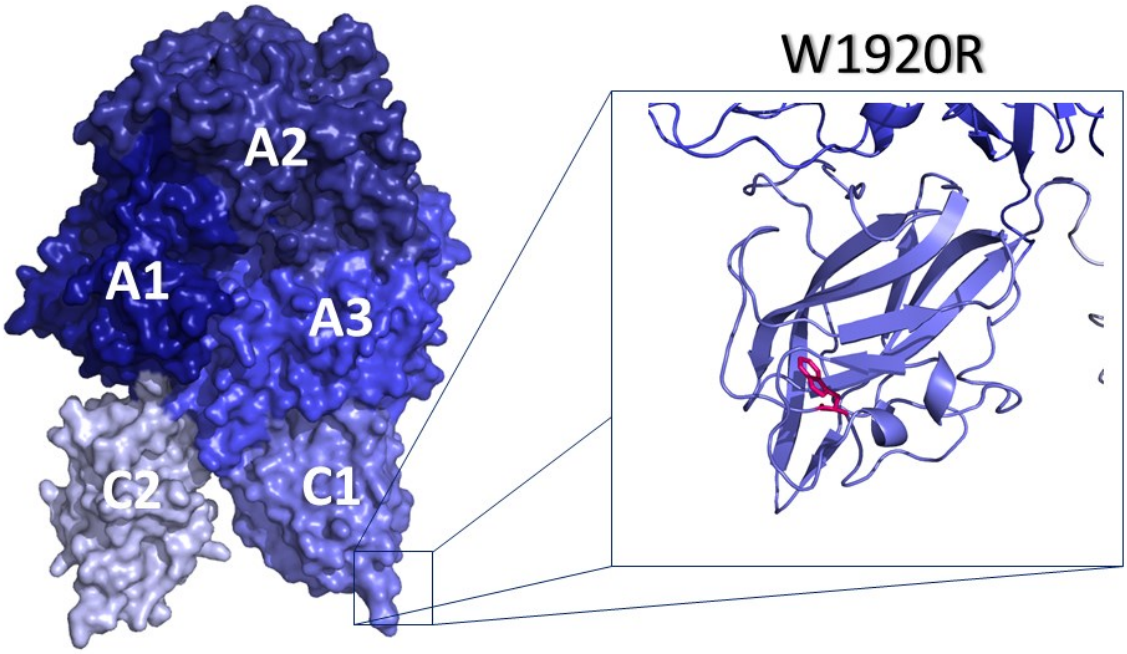


Figure 1.7. Molecular model of FVa with Trp1920R (mutated in FV Nara) highlighted.
The mutation at W1920R is localised in the C1 domain of FV and leads to reduced inactivation by APC. It has been suggested to alter APC binding site on FV. Interestingly, it is not surface exposed. This unpublished model was kindly provided by Prof. Jim Huntington, University of Cambridge.

1.3 Protein C/APC

Protein C is a vitamin K-dependent serine protease circulating in plasma as a zymogen at a concentration of 70nM (4 µg/ml). Protein C is activated by the thrombin/thrombomodulin complex on the endothelium surface. Once activated, activated protein C (APC) exerts its anticoagulant and non anticoagulant functions as outlined in the following sections.[119]

1.3.1 Protein C expression and structure

Protein C is mainly synthesised in the liver and is expressed as a single-chain polypeptide. Mature protein C is 419 amino acids long. The gene of protein C (*PROC*) is located on chromosome 2q13-14 and comprises of nine exons and seven introns.[120,121] Protein C undergoes a number of post-translational modifications such as β-hydroxylation, ɣ-carboxylation, glycosylation and proteolysis. Approximately 85% of protein C in human plasma consist of a heavy (41kDa) and light (21kDa) chain, connected by a disulphide bond. The remaining 15% circulates in plasma as a single chain protein. Despite the structural differences, both forms of protein C exert the same activities.[122] Protein C consists of an N-terminal Gla domain, followed by two epidermal growth factor (EGF)-like domains, a short activation peptide (AP) and a serine protease (SP) domain (see Figure 1.8).

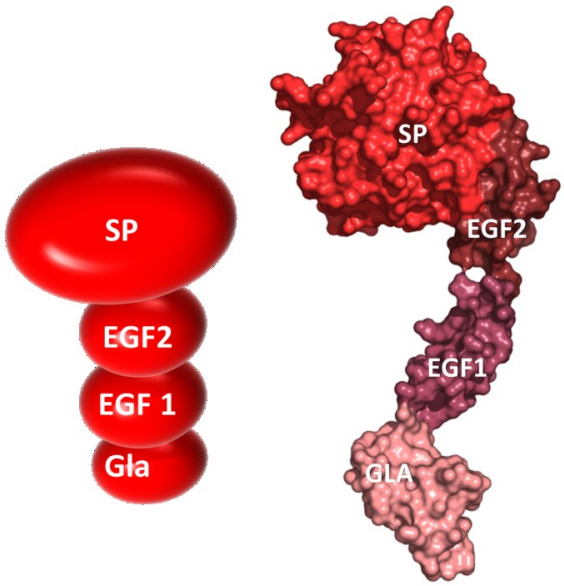


Figure 1.8. Molecular model of protein C.
The majority of protein C circulates in plasma as a two chains protein. A light chain of protein C, composed of a Gla domain and two EGF domains, is linked to a heavy chain (serine protease domain) by a disulphide bond. This molecular model of protein C was adapted from Mather *et al.* and visualised with PyMOL software.[122]

The Gla domain and two EGF domains form the light chain of protein C while the AP and SP domain form the heavy chain.[122,123] The Gla domain mediates its binding to negatively charged phospholipid membranes as well as to the endothelial protein C receptor (EPCR). Direct or indirect binding of APC to phospholipid membranes is essential for its anticoagulant functions. There are 9 ɣ-carboxyglutamic (Gla) residues on the Gla domain of protein C. They are generated by carboxylation of glutamic acid residues. These carboxyl groups coordinate the association of Gla residues with Ca^{2+}. Binding of Ca^{2+} is crucial for correct folding of the Gla domain, anticoagulant properties, as well as for binding to the endothelial protein C receptor (EPCR).[124]

Furthermore, residues Phe4, Lys5 and Lys8 and His10 have been identified to take part in the phospholipid interaction as well as EPCR binding.[125-127] They form a hydrophobic cluster, also called a ω-loop, which are inserted into phospholipids membranes upon binding. The Gla domain has also been shown to be essential for the ability of APC to be enhanced by its cofactor, protein S. Work carried out in my lab by Preston *et al*. demonstrated that an APC variant with amino acid substitutions at positions Asp36, Lys38 and Ala39 was not enhanced by protein S, suggesting that these residue are important for the APC/PS interaction.[127]

In contrast to the Gla domain and the serine protease domain, the functions of the EGF domains are not fully defined. However, it has been suggested that also these domains are involved in the interaction with Ca^{2+} and with protein S.[128,129]

The activation peptide of APC is localised at residue 158-169. Upon cleavage by the thrombin/thrombomodulin (TM) complex, the activation peptide is removed, leading to a conformational change in the protein C serine protease domain, turning the zymogen into the active protease, APC.[119,130]

The serine protease domain of APC contains the catalytic triad forming the proteolytic active site. The catalytic triad is comprised of His211, Asp257 and Ser360.[122,123,131] The serine protease domain also has a positively charged region with a large number of positively charged amino acids. This region includes the 37 loop, the 60 loop and the 70-80 loop which have been found to mediate interaction with heparin,[132] thrombomodulin,[133] FVa[134] and FVIIIa.[119,135] The autolysis loop (143-154) is thought to stabilise the activation peptide and interactions with inhibiting proteins, the serpins.[136,137]

1.3.2 Protein C activation

Protein C is activated by a high affinity thrombin/TM complex (Kd 1-10nM, depending on presence of chondroitin sulphate).[138,139] Free thrombin can also activate protein C, but very inefficiently.[140] The presence of TM enhances the activation by at least three orders of magnitude.[141] Upon its binding to TM, thrombin undergoes conformational changes which modulates its function. Thrombin bound to TM is not able to activate clotting factors such as FV and FVIII, or convert fibrinogen to fibrin. Thrombin binds to TM EGF 4-6 domains through its anion binding exosite 1, see Figure 1.9.[141,142] A chondroitin sulphate moiety, although not mandatory for TM function, interacts with anion-binding exosite 2 on thrombin, and by doing so enhances its affinity to thrombin by 10-fold. TM interact with protein C through a low affinity binding site in its EGF4 domain. Activation of protein C is achieved by the removal of the activation peptide (APC Asp158-Arg169). It has been demonstrated that TM, as a transmembrane protein, can recruit thrombin to membrane surfaces. It has also been proposed that TM relocates the thrombin active site to optimal position for the cleavage and activation of protein C.[143] The activation reaction can be further enhanced by endothelial protein C receptor (EPCR).[144] EPCR is believe to increase the local concentration of protein C and decrease the K_m of the activation at least by 5-fold.[145-147]

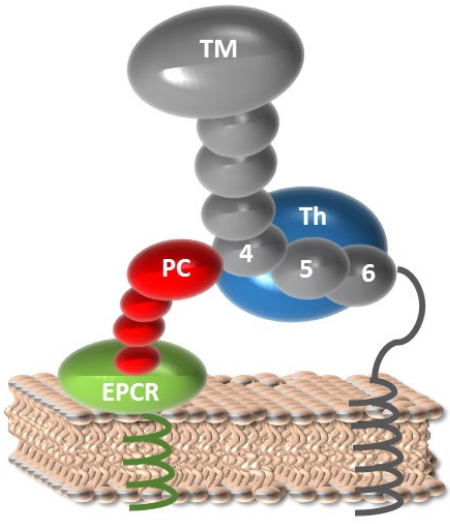


Figure 1.9. Activation of protein C
Protein C is activated by the complex of thrombin (Th) and thrombomodulin (TM). The Th/TM complex mediated activation of protein C is at least 10^3 times more efficient than that catalysed by thrombin on its own. In this model TM binds thrombin with high affinity in its EGF4-6 domain (labelled 4-6). It repositions the Th active site so that it favours activation of PC (bound weakly to EGF4). PC binds only weakly to phospholipid but its membrane receptor acts to increase the affinity of the binding.

1.3.3 APC functions

1.3.3.1 Anticoagulant function of APC

The APC pathway plays an important role in regulation of coagulation. Homozygous protein C deficiency is a life threatening condition which often cause rapidly progressing purpura fulminans or necrosis of the skin already during the neonatal period.[148] Individuals with heterozygous deficiency have ~5-fold increased risk of venous thrombosis.[149] APC is a serine protease that inhibits thrombin generation in the propagation phase of coagulation. This anticoagulant effect is achieved through inactivation of coagulation factors FVa and FVIIIa.[45,131] Both proteolytic reactions depend on APC interacting with anionic phospholipids surfaces, provided by activated platelets or endothelium.[61,62,150] Inactivation of both coagulation factors is further enhanced by the cofactor of APC, protein S.[150] APC only down-regulates thrombin generation efficiently in the presence of protein S. In its absence, APC is not an effective anticoagulant.

APC-mediated inactivation of FVa is achieved by proteolytic cleavage at positions Arg506, Arg306 and Arg679, while inactivation of FVIIIa occurs through cleavage at Arg336, Arg362 and Arg740.[151,152] These anticoagulant activities of APC are described in more detail in sections 1.3.3.1.1 and 1.3.2.1.2.

1.3.3.1.1 FVa inactivation

Once activated, FVa is incorporated into the prothrombinase complex where it functions as a cofactor for FXa in the activation of prothrombin to thrombin. The activity of FVa is regulated through inactivation by APC. Both free FVa, as well as FVa assembled in the prothrombinase complex, can be proteolytically inactivated by APC. APC-mediated inactivation of FV is achieved by proteolytic cleavages at three sites in the FVa A2 domain of the heavy chain, Arg306, Arg506 and Arg679[150] (see also Figures 1.10 and 1.11). FVa inactivation can proceed though a biphasic reaction, with rapid cleavage at Arg506, leading to an intermediate product with 60% reduced FXa cofactor activity. Cleavage at Arg506 (k = 4.3x10^7 M^{-1} s^{-1}) is followed by a slow cleavage at Arg306 (k = 2.3x10^6 M^{-1} s^{-1}) which completely inactivates FVa.[151] It has been suggested that the loss of FXa cofactor function arises from the cleavage at Arg506, disturbing the interaction and decreasing the affinity for FXa.[151] In agreement with this finding, Rosing

et al. showed that FXa protects FVa from inactivation by preventing the APC-mediated proteolysis at Arg506.[153] Subsequent inactivation cleavage occurs at Arg306. In contrast to what was initially believed, the Arg506 cleavage is not required prior to cleavage at Arg306 as FVa inactivation also can proceed via a monophasic reaction where only cleavage at Arg306 occurs.The Arg306 cleavage occurs in partially inactivated FVa as well as in intact FVa. The rate constants for the Arg306 cleavage in activated WT FVa and FVa Leiden (which cannot be cleaved at Arg506) were found to be quite similar.[151]

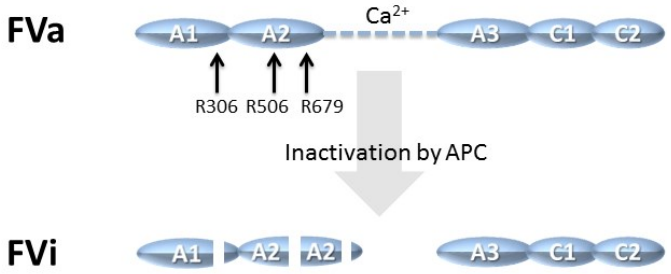


Figure 1.10. Schematic representation of the APC-mediated FVa inactivation.
APC-mediated inactivation of FVa is achieved by proteolytic cleavage at residues R306, R506 and R679.First rapid APC clavage occurs at Arg506 and it is followed by slow clavage at Arg306. After the Arg306 clavage, FVa is completely inactivated.

FVa can also be cleaved at Arg679, however, the function and the physiological relevance of this cleavage site is still unknown. While FVa can be fully inactivated in the absence of phospholipid membranes, APC-mediated inactivation is greatly enhanced in the presence of phospholipids. Cleavage at Arg306 particularly, is highly phospholipid dependent.

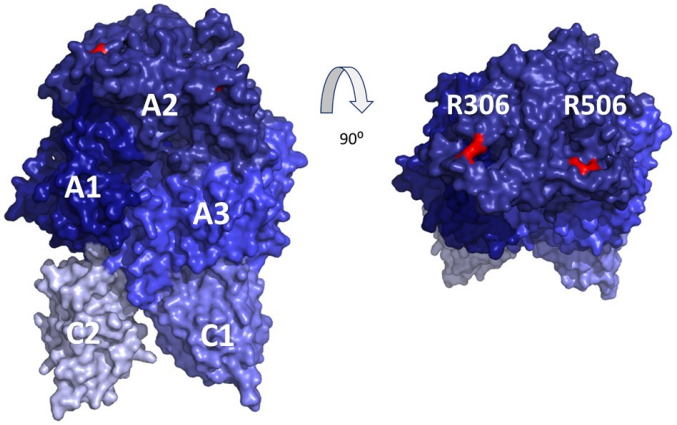


Figure 1.11. Model of FVa with the two APC cleavage sites (Arg306 and Arg506) shown in red.
The unpublished coordinates for this model were kindly provided by Prof. Jim Huntington, University of Cambridge.

1.3.3.1.2 FVIIIa inactivation

Free FVIIIa is readily inactivated by a combination of spontaneous dissociation of the A2 domain (accounts for 60-80%) and proteolytic cleavage by APC at Arg336 and Arg562 (accounts for 20-40%), see Figure 1.12. The relatively short half-life of FVIIIa of approximately 2 minutes is due to spontaneous dissociation of the A2 domain.[154] However, FVIIIa is more stable once incorporated into the tenase complex. APC can inactivate FVIIIa even when it is bound to FIXa. Since free FVIIIa can be inactivated by spontaneous dissociation of the A2 domain, APC mediated inactivation might even be more physiologically relevant for FVIIIa in the tenase complex. Protein S was found to enhance the FVIIIa inactivation, but not to the same extent as the inactivation of FVa (see discussion in section 1.4.2.1.2). However, Shen *et al*. demonstrated that FV enhances APC-dependent proteolysis of FVIIIa together with protein S.[49] Overall, protein S and FV enhance FVIIIa proteolysis by APC ~11-fold, with cleavage at Arg336 being stimulated 3-5 fold and cleavage at Arg562 by 7-8 fold.[105,155,156] It was suggested that individuals with FV Leiden did not have synergistic enhancement by FV, which lead to the finding that Arg506 cleavage is required for APC cofactor function of FV (see discussion in section 1.2.3.2).[105,107,151]

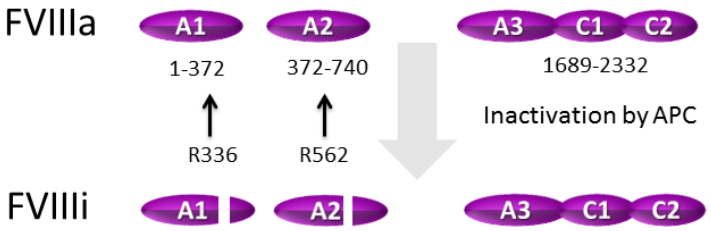


Figure 1.12. Schematic model of APC-mediated FVIIIa inactivation
FVIIIa is inactivated by APC through proteolysis at residues Arg336 and Arg562. Both cleavages are enhanced by the presence of APC cofactors, protein S and FV.

1.3.3.2 Cytoprotective activity of APC

APC cytoprotective effects arise from interactions with a number of receptors and proteins, resulting in anti-inflammatory and anti-apoptotic properties. APC also exhibits protective effect on the endothelium barrier function.[119] There are different mechanisms of APC induced signalling depending on the type of the cell, tissue and organ. APC cytoprotective effects depend on its binding to EPCR.[157] EPCR-bound APC is able to activate the G protein-coupled

protease activated receptor (PAR) 1, 2 and 3.[158] Of these, the best studied and described is the APC mediated activation of PAR1. APC proteolytically cleaves PAR1 at Arg46 of its N-terminus, exposing a tethered ligand which activates the receptor. Interestingly, APC-mediated activation of PAR1 triggers a different signalling response in comparison to thrombin-initated activation of PAR1.[159] While thrombin-mediated activation leads to endothelial barrier distruption and pro-inflammatory response, activation of PAR1 by APC promotes endothelial barrier stability and results in anti-inflammatory and anti-apoptotic effects.[160] Activation of PAR3 by APC is belived to be essential for therapeutic properties of APC.[159] In particular APC cytoprotective effect in the brain and in the kidney involves PAR3 activation.[161]

APC reacts with a number of different receptors. The most important interaction are described in the section below.

1.3.3.2.1 Stabilisation of endothelial barrier

One of the roles of the endothelium is to control a passage of molecules in and out of the tissue. During inflammation, permeability of the endothelial layer is increased. APC exhibits a protective effect by improving integrity and minimizing vascular permeability of endothelium. This is achieved by up-regulation of sphingosine kinase 1 (Sphk1) and bioactive lipid sphingosine 1 phosphate (S1P). Binding of S1P to its receptor (S1P1) activates the PI3-kinase/Akt pathway, which upregulates Rac 1 and angiopoetin 1 (Ang1). PI3-kinase is also activated by binding of Ang1 to Tie2.[162] Recent report suggests that APC can also directly bind to Tie2.[163] Together these processes lead to increased endothelial barrier integrity, followed by a reduction of the passage of inflammatory cells through the endothelium.[119,162]

1.3.3.2.2 Anti-inflammatory function

APC-mediated up regulation of Sphk1 and S1P can also occur in leukocytes, which reduces the inflammatory response. APC is able to decrease expression and activity of nuclear transcription factors. This, together with increased levels of S1P, results in inhibition of cytokine signalling, reduction of cell surface adhesion and stimulation of anti-inflamatory interleukin such as IL10. Passage of leukocytes through the endothelium layer as well as tissue

damage is reduced.[3] APC is also believed to reduce adhesion of neutrophils to the endothelium and inhibit their migration as a result of interaction with β1 and β3 integrins.[164]

APC also exerts anti-inflamatory functions by inhibiting PAR2 activation. PAR2 can be activated by the tenary TF-FVIIa-FXa complex in an EPCR-dependent reaction, resulting in pro-inflamatory signalling.[158,165] This activation is inhibited by APC, in contrast to APC-L8V, an APC variant with impared EPCR interaction. Intriguingly, APC-mediated inhibition requires the presence of two cofactors, protein S and FV. It also appears that cleavage of FV at Arg506 is required for its anti-inflamatory cofactor function, as FV-Leiden failed to support inhibition of PAR2 activation by APC.[158]

1.3.3.2.3 Anti-apoptotic function

Apoptosis is a process of programmed cell death. It can be activated by the death receptor pathway upon its binding to TNFα or FasL, or the mitochondrial pathway as a result of DNA damage, hypoxia. These result in activation of caspases and apoptosis.[3] APC exerts its anti-apoptotic function by reduction of caspase activation and pro-apoptotic genes expression (such as p53 and Bax). Moreover, APC has been suggested to up regulate transcription of anti-apoptotic genes Bcl2.[119,166]

Multiple studies using human and murine APC, as well as variants thereof, have been conducted. One of the most interesting variants of APC is 3K3A-APC, with three Lys to Ala substitutions in the surface loop at positions 191-193. This APC derivative was shown to have reduced anticoagulant activity and yet retained neuroprotective properties and is a potential new treatment in ischemic stroke. tPA is a drug currently used in ischemic stroke treatment. However, it has neurotoxic side effects and may induce bleeding. APC was found to block the neurotoxic effect of tPA and reduce tPa-induced bleeding. At the moment the APC-3K3A variant is in phase II clinical trials for use in treatment of ischemic stroke.[167,168]

1.4 Protein S

Protein S is a 73kDa vitamin K-dependent glycoprotein circulating in plasma at a concentration of 20-25 mg/ml (300-350nM).[169] Approximately 60% of plasma protein S forms a non-covalent high-affinity complex with C4b-binding protein (C4BP). A population of protein S circulating as a free protein serves as a cofactor for the TFPI and APC anticoagulant pathways.[36,46]

1.4.1 Protein S expression and structure

Protein S is mainly produced by hepatocytes. Endothelial cells, testicular Leydig cells, sertoli cells, dendritic cells and osteoblasts also synthesised protein S.[170-174] The protein S gene (*PROS1*) is located near the centromere of chromosome 3q11.2, and contains 15 exons that encode the 676 amino acid long protein precursor.[175] Removal of the signal peptide (aa 1-24) and propeptide (aa 25-41) by proteolytic cleavage result in secretion of a 635 amino acid mature protein. Mature protein S consists of an N-terminal Gla domain, thrombin sensitive region (TSR), four EGF-like domain and a sex hormone-binding (SHBG)-like domain, see Figure 1.13. Protein S shares domain organization and ~44% sequence homology with growth arrest specific 6 (GAS6) which has no anticoagulant functions.[174,176] The Gla domain contains 11 Gla residues and is responsible for the interaction with the negatively charged phospholipid membranes of activated platelets, endothelial cells and microparticles.[177] The importance of the binding to phospholipid membranes is illustrated by a naturally occurring mutation, Gly11Asp, which is associated with reduced membrane binding and consequently impaired protein S function.[64] As was described above for protein C, coordination of Ca^{2+} to the Gla-residues within the Gla domain leads to correct folding and exposure of hydrophobic residues, crucial for phospholipid binding.[178] In addition to the phospholipid binding, the Gla-domain has been shown to have additional functions required for APC-cofactor function. The importance of the Gla-domain for protein/protein interactions was first suggested by Saller *et al*.[177] In their study, amino acid residues within the Gla-domain of protein S were substituted to corresponding residues of prothrombin. The resulting protein S variant did not express APC cofactor function despite efficient binding to phospholipids, suggesting that these residues were instead involved in a protein/protein interaction within this pathway.

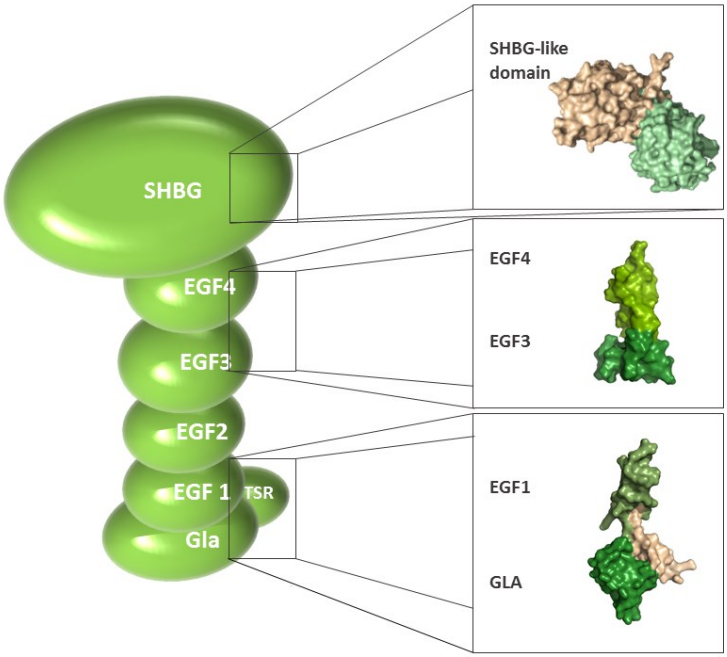


Figure 1.13. Schematic representation of protein S structure and available molecular models.
Protein S is composed of N-terminal Gla domain, TSR, four EGF domains and SHBG-like domain.The models of protein S Gla-TSR-EGF1, EGF3-EGF4 and the SHBG-like domain were adapted from Giri et al., Drakenberg et al., and Villoutreix et al., respectively[179-181]

Following on from this work, my supervisor, Dr Josefin Ahnström, showed that protein S Gla36 is essential for APC-cofactor function whilst being dispensable for phospholipid binding (see Figure 1.14).[182] Interestingly, in contrast to the other Gla residues in protein S, Gla36 is not predicted to coordinate calcium. Heeb *et al*. have suggested that protein S residue 37-50 function as a potential binding site for FV, in a study using monoclonal antibodies and synthetic peptides.[183]

The thrombin sensitive region (TSR) is a loop between the Gla and EGF1-domains, also called a thumb loop. TSR was shown to stabilize the protein S structure.[179] The importance of the TSR for APC cofactor function was suggested by Dahlbäck *et al.*[184] who demonstrated that monoclonal antibodies against TSR and EGF1 inhibit APC cofactor function of PS. The TSR region contains a disulphide bridge and is susceptible to thrombin and FXa cleavage.[184,185] The thrombin cleavage sites are localized at Arg49 and Arg70, while FXa mediated proteolysis occurs at Arg60. Proteolysis of TSR results in reduction of APC cofactor function. A recombinant protein S variant in which the TSR was deleted, bound to anionic phospholipid with 25-fold reduced affinity.[186] The same group later reported that TSR is not directly

involved in phospholipid binding, but rather affeced it through changing the conformation of the Gla domain.[187]

Protein S contains four EGF-like domains, building blocks found in many plasma proteins, see Figure 1.13. They physically separate the Gla domain from the SHBG-like domain. The EGF-like domains contain 6 cysteines which form 3 disulphide bonds between cysteines C1-C3, C2-C4 and C5-C6. The first EGF domain is important for APC cofactor function. A naturally occurring mutation Thr103Asn leads to qualitative protein S deficiency.[188] In addition, Dr Helena Andersson, a former member of our lab, showed that Asp95 has a crucial role in APC cofactor function (see Figure 1.14).[189] The exact role of this residue is however not clear. The importance of EGF2 was shown by Mille-Baker, also a previous member of our lab, who demonstrated that a protein S variant where EGF2 had been deleted only retained 11% activity compared to WT protein S in a FVa inactivation assay.[190]

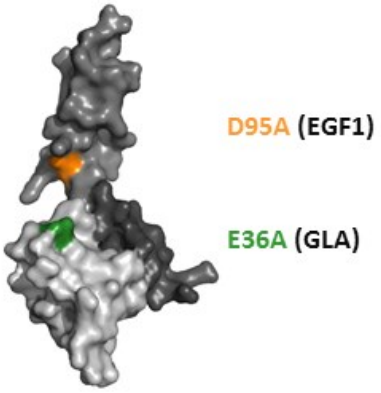


Figure 1.14. The models of protein S Gla-TSR-EGF1 domains with highltered residues Gla36 and Asp95.
It has been demonstrated that protein S variants, E36A and D95A, have strongly reduced APC cofactor function. However, it is not established whether the substituted protein S residues are important for the interaction with APC or FVa. The model was adopted from Giri et al.[179]

EGF2-4 all contain high-affinity Ca^{2+} binding sites. A lack of calcium binding in the EGF4 has been shown to have an effect on the conformation and function of EGF1 domain, which suggests a conformational dependency between the different EGF domains.[191] The importance of these calcium binding sites is further shown by a naturally occurring protein S variant, in which a residue known to be important for the formation of a calcium binding site

has been subsituted, Asn217Ser.[191] The Asn217Ser mutation is associated with an increased risk of thrombosis in affected individuals.

The C-terminal SHBG-like domain of protein S consists of two subdomains, LG1 and LG2 (see Figure 1.13). These are similar to the laminin-G-like domain-like modules found in a number of multidomain proteins, such as components of the extracellular matrix, signalling proteins and plasma proteins such as Gas6 and sex hormone binding globulin.[181] There are three N-linked glycosylation sites in the SHBG-like domain at Asn residues 499, 509 and 530.[192] The SHBG-like domain is responsible for binding of protein S to C4BP, which has been shown to result in a decrease ability of protein S to enhance TFPI as well as APC.[193-195]

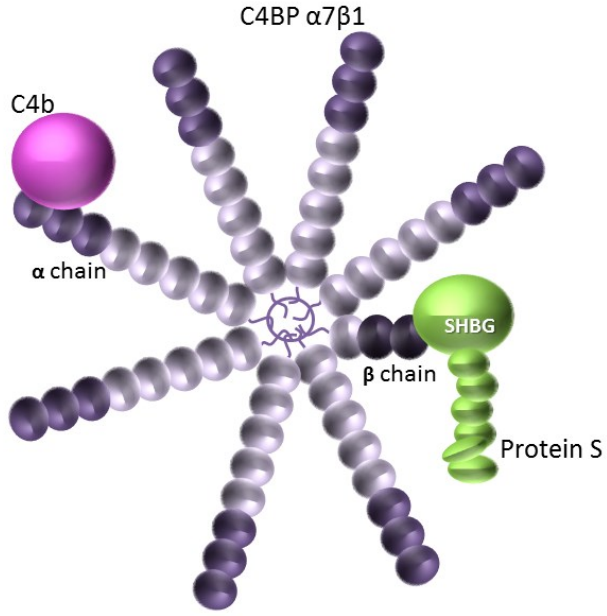


Figure 1.15. Schematic model of C4BP-protein S complex
Protein S forms a non-covalent, high affinity complex together with C4BP. The interaction is mediated between the SHGB-like domain of protein S and the β-chain of C4BP.[196]

C4BP, synthesised in liver, is a cofactor of factor 1 (FI) during inactivation of C4b and C3b in the classic complement pathway. C4BP circulates in plasma in several forms, with different combinations of α and β-chains linked together by disulphide bound in a spider like structure (see Figure 1.15). The predominant C4BP form is composed of seven α-chains and one β-chain. The β-chain is always single and it is present in 80% of C4BP molecules under normal conditions.[193] Both α and β-chains consists of short complement control protein domains (CCP), also called Sushi domains. Protein S binding sites are localized on the β-chain of C4BP, thereby only the β-chain containing form of C4BP can form a complex with protein S. More

precisely, β-chains residues located within CCP1, namely Ile16, Val 18, Val 31 and Ile 33, were found to mediate the interaction with protein S.[196] It has also been suggested that CCP2 may contribute to the interaction between protein S and C4BP.[197] Protein S residues involved in the interaction with C4BP are localized in SHBG-like domain and are suggested to include residues 420-434, 447-460 and 605-615.[198-202]

Interestingly, these residues are localised in close proximity to the proposed FV binding site on protein S, [203] (see figure 1.16). Under normal physiological conditions, C4BP is present in plasma at concentration of 340nM. However, its concentration increases dramatically during the acute phase of inflammation. Yet only the C4BPα form is elevated and the level of free protein S is not affected.[193,204]

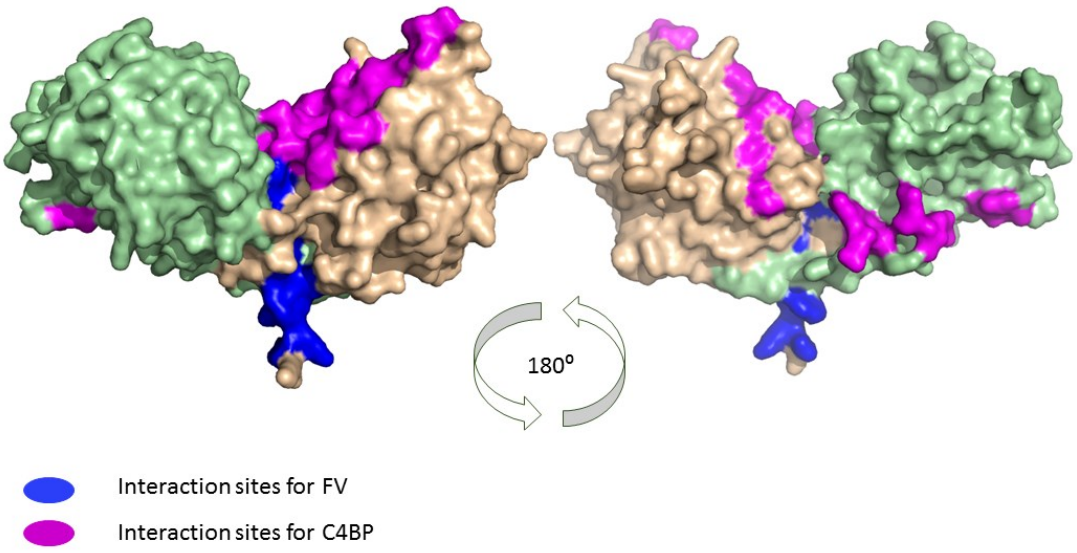


Figure 1.16. A model of the SHBG-like domain of protein S with proposed FVand C4BP binding sites. Residues 420-434, 447-460 and 605-615[198-202] were proposed as binding sites for C4BP while residue 621-635[203] were suggested to be FV/FVa interaction site.

In addition to C4BP, the SHBG-like domain has been shown to interact with several other proteins, such as TFPI and FV/FVa.[203,205] My former colleague, Dr Reglinska-Matveiyev, showed that the LG1 subunit contains an interaction site for TFPI.[205] Heeb *et al*. identified a potential interaction site for FV at residues 621-635.[203] Further studies have also suggested that the SHBG-like domain plays a role in the APC pathway. A protein S derivative lacking the SHBG-like domain had reduced APC cofactor function in plasma but normal in purified systems.[206] In agreement with their findings, Nyberg *et al*. generated chimera of protein S

where the SHBG-like domain had been substituted with that of Gas6. This chimera also showed impaired APC cofactor function in plasma.[207]

1.4.2 Protein S functions

1.4.2.1 Protein S anticoagulant function

Protein S plays an important role in thrombin regulation by functioning as a cofactor both in the TFPI and APC anticoagulant pathways. Both pathways are essentially dependent on protein S for efficient regulation of coagulation.

1.4.2.1.1 TFPI- cofactor function

TFPI cofactor function by protein S was first discovered by Heckeng *et al.* who demonstrated that addition of anti-protein S antibodies to plasma in the presence of anti-protein C antibodies led to an increase in thrombin generation.[36] The effect appeared to be TFPIα specific as it was not observed in TFPI depleted plasma. A later study demonstrated that protein S directly interacts with kunitz domain 3 of TFPIα.[208] My supervisor, Dr Ahnström and colleagues, showed that the interaction site involved TFPI kunitz domain 3 residues Arg199, Glu226, Glu234 and Arg237 and that the direct interaction was necessary for protein S to enhance TFPI.[37] Protein S is believed to enhance TFPI by reducing the TFPI concentration required for efficient inhibition of FXa.

1.4.2.1.2 APC cofactor function

The observation that APC-mediated inactivation of FVa is enhanced by addition of plasma, resulted in the discovery of protein S as a cofactor for APC.[46] Protein S exerted its function only in the presence of phospholipids but had no effect upon FVa inactivation at supra-high phospholipid concentrations.[209] Due to this finding, it was suggested that protein S exhibits its cofactor function by increasing the affinity of APC to phospholipid membrane and thereby amount of APC bound to phospholipids.[209] Using light scattering, Walker demonstrated that APC, in the absence of protein S, binds to phospholipids vehicles with a K_d of approximately 150nM. Addition of protein S to the assay lowered the K_d by more than 10-fold to 14nM (see Figure 1.17).

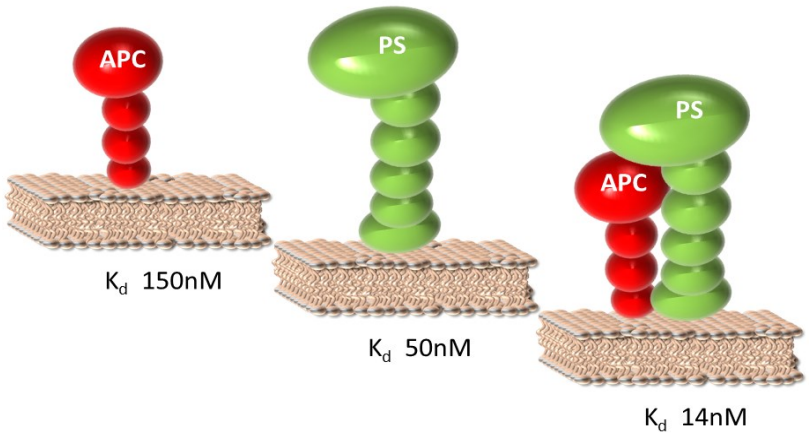


Figure 1.17. Schematic model of protein S enhancement of APC/phospholipids association.
It was proposed that protein S increases affinity of APC to phospholipids vehicles by ~10 fold.[209]

Many findings, several contradicting each other, regarding the mechanism and level of protein S enhancement have since then been published. Bakker *et al*. reported a moderate 2-fold enhancement of APC-catalysed FVa proteolysis by protein S, independent of phospholipid concentration and composition.[47] In contrast, Rosing *et al*. reported a much more profound effect of protein S upon the FVa inactivation. They identified a 20-fold enhancement by protein S, which was specific for the cleavage at Arg306.[153] The same group also suggested that the discrepancy in the protein S enhancement observed by different research groups may be caused by insufficient inactivation time. If only the initial rate of FVa inactivation was monitored, only cleavage at Arg 506 would have a chance to occur, and this is not stimulated by the presence of protein S. The findings by Rosing *et al*. were later confirmed by those of Norstrøm *et al*. who demonstrated that protein S increased the rate of proteolysis at Arg306 by 20-30 fold, while the Arg506 cleavage is stimulated only by 1-5 fold.[62] In 1982 it was demonstrated that FXa protects FVa from inactivation by APC.[210] Solymoss *et al*. built an alternative hypothesis for the mechanism of APC enhancement by protein S on this observation.[211] In that paper, it was suggested that protein S removes the ability of FXa to protect FVa from APC-mediated inactivation.[211] This hypothesis was later supported by Norstrøm *et al*. who showed that protein S prevents the FXa-mediated inhibition of the FVa Arg506 cleavage by APC, using FV variants which can only be cleaved at either Arg306 or Arg506.[151]

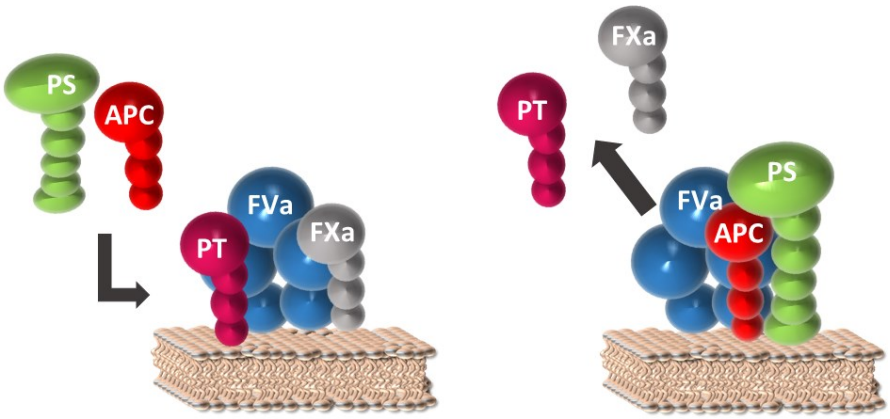


Figure 1.18. Speculative model of protein S enhancement of APC mediated FVa inactivation
Binding of FVa to FXa prevents APC-mediated degradation of FVa. It has been suggested that protein S is able to reverse the protective effect of prothrombinase on FVa and instead stimulate FVa inactivation even when part of the prothormbinase complex.

However, the same hypothesis was later challenged by Rosing and colleagues who concluded that the protein S enhancement is independent of the protective effect of FXa and may occur even at concentrations of FXa which blocks the Arg506 cleavage. They reported that FXa inhibits the Arg506 cleavage while protein S enhances proteolysis at Arg306.[47] Matters were even further complicated when Heeb *et al.* suggested that FXa and protein S have overlapping binding sites on FVa.[212]

The third hypothesis of the molecular mechanisms involved in protein S enhancement of APC-mediated inactivation of FVa was described by Yegneswaran *et al*.[213] They proposed that protein S relocates the active sites of APC closer to phospholipid surface to create optimal orientation for cleavage. Using fluorescence resonance energy transfer between APC, labelled in the active site with fluorescein, and phospholipids containing occtadecylrhodamine,they showed that the active site of APC moved closer to phospholipid membrane upon addition of protein S. Thrombin treated protein S did not change the location of APC active site, in agreement with the inefficient APC enhancement by cleaved protein S.[213] However the relocation hypothesis was questioned by Segers *et al.*[214] who pointed out that both cleavage sites, Arg306 and Arg506, are predicted to be localised 80Å above the membrane and that only Arg306 cleavage is enhanced by protein S. By substituting amino acid residues localised closely to Arg306, these investigators managed to increase the inactivation rate of the Arg306 cleavage. They therefore suggested that protein S instead exerts its cofactor function by

improving the contact between a highly positive FVa region (Lys320, Arg321, Arg400) and the serine protease domain of APC.[214]

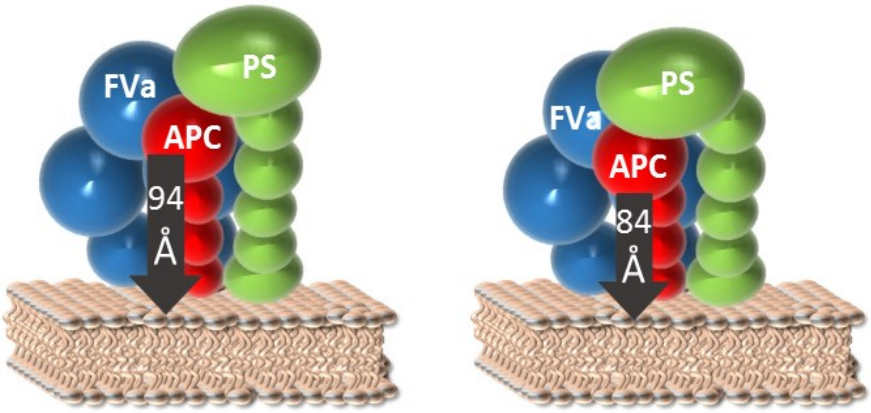


Figure 1.19. Schematic model of Protein S mediated relocation of APC active site.
Active site of APC was moved closer to the phospholipid membrane upon addition of protein S.

Protein S also functions as a cofactor for APC mediated inactivation of FVIIIa. However, in this case the enhancement is moderate (~ 3-fold).[215] As mentioned in previous sections, FV has been identified as a synergistic cofactor, together with protein S, for APC-mediated inactivation of FVIIIa leading to an overall 11-fold enhancement of inactivation.[105,49] The moderate enhancement by protein S and the need of a synergistic cofactor suggests that the mechanisms involved in the inactivation of FVa and FVIIIa, and the role protein S plays in these complexes, are different. However, to date there is very little discussion in the literature, describing the difference in mechanism between the inactivations of these important cofactors.

As mentioned in earlier sections, it has been demonstrated that C4BP-bound protein S exhibits reduced APC cofactor function. The protein S-C4BP complex has been shown to enhance APC mediated FVa inactivation by only 6 to 8-fold.[216] Somewhat surprisingly, C4BP-bound protein S exhibit full cofactor function during FVIIIa inactivation, further illustrating likely differences in mechanisms involved in APC-mediated inactivation of FVa and FVIIIa.[217] Many groups have therefore tried to elucidate the mechanism of protein S cofactor function over the last ~35 years. However, the mechanisms involved still remain unclear.

1.4.2.1.3 Protein S direct anticoagulant properties

Protein S also exhibits anticoagulant function which are independent from APC and TFPI. This has been termed the protein S direct anticoagulant effect. It has been reported that protein S directly inhibits the prothrombinase and tenase complexes in plasma.[218] Heeb *et al*. demonstrated that the protein S direct inhibitory function is associated with binding to FVa or FXa.[219,220] It has also been suggested that this anticoagulant protein S activity is phospholipid dependent. Since protein S binds to phospholipid vehicles with high affinity it is possible that protein S could compete for limited phospholipid surface with other coagulation factors.[221] If this is the case, it could suggest a likely artefact in the functional assays and the physiological relevance of this function of protein S would then be questionable. Some groups have explained the observed direct anticoagulant effect of protein S by its multimerisation, an artefact caused during its purification.[222] Others claim that the protein S direct effect is physiologically relevant as both forms of protein S, monomeric and multimeric, exert direct anticoagulant effects.[223,224] Heeb and colleagues have suggested that the protein S direct effect is dependent on Zn^{2+}. Many methods of protein S purification remove Zn^{2+} from the final preparation, therefore impairing the protein S direct function. They claim that protein S containing Zn^{2+} exhibits 10-fold higher direct anticoagulant function than protein S where the Zn^{2+} has been lost during purification.[225]

While C4BP-bound protein S is an inefficient cofactor in for TFPI and APC, its direct ability to inhibit the prothrombinase complex is not affected by the presence of C4BP.[221] The direct inhibition of the tenase complex by protein S is even enhanced in the presence of C4BP.[226]

1.4.2.2 Protein S non-anticoagulant function

Protein S has suggested biologic functions distinct from its anticoagulant activities. It has been suggested that protein S participates in cell proliferation, apoptosis, and regulation of inflammation, atherosclerosis, vasculogenesis and cancer development.

1.4.2.2.1 Protein S and C4BP

As mentioned previously, around 60% of plasma protein S form high affinity complex with C4BP with a K_d ~0.1nM. It appears that the most prominent function of protein S in the protein S-C4BP complex is recruitment of C4BP to negatively charged phospholipids.[227] Anionic

phospholipids are present on the surface of apoptotic cells, activated platelets and microparticles. C4BP, unable to bind to apoptotic cells, localise on their surface by protein S.[228,229]

1.4.2.2.2 Protein S and TAM receptors

A number of protein S functions arises from its ability to activate three TAM receptors, namely Tyro3, Axl, Mer. Apart from hepatocytes, protein S is secreted by a wide range of cells including endothelial, Leydig, Sertoli cells, osteoblasts, dendritic cells, T cells and tumor cells where it serves as a ligand for TAM receptors.[230]

TAM receptors belong to one of 20 different families of tyrosine kinases receptors, which transmits signals from cytoplasm across the cell membrane to the extracellular environment.[230,231] They have been found to stimulate cell proliferation, inhibit apoptosis, regulate inflammation, mediate efferocytosis, stimulate haemostasis as well as stimulate cancer progression.[232] They dimerise and become activated upon ligand binding.[233]

TAMRs are able to recognise and mediate phagocytosis and protein S potentially facilitate this process by calcium dependent binding to phosplatidylserine on apoptotic cells. Protein S which is bound to the apoptotic cell is recognized by TAMR expressed on phagocytic cells and can facilitate uptake. In fact, TAMRs deficient mice were found to have increased level of apoptotic cells.[232]

It has been demonstrated that TAMRs inhibit rapid inflammatory response induced by Toll-like receptors (TLR) and it has been proposed that protein S participate in this process by mediating clearance of apoptotic cells. Protein S was found to down-regulate expression of TNF, Interleukin 6 (IL6) and Interleucin IL-1b induced by LPS interaction with TLR.[234]

TAMRs are overexpressed in number of different human cancers. Moreover, TAMRs has been shown to mediate cell migration.[232] Lung carcinoma cell lines which overexpressed all three TAMRs were found to secrete protein S. Protein S has been suggested to have a role in cancer development.[235]

As mentioned previously around 60% of plasma protein S form high affinity complex with C4BP. It appears that the most prominent function of protein S in the protein S-C4BP complex

is recruitment of C4BP to negatively charged phospholipids.[227] Anionic phospholipids are present on the surface of apoptotic cells, activated platelets and microparticles. C4BP, unable to bind to apoptotic cells was localized on their surface by protein S.[228,229] In contrast to free protein S, protein S-C4BP complex does not bind to TAM receptors and inhibits rather than stimulate phagocytosis of apoptotic cells.[236] Moreover, the complex protect apoptotic cells by inhibition of the complement activation system.[237]

1.5 My hypothesis and aims

Despite its physiological and clinical importance, the mechanisms underlying APC-mediated inactivation of FVa are not yet fully understood. In this thesis I have aimed to investigate complexes that are formed in this process, determine interactions involved, and potential conformational changes that might occur during FVa inactivation.

I hypothesised that FVa, together with protein S enhances the binding of APC to negatively charged phospholipid surfaces, forming a FVa/APC/protein S tri-molecular complex. I also hypothesised that FVa, through this mechanism, increases the efficiency of its own APC-mediated inactivation in the presence of protein S.

Furthermore, I hypothesised that, rather than protein S causing conformational changes in APC to enhance FVa inactivation, protein S and/or APC interaction leads to conformational changes within the FVa heavy chain, exposing the APC-cleavage sites for more efficient proteolysis by APC/protein S.

My precise objectives were:

- Prepare high quality recombinant proteins for binding studies: FV, protein S , protein C and their variants.
- Investigate binding of these proteins in the presence of phospholipids.
- Assess whether the mechanism of APC-mediated FVa proteolysis is specific or whether the same mechanism occur also in the proteolysis of FVIIIa.
- Investigate influence of C4BP upon molecular interaction between APC, FVa, protein S and phospholipids.
- Study importance of protein S mutations resulting in reduced APC cofactor function for the formation of the inactivation complex with APC and FVa.
- Characterise a variant of FV, FV Nara, which is strongly resistant to APC and assess whether it can be incorporated into the inactivation complex.
- Evaluate structure related properties of FV-810, the kinetics of FV-810 inactivation by APC and its ability to assemble the complex with APC and protein S.

2 METHODOLOGY

2.1 Materials

Cell lines stably expressing WT FV , WT protein S and protein S variants E36A, D95A and CHIII had been generated in my research group and were available to me. My supervisor Dr Josefin Ahnström generated vectors encoding mutations of protein S spanning EGF2-4 domain. I used them to create HEK293 cell lines stably expressing the protein S variants. A vector encoding WT FV was a kind gift from Dr Camire which I used to construct FV Nara through site-directed mutagenesis. Vectors encoding WT protein C and the protein C C D36A/L38A/A39V were created by Dr Roger Preston when he worked in my lab. I prepared stable HEK293 cell lines expressing both proteins using Lipofectamine 2000. All recombinant proteins expressed and purified during the course of my study are listed in Table 3.1. Protein S-free β-chain containing C4BP was purified from pooled fresh frozen citrated human plasma by my colleague Adrienn Teraz-Orosz.

2.2 Protein expression

2.2.1 Mammalian cell culture

Baby Hamster Kidney (BHK) cells were used for the stable transfection and expression of WT FV and its variants. WT protein S, WT protein C and their mutants were stably transfected and expressed in Human Embryonic Kidney (HEK) 293 cells (ATCC). Typically, the cells were cultured in T175 culture flasks in humidified incubators at 37°C, 5% CO_2. Confluent cells were washed with phosphate buffered saline (PBS; 10mM phosphate buffer, 2.7mM potassium chloride, 137 mM sodium chloride, pH7.4; Sigma) and split using 1.5 ml trypsin/EDTA (Sigma) per T175 flask. The cells were scaled up and transferred into T175 triple flasks.

2.2.1.1 BHK cells

BHK cells were grown in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) with phenol red (Invitrogen) supplemented with 10% foetal bovine serum (FBS; Invitrogen), 2mM L-glutamine (Invitrogen), 1U/ml penicillin, 0.1mg/ml streptomycin (Sigma) and 100mg/ml $CaCl_2$ (Sigma).

2.2.1.2 HEK293 cells

HEK cells were cultured in minimum essential media (MEM;Invitrogen) supplemented with 10% FBS, 2mM L-glutamine, 1U/ml penicillin , 0.1mg/ml streptomycin, non-essential amino acids (Invitrogen), and 10µg/ml of vitamin K (Konakion® Roche).

2.2.1.3 Cell line cryopreservation and revival

All cell lines were cryopreserved in liquid nitrogen. For this, confluent cells were washed with PBS, detached with 1ml of trypsin/EDTA solution and diluted in 10ml of complete media, see specified above for respective cell type. Following centrifugation at 1200 rpm for 5 minutes, supernatants were removed and cells were resuspended in 3ml of 10% dimethyl sulfoxide (DMSO) in FBS. Cells were aliquoted in cryovials (1ml/vial) and stored in a cryo freezing container (Biocision) at -80°C for at least 12 hours. After that the cells were transferred to liquid nitrogen.

To revive cells from liquid nitrogen, the cells were thawed at 37°C and seeded into T175 flasks supplemented with a minimum of 25ml complete media. Once the cells were attached to the flask, typically after 24 hours, the media was changed to remove any residual DMSO.

2.2.2 Expression of factor V

2.2.2.1 Generation of FV variants by site-directed mutagenesis

FV variants (FVNara , FV-810^{2Q}) were created by site-directed mutagenesis, using the WT FV and FV-810 vectors (pED; kind gift from Dr Rodney Camire, University of Pennsylvania) as a template and complementary oligonucleotides containing mutations. The dsDNA was generated and amplified in a polymerase chain reaction (PCR) using KOD hot start kit (Novagen) according to the manufacturers instructions. Since the primers introducing the FV Nara mutation had high G and C bases content, conditions of the PCR reaction had to be adjusted. The details of the PCR reaction and sample preparation are reported in Tables 2.1 and 2.2. After the PCR reaction, the template cDNAs, encoding FV-810 and WT FV, were digested using Dpn 1 (NEB) restriction enzyme for one and two hours, respectively, at 37°C according to the manufacturer's instructions. Following digestion, the generated cDNA was purified on 1.5% agarose gel and extracted using a QIAgen kit.

Table 2.1. Composition of PCR reactions to generate FV variants
The mutated cDNAs encoding FV-810^{2Q} and FV Nara were produced in PCR using the complementary oligonucleotides containing the mutations.

Component	FV-810^{2Q}	FVNara
10x reaction buffer	2.5 µl	2.5 µl
25mM $MgSO_4$	1.5 µl	1.5 µl
Forward primer (10µm)	1.5 µl	1.5 µl
Reverse primer (10µm)	1.5 µl	1.5 µl
dNTP	2.5 µl	2.5 µl
DMSO	0.5 µl	2.0 µl
cDNA	4.0 µl	0.5 µl
KOD hot start	0.5 µl	0.5 µl
dH_2O	10.5 µl	12.5 µl
Total	25 µl	25 µl

Table 2.2. Steps and conditions of PCR reaction

Steps	FV-810^{2Q}			FVNara		
Step	**Cycles**	**Temperature**	**Time**	**Cycles**	**Temperature**	**Time**
Denaturation	18	94°C	15s	30	99°C	15s
Annealing		60°C	30s		60°C	30s
Extantion		72°C	20s		72°C	20s
Hold		4°C			4°C	

2.2.2.2 Transformation of competent cells and sequencing

Transformation of competent cells (Top10, NEB10 beta; Invitrogen) with mutated cDNA was conducted according to the manufacturer's instruction. 50 µl of cells were thawed on ice and gently mixed with 1 µl of cDNA. Following 20 minutes incubation on ice, the cells were heat-pulsed for 30 seconds at 42°C and incubated on ice for another 2 minutes. Transformed bactiria were mixed with 500 µl of S.O.C. (Sigma) medium and were allowed to grow for one hour at 37°C with shaking. The transformed cells were plated on a Luria-Bertani medium (LB) agar plates containing 100 µg/ml ampicillin and incubated overnight at 37°C. Ampicilin-resistant colonies were picked and grown in 5 ml LB broth (Invitrogen) containing100 µg/ml ampicillin for 8-15 hours, with shaking. Plasmids were extracted using MiniPrep kit (Qiagen) according to the manufacturers instructions. Plasmid cDNA was quantified by measuring the absorbance at 260nm using a NanoDrop spectrophotometer (ThermoScientific).

2.2.2.3 Stable transfection of BHK cells

A stably transfected BHK cell line expressing WT FV had already been generated in our lab by Dr Helena Andersson. Stable cell lines expressing variants of FV were prepared using Lipofectamine 2000 (Invitrogen). For this, BHK cells were seeded in a 6-well plate in complete DMEM/F12 media and grown until reaching 40% confluency. 4 µg of vector containing the mutation of interest and an antibiotic selection plasmid were mixed together at ratio of 2:1 or 4:1 in 250 µl of transfection media (DMEM/F12 supplemented with 2mM of L-glutamine). 10 µl Lipofectamine 2000 were diluted in transfection media in the same maner. After 5 minutes incubation at RT, both solutions were gently mixed together and further incubated for 5 minutes at RT. During this time the cells were washed with transfection media and 2.5 ml of transfection media was applied onto the 40-50% confluent well. The DNA-lipofectamine 2000 mixture was added to the cells which were then incubated at 37°C, 5% CO_2 overnight. The transfected cells were split hard and diluted in media into Petri dishes. At this stage the media was changed into complete DMEM/F12 media containing selective antibiotic (G418 450 µg/ml) and was replaced twice a week until colonies appeared (usually around 1.5-2 weeks). Around 30-40 colonies per each FV variant were transferred to 48 well plate using cloning rings (Sigma). The 15 colonies that first reached confluency were transferred into T75 flasks. The clones were allowed to reach confuency before protein expression was checked. Protein expression was checked twice using Western blot analysis. To enable detection of FV on Western blotting despite the low level of expression, trichloroacetic acid (TCA) precipitation was used. Proteins from 1ml of expression media were precipitated with 50 µl of TCA acid for minimum 4h at 4°C, followed by centrifugation at 13 000g for 15 minutes at 4°C . Supernatants were removed and the pellets were dissolved in LDS sample buffer. The clones with the higest expression levels were used for protein production.

2.2.2.4 FV expression and harvesting

Stably transfected cells expressing WT FV, or its variants, were cultured in triple flasks in DMEM/F12 without phenol red supplemented with penicillin/streptomycin, 2mM $CaCl_2$, 1.5 mg/ml albumax, 4.2 mg/ml insulin, 3.8 mg/ml transferrin and 5µg/ml sodium selenite (ITS; Roche). The conditioned media was harvested for the first time after 72 hours, then every 24 hours for 4 days. The harvested media was supplemented with 10mM benzamidine (Sigma-

Aldrich), 50µM amidinophenylmethanesulfonyl fluoride hydrochloride (APMSF; Sigma-Aldrich), 20mM Tris, pH 7.4 and centrifuged at 10 000g for 10 minutes at 4°C to remove cell debris. The media was partially purified on SP sepharose Fast Flow cation exchange column on the day of the harvest (see section 2.3.2.1).

2.2.3 Expression of protein S and protein C

2.2.3.1 Stable transfection of HEK293 cells

HEK293 stable cell lines expressing WT protein S, WT protein C and their variants were prepared using Lipofectamine 2000, using the same protocol as described for BHK stable cell lines (section 2.2.2.3).

2.2.3.2 Expression and harvesting of protein C and protein S

Protein C and protein S were expressed by stably transfected HEK293 cells in triple flasks in reduced serum medium OptiMem (Invitrogen) supplemented with 1 U/ml penicillin, 0.1 mg/ml streptomycin, and 10 µg/ml vitamin K (Konakion® Roche). The medium containing the protein of intetrest was harvested after 72 hours of protein expression. The media was centrifuged, filtered through 0.45 µm membrane filters (Millpore) and concentrated using a tangential flow filtration system (TFF; Millipore) with a 10kDa cut-off membrane. Concentrated protein was subjected to purification (see section 2.3.1).

2.3 Purification of proteins

2.3.1 Purification of FV and its mutants

2.3.1.1 Initial purification of FV on a cation exchange SP column

Spun media containing FV was loaded onto a SP sepharose Fast Flow cation exchange column (5ml; GE Healthcare) equilibrated in 20mM Tris-HCl pH 7.4, 150mM NaCl (TBS) supplemented with 5mM $CaCl_2$ and 4mM benzamidine. The media was loaded onto the column on the day of the harvest, at a flow rate of 5 ml/minute. After washing with equilibration buffer, FV was eluted with 0.3M NaCl in 20mM Tris pH 7.4, 5mM $CaCl_2$, 4mM benzamidine. The column was regenerated with 2M NaCl, washed with water and stored in 20% ethanol at 4°C. Peak

fractions were collected and stored at -80°C. The procedure was repeated for each harvest separately.

2.3.1.2 Further purification of FV on an anion exchange QFF column

Subsequently FV was further purified using a Q-sepharose FF anion exchange column (5ml; GE Healthcare). The SP-peak fractions containing FV were thawed at 37°C, pooled, diluted 3 times in 20mM Tris, 5mM $CaCl_2$, pH 7.4 and loaded onto the column equilibrated with TBS, 5mM $CaCl_2$ buffer, (TBSCa2+). The column was washed with equilibration buffer and FV was eluted with a linear gradient of 0.15-0.42M NaCl in 20mM Tris, 5mM $CaCl_2$, pH 7.4 over 10 CV. Fractions containing FV were identified by SDS-PAGE using 4-12% NuPAGE (Invitrogen), followed by silver staining and Western blotting. Fractions containing FV were pooled together and concentrated on a Amicon spin column (Merck) with 100kDa cut off which lead to further purification from low molecular weight species. The concentration of WT FV was determined using prothrombinase assay. FV Nara concentration was measured using A280 (E1% 1cm; 0.96) and confirmed using semi-quantitative Western blotting compared to WT FV.

2.3.2 Purification of protein S and protein C

Protein S, protein C, as well as variants of these proteins were purified using a two step protocol. Initially the concentrated media was barium citrate precipitated in order to select fully ɣ-carboxylated protein. The precipitated protein was further purified by anion-exchange chromatography on a HiTrap DEAE column (GE Healthcare). Fractions containing protein were further concentrated using Amicon® Ultra column with 50kDa cut off. The concentrations of purified proteins were determined by measurement of A280. The following extinction coefficient, ε 0.1%, were used: WT protein S, single amino acid and composite variants of protein S: 0.98, protein S chimera III: 0.99, and protein C: 1.45. (ε 0.1% 1cm; 0.98).

2.3.2.1 Barium citrate precipitation

The TFF concentrated media was aliquoted into two 25 ml samples. 565 µL of a 1M trisodium citrate was added to each sample and incubated on ice for 10 minutes. Subsequently 1.13 ml of 1M barium chloride ($BaCl_2$) was added to each sample. The media was vortexed and incubated on ice for one hour before centrifugation at 8 500g for 15 minutes at 4°C. After that the supernatant was removed and kept for analysis and the pellet was resuspended in 25 ml

of 0.1M $BaCl_2$/0.1M NaCl. The sample was incubated and spun as before. After the second centrifugation the pellet was resuspended in 25 ml of 0.15M sodium citrate in TBS. The precipitated protein was dialysed in TBS (two buffer changes: 5L), for two hours at RT then overnight at 4°C.

2.3.2.2 Purification of protein C and protein S on an anion exchange DEAE column

Barium citrate precipitated protein was further purified on a diethylaminoethyl (DEAE) cellulose anion exchange column (GE Healthcare). The dialysed and filtered sample was loaded on the column already equilibrated with TBS at 2 ml/minute. The column was washed with TBS and bound protein was eluted with linear gradient of 0-30mM $CaCl_2$ in TBS over 7 CV. The column was regenerated with 2M NaCl, washed with water and stored in 20% ethanol at 4°C. Fractions containing protein of interest were identified by SDS-PAGE using 10% gels, followed by silver staining and western blot.

2.4 Characterisation and quantification of proteins

2.4.1 SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reduced and non-reduced conditions was performed to analyse purification fractions, assess rate of cleavage and purity of protein S, protein C and FV. Samples diluted in LDS samples buffer were heated for 5 minutes at 95°C and separated on 10% in-house cast polyacrylamide gel (Table 2.1), 10% and 4-12% pre-cast Bis-Tris gradient gels (Bio-Rad and Invitrogen). Pre-cast gels were run in MES or MOPS buffer (Invitrogen) at 200V. The in house cast gels were run in 400mM glycin, 5mM SDS in 50mM Tris buffer at 100V and 200V through the stacking and running gel, respectively. HiMarkTM Protein Standard (Life Technologies), MagicMark™ XP Western Protein Standard, were used to assess the molecular weights of analysed proteins.

Table 2.3. Composition of gels used for SDS-PAGE
In house polyacrylamide gels were prepared by mixing PROTOGEL (National Diagnostics), 2M Tris pH 8.8 or 0.5M Tris pH 6.8 tetramethylethylenediamine (TEMED, Sigma), 20% SDS, 60% Sucrose, dH_2O and ammonium persulfate (APS, Sigma) at indicated volumes.

Component	Running gel (10%)	Stacking gel (3.7%)
PROTOGEL	3.3 ml	1.2 ml
2M Tris pH 8.8	2 ml	-
0.5M Tris pH 6.4	-	1.4 ml
TEMED	5 µl	10 µl
20% SDS	25 µl	25 µl
60% Sucrose	2 ml	2 ml
dH_20	2.67 ml	5.37 ml
Amonium persulfate (10%)	50 µl	50 µl

2.4.2 Silver staining

The type of silver staining depended to the type of gel used for electrophoresis.The detailed buffers compositions for in-house and pre-cast gels are provided in Table 2.4. Both protocols consist of the following phases: fixation, sensitization, silver impregnation and development. In both protocols, a volume of 50ml of solution per gel was used thoughout of the procedure. All steps were performed at RT with shaking.

2.4.2.1 Silver staining for in-house gels

Proteins were fixed in the in-house gel with 40% ethanol, 10% acetic acid (Buffer A) for 30 minutes. After that the gel was incubated with 30% ethanol, sodium acetate, sodium thiosulfate and glutaraldehyde (Buffer B) for at least 30 minutes. After three 20 minute washes with water, the gel was stained with silver nitrate (Buffer C) for 40 minutes. Afterwards, the gel was developed with sodium carbonate and formaldehyde (Buffer D). When a adequate degree of staining was achieved, the process was stopped with EDTA solution (Buffer F).

Table 2.4.Composition of buffers for silver staining of pre-cast and in house gels
Silver staining included the following steps: fixation, sensitization, silver staining and development which was stopped once suitable staining was achieved. Buffer compositions for each step of the protocol are listed.

		Silver staining for in house gel		Silver staining for pre-cast gel
Fixation	A	40%C_2H_5OH, 10% CH_3COOH	A	50%CH_3OH, 10% CH_3COOH
			B	5%CH_3OH
Sensitization	B	30% CH_3COOH, 8.1mM $Na_2S_2O_3$*$5H_2O$ 500mM CH_3OONa *$3H_2O$, 260 µl of 25% $CH_2(CH_2CHO)_2$	C	1.26 mM $Na_2S_2O_3$*$5H_2O$
Silver staining	C	0.1% $AgNO_3$, 20 µl of 37% HCOH	D	0.2% $AgNO_3$
Development	D	2.5% Na_2CO_3, 15 µl of 37% HCOH	E	3% Na_2CO_3 25 µl of 37% HCOH
Stopping	E	1.4% Na_2EDTA	F	1.4% Na_2EDTA

2.4.2.2 Silver staining for pre-cast gels

The pre-cast gel was fixed with 50% methanol, 10% acetic acid (Buffer A) for 30 minutes, followed by 15 minutes incubation with 5% methanol (Buffer B). The gel was then washed with water three times 5 minutes and sensitized with sodium thiosulphate (Buffer C) for 2 minutes. Afterwards, the gel was washed with water and stained with silver nitrate for 25 minutes (Buffer D). After that the gel was washed with water and developed with sodium carbonate, sodium thiosuphate and formaldehyde solution (Buffer E). When the sufficient degree of staining was achieved, the process was stopped with EDTA solution (Buffer F).

2.4.3 Western blot

Proteins separated by SDS-PAGE were transferred onto Hybond-ECL nitrocellulose membranes (Amersham Bioscience) in a transfer buffer containing 25mM Tris, 190mM glycin, 20% methanol or using a Trans blot mini nitrocellulose transfer Kit (BioRad). Protein transfers were conducted at 25V for 7 or 30 minutes, dependent on the size of analysed proteins the using Trans-Blot Turbo transfer system (BioRad) or at 35V for 1.5 hour using a XCell II Blot system (Invitrogen). Membranes were blocked with 2% bovine serum albumin (BSA, Sigma) in TBS for 45-60 minutes. Proteins were detected by incubation with suitable primary antibodies diluted in 0.5% BSA in TBS. Membranes were incubated 1 hour at RT or overnight

at 4°C, followed by washing (3x5 minutes) with TBS, 0.1% Tween 20. Subsequently the membranes were incubated with suitable secondary horseradish peroxidase (HRP)-conjugated antibodies. Antibodies used thoughout the project are listed in Table 2.5. When needed the buffers and antibodies diluttions were supplemented with 5mM $CaCl_2$.

Table 2.5. Primary and secondary antibodies used for Western blotting during this project
Protein S, protein C and FV were detected with primary antibodies which were subsuqeuntly recognized by HRP conjugated secondary antibodies. All dilutions were prepared in$TBSCa^{2+}$ suplemented with 0.5% BSA and 5mM $CaCl_2$.

Protein	Primary antibody	Secondary antibody
Protein S	Polyclonal rabbit anti-protein S (DAKO); 1:7000	Polyclonal goat anti-rabbit (A0545, DAKO); 1:10 000
	Monoclonal anti-protein S (AHPS5092, HTI); 10 µg/ml	Polyclonal goat anti-mouse (P0447, DAKO); 1:10 000
Protein C	Monoclonal anti-protein C (AHPC-5072, HTI); 10 µg/ml	Polyclonal goat anti-mouse (P0447, DAKO); 1:10 000
	Polyclonal rabbit anti-protein C (P4680, Sigma); 1:2000	Polyclonal goat anti-rabbit (A0545, Sigma); 1:10 000
FV	Polyclonal sheep anti-FV (PAHFV, HTI); 20 µg/ml	Mouse anti-goat/sheep (A9452, Sigma); 1:160 000
	Monoclonal anti-FV (AHV5146, HTI); 10 µg/ml	Polyclonal goat anti-mouse (P0447, DAKO); 1:10 000

Proteins were detected with HRP substrate (IMMOBILON ECL; ThermoFisher) according to the manufacturer's instractions. The blots were acquired using a Chemidoc™ imaging system (BioRad) and analyzed using the Image Lab™ software.

2.4.4 Protein S Enzyme-linked immunosorbent assay (ELISA)

An in house ELISA was employed to measure the concentration of protein S in concentrated conditioned media. For this, a 96-well microplate (Nunc Maxisorp) was coated with polyclonal anti-protein S antibodies (5 µg/ml, DAKO) in 50mM sodium carbonate buffer, pH 9.6 After overnight incubation at 4°C and all subsequent incubation steps the wells were washed 3 times with 200 µl of 20mM Tris, pH 7.4, 150mM NaCl, 5mM $CaCl_2$ and 0.1% Tween 20 ($TBSTCa^{2+}$). All of the following incubation steps were performed at 37°C . The wells were blocked with 3% BSA in $TBSCa^{2+}$ for 2 hours. Protein S samples and standard curve (0-5nM) were diluted in 1% BSA in $TBSTCa^{2+}$ and were incubated in the wells for 1 hour (100 µl/ml). Bound protein S was detected with monoclonal anti-protein S antibodies (8nM) (391609;

R&D), followed by 8nM of HRP-conjugated goat anti-mose (DAKO) antibody. Both antibodies were diluted in 0.5% BSA in TBSCa^{2+} and incubated in the wells for one hour at 37°C. The plate was developed with 100 µl/well HRP chromogenic substrate o-phenylenediamine dihydrochloride (OPD; Sigma). Once sufficient color was obtained, the reaction was stopped with 50 µl/well of 3.2M H_2SO_4 and the absorbance was measured at 492 nm using microplate spectrophotometer (BioTek). The data analysis was performed using GraphPad Prism and a sigmoidal dose respone equation was used to determine concentrations of protein S samples.

2.4.5 Protein quantification based on optical density

In order to quantify the total protein concentration of protein S, protein C and FV preparations the absorbance was measured at 280nm using a NanoDrop spectrophotometer. The Beer Lambert law for concentration C=A/εL was used. Protein concentrations were determined by dividing the absorbance of a protein by its extinction coefficient, ε. The following ε 0.1% were used during the project: WT protein S and single amino acid and composite variants: 0.98, protein S chimera III: 0.99, WT FV, FV Nara: 0.96, FV-810: 1.54 and protein C: 1.45.

2.4.6 APC activation and quantification using chromogenic substrate

Protein C was activated by Protac at a ratio of 0.1U/ml of Protac per 100nM of protein C. Activation was performed for 2 hours at 37°C, followed by 16 hours incubation at 4°C. The concentration of APC was determined using an APC chromogenic substrate, S2366 (Chromogenix). Activated recombinant APC as well as commercial plasma derived APC (HTI) were diluted in 0.1% BSA in 20mM Tris, pH 7.4, 100mM NaCl, 1 mg/ml of PEG 6000 buffer. 100 µl of each preparation was transferred into 96-well plate. Following addition of the APC substrate (400 µM), the rate of the cleavage was measured at 405nm for 20 minutes using a microplate spectrophotometer (BioTek). The data was analysed using GraphPad Prism. The concentration of APC was derived from the linear standard curve of plasma derived APC.

2.5 Functional assays

2.5.1 Phospholipid preparation

Synthetic phospholipids (Avanti Polar Lipids) 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-Dioleoyl-sn-glycero-3-phosphoserine (DOPS), and 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE),1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine-N-biotinyl (biotinylated DOPE) were prepared to provide a negatively charged cell membrane in functional, pull-down and flow cytometry assays. Phospholipid vesicles with ratio DOPS/DOPC/DOPE 20:60:20 were used in plasma based-thrombin generation assays and FVa inactivation assays. A molar ratio of DOPS/DOPC 10:90 was used in prothrombinase assays. Phospholipid vesicles DOPC/DOPS/DOPE/biotinylated DOPE 60:20:18:2 were used to prepare phospholipid-coated magnetic beads for pull-down and flow cytometry assays. The phospholipids, dissolved in chloroform, were mixed at appropriate ratio and the chloroform was then removed under a stream of nitrogen gas. The phospholipids were resuspended in TBS and extruded by pushing through a 0.1 μm filter at least 19 times. Extruded unilamellar phospholipids with final concentration of 1.25mM were stored at 4^{o}C and used within 4 days.

2.5.2 The calibrated automated thrombogram (CAT) assay

2.5.2.1 The principle of CAT assay

Thrombin generation assays by calibrated automated thrombography (CAT) were used to assess APC cofactor function of protein S variants. CAT is a plasma-based assay in which thrombin generation is initiated by addition of TF (Dade Innovoin). Thrombin formation is monitored by the cleavage of a thrombin-sensitive fluorogenic substrate Z-GlyArg_AMC-HCl (Bachem) over time using a Fluoroscan Ascent FL Plate Reader (Thermo Lab System) and Thrombinoscope software (SYNAPSE BV). The amount of thrombin generated is quantified by reference to a standard thrombin calibrator (Diagnostics Stago UK Ltd). To initiate coagulation with the citrated plasma, it is supplemented with calcium. Addition of phospholipid vesicles provides a surface for coagulation. Corn trypsin inhibitor (CTI) is added to inhibit FXIIa and thereby contact activation. The most important parameters used to characterise thrombin generation are: lag time, thrombin peak height and the endogenos thrombin potencial (ETP). The lag time is an equivalent to clotting time and represents the time to the burst of thrombin

generation. Thrombin peak height is the highest rate of thrombin generation. The ETP refers to the total amount of thrombin generated during the assay.

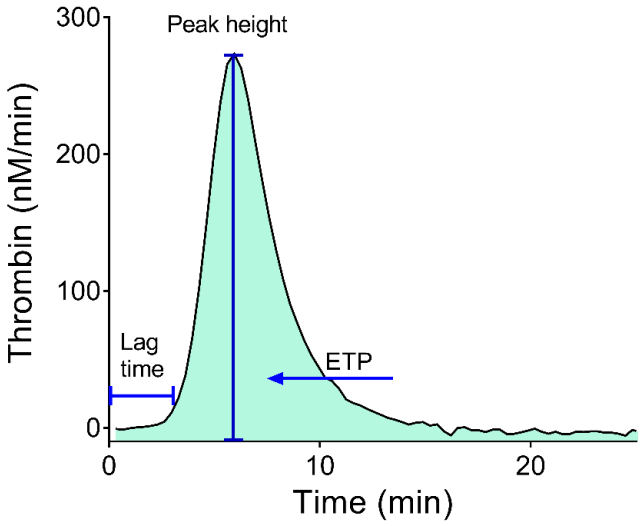


Figure 2.1. Parameters of thrombin generation in CAT assays.
Thrombin generation in CAT is characterised by lag time, peak height and ETP.

2.5.2.2 Evaluation of APC cofactor function of protein S

The abilities of protein S, WT and variants, to enhance APC-mediated inhibition of thrombin generation were assessed in protein S depleted plasma (Enzyme Research Laboratories). The plasma was supplemented with the increasing concentration of WT protein S, protein S variants spanning the EGF3 and EGF4 domains, protein S chimera III (CHIII) and variants E36A, D95A for which reduced APC cofactor function has been previously established.[182,189,207] Thrombin generation was initiated by addition of 1pM TF, 50µM phospholipid vesicles (DOPC/DOPS/DOPE; 60:20:20) and 16.6mM $CaCl_2$. Thrombin generation was monitored in the presence or absence of 9nM APC and protein S variants (0-120nM). The assay was also performed in the presence of anti-protein S antibodies to demonstrate that the anticoagulant properties were specific.

2.5.3 Prothrombinase assay

2.5.3.1 The principle of the prothrombinase assay

Prothrombinase assays were used to study WT FVa, $FVa_{Nara,}$ FV-810 and FVa-810 cofactor functions for FXa and to quantify FVa activity remaining after inactivation by APC in FVa inactivation assay. It was also used to quantify WT FV after expression and purification. In this assay, thrombin generation was induced by addition of prothrombin (Enzyme Research Laboratories) to a reaction mix consisting of FXa, FVa, phospholipids and calcium. The reaction was allowed to proceed for 2 minutes at 37°C. After that time thrombin generation was stopped by dilution into 50mM Tris, pH 7.4, 100mM NaCl, 20mM EDTA, 1% PEG 6000. The rate of thrombin generation was measured by cleavage of the chromogenic substrate S-2238 (Chromogenix) at 405nm for 20 minutes. The amount of thrombin generated was quantified using a standard curve with a known amount of thrombin (Enzyme Research Laboratories).

2.5.3.2 Evaluation of the FXa cofactor function of FVa

To asses the FXa cofactor function of FVa, FVa Nara, FV-810 and FVa-810 , thrombin generation was initiated by addition of prothrombin (500nM or 0-600nM) to a mixture of FVa (8pM), FXa (5nM), phospholipid vesicles (DOPC/DOPS, 90:10) in the presence of $CaCl_2$ (2mM).

2.5.3.3 Quantification of FVa inactivation assay

In order to quantify FVa activity remaining after FVa inactivation assay, the prothrombinase assays were performed with 500nM prothrombin and 8pM FVa and 5nM of FXa. In assays used to quantify FVa Nara activity, 16pM of FVa was used due to its low activity.

2.5.4 FVa inactivation assay

2.5.4.1 The principle of the FVa inactivation assay

FVa inactivation assays were employed to study the APC-mediated inactivation of WT FVa, FVa Nara, FV-810 and FVa-810 as well as the ability of protein S and its variants to enhance APC-mediated FVa degradation. To specifically determine the protein S enhancement of cleavage at FVa Arg306, a FV R506Q/R679Q variant was used. FVa, activated by thrombin,

was inactivated by APC in the presence and absence of WT protein S or its variants. Efficiency of the inactivation was quantified by remaining FVa activity in prothrombinase assays.

2.5.4.2 FV and FVIII activation

WT FV and its variants, FV Nara, FV R506Q/R679Q and FV-810, were activated by human thrombin (Enzyme Research Laboratories) under two different conditions. For the purpose of pull-down and flow cytometry assays, FV was activated at a FV to thrombin ratio of 1:0.25. For functional assays 1.336nM of FVa was activated with 0.5U/ml of thrombin (1:3.3 ratio). In both cases the activation was allowed to proceed for 10 minutes at 37°C and was subsequently stopped with 2-fold excess of hirudin to thrombin. The activation reaction was performed in 0.5% BSA in 50mM Tris, pH 7.4, 150mM NaCl and 5mM $CaCl_2$ ($BTBSCa^{2+}$). FVIII was activated with thrombin at ratio of 1:1 for 3 minutes at 37°C in $BTBSCa^{2+}$. The FVIII activation was stopped with 2-fold exess of hirudin to thrombin. Full activation of both proteins was assessed by Western blotting.

2.5.4.3 FVa inactivation

0.8nM FVa was inactivated by APC (0.25nM or 0-0.25nM) in the presence of protein S (0-100nM or 100nM) or by 0-4nM APC in the absence of protein S. The inactivation was conducted in the presence pf 25µM of phospholipids (DOPC/DOPS/DOPE; 60:20:20) for 10 minutes at 37°C in $BTBSCa^{2+}$buffer. The inactivation was stopped by 20-fold dilution in the same buffer. To specifically characterise the enhancement of cleavage at Arg306 of FVa mediated by protein S, FV R506Q/R679Q was used. Remaining FVa activity after APC-mediated inactivation was measured in prothrombinase assays as described in section 2.5.3.1.

2.5.5 Pull-down to phospholipid coated beads

2.5.5.1 Preparation of phospholipid coated magnetic beads

Phospholipid coated magnetic beads were used in pull-down and flow cytometry experiments. For this, streptavidin coated magnetic beads (Invitrogen) were washed twice with 0.5% BSA in TBS, 5mM $CaCl_2$ and quenched with 2% BSA in TBS, 5mM $CaCl_2$ for 4 hours at 37°C at constant rotation. Subsequently, the beads (2.5 mg/ml) were mixed with 2mM

phospholipid vesicles (DOPC/DOPS/DOPE/biotinylated DOPE; 60:20:18:2) at RT, overnight with rotation. The beads were then washed twice and re-suspended in 0.5% BSA in TBS, 5mM $CaCl_2$ at a concentration of 2.5mg/ml.

2.5.5.2 Pull-down of APC/protein S/FVa to phospholipid coated beads

The effects of protein S and/or FVa on APC binding to phospholipids were assessed using pull-down experiments. For this, phospholipid-coated magnetic beads (250 µg/ml) were pre-incubated with protein S (100nM) and/or FVa (25nM) for 25 minutes prior to addition of APC (Haematologic Technologies Inc.) or active site inhibited, FITC labelled APC (APC-FEGRCK) (Haematologic Technologies Inc.). Supernatants and the beads were separated by a magnet after 0.5 minute or 2 minutes incubation with APC and APC-FEGRCK, respectively. After separation, the beads were briefly washed with 400 µl TBS, 5mM $CaCl_2$. Bound proteins were eluted with 30 µl LDS buffer (Invitrogen). Eluted samples were loaded on a 4-12% SDS-PAGE under unreduced conditions and transferred to a nitrocellulose membrane. Proteins were detected using polyclonal anti-protein C (Sigma), monoclonal anti-FV (AHV-5146; Haematologic Technologies Inc.) or polyclonal anti-protein S (DAKO) antibodies. The blots were acquired using a Chemidoc™ imaging system (BioRad) and analyzed using the Image Lab™ software.

2.5.5.3 Pull-down of WT FVa and FVa Nara to phospholipid coated beads

The ability of WT FVa and FVa Nara to bind to phospholipids was assess in pull-down experiment where phospholipid coated magnetic beads were incubated with 5, 10 or 25nM of FVa. After 25 minutes incubation, supernatants (100 µl) were collected and the beads were washed with 400 µl TBS, 5mM $CaCl_2$. Bound proteins were eluted with 30 µl LDS buffer and run alongside corresponding supernatants on 4-12% SDS-PAGE, blotted and detected with anti-FV (AHV-5146) antibodies.

2.5.6 Flow cytometry assay

2.5.6.1 The principle of the flow cytometry assay

I have optimised a flow cytometry assay to study the effect of protein S and/or FVa and their variants on binding of FEGRCK-active site labelled APC to phospholipid-coated magnetic

beads. Binding of APC-FEGRCK to phospholipid vehicles was evaluated in the presence and absence of protein S and/or FVa. The enhancement of the APC-phospholipids interaction was reflected in the increase of the mean fluorescence intensity (MFI). MFI was measured using a flow cytometer (BD FACSCalibur) either at 1 minute or at defined time points after addition of APC-FEGRCK to phospholipid coated magnetic beads. The data was analysed using Flowlogic and Flowing software(version 2.5.1).

2.5.6.2 Evaluation of APC-FEGRCK interaction with phospholipids

The beads (0.125 mg/ml) were pre-incubated with 100nM protein S and/or 0-75nM FVa at room temperature for 25 minutes. The mean fluorescence intensity (MFI) was measured at precisely defined time points after addition of APC-FEGRCK (0-75nM). Specificity of the enhancement by protein S and FVa upon the binding was demonstrated with anti-protein S (1µM; DAKO) and anti-FV (0.25µM; AHV-5101, Haematologic Technologies Inc.) antibodies. The antibodies were pre-incubated with the respective antigen for a minimum of 10 minutes before addition of APC.

The effect of C4BP upon the enhancement induced by protein S and FVa on the APC binding to membranes was studied with 200nM ß-chain containing C4BP (2:1 ratio to protein S). C4BP was preincubated, together with protein S and FVa for 10 minutes, prior to the addition of APC. The MFI was measured 1 minute after addition of APC.

The importance of protein S residues for the direct interaction between APC, protein S and FVa, was studied using protein S variants previously known to have reduced APC cofactor function, protein S E36A, D95A, CHIII, as well as three newly identified EGF3-4 variants with decreased APC cofactor function, protein S DEEE, DK and REDD. The assays were run with 50nM protein S to make the assay more sensitive to any decrease in protein S enhancement. The MFI was measured immediately and 1, 3, and 5 minutes after addition of APC.

For proteins E36A, D95A and CHIII, binding of increasing concentrations (0-75nM) APC-FEGRCK to phospholipid-coated beads was measured. This was done in the presence and absence of 25nM FVa 100nM WT protein S and its variants. For this, the MFI was measured 1 minute after addition of APC. Binding of 50nM APC-FEGRCK to phospholipid-coated magnetic beads was also characterised in the presence and absence of 100nM WT protein S and/or

25nM WT FVa, FV-810 and its activated form FVa-810 or FVa Nara. Additionally, WT FVa and FVa Nara were titrated (0-75nM) to assess the concentration dependency upon this protein for complex formation. The MFI was measured at 1 minute after addition of APC.

3 RESULTS

3.1 Purification and quantitation of proteins

This main aim of this project is to establish the molecular mechanisms involved in the inactivation of FVa by APC. For this type of project, high quality reagents are key, in particular working with pure and well characterised proteins. Therefore, recombinant FV, protein S and protein C were produced as described in detail in Methods section 2.3 of this thesis. All recombinant proteins expressed and purified during this PhD project are listed in Table 3.1.

Table 3.1. Recombinant proteins investigated during this thesis.
Variants of FV, Protein S and Protein C that were stably expressed, purified and quantified during this PhD project are listed.

Protein	Substitution, deletion of fragment	cDNA	Stable cell line	Pure protein
Factor V WT				
Factor V 2Q	R506Q/R679Q			
Factor V-810	Lacking residues 811-1491			
Factor V-810 2Q	Lacking residues 811-1491 R506Q/R679Q			
Factor V Nara	W1920R			
Protein S WT				
Protein S E36A	E36A			
Protein S D95A	D95A			
Protein S DEEE	D182N/E184Q/E186Q/E189Q			
Protein S REDD	R192Q/E201Q/D202N/D204N			
Protein S DK	D227N/K233Q			
Protein S CHIII	SHBG-like domain			
Protein C				
Protein C D36A/L38A/A39V	D36A/L38A/A39V			

Available in the lab
Generated during PhD project

3.1.1 Purification, quantification and activation of FV

3.1.1.1 Purification of FV and its variants

A main focus of this thesis is to determine the role/s of FVa in its own inactivation by APC and its cofactor, protein S. To enable this, I expressed and purified various versions of FV . To study the role of FVa in its inactivation mediated by APC, pure WT FV was required. I also produced FV Nara, a naturally occurring FV variant reported to be strongly resistant to APC. I hypothesised that studying this variant might shed light on the mechanisms involved in FVa inactivation. A FV variant, FV R506/R679Q, was produced to evaluate the ability of WT protein S and its variants to specifically enhance FVa cleavage at residue Arg306. FV-810, a FV with partially removed B domain, originally designed and produced by Toso *et al*.[238], was employed to study the kinetics and molecular mechanisms of APC mediated inactivation. This deletion variant was also prepared with the double substitution, R506Q/R679Q.

FV and its variants were expressed by stably transfected BHK cells in triple flasks (described in section 2.2.2). FV is an unstable protein, prone to proteolysis. To reduce the degree of FV proteolysis I kept all samples and columns on ice during the whole purification procedure. For the same reason, I harvested FV containing media in the presence of saturating amount of protease inhibitors. The media was first spun down to remove cell debris. The FV was then partially purified using cation exchange chromatography. Importantly this was done on the day of the harvest. Figure 3.1A presents a representative chromatogram of the first step of FV purification. FV that was bound to the SP-column was eluted with 0.3M NaCl in 20mM Tris buffer as can be seen by the increase in absorbance (A280). Reusing the same cells, new media was harvested every 24 hours for 5-6 days. At the end of the expression phase, all FV elutions from the SP columns were quality assessed on Western blotting for proteolysis. As illustrated in Figure 3.1B (see lanes H5 and H6), the amount of FV cleavage increased in the 5th and 6th harvests. Peak fractions, which were not substantially cleaved, were pooled together and purified further using anion exchange chromatography (Figure 3.1C). For this, partially purified FV was loaded on a QFF column and eluted with a linear gradient of NaCl (0.15-0.7M). Fractions were collected throughout the elution and were analysed by SDS-PAGE. To evaluate purity of the eluted FV, proteins were detected by silver staining, see Figure 3.1D. FV was identified and the level of cleavage was assessed by Western blot analysis using

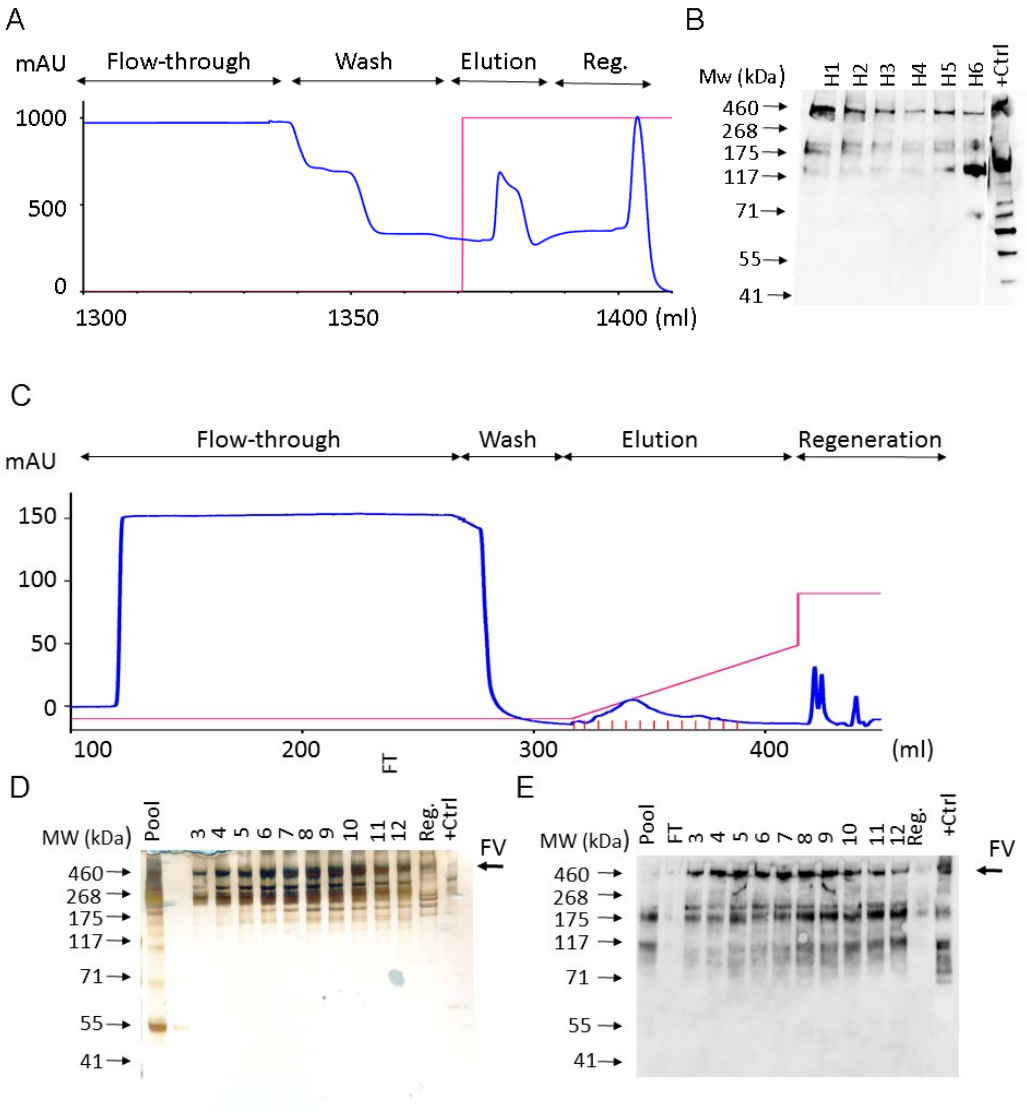


Figure 3.1.Purification of FV using a two stage ion-exchange chromatography protocol.
(A) Conditioned media was applied onto a SP-sepharose fast flow (SPFF) column. The column was washed with TBSCa containing 4mM benzamidine. Proteins were eluted with 0.3M NaCl in 20mM Tris pH7.4. Peak fractions were further purified on a Q-Sepharose fast flow (QFF) column. **(B**) Peak fractions from SP purifications were analysed by Western blot and detected with monoclonal anti-FV antibodies (AHV-5146). **(C)** Peak fractions from the SP-sepharose purification were together, diluted 3 times with 20mM Tris pH7.4 and 5mM CaCl2 and applied to a QFF column. After washing with TBSCa2+, FV was eluted with linear gradient of 0.15-0.7M NaCl. **(D-E)** Elution fractions containing FV were identified by SDS-PAGE and Western blot analysis. For this, fractions were loaded into a 4-12% gradient gel. Electrophoresis was run under non-reduced conditions. Proteins were visualised using silver stain **(D)** or blotted and detected with monoclonal anti-FV antibodies **(E).** The samples assessed comprised: regeneration fraction (Reg.), SP peak fractions (H1-H6), positive control (+Ctrl), high molecular weights marker (HiM), fractions from SP purification pooled together (Pool), elution fractions from QFF column (3-12), flow through from QFF column (FT).

monoclonal anti-FV antibodies (AHV-5146), Figure 3.1E. Although the results from the Western blot result suggests appreciable cleavage of FV, it should be noted that antibody AVH-5146 has substantially higher affinity for cleaved FV than for full-length FV and therefore

overestimated the amount of cleavage. Fractions containing FV were pooled together and concentrated.

While multiple contaminating proteins were present on the silver stained gel of the elution fractions from the QFF (Figure 3.1D) many were not present in the final preparation, as can be seen in Figure 3.2. The majority of contaminants were removed during the concentration step on a spining column, due to the high molecular weight cut off (100kDa) used.

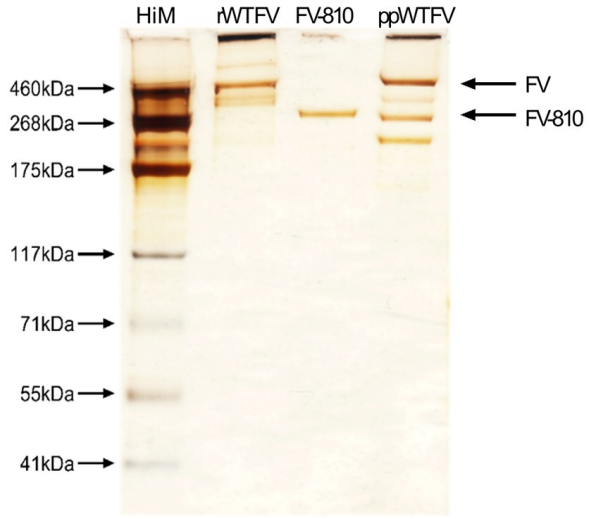


Figure 3.2. SDS-PAGE analysis of WT FV and FV-810.
500 ng of purified recombinant FV (rWTFV), plasma purified FV (ppWTFV) and partially B domain truncated FV (FV-810) were analysed on SDS-PAGE (4-12% gradient gel) under non reducing conditions. Proteins were visualised using silver-staining. HiM, High molecular weight marker.

Production of FV and its variants proved to be problematic due to low yields. The yield from 7.5L expression media was approximately 50-150 µg of WT FV. The low yield arose from the fact that FV is a highly glycosylated, large protein, which therefore is expressed at low levels. In addition, as mentioned above, FV is very easily proteolysed. Since the majority of the assays used during my work required high amounts of FV, expression and purification of FV required large amounts of time spent expressing the protein under conditions which minimised proteolysis.

3.1.1.2 Quantitation of FV

The concentration of purified FV was first determined by measurement of optical density at A280. However, it soon became clear that there were inconsistencies in the quality of the

purified FV in different batches. Quantitation through A280 measurement was therefore no longer suitable. The FV concentrations used throughout this thesis have therefore been determined in prothrombinase assays following FV activation by thrombin (see methodological details in section 2.5.3 and 2.5.4.2). For this, commercial plasma purified FV, with a known concentration, was activated with thrombin alongside recombinant purified FV. FVa was then diluted and thrombin generation initiated by addition FXa to prothrombin in the presence of increasing concentrations of plasma purified FVa (0-32pM) or recombinant FVa. FVa effectively enhanced thrombin generation in a dose-dependent manner as shown in Figure 3.3A. The generated thrombin cleaved a chromogenic substrate, generating absorbance (A405). The rate of substrate cleavage (mA405/minute) was then used as a measurement of thrombin concentration which was plotted against FVa concentration (Figure 3.3B). As demonstrated in Figure 3.3B, a linear relationship between FVa concentration and thrombin generation was obtained. The graph also shows that the assay is very sensitive to FVa in this pM concentrations range. Recombinant FVa concentrations were derived by reference to slopes obtained for plasma purified FVa. For the most pure batches of FV, the concentration determined using this prothrombinase assay corresponded well to the concentration quantified by A280.

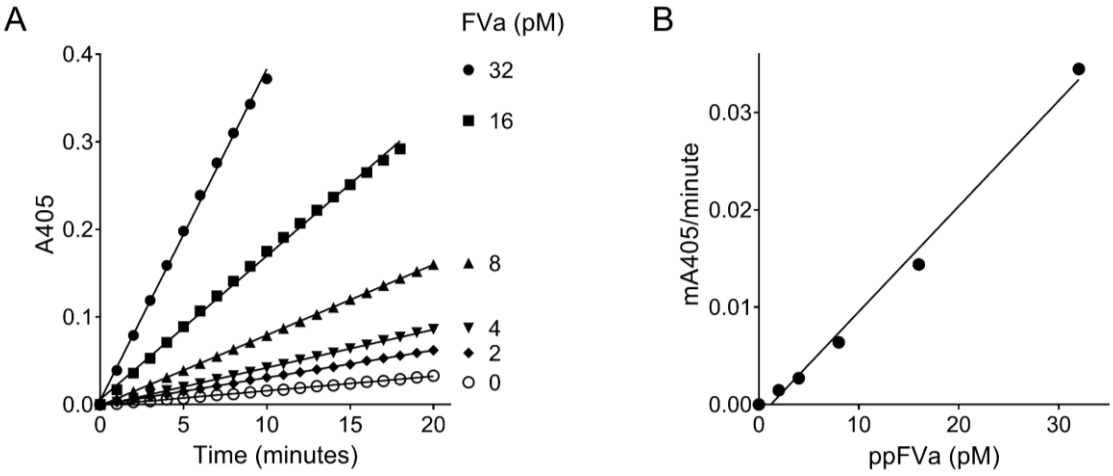


Figure 3.3. Standard curve of plasma purified FVa (pp) for quantification of recombinant FVa in a prothrombinase assay.

Plasma purified and recombinant FV (1.3nM) were both activated by 0.5U/ml of human thrombin. The activation was stopped by addition of 1.5 U/ml of hirudin. **(A)** 0-32pM of plasma purified FVa was incubated with 5nM FXa in the presence of phospholipids (DOPS/DOPC/DOPE; 10:90) and $CaCl_2$. Thrombin formation was trigered by addition of 0.5um prothrombin. After 2 minutes the reaction was stopped by dilution in EDTA and thrombin generation was quantified using a chromogenic substrate (S2238) for 20 minutes.**(B)** Slopes obtained at A405 were derived using linear reggresion and plotted against the concentration of activated plasma purified. The concentrations of recombinant FV were derived using the ppFVa curve.

The FV Nara mutation (W1920R) is localised within the FV C1 domain, close to the proposed phospholipid binding sites of FV.[118] I thought it was likely that the mutation could affect the ability of FV Nara to bind phospholipids. Moreover, in preliminary experiments, FV Nara did not appear to enhance thrombin generation as efficiently as WT FV. There was a systematic difference between FV Nara concentrations derived from prothrombinase assay and A280. For these reasons, FV Nara was initially quantified by A280, assuming the same level of purity as WT FV, purified in parallel with FV Nara. The concentration of FV Nara was confirmed by semi-quantitative Western blot using polyclonal anti-FV antibodies, as shown in Figure 3.4.

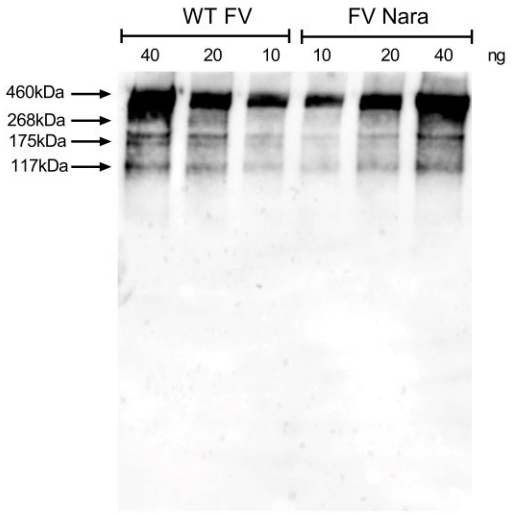


Figure 3.4. Semi-quantitative Western blot of WT FV and FV Nara.
10, 20, 40 ng of WT FV and FV Nara were separated on 4-12% gradient gel under non-reducing conditions. Following SDS-PAGE, samples were analysed by Western blotting using polyclonal anti-FV antibodies

3.1.1.3 Activation of FV

In order to study the activated forms of FV and FV-810, it was essential to determine the conditions under which FV was completely activated. For this, 1.34nM of WT FV and FV-810 were activated with different thrombin concentrations. As can be seen from Figure 3, FV-810 was successfully activated even with a ratio of 50:1 of FV:thrombin. In contrast, activation of WT FV was slightly less efficient. Full activation of WT FV occurred at ratio 4:1 (FV:thrombin).

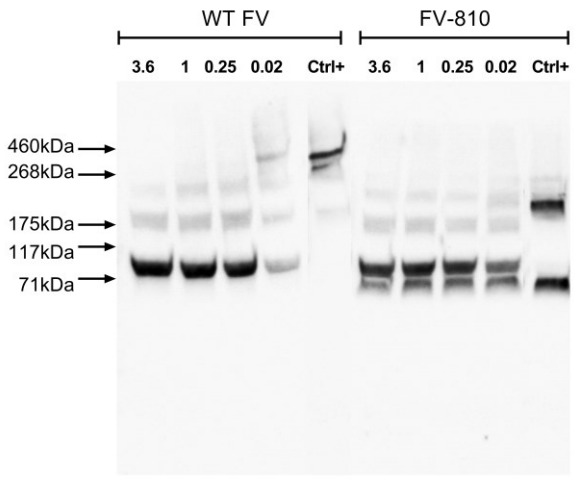


Figure 3.5. Activation of FV and FV-810 with different concentration of thrombin.
FV (1.34 nM) was activated with 0.027, 0.334, 1.33 or 4.81 nM of thrombin, corresponding to a FV:thrombin ratio of 50, 4, 1, 0.28. The activation was performed for 10 minutes at 37°C. The reaction was stopped with 2-fold access of hirudin over that of thrombin. All activation reactions were analysed on SDS-PAGE under non reducing conditions followed by Western blot analysis. Activated and non-activated forms of FV/FV-810 were detected with monoclonal anti-FV antibodies (AHV-5146).

3.1.2 Purification and quantification of protein S

Protein S, the cofactor of APC, is essential for efficient inactivation of FVa. Stably transfected HEK293 cells expressing WT protein S, protein S E36A, D95A and CHIII were available in our laboratory. The expression vectors containing substitutions spanning the EGF3 and EGF4 domains, protein S DEEE, REDD and DK, were stably transfected into HEK293 cells. All variants were successfully expressed and purified.

3.1.2.1 Purification of protein S by barium citrate precipitation and DEAE anion exchange chromatography

Protein S was expressed by stably transfected HEK293 cells in triple flasks. I harvested media containing protein S after 2-3 days of expression and purified the protein using protocols previously optimised by my supervisor, Dr Josefin Ahnström. Binding of protein S to negatively charged phospholipid membranes is key for all of its functions, a process which is highly dependent on its Gla residues. Therefore, in order to selectively purify fully ɣ-carboxylated protein S, the media was precipitated with barium citrate. During the first step of the precipitation, sodium citrate was added to chelate the calcium ions in the Gla-domain of ɣ-carboxylated protein S. Once barium chloride was added, the barium ions replaced the

sodium ions and precipitated the ɣ-carboxylated protein S. Partially purified protein S was dialysed overnight against TBS and then purified further by anion exchange chromatography using a DEAE column. Protein S that was bound to the column was eluted with a linear gradient of 0-30mM $CaCl_2$ as illustrated in the chromatogram in Figure 3.6A. Fractions from different stages of purification were analysed by SDS-PAGE, followed by silver staining, Figure 3.6B or Western blot, Figure 3.6C. As can be seen on the silver stained gel, there was a single protein band in elution fractions with MW of around 73 kDa, which was confirmed by Western blot analysis to be protein S. Protein S was purified to homogeneity, with a yield ranging between 0.1-0.5 mg from 1.125L of expression media

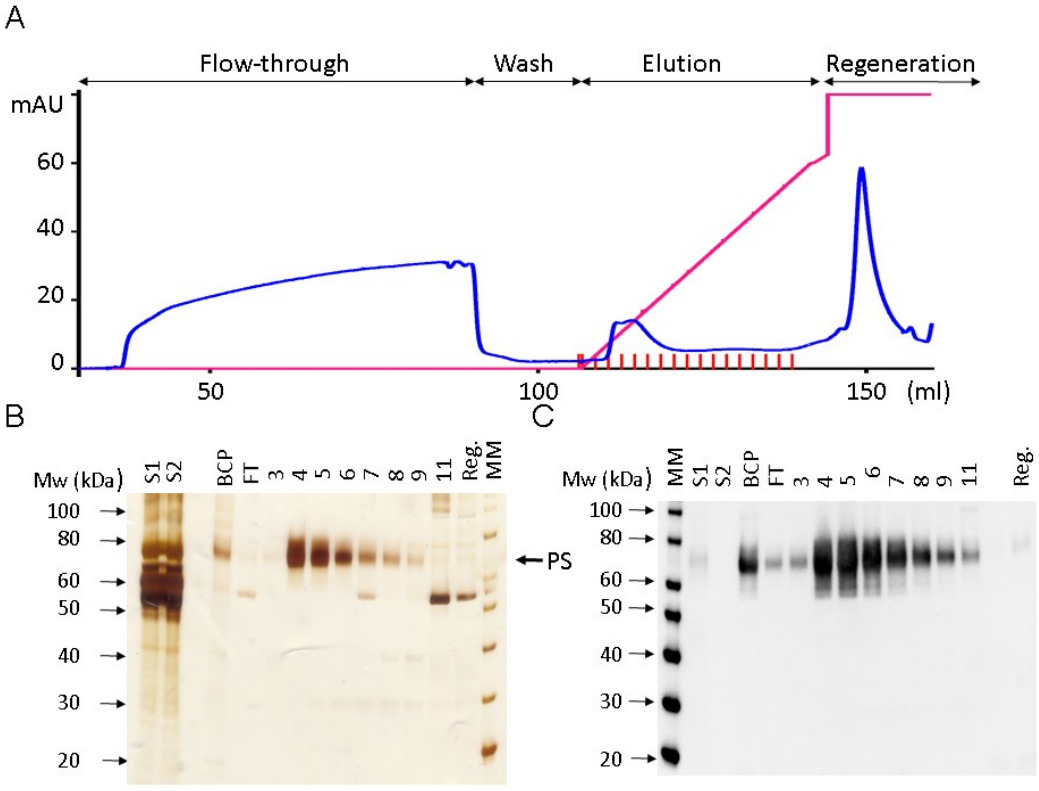


Figure 3.6. Purification and analysis of WT protein S.
Concentrated conditioned media from stably transfected HEK293 cells expressing protein S was barium citrate precipitated. Barium citrate precipitated proteins were further purified on a 5 ml HiTrap DEAE column **(A)** followed by analysis on SDS-PAGE **(B and C). (A)** Protein S bound to the column was eluted with a linear gradient of $CaCl_2$ (0-30mM) in TBS. The gradient is shown in purple. **(B and C)** The fractions were analysed using SDS-PAGE **(B)** and Western blot analysis **(C)**. 15 µl of each fraction was loaded onto 10% SDS-PAGE under unreduced conditions. The gel was either silver stained or used for Western blotting. On the Western blot, protein S was detected with polyclonal anti-protein S antibodies. Fractions containing protein S (3-5) were pooled together and concentrated on a spin column with a 50 kDa cut off. The samples assessed comprised: supernatant 1 (S 1) and supernatant 2 (S2) from the barium citrate precipitation, Barium Citrate Precipitated proteins (BCP), flow through (FT), elution fractions from the DEAE column (3-11), regeneration fraction (Reg.) and a molecular weight marker (MM).

3.1.2.2 Quantitation of protein S

The concentration of purified protein S was quantified both by ELISA and by measurement of optical density at A280. Quantitation by absorbance at 280 nm was reliable, more cost- and time-effective and was therefore used for the remaining of the project.

The concentrations of protein S and its variants were measured and the samples were then examined by SDS-PAGE and Western blotting (Figure 3.7). As can be seen on Figure 3.7A, all protein S variants were purified to homogeneity. Although the variants REDD and E36A had reduced intensities, there was broad agreement between the absorbance and Western blot method (Figure 3.7B).

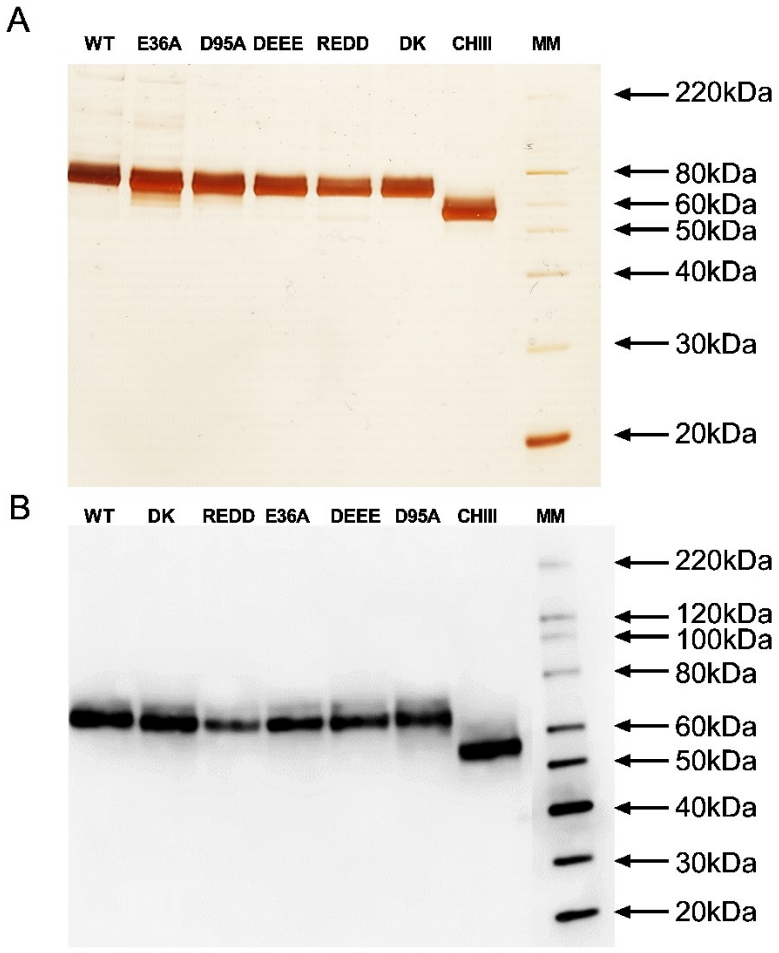


Figure 3.7. SDS-PAGE and Western blot analysis of WT protein S and its variants.
1 µg **(A)** or 500 ng **(B)** of purified WT protein S and protein S variants (E36A, D95A, DEEE, REDD, DK, CHIII) were separated on 10% SDS-PAGE under unreduced conditions. Protein S on Western blot S was detected with monoclonal anti-PS antibodies (AHPS 5092).MM, Molecular weights markers.

3.1.3 Purification, activation and quantitation of Protein C

Similarly to FV and protein S, high quality recombinant protein C was also needed for this project. I therefore set up stably transfected HEK293 cells, expressing WT protein C as well as a variant, protein C D36A/L38D/A39V, which cannot be enhanced by protein S during this project. This was followed by expression and purification of the proteins. The variant was first expressed by Dr Roger Preston during his PhD in our lab, examined in 2004.

3.1.3.1 Purification of protein C by barium citrate precipitation and anion exchange DEAE column

Protein C, like protein S, contains a ɣ-carboxylated Gla-domain and is also dependent on this for its functions. I therefore successfully employed the same two step purification protocol as that used for protein S. The media containing protein C was first barium citrate precipitated and dialysed overnight against TBS. Following BCP, protein C was then further purified by anion exchange chromatography using a DEAE column. Protein C was eluted from the column with a linear gradient of 0-50mM $CaCl_2$. Figure 3.8A is a representative chromatogram of DEAE purification. Fractions from the BCP step as well as fractions from the anion exchange purification were examined with SDS-PAGE followed by silver stain (Figure 3.8B) and Western blot analysis (Figure 3.8C). There was a strong band present on the silver stained gel migrating with the same mobility as plasma derived protein C (Figure 3.8B). Similarly to protein S, the purification protocol was therefore efficient in removing the majority of the contaminants. The ~60kDa band was identified as protein C by rabbit anti- protein C antibody with Western blotting (Figure 3.8C). There were additional bands present with mass of ~ 35-40kDa, which correspond to different subforms of the heavy chain of protein C. Fractions containing protein C were concentrated on a spin column and assessed using SDS-PAGE followed by silver staining and Western blotting. Figure 3.9 presents a comparison of recombinant WT protein C, protein C D36A/L38A/A39V and commercial plasma purified protein C. In contrast to WT protein C, which was essentially pure, the final preparation of the variant contained a number of contaminants. For that reason, it was decided to quantify protein C and its variants following their activation to APC. A chromogenic substrate was used for this purpose. In what follows, protein C D36A/L38A/A39V was used only for preliminary experiments.

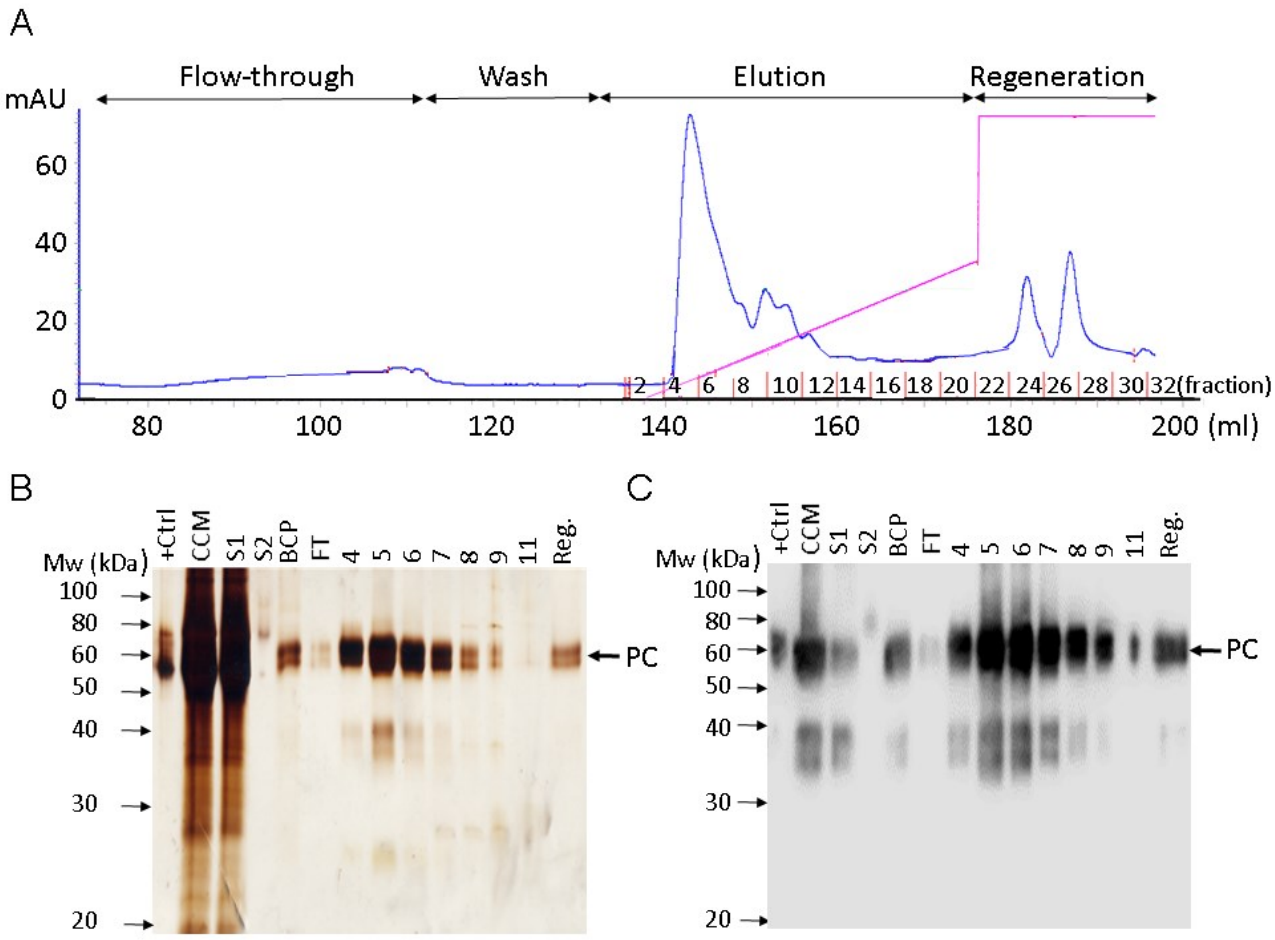


Figure 3.8. Purification and analysis of WT protein C

WT protein C was purified from media from stably transfected HEK293 cells by barium citrate precipitation followed by anion exchange chromatography on a 5 ml HiTrap DEAE column **(A)**. After loading of barium-precipitated proteins onto the column it was washed with TBS. Protein C was eluted with linear gradient of $CaCl_2$ (0-50mM) in TBS. The eluted fractions were analysed on SDS-PAGE. 5 µl of each fraction was loaded onto a 10% SDS-PAGE under unreduced conditions. The gels were used for silver staining **(B)** and Western blotting **(C)**. On Western blotting, protein C was detected using a monoclonal mouse anti-human protein C antibody. The samples assessed comprised: positive control (+Ctrl), concentrated conditioned media (CCM), supernatant 1 (S1), supernatant 2 (S2), barium citrate precipitated proteins (BCP), flow through (FT), eluted from DEAE column fractions corresponding to those in Figure 3.8A (4-11), regeneration fraction (Reg.).

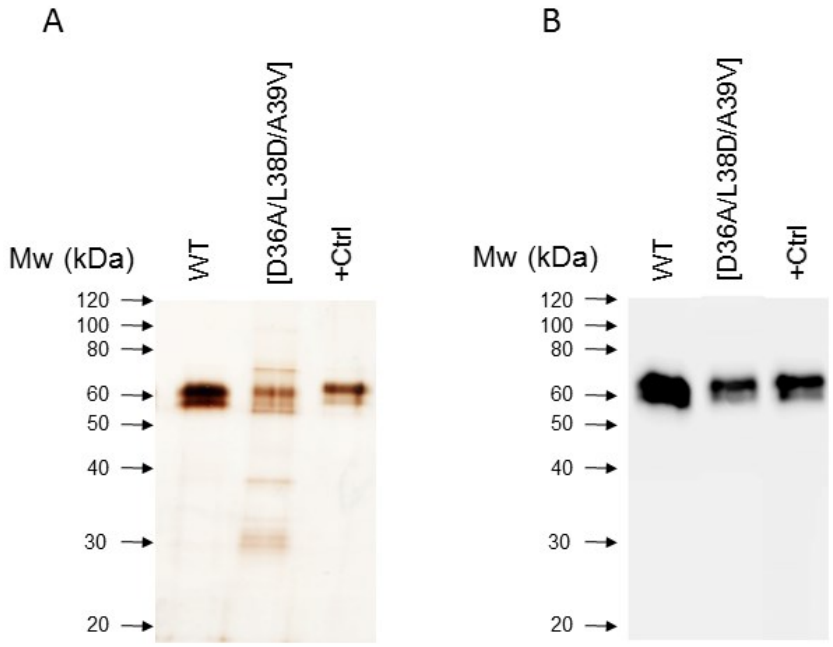


Figure 3.9. Comparison of recombinant protein C with commercial plasma purified protein C.
300 ng of WT protein C and 150ng of protein C D36A/L38D/A39V were analysed on a 10% SDS-PAGE under unreduced conditions and compared to plasma purified protein C (150ng) (+Ctrl). Protein concentration was determined by measurement of absorbance at 280 nm. The gels were used for silver staining **(A)** and Western blotting **(B)**. Protein C was detected in **(B)** using monoclonal mouse anti-human protein C antibodies**.**

3.1.3.2 Activation and quantification of protein C

I activated protein C by Protac, a serine proteinase isolated from Agkistrodon contortrix snake venom using a ratio of 0.1U/ml of Protac per 100nM of protein C (see Methods section 2.4.6). Activation was performed for 2 hours at 37°C, followed by 16 hours incubation at 4°C. The efficiency of the process was determined by cleavage of a chromomeric substrate specific for APC (S2366). Cleavage of the APC chromogenic substrate by increasing concentration of APC was followed over 10 minutes as shown in Figure 3.10A. A linear association between the rate of substrate hydrolysis and APC concentration can be observed in Figure 3.10B. Hydrolysis of the chromogenic substrate was used for quantification of both APC and APC D36A/L38A/A39V. The rate of substrate cleavage was measured for known concentrations of plasma purified APC. The concentration of the recombinant protein C preparations was determined by reference to the generated standard curve, as illustrated in Figure 3.10B.

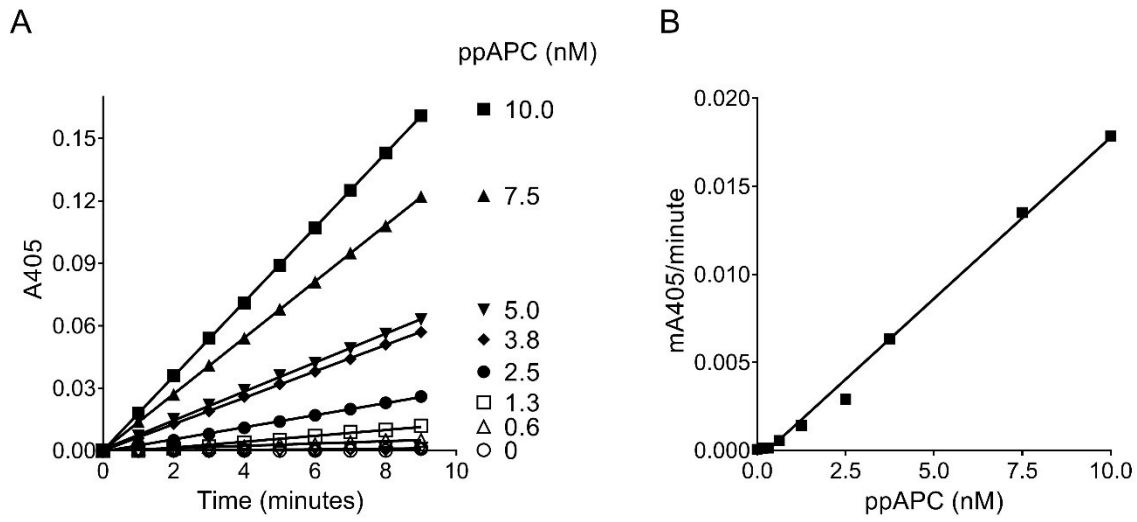


Figure 3.10. Determinantion of the concentration of recombinant APC.
Serial dilutions of activated plasma derived protein C were prepared together with dilutions of recombinant APC and APC D36A/L38A/A39V. The rate of chromogenic substrate hydrolysis was measured at A405 nm. **(A)** The cleavage of chromogenic substrate (S2366) by 0-10nM of plasma purified APC was measured for 10minutes. **(B)** The rate of the substrate hydrolysis was plotted against concentration of plasma purified APC. Curves such as this were then used to derive the concentration of the recombinant APC proteins.

3.2 Evaluation of cofactor function of protein S and FVa

3.2.1 APC-dependent cofactor function of protein S

The overarching aim of my project is to determine the molecular mechanisms involved in the inactivation of FVa by APC. Initially the importance of cofactor function of protein S for APC anticoagulant activity was studied using CAT thrombin generation assay. For this, the CAT assay was conducted in protein S depleted plasma as described in details in section 2.5.2. As can be seen from the curves in Figure 3.11, 9nM plasma derived APC did not affect thrombin generation in the absence of protein S. When APC was added to the plasma together with protein S, peak height and area under the generated curve, ETP, were substantially reduced. When anti-protein S antibodies were added together with APC and protein S, the anticoagulant effects of APC and protein S were completely reversed, showing the importance of protein S as a functional cofactor for APC.

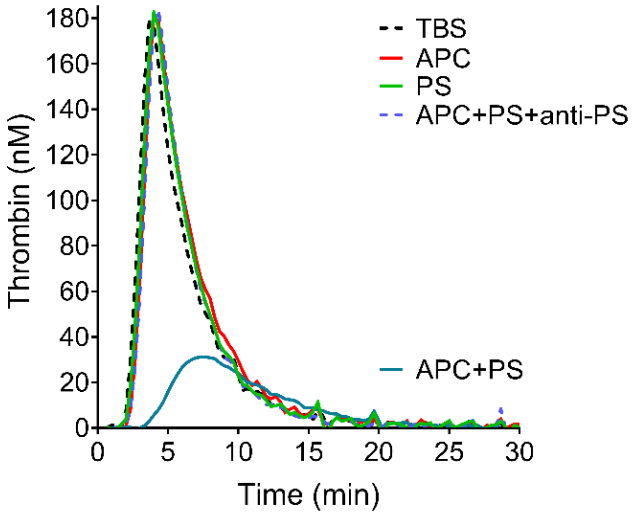


Figure 3.11. APC cofactor function of protein S demonstrated in thrombin generation assays.
Thrombin generation was measured using calibrated automated thrombography (CAT) after initiation by 1pM TF and $CaCl_2$ in protein S-depleted plasma reconstituted with phospholipids, TBS, 9nM APC (plasma derived) and protein S. The protein S enhancement of APC by 60nM WT protein S was reversed by the addition of polyclonal anti-protein S antibodies (600nM).

The functionality of the purified recombinant APC was also evaluated by thrombin generation assays. While thrombin generation was not reduced even at 70nM recombinant APC in the absence of protein S (Figure 3.12A), 9nM APC together with 80nM protein S reduced the peak height and ETP by up to 80% (Figure 3.12B). This demonstrates that the purified, recombinant APC is active, and like the plasma derived APC used in Figure 3.11, exerts its anticoagulant activities through an interaction with protein S. Moreover, the results presented in Figure 3.11 and 3.12 show how strongly APC anticoagulant function depends on the presence of protein S.

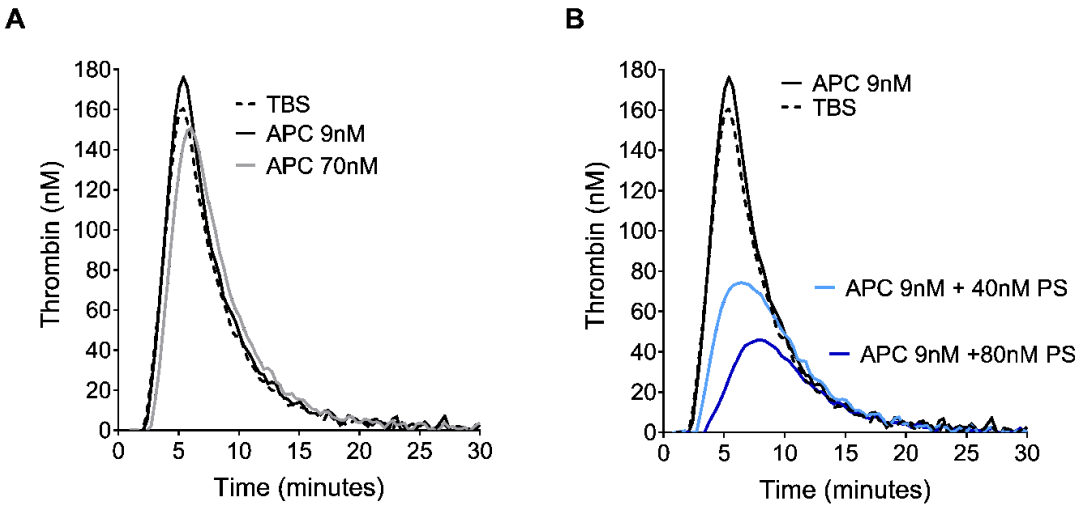


Figure 3.12. Anticoagulant activity of recombinant WT APC in the presence and absence of protein S.
Thrombin generation was initiated by addition of 1pM TF, $CaCl_2$ and phospholipids in protein S depleted plasma in the presence of 9nM or 70nM recombinant WT APC **(A)** and 9nM WT APC and 40 or 80nM protein S **(B)**. Representative results from n=2 are shown.

3.2.1.1 Evaluation of protein S enhancement of APC mediated FVa inactivation using a FVa inactivation assay

I studied the dependency of APC on protein S for FVa inactivation in more detail using pure-component FVa inactivation assays. To allow me to specifically investigate the protein S enhancement of cleavage of FVa at Arg 306, FVa R506Q/R679Q was employed, in addition to WT FVa. In principle, FVa was inactivated by APC in the presence or absence of protein S, with and without phospholipids. The remaining FVa activity was quantified by prothrombinase assays (see section 2.5.3 and 2.5.4).

The significance of protein S cofactor function, especially for cleavage at Arg 306, was once more demonstrated (Figure 3.13). While APC on its own reduced WT FVa activity, the presence of protein S was required to obtain optimal inactivation (Figure 3.13A). In contrast, APC on its own had very little effect on FVa R506Q/R679Q activity (Figure 3.13B). However, when APC was added together with protein S, APC inactivated FVa R506Q/R679Q efficiently as can be seen in the dramatic decrease in FVa activity (Figure 3.13B). WT FVa and FVa R506Q/R679Q were both inactivated with increasing concentrations of protein S (0-100nM) (Figure 3.13C and D). WT FVa activity was reduced to 60% by APC itself and 6nM protein S was sufficient to decreased FVa activity to 20% (Figure 3.13C). In contrast, in the absence of

protein S only 10% of FVa R506/R679Q was inactivated and at least 50nM protein S was required to achieve 80% reduction (Figure 3.13D). APC was titrated in the assay in the absence and presence of protein S. As shown in Figure 3.13 E and F, only 0.25nM APC was required for complete inactivation of WT FVa when 100nM of protein S was present, while even concentrations as high as 4nM APC was not sufficient to fully inactivate FVa in its absence.

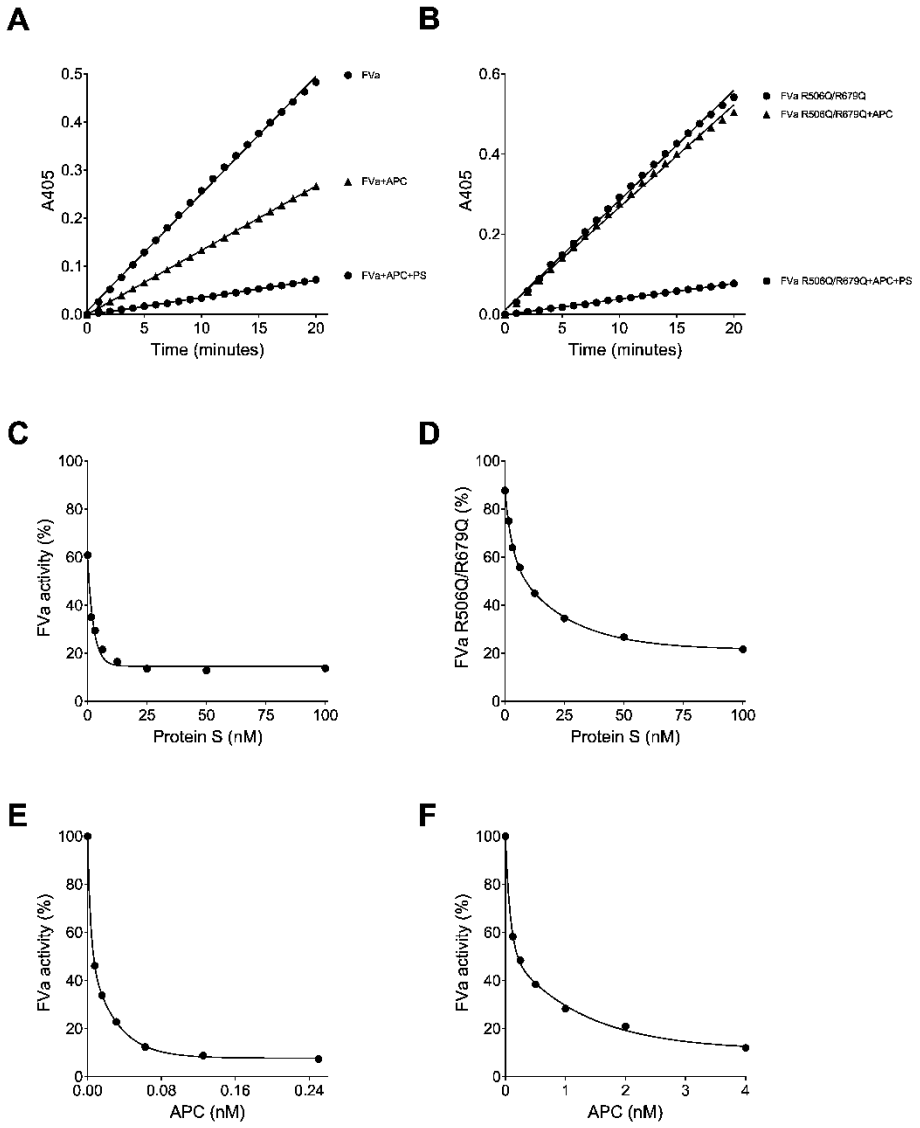


Figure 3.13. Protein S enhancement of APC mediated cleavage of FVa and FVa R506Q/R679Q.
WT FV and FV R506Q/R679Q (1.3nM) were activated by 0.5 U/ml of human thrombin. The activation was stopped by addition of 1.5 U/ml of hirudin. Subsequently, WT FVa **(A, C)** and FVa R506Q, R679Q **(B, D)** were inactivated by 0.25nM APC in the presence or absence of 100nM of protein S **(A and B)** or 0-100nM protein S **(C and D)** for 10 minutes at 37°C. Remaining FVa activity was measured in a prothrombinase assay. The percentage of remaining FVa activity was plotted against the protein S concentration **(C and D).** WT FVa was also inactivated with increasing concentrations of APC (0-4nM) in the presence **(E)** and absence **(F)** of protein S (100nM). Representative results from 3 independent experiments are shown.

3.3 A tri-molecular complex between APC, FVa and protein S

3.3.1 Evaluation of the binding of APC to negatively charged phospholipid surfaces using a pull-down method

While the APC cofactor function of protein S can be demonstrated readily (see above), its molecular mechanism, causing the enhancement of APC, remains unresolved. It has been hypothesised that protein S exerts its cofactor function through enhancing the affinity of APC to phospholipids.[209] Some reports have also suggested that protein S directly binds to FV/FVa.[183,203,212] This interaction could potentially be important since FVa also has much higher affinity to phospholipid surfaces than either APC or protein S.[209] Moreover, naturally occurring FV mutations such as W1920R lead to strong resistance to APC inactivation. Collectively, these findings suggest that FVa could enhance its own proteolysis, through mechanisms involving its high affinity towards phospholipid membranes and direct interactions with protein S and FVa. In fact, it is intuitive that FVa, APC and protein S would, at some point, all be bound together in a tri-molecular complex. Formation of such a complex might be necessary for optimal assembly of the proteins on phospholipid membranes. However, to date formation of such a complex has never been shown.

To study the potential assembly of the APC/protein S/FVa tri-molecular complex I performed pull down experiments as described in the Methods section 2.5.5. For this, I pre-incubated phospholipid coated magnetic beads with 100nM protein S and/or 25nM FVa prior to addition of plasma derived APC. APC was incubated with the beads for 30 seconds before pull-down with a magnet and subsequent washing. Bound proteins were then eluted with LDS buffer and analysed by Western Blot analysis (Figure 3.14A). Little APC bound to the beads in the absence of protein S and FVa, see 1st lane on figure labelled anti-PC. The binding was moderately increased in the presence of protein S, which is in agreement with previously reported findings.[209] In contrast, the addition of FVa alone had no effect upon the amount of APC bound to the beads. A larger increase of APC association with the phospholipid coated beads was observed in the presence of both protein S and FVa. This result suggests that protein S and FVa synergistically enhance the association of APC to phospholipids and importantly agrees with my hypothesis that all three proteins interact with each other at the same time in a tri-molecular complex. Re-probing the membranes with Anti-FV antibody

revealed almost complete cleavage of FVa. Comparable amount of protein S was detected with anti-protein S antibodies in the presence and absence of FVa, indicating that FVa did not enhance protein S-phospholipid association (see anti-PS row). To enable a more stable complex formation, by limiting APC-mediated inactivation of FVa and thereby complex dissociation, active site inhibited APC (APC-FEGRCK) was employed (see Figure 3.14B). As in the experiments using active APC, association of 50nM APC-FEGRCK to phospholipid-coated beads was evaluated after pre-incubation with protein S and/or FVa. The binding of APC-FEGRCK was examined after 2 minutes incubation with the beads. While the main pattern of APC-FEGRCK enhancement was comparable to that of APC (see anti-PC rows), the synergistic enhancement of APC-FEGRCK by protein S and FVa was more pronounced. With APC-FEGRCK, the FVa detected showed only limited signs of proteolysis (see anti-FV row). As in experiments using active APC, re-probing the membranes with anti-protein S antibodies suggested that the presence of FVa did not increase the amount of protein S bound to the beads (see anti-PS row).

Results of these pull-down experiments demonstrated that the association of APC to the phospholipids was just moderately enhanced by protein S alone. While FVa by itself did not increase APC binding to phospholipids, the presence of protein S and FVa together strongly increased the APC-phospholipid association. It is possible, therefore, that protein S and FVa enhance binding of APC to phospholipids by forming an APC/protein S/FVa complex on the phospholipid surface.

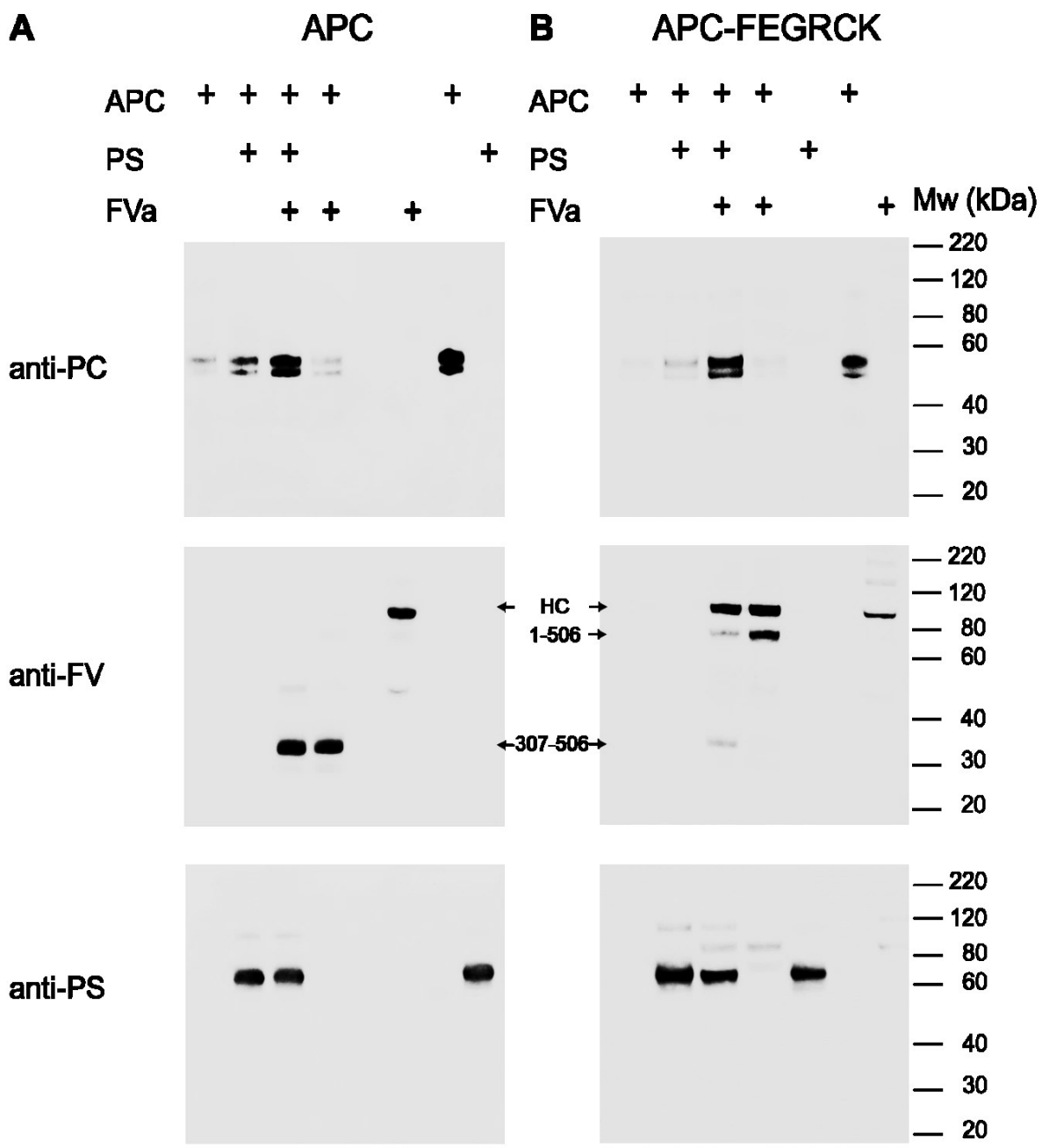


Figure 3.14. Enhancement of APC binding to phospholipids by protein S and FVa.
Binding of 50nM APC **(A)** and APC-FEGRCK **(B)** to phospholipid coated magnetic beads was analysed in pull-down experiments. The APC binding was assessed in the presence and absence of 100nM protein S and/or 25nM FVa. Phospholipid-bound proteins were pulled-down using a magnet and eluted after 0.5 min **(A)** or 2 minutes **(B)** incubation. Bound proteins were separated by SDS-PAGE (4-12% gradient gel), transferred to nitrocellulose membranes and bound APC was detected using rabbit polyclonal anti-protein C antibodies (anti-PC). Subsequently the membranes were stripped and re-probed using anti-FV (anti-FV) and anti-protein S (anti-PS) antibodies for FVa and protein S detection, respectively. Representative blots are shown (n=3). Abbreviations: heavy chain (HC), fragment of FVa obtained by cleavage at residue Arg506 (1-506), fragment of FVa achieved by cleavage at residue Arg506 followed by cleavage at residue Arg306 (307-506).

3.3.2 Evaluation of the APC binding to negatively charged phospholipid surfaces using flow cytometry

Following on from the promising results from the pull-down assays, I optimised flow cytometry assays to study the association of protein S, FVa and APC with phospholipids in more detail. A major advantage of this technique is that it is quantitative and therefore allows a deeper insight into complex formation on phospholipids.

In preliminary experiments, binding of 50nM APC-FEGRCK (FITC-labelled) to phospholipid-coated magnetic beads was evaluated in the presence and absence of protein S and/or FVa (see Method section 2.5.6 for further details). Representative histograms of APC binding to phospholipids after 1 minute incubation are shown in Figure 3.15. Protein S enhanced APC-FEGRCK binding to phospholipids minimally. The presence of FVa (25nM) alone did not increase APC-FEGRCK association. However, FVa in combination with protein S enhanced APC binding to phospholipids appreciably, again suggesting the formation of an APC/protein S/FVa tri-molecular complex. Conditions of the assay were next varied to ensure optimal formation of the complex.

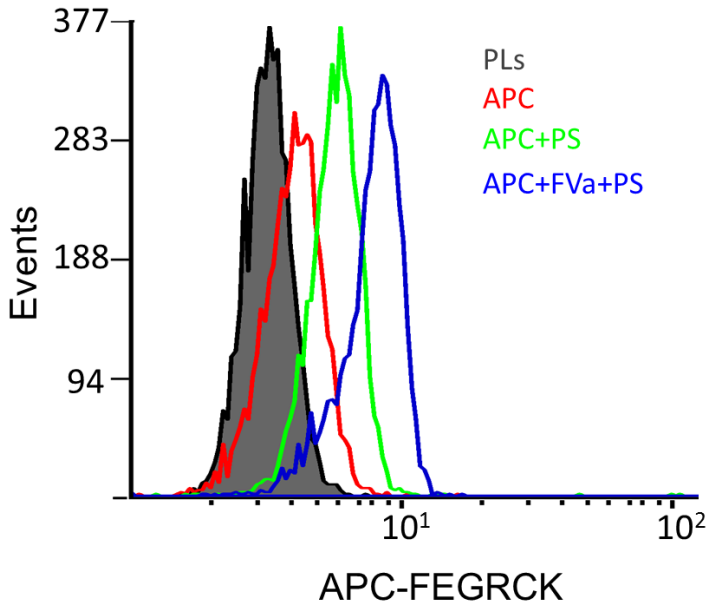


Figure 3.15. FVa together with protein S enhances the binding of APC to negatively charged phospholipids.
Histograms are shown of binding of 50nM APC-FEGRCK to phospholipid-coated magnetic beads in the presence and absence of 100nM protein S and/or 25nM FVa after 1 minute incubation.

3.3.2.1 Optimisation of phospholipid coated magnetic beads

To ensure that the phospholipid surface was not limiting or, conversely, that its concentration was not high enough to interfere with the interactions, the binding of 50nM APC-FEGRCK was analysed with increasing concentrations of phospholipid coated beads (31.3-500µg/ml) in the presence and absence of protein S and/or FVa (Figure 3.16). The highest change in MFI was achieved for the APC binding to the beads at a beads concentration of 62.5-125µg/ml. To avoid the possibility that phospholipid surface might be a limiting factor, the concentration of 125µg/ml was selected and used in all the following experiments.

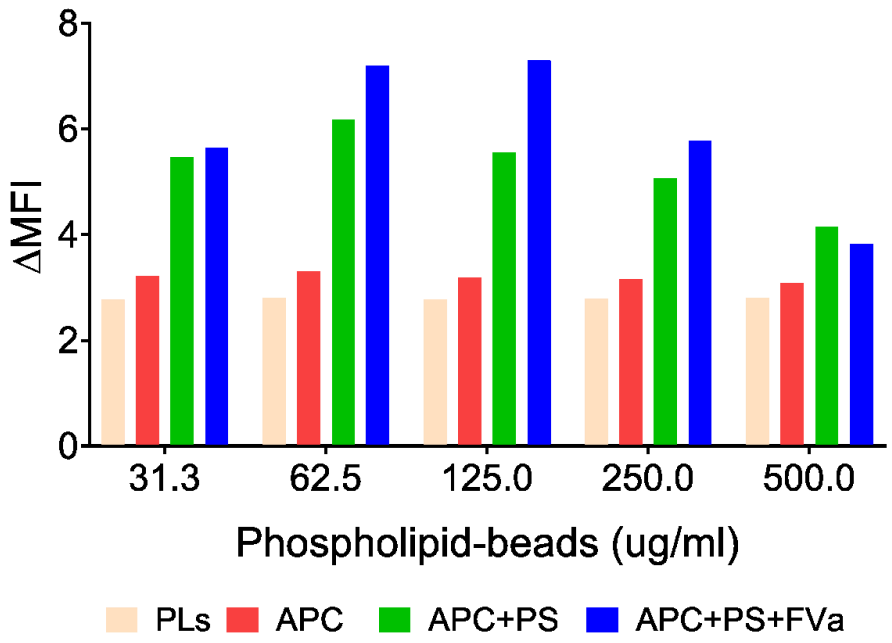


Figure 3.16. Optimisation of the concentration of phospholipid coated magnetic beads.
Binding of 50nM APC-FEGRCK to phospholipid-coated magnetic beads was analysed in the presence and absence of 100nM protein S and/or 25nM FVa with increasing concentration of phospholipid coated magnetic beads (31.3-500 µg/ml). The phospholipids were prepared at ratio DOPS/DOPC/DOPE/ bDOPE of 60:20:18:2.

3.3.2.2 Comparison of recombinant and plasma purified FVa

To ensure that the synergistic enhancement by protein S and FVa was not restricted to one form of FVa, I studied APC-FEGRCK binding to phospholipid coated magnetic beads using both recombinant FVa (rFVa) and plasma purified FVa (ppFVa). The enhancement of APC-phospholipid association by recombinant FVa in the presence of protein S was comparable to that of pp FVa (Figure 3.17).

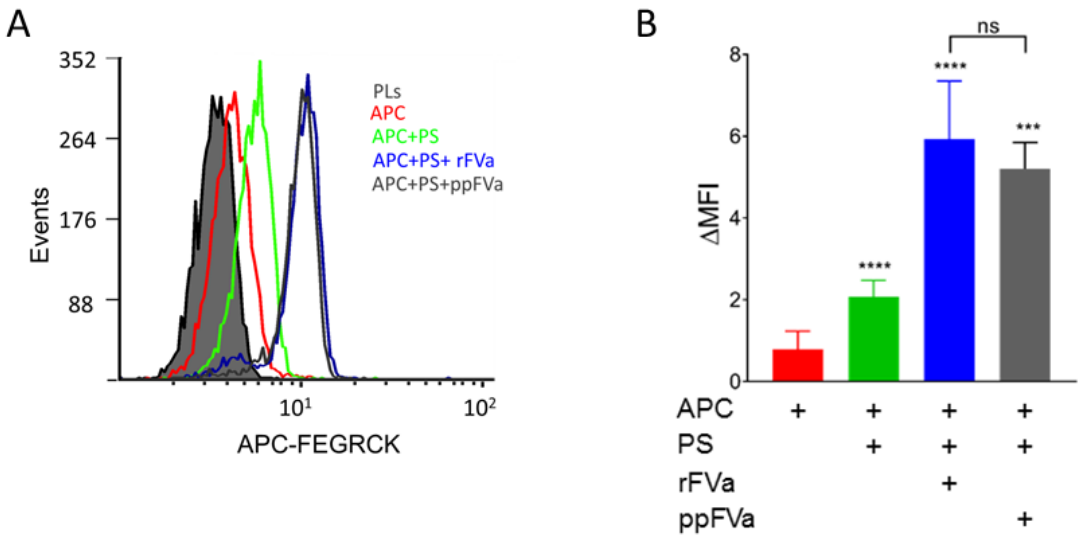


Figure 3.17. Comparison of the enhancement of APC-FEGRCK binding to phospholipids by recombinant and plasma purified FVa in the presence of protein S.
(A) Representative histogram and (B) quantification plot of binding of 50nM APC-FEGRCK to phospholipid-coated magnetic beads was analysed in the presence and absence of 100nM protein S and/or 25nM of recombinant and plasma purified FVa (rFVa and ppFVa, respectively). n≥3-23. ***p<0.0002; ****p<0.0001 using to Mann-Whitney tests compared to APC alone, unless otherwise indicated. (A) Representative histogram of

3.3.2.3 Influence of protein S on the APC-phospholipid interaction

In all preliminary experiments, a protein S concentration of 100nM was used. This concentration corresponds to the amount of free protein S present in circulation. To establish whether protein S enhancement of APC-phospholipid association is dose dependent and to choose a protein S concentration suitable for further investigations, I analysed the binding of APC-FEGRCK to phospholipids in the presence of increasing concentrations of protein S (0-100nM) (Figure 3.18). The MFI was measured immediately after addition of fluorescently labelled APC. A modest and dose dependent enhancement by protein S was observed. Like in my previous experiments, the APC-phospholipid binding was further increased in the presence of FVa. Furthermore, the enhancement increased with increasing concentrations of protein S.

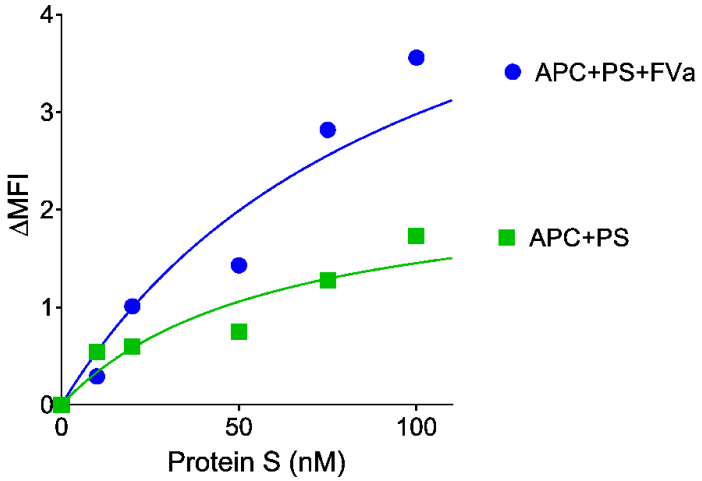


Figure 3.18. Protein S titration.
Association of 50nM APC-FEGRCK to phospholipid-coated magnetic beads was analysed in the presence of increasing concentrations of protein S (0-100nM) in the presence or absence of 25nM FVa, immediately after addition of APC. The MFI observed in the absence of protein S was substracted.

3.3.2.4 Time-dependency of APC association with phospholipids

To study the enhancement of APC binding to phospholipids over time, I performed time course experiments (Figure 3.19A). Association of 50nM APC-FEGRCK to phospholipid-coated beads was investigated in the presence and absence of protein S (100nM) and/or FVa (25nM). The increase in MFI was measured immediately and 1, 3 and 5 minutes after addition of 50nM APC-FEGRCK. Binding of APC-FEGRCK alone only slightly increased over time. Whereas protein S alone provided a stable ~2-fold enhancement at all-time points, the enhancement by protein S together with FVa was very rapid. In fact, the fold enhancement induced by protein S and FVa in combination was higher at earlier time points.

To ensure that the same result was obtained also with plasma purified FVa, I tested recombinant FVa and plasma purified FVa in parallel. Both variants of FVa behaved the same (Figure 3.19B).

The decrease in enhanced binding of APC-FEGRCK by protein S and FVa at later time points observed in Figure 3.19B suggested partial FVa proteolysis. A possible explanation for this result may be the presence of some residual active APC in the inhibited, APC-FEGRCK preparation. To explore this possibility, the level of active APC in APC- FEGRCK was measured by following the cleavage of chromogenic substrate S2366 (data not shown). According to my

preliminary substrate determination, APC-FEGRCK did contain some active APC. I estimated that ~ 0.2% of this APC preparation was active. Although, the level of residual active APC is expected to vary between different batches. Together, this strongly suggests that the enhancement by protein S and FVa decreases over time due to the FVa cleavage by APC (Figure 3.19B).

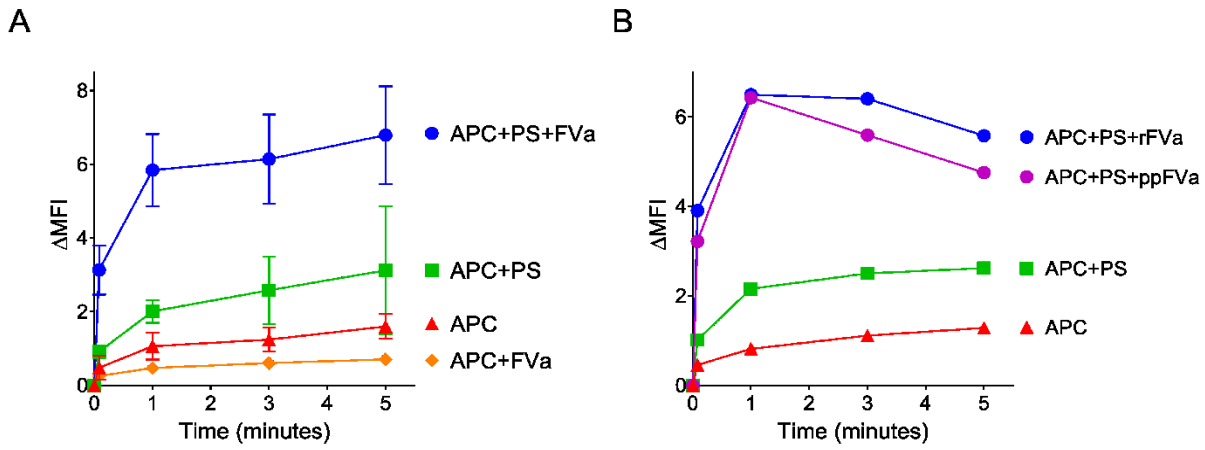


Figure 3.19. Time dependency of the assembly of the FVa inactivation complex.
The APC-FEGRCK (50nM) association to phospholipid coated magnetic beads was characterised over time. The MFI of the phospholipid-coated beads was measured over time after addition of APC. The association of 50nM APC-FEGRCK to phospholipid-coated beads was determined in the presence and absence of protein S (100nM) and /or **(A)** 25nM of recombinant FVa (rFVa), **(B)** 25nM of both recombinant and plasma purified FVa (ppFVa). **(A)** The results are presented as mean ± SD, n=3, **(B)** An example of decrease in protein S and FVa enhancement of APC-FEGRCK binding to phospholipids at later time points (n=1).

3.3.2.5 Incubation time and the binding of APC to phospholipids

To verify whether preincubation of protein S and FVa with phospholipid-coated beads influences the APC binding to phospholipids vehicles, I either incubated FVa and protein S with the beads for 10 and 15 minutes, respectively, or added the proteins to the beads immediately prior to addition of APC. The MFI was measured immediately, 1, 3 and 5 minutes after APC addition (Figure 3.20). I observed no difference between the APC enhanced binding, suggesting that the enhancement is independent on pre-incubation of protein S and FVa with the phospholipid coated beads. However, to ensure that all samples were analysed as identically as possible, I preincubated the beads with FVa and protein S for 10 and 15 minutes respectively before addition of APC throughout this project.

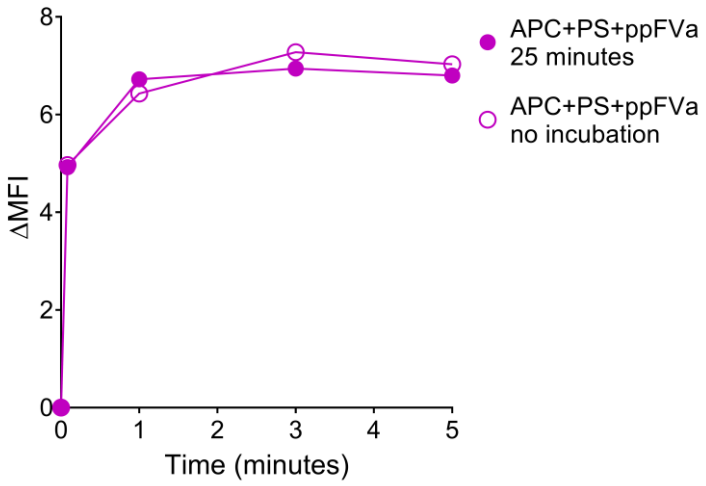


Figure 3.20. Incubation time and enhanced binding of APC-FEGRCK.
Protein S and FVa were either incubated with phospholipid-coated beads for 10 and 15 minutes ,respectively, or added to the beads just before addition of APC-FEGRCK. The MFI was measured immediately, then 1, 3 and 5 minutes after addition of APC-FEGRCK.

3.3.2.6 Influence of thrombin and hirudin on APC-phospholipid association.

To rule out the possibility that proteins used in FV activation (thrombin and hirudin) may influence APC-phospholipid association, I evaluated the binding of 50nM APC-FEGRCK to the beads with and without protein S in the presence and absence of thrombin (6.25nM) and hirudin (1.4U/ml) (Figure 3.21). No difference in the binding was observed in the presence of thrombin/hirudin, compared to their absence, suggesting that these activation agents do not have any direct influence on the binding of APC to phospholipids. It is worth noting that the experiments in Figure 3.20 provide further support for this. ppFVa and rFVa enhanced APC-binding to phospholipids to the same extent. While rFVa contains residual thrombin and hirudin, ppFVa was affinity purified after the FV activation step and does not contain thrombin.

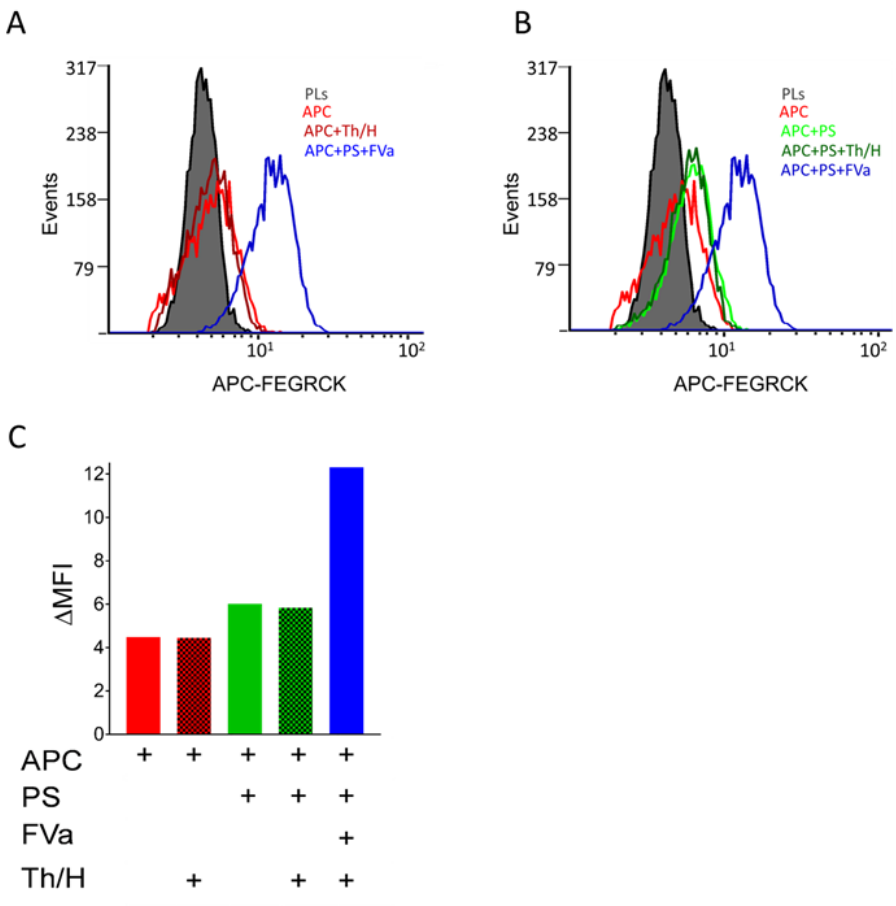


Figure 3.21. Influence of thrombin/hirudin on APC-phospholipid association.
(A and B) Representative histograms and (C) quantification plot of binding of 50nM APC-FEGRCK to phospholipid-coated magnetic beads was evaluated in the presence and absence of 100nM protein S, 25nM FVa, thrombin (6.25nM) and hirudin (1.4 U/ml). Abbreviations: thrombin (Th), hirudin (H).

3.3.2.7 Specificity of the enhancement of APC association with phospholipids

I used specific anti-protein S and anti-FVa antibodies to verify the specificity of APC enhanced binding to phospholipids, (Figure 3.22). While the enhancement induced by protein S was reversed with 1µM anti-protein S antibodies, that produced by both FVa and protein S was only partially inhibited by anti-protein S or anti-FV antibodies added separately. A combination of both type of antibodies successfully fully reversed this enhancement.

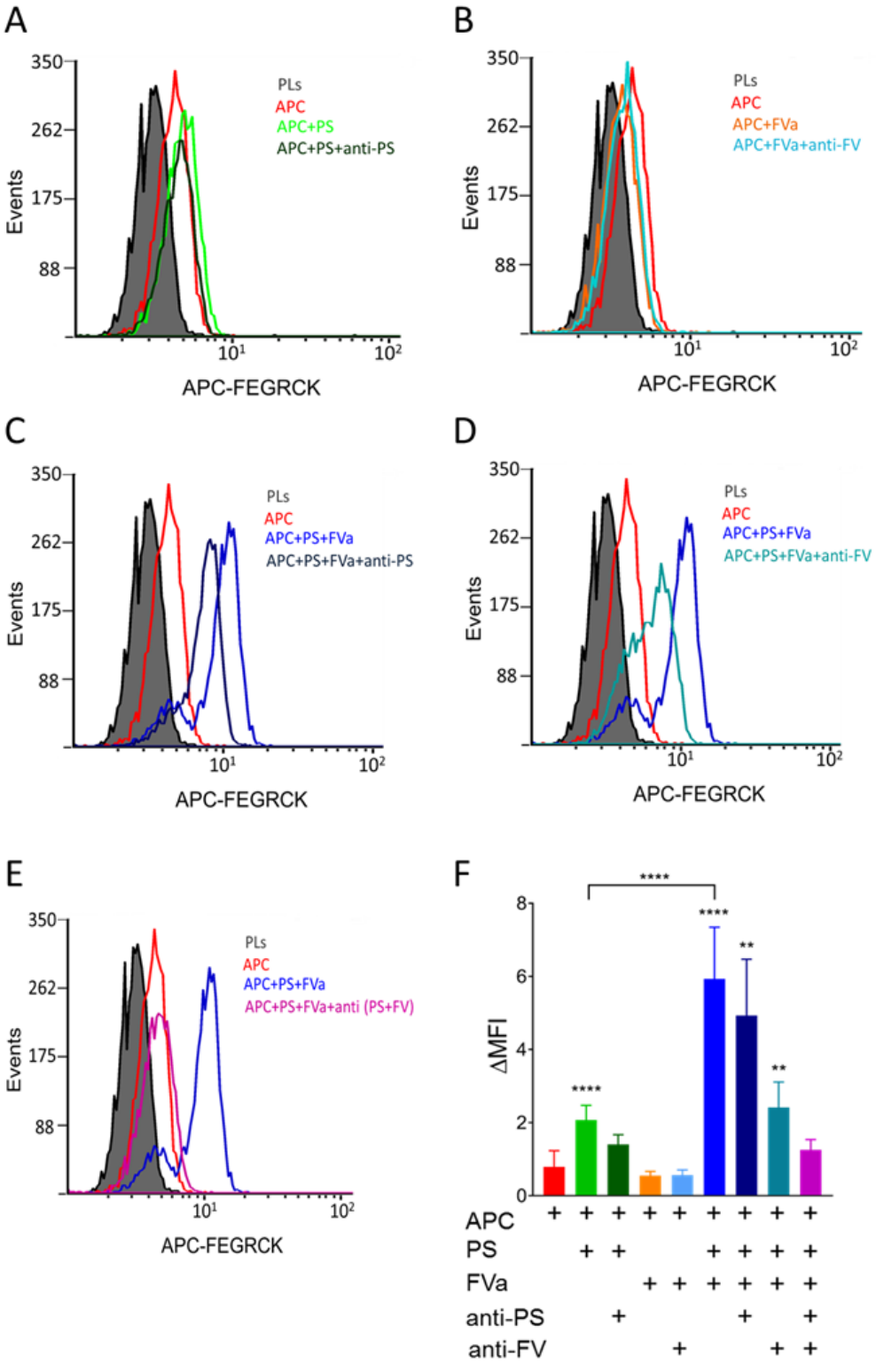


Figure 3.22. Specificity of enhanced binding of APC to phospholipids induced by protein S and FVa. **(A-E) Representative histograms and (F) quantification plot of** binding of 50nM APC-FEGRCK to phospholipid-coated magnetic beads was determined in the presence and absence of 100nM protein S and/or 25nM FVa using flow cytometry. To show specificity of enhanced binding by protein S (100nM) and FVa (25nM), binding to phospholipids was assessed after incubation with 1µM anti-protein S and 250nM anti-FV antibodies n≥3-23. ** p<0.002 **** p<0.0001 according to Mann-Whitney tests compared to APC alone, unless otherwise indicated.

3.3.2.8 Evaluation of the influence of FVIIIa on APC binding to phospholipids

APC proteolytically inactivates not only FVa but also FVIIIa, the cofactor of FIXa. Protein S functions as a cofactor for APC also in this inactivation, although less efficiently. In fact, the enhancement of APC-mediated inactivation of FVIIIa compared to that of FVa is ~10-fold less efficient, suggesting different mechanisms involved. To assess whether both substrates of APC are able to enhance its association to phospholipids, APC-phospholipid binding was examined in the presence of FVIIIa, either in the absence or presence of protein S. FVIII (100nM) was activated with 100nM of thrombin as described in the Methods section 2.5.4.2, similarly to the activation procedure of FV. Full activation of FVIII was confirmed by Western blotting (Figure 3.23A). FVIII (495ng) and 415ng of FVIII treated with thrombin were loaded onto 4-12% gradient gel. After SDS-PAGE separation and Western blotting, FVIII/FVIIIa was detected with polyclonal anti-FVIII antibodies. FVIII consists of an 80kDa light chain linked by metal ions to a heavy chain (200-90kDa). The heavy chain of FVIII (200-90kDa) might have different sizes due to multiple cleavages of the B-domain before FVIII secretion. Thrombin activates FVIII by proteolytic cleavages at residues Arg372, Arg740 and Arg1689 (see Figure 3.23B). Activated FVIII (FVIIIa) is a trimer composed of A1 (a.a. 1-372), A2 (a.a. 373-740) domains and a light chain (A3-C1-C2; a.a. 1689-2332). Molecular masses of bands visualised on the Western blot in the FVIIIa lane corresponded well to thrombin activation products and no bands corresponding to the heavy and lights chains of FVIII are visible after activation by thrombin (see Figure 3.23 B and C).

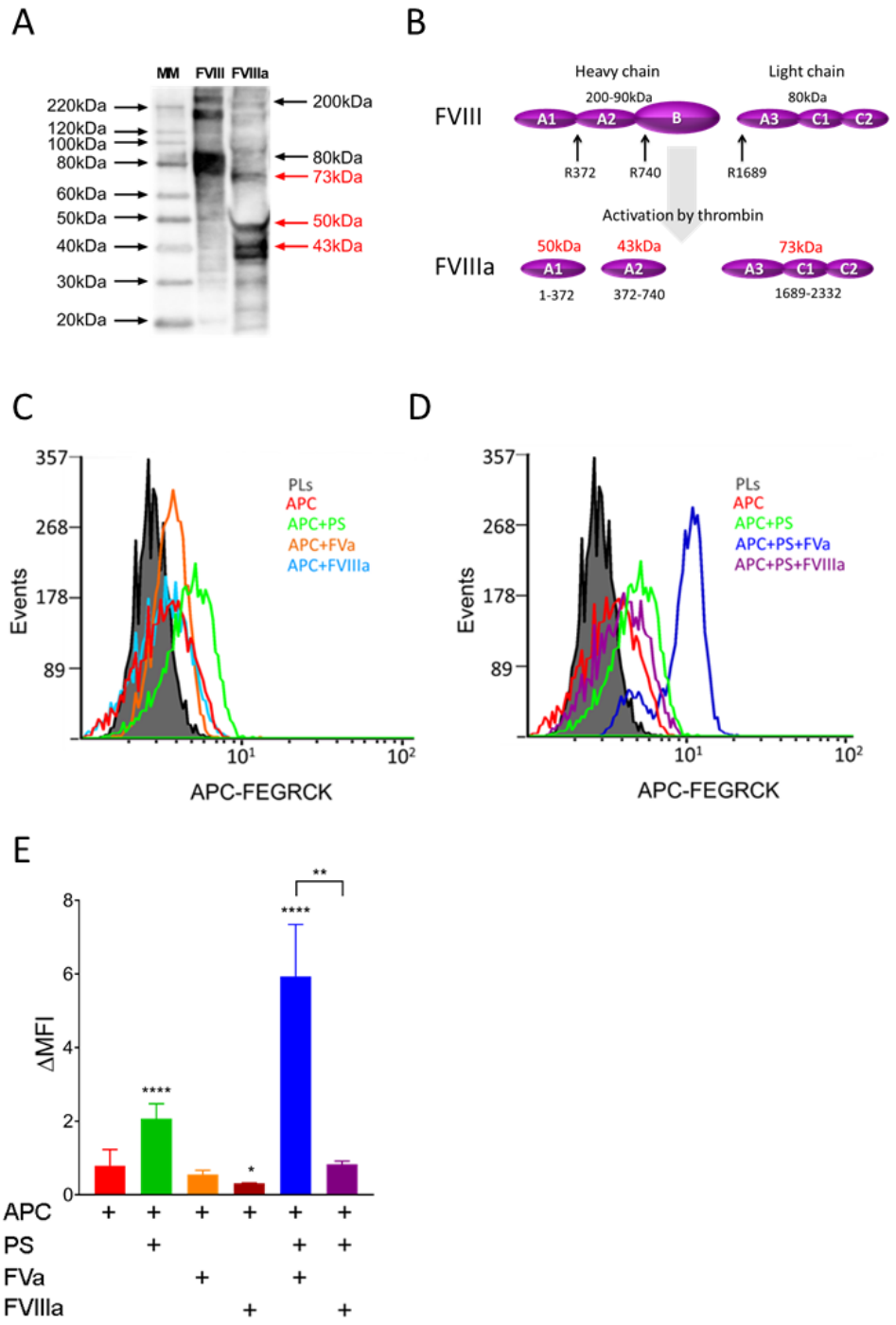


Figure 3.23. FVIIIa does not synergistically enhance the binding of APC to negatively charged phospholipids together with protein S.

(A) Activation of FVIII (100nM) by thrombin (25nM) was performed for 3 minutes at 37 ^{o}C. 495 ng of FVIII and 395 ng of FVIIIa were loaded onto 4-12% gradient gel. After separation by SDS-PAGE, proteins were transferred to nitrocellulose. FVIII/FVIIIa were detected with polyclonal anti-FVIII antibody. **(B)** Schematic illustration of thrombin cleavage site on FVIII. Thrombin activation products and their masses are also indicated. **(C-D)** Representative histograms and **(E)** quantification plot of binding of 50nM APC-FEGRCK to phospholipid-coated magnetic beads was characterised in the presence and absence of 100nM protein S and/or FVIII (25nM) using flow cytometry. The effect of 25nM FVIIIa upon APC-phospholipids association, n≥3-23. *p<0.033 **p<0.0021, ****p<0.0001 according to Mann-Whitney tests compared to APC alone, unless otherwise stated.

It can, therefore, be concluded that FVIII was fully activated by thrombin. Following FVIII activation, binding of APC-FEGRCK to phospholipids was analysed in the presence and absence of 25nM FVIIIa (with and without protein S) using flow cytometry. From the results shown in Figure 3.23C, it is apparent that, in contrast to FVa, FVIIIa does not increase APC-phospholipid association in the presence of protein S. This was also the case in the absence of protein S. In fact, APC binding in the presence of FVIIIa appears to be somewhat decreased. These results suggest that FVIIIa does not form a complex with APC and protein S and that the molecular mechanisms involved in APC-mediated inactivation of FVIIIa and FVa are different. Furthermore, the lack of synergistic enhancement by FVIIIa and protein S upon APC binding to negatively charged phospholipid membranes may therefore in part explain the relatively moderate protein S enhancement of FVIIIa inactivation.

3.3.2.9 Titration of APC in the presence and absence of protein S/FVa

Next, I assessed the dose-dependent binding of APC to phospholipid membranes. For this, APC binding was analysed by increasing the concentrations of APC-FEGRCK (0-75nM) (Figure 3.24). A moderate, dose dependent increase in MFI, corresponding to increasing amounts of APC binding, was observed with APC alone (Figure 3.24A). The presence of FVa (25nM) produced little or no effect on APC binding. Bound APC increased progressively in the presence of 100nM protein S. However, protein S in combination with FVa enhanced the APC-phospholipid association greatly. Under the conditions used, half-maximal binding of APC-FEGRCK decreased appreciably to less than 20nM. As the binding affinity of protein C/APC to phospholipids has been determined to be in the µM range, this suggests an estimated ~50-fold increase in affinity induced by protein S/FVa.[63,239,240] As expected, the enhancement was stronger at lower concentrations of APC, or at equimolar levels to those of FVa. To therefore determine whether the enhancement depended on the FVa concentration, FVa was titrated in the assay. A dose dependent enhancement of the APC binding to phospholipids was observed suggesting that this is the case (Figure 3.24B). This could be explained by the high affinity of FVa for negatively charged phospholipid membranes and suggest that the FVa/phospholipid interaction is essential for the tri-molecular complex formation. Together, the enhanced affinity caused by protein S and FVa strongly suggests the formation of an APC/protein S/FVa complex and that FVa plays an essential role in its formation.

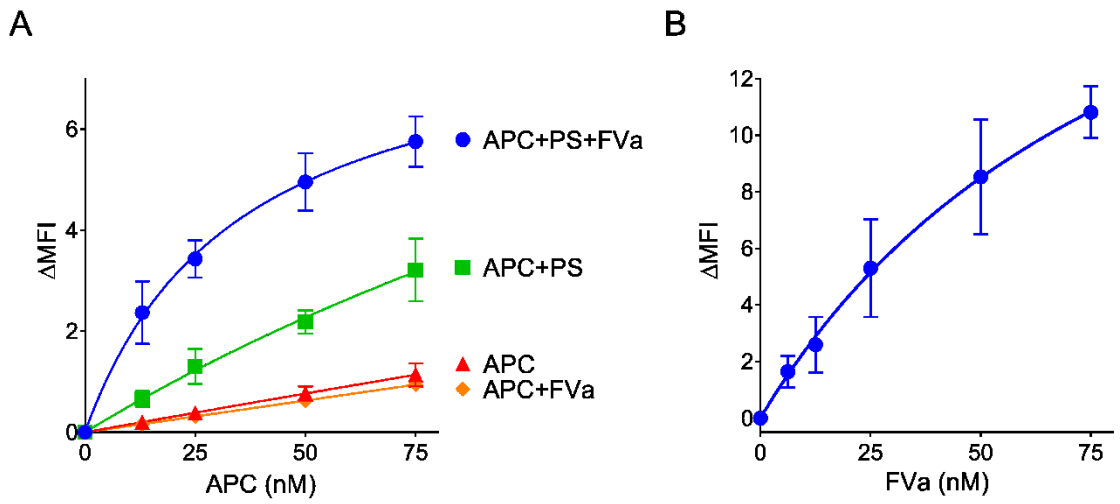


Figure 3.24. Titrations of APC and FVa in flow cytometry assay.
(A) APC-FEGRCK binding to phospholipids was analysed at increasing concentrations (0-75nM) in the presence and absence of 100nM protein S and/or 25nM FVa. **(B)** 50nM APC-FEGRCK binding to phospholipid was analysed at increasing concentrations of FVa (0-75nM). The measurements were taken 1 minute after addition of APC (n=3).

3.3.2.10 C4PB-bound protein S cannot be incorporated in the tri-molecular complex

It has previously been demonstrated that protein S bound to C4BP has limited APC cofactor function.[216,241] It has also been shown that protein S bound to C4BP does not efficiently bind FVa.[203] In what follows, the influence of C4BP upon complex formation was investigated (Figure 3.25). For this, protein S was pre-incubated with saturating amounts of C4BP prior to addition to the phospholipid coated magnetic beads in my flow cytometry assay, to form a protein S/C4BP complex. In agreement with the results from previously published functional data,[40,242] C4BP strongly inhibited both protein S-mediated enhancement as well as the synergistic enhancement induced by FVa and protein S upon APC binding to phospholipids (Figure 3.25). In fact, C4BP reversed the enhancement more efficiently than that observed using anti-protein S antibodies (Figure 3.22). These findings suggest that C4BP inhibits the formation of the APC/protein S/FVa complex and may therefore explain the reduced ability of C4BP-bound protein S to enhance APC mediated FVa inactivation.

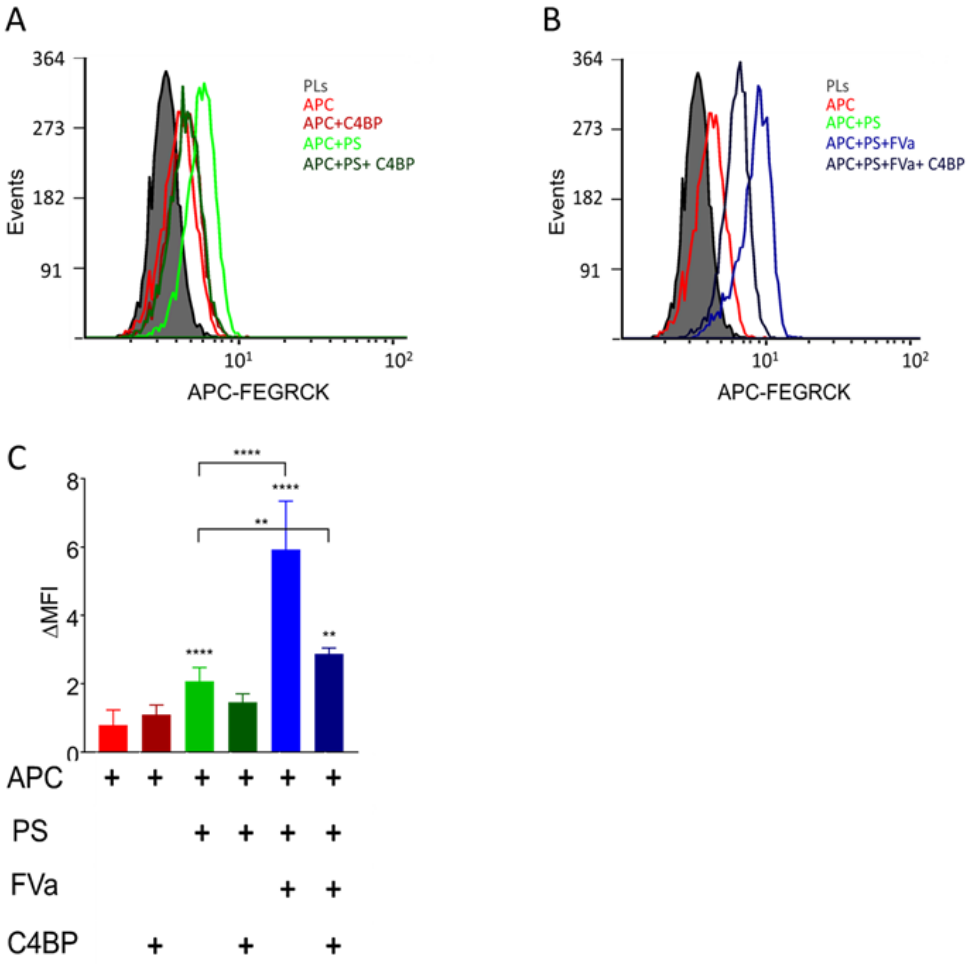


Figure 3.25. The influence of C4BP on protein S and/or FVa enhancement of APC binding to phospholipids.

(A and B) Representative histograms and (C) quantification plot of binding of 50nM APC-FEGRCK to phospholipid-coated magnetic beads was measured in the presence and absence of 100nM protein S and/or 25nM FVa using flow cytometry. Measurements were taken 1 min after addition of APC. The effect of C4BP on the enhancement by protein S and FVa was investigated by addition of 200nM β-chain containing C4BP. Results are presented as mean ± SD, n=3 $^{**}p<0.002$; $^{****}p<0.0001$ according to Mann-Whitney tests compared to APC alone, unless otherwise indicated.

3.4 Evaluation of protein C D36A/L38D/A39V

It was demonstrated by Preston *et al.* that APC residues Asp36/Leu38/Ala39, localised in the Gla domain, are essential for protein S cofactor function[191]. This was shown using a protein C variant where residues Asp36/Leu38/Ala39 were substituted for corresponding residues in prothrombin (protein C D36A/L38D/A39V). These substitutions resulted in complete lack of enhancement by protein S. Following up on this study by Preston and colleagues, I wished to assess whether the lack of protein S cofactor function of this variant is related a potential

inability to take part in the formation of the inactivation complex. Initially, the activity of APC D36A/L38D/A39V was investigated by CAT (Figure 3.27). For this WT protein C and protein C D36A/L38D/A39V were first activated using PROTAC (See Method section 2.4.6). Thrombin generation was initiated by addition of tissue factor, phospholipids and Ca^{2+} in protein S depleted plasma, as described in Method section 2.5.2. The assays were run in the presence of 9nM of WT APC or APC D36A/L38D/A39V and in the presence and absence of protein S (40 or 80nM).

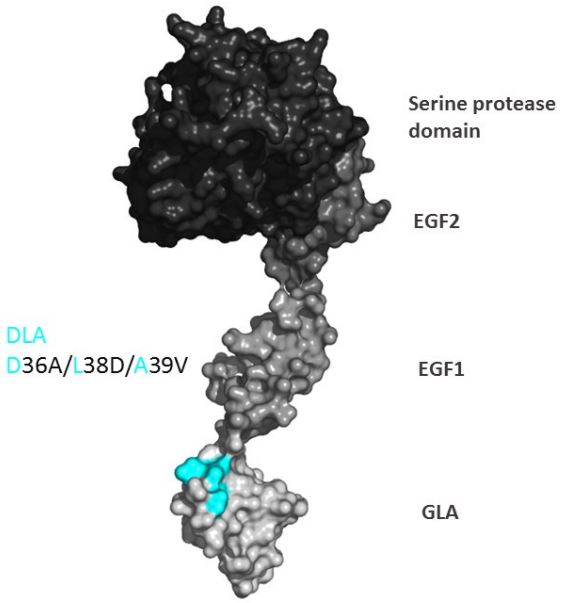


Figure 3.26. Molecular model of protein C with highlighted residues Asp36/Leu38/Ala39.
Anticoagulant activity of protein C variant with substitutions D36A/L38D/A39V was not enhanced by protein S. Molecular model of protein C was adopted from Mather et al.[122]

Similarly to WT APC, addition of 9nM APC D36A/L38D/A39V did not have any effect on thrombin generation (see Figure 3.27 A and B). However, in contrast to WT APC, the activity of APC D36A/L38D/A39V was not enhanced by protein S at concentrations up to 80nM (see Figure 3.2B).

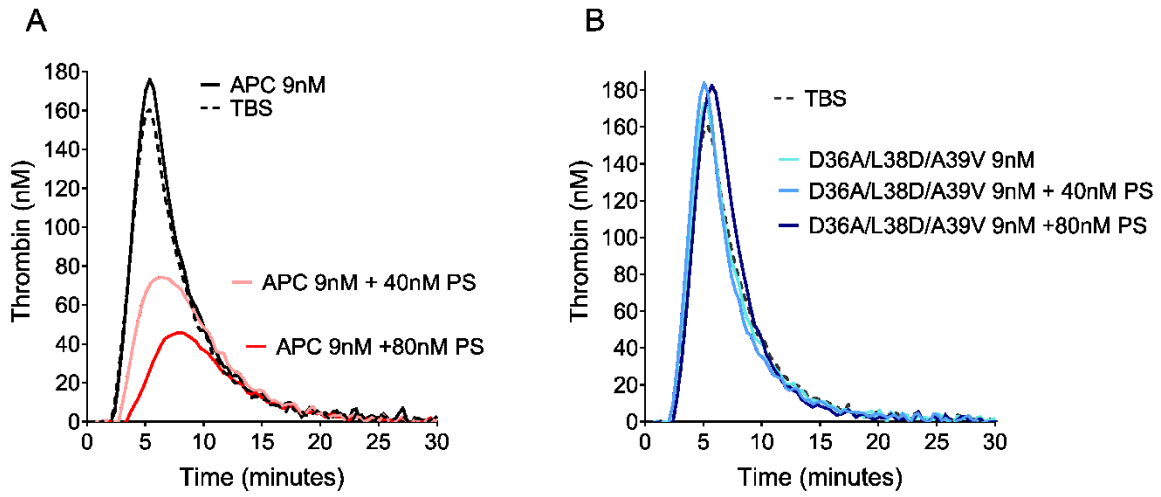


Figure 3.27. Anticoagulant activity of the protein C variant D36A/L38D/A39V in the presence and absence of protein S.
Thrombin generation was initiated by addition of 1pM TF, $CaCl_2$ and phospholipids in protein S depleted plasma in the presence of 9nM APC D36A/L38D/A39V and 40nM or 80nM protein S. Representative results from n=2 are shown.

My results confirmed previous findings and showed the crucial role of residues Asp36/Leu38/Ala39 for protein S cofactor function. Unfortunately, due to insufficient time, I was not able to further investigate the protein C D36A/L38D/A39V variant as part of this PhD. At the moment, the question whether protein C residues Asp36/Leu38/Ala39 are important for assembly of the inactivation complex remains unanswered. However, when time permits I plan to study the variant D36A/L38D/A39V ability for formation of complex with protein S and FVa using flow cytometetry.

3.5 Evaluation of protein S variants

The experiments reported above have shown the importance of protein S in the formation of the inactivation complex of FVa. To date, results have shown that protein S Gla-EGF1 and the SHBG-like domain are involved in the enhancement of APC.[177,207,243] There are three variants of protein S available in the lab which all have been reported to have reduced anticoagulant activity, namely protein S E36A, D95A and chimera III (CHIII).[182,189,207] I wished to investigate these variants to try to find an explanation for the lack of activity in the context of formation of the inactivation complex. I also had three composite variants of protein S which never had been investigated for their APC cofactor function available and I included these in my

investigations. Figure 3.28 shows all protein S variants investigated during this project. In order to identify protein S variants with reduced APC cofactor function, two functional assays were employed; thrombin generation assay using CAT and FVa inactivation assays. WT protein S, protein S E36A, D95A and protein S CHIII were analysed alongside three protein S variants spanning the EGF3-4 domains DEEE (D182N/E184Q/E186Q/E189Q), REDD (R192Q/E201Q/D202N/D204N) and DK (D227N/K233Q)).

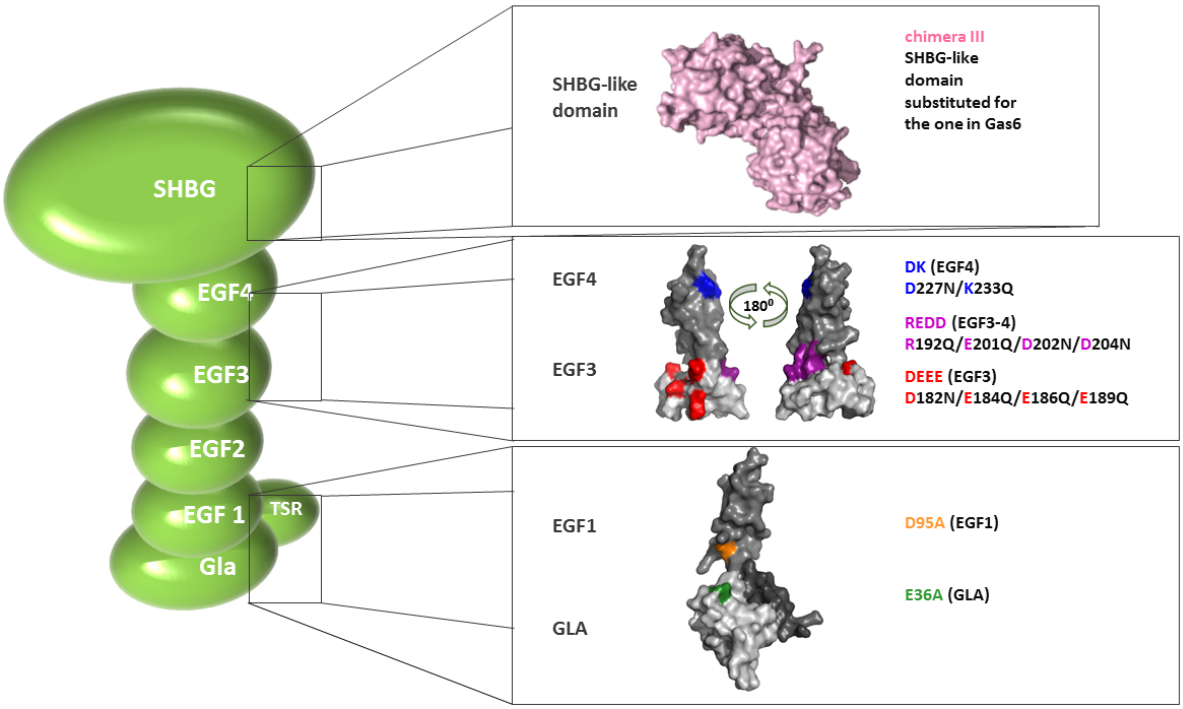


Figure 3.28. Molecular models demonstrating protein S variants studied during this PhD.
APC cofactor function of the protein S variants spanning almost entire protein S was assessed in thrombin generation assay and FVa inactivation assay. The variants with reduced anticoagulant function were studied further by flow cytometry. The models of protein S Gla-TSR-EGF1, EGF3-EGF4 and the SHBG-like domains were adopted from from Giri et al., Drakenberg et al., and Villoutreix et al., respectively[179-181]

3.5.1 Evaluation of protein S variants in thrombin generation assay

I first compared the APC-dependent cofactor function of WT protein S and its variants in thrombin generation assays using CAT (see section 2.5.2). For this, protein S-depleted plasma was supplemented with increasing concentration of protein S (0-120nM) in the presence of 9nM of APC. Thrombin generation was initiated by addition of 1pM TF, see figure 3.29A. As shown in Figure 3.29B and C, protein S E36A and D95A demonstrated severely impaired APC cofactor function. Whereas the DEEE variant showed only slight decrease in ability to enhance APC (Figure 3.29D), the REDD variant exhibited a moderate decrease in anticoagulant function

(Figure 3.29E). The APC enhancement by protein S DK was only minimally reduced as can be seen in Figure 3.29F. In contrast to the variants spanning the EGF3-4, protein S CHIII showed a large reduction in APC cofactor function (Figure 3.29G). The relative abilities of these variants to function as anticoagulant proteins can be compared in Figure 3.29H where their peak height of thrombin generation are plotted against protein S concentration. Protein S CHIII at a highest concentration used in the assay (120nM) decreased the peak height by only 30%. The same decreased in the peak height was obtained with 90nM of D95A variant while protein S E36A at this concentration reduced thrombin generation by 40%. APC cofactor function of the REDD variant was less reduced and 70% reduction in thrombin generation was achieved with 120nM of the variant. The variants DK and DEEE showed almost the same APC cofactor function and at concentration of 60nM decreased peak height by 75%.

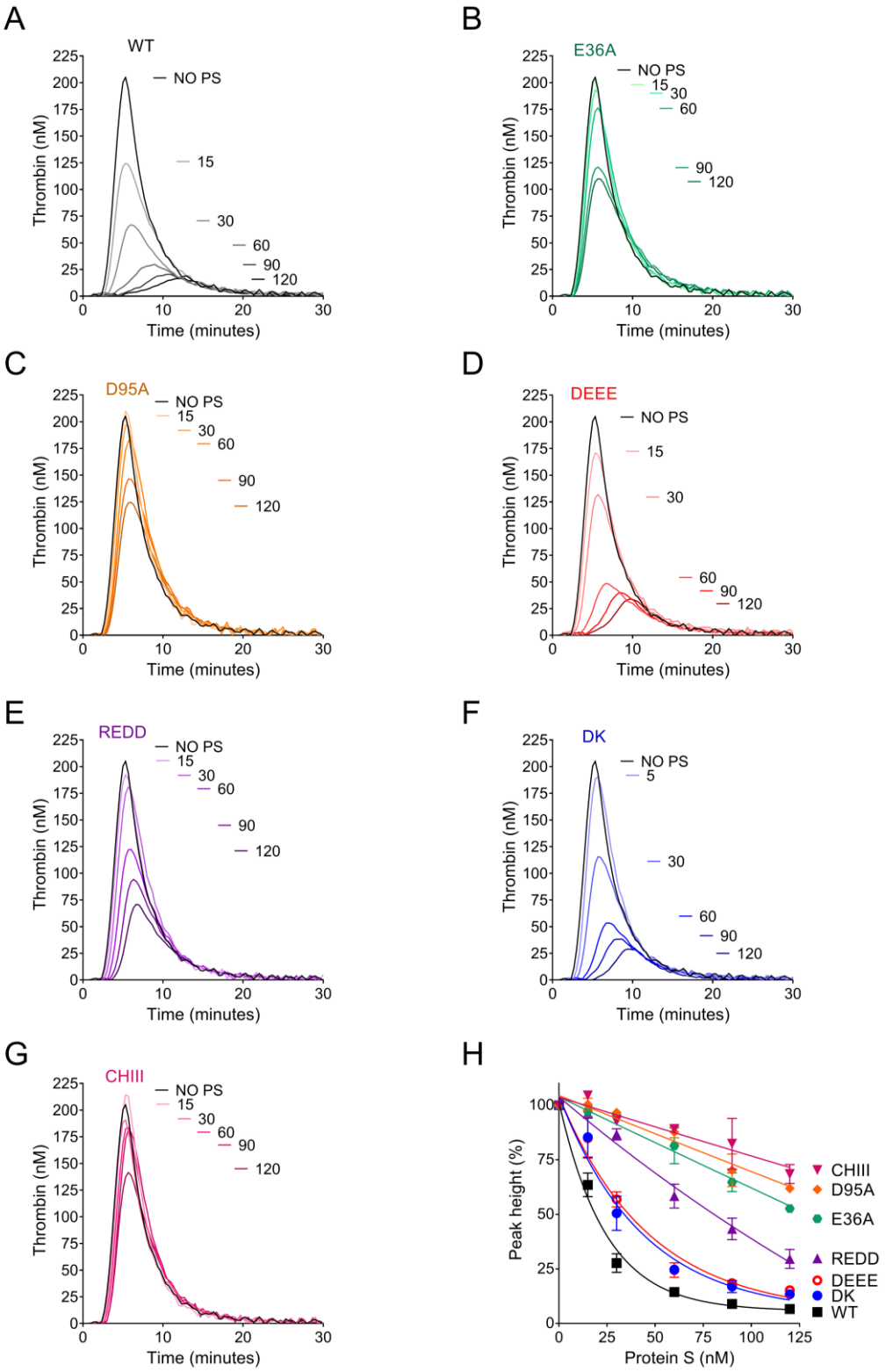


Figure 3.29. Protein S APC cofactor function determined using the thrombin generation assays.
Thrombin generation was measured using calibrated automated thrombography after initiation by 1pM TF in protein S-depleted plasma reconstituted with phospholipids, 9nM APC and supplemented with protein S at various concentrations. WT protein S **(A)** and protein S E36A **(B),** protein S D95A **(C),** protein S DEEE **(D),** protein S REDD **(E),** protein S DK (F), protein S CHIII **(G)** were titrated in the assay (0-120nM). Representative experiments are shown (n=3). The peak heights were plotted against protein S concentration **(H).** Results are expressed as a mean ±SD (n=3).

3.5.2 Anticoagulant activities of protein S variants in a FVa inactivation assay

To directly evaluate the ability of WT protein S and its variants to enhance APC-mediated FVa cleavage, I performed FVa inactivation assays (see Methods section 2.5.4). FVa (0.8nM) was inactivated using 0.25nM APC for 10 minutes in the presence of increasing concentration of protein S (0-100nM). To study the overall protein S cofactor function, the assay was conducted using WT FVa (Figure 3.30A), while $FVa^{R506Q,R679Q}$ was used to specifically analyse the enhancement of APC cleavage at Arg306 (Figure 3.30B). Remaining FVa activity was measured by prothrombinase assays. In contrast to what was observed in the CAT assays, all three EGF3-4 variants appeared to have normal APC cofactor function in the FVa inactivation assays.

Protein S E36A and D95A showed reduced enhancement of APC proteolysis of both WT FVa (Figure 3.30A) as well as $FVa^{R506Q,R679Q}$ (Figure 3.30B). As much as 70% and 85% of FVa activity remained after inactivation with 100nM of protein S E36A and D95A, respectively. These results confirm the previous findings from my lab by Drs Josefin Ahnström and Helena Andersson, that Gla36 and Asp95 are essential for APC cofactor function of protein S. Interestingly, the reduction in enhancement of APC by protein S CHIII was somewhat more pronounced in the inactivation of WT FVa. However, the decrease in enhancement is relatively mild compared to what I observed in the CAT assays (Figure 3.29).

The discrepancy in results obtained for the variants spanning EGF3-4 and protein S CHIII in CAT and FVa inactivation assay can potentially be partially explained by the different nature of the FVa inactivation and CAT assays. CAT is a plasma based assay where APC-mediated inactivation of FVIIIa as well as FVa occurs. In contrast, in FVa inactivation assays APC-catalysed FVa inactivation is specifically assessed. Since protein S serves as a cofactor for inactivation of both FVa and FVIIIa it is possible substituted residues can have a great effect upon FVIIIa inactivation which is not accessed in FVa inactivation assays.

Interestingly, the APC cofactor function of protein S CHIII was more decreased in inactivation of WT FVa than that of FV R506Q/R679Q. This result may suggest that the protein S SHBG-like

domain, substituted in protein S CHIII, plays an important role for the enhancement of first initial cleavage at residue Arg506 of FVa.

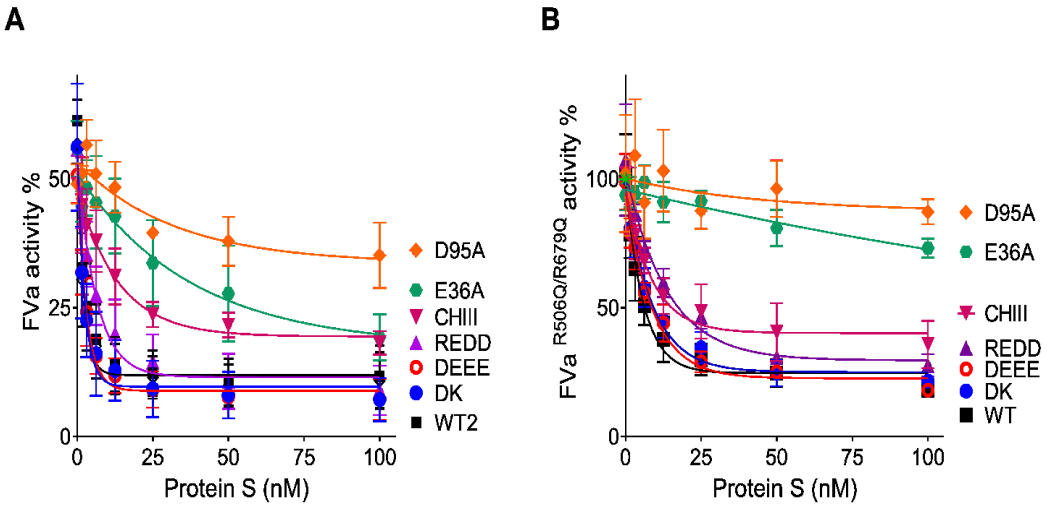


Figure 3.30. APC cofactor function of protein S variants in FVa inactivation assays.
FVa **(A)** and FVa $^{R506Q/R679Q}$ **(B)** (0.8nM) were inactivated by 0.25nM APC in the presence of increasing concentrations of protein S (0-100nM) for 10 minutes. Remaining FVa activity was quantified using prothrombinase assays. The results are plotted as a mean ± SD (n=3).

3.5.3 The importance of protein S variants in the formation of the FVa inactivation complex

My next aim was to determine the importance of substituted protein S residues for the formation of the FVa inactivation tri-molecular complex. For this, the protein S variants were analysed in my flow cytometry assay, used in previous sections, to study the assembly of the APC/protein S and FVa complex on phospholipids (Figure 3.31). For these assays I added 50 rather than 100nM protein S to make the assay more sensitive to any decrease in protein S enhancement. I first studied the time course of assembly of the inactivation complex. As can be seen in Figure 3.31A, I again observed rapid assembly of the FVa inactivation complex with APC, WT protein S and FVa. While less complex was formed in the absence of FVa, the enhanced binding of APC by protein S was evident. A reduction in the enhancement of APC binding to phospholipids binding by protein S (in the absence of FVa) was observed for the E36A (Figure 3.31A), D95A (Figure 3.31B), REDD (Figure 3.31D), and CHIII (Figure 3.31F) protein S variants. Although all protein S variants demonstrated decreased enhancement of

APC binding in the presence of FVa, the decreases in DEEE (Figure 3.31C) and DK (Figure 3.31E) were less pronounced. Considering that mutations D182N/E184Q/E186Q/E189Q, D227N/K233Q did not have any effect on protein S enhancement in the absence of FVa, the DEEE and DK variants were withdrawn from further study. Although the REDD variant had strongly reduced enhanced binding of APC in the presence and absence of FVa, it was also excluded from further analysis. Mutations R192Q/E201Q/D202N/D204N included one calcium binding site (Asp 202) and are localised very closely to two others, at Ile203 and Glu205. Since calcium binding sites are important for the maintenance of protein S structure, it would be difficult to conclude whether the impaired REDD cofactor function arose from interrupted protein-protein interaction or a major structural change. I next evaluated the importance of three protein S variants, protein S E36A, D95A and CHIII upon formation of the APC/protein S/FVa complex in more detail.

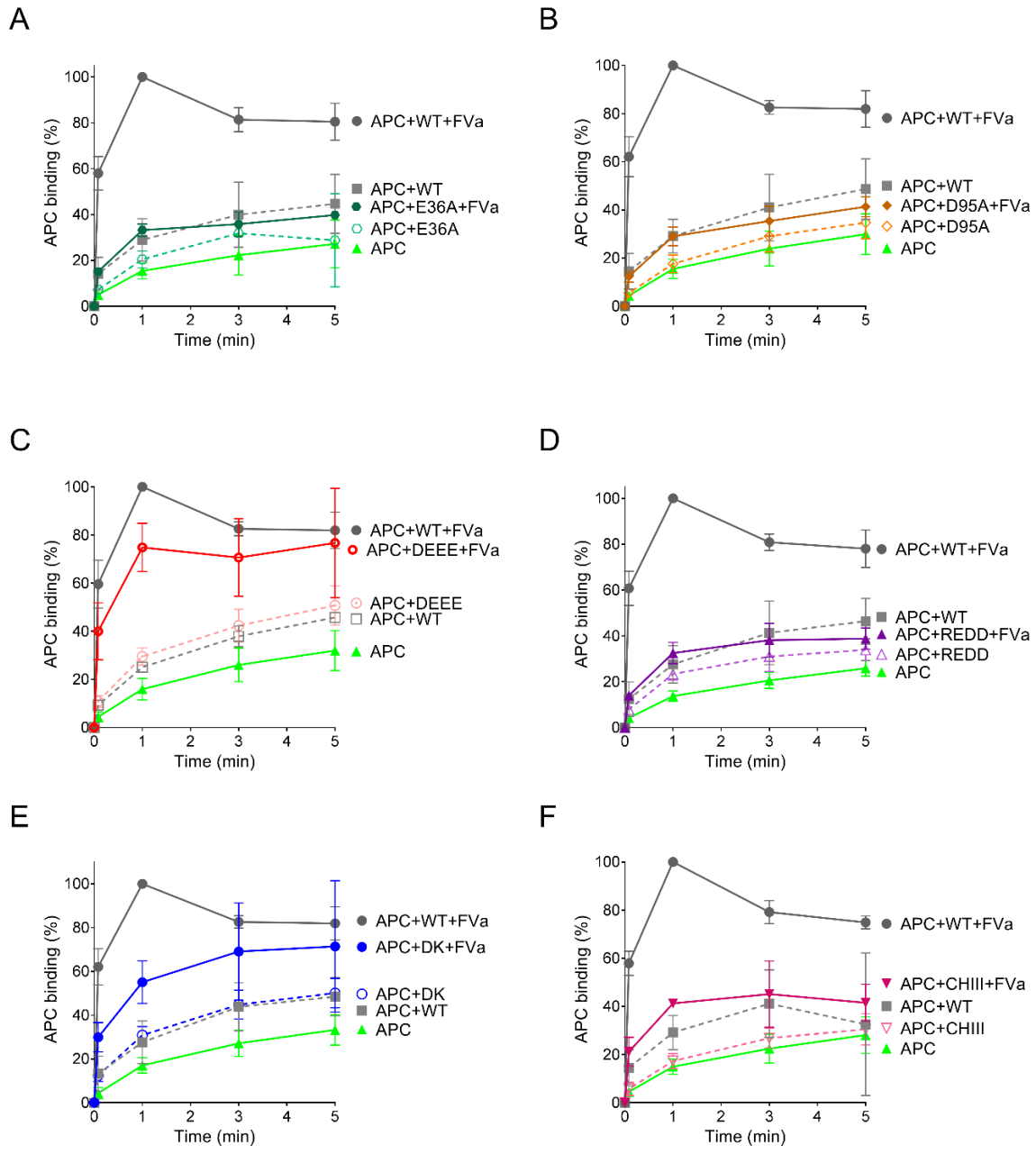


Figure 3.31. Protein S variants (E36A, D95A, DEEE, REDD, DK, CHIII) and the binding of APC to negatively charged phospholipids in the presence of FVa.

(A-F) Binding of 50nM APC-FEGRCK to phospholipids coated magnetic beads was evaluated in the presence and absence of 25nM FVa and 50nM of protein S E36A **(A)** protein S D95A **(B)** protein S DEEE **(C)** protein S REDD **(D)** protein S DK **(E)** or protein S CHIII **(F)** using flow cytometry. Results are compared to those of WT protein S (in grey) run in parallel experiments. **(A-F)** The MFI was measured immediately, 1, 3, and 5 minutes after addition of APC. All results were normalised, with 100% binding corresponding to APC binding in the presence of WT protein S and FVa measured 1 min after addition of APC. 0% corresponds to the autofluorescence of phospholipid coated magnetic beads. Results are expressed as a mean±SD, n=3

3.5.3.1 Titration of APC in the presence of protein S variants and FVa

As was shown in Figure 3.31, protein S E36A, D95A and CHIII displayed severely decreased ability to enhance APC binding to negatively charged phospholipid membranes, either in absence or presence of FVa. To further investigate the role of these variants upon APC/protein S/FVa complex formation, APC titrations (0-75nM) were performed in the presence of these protein S variants (100nM), in the presence or absence of 25nM of FVa (Figure 3.32-3.34). For protein S variants E36A (Figure 3.32) and D95A (Figure 3.33) the enhancement of APC binding observed in the absence of FVa was essentially normal. In contrast, binding in the presence of protein S CHIII variant was noticeable reduced (Figure 3.34).

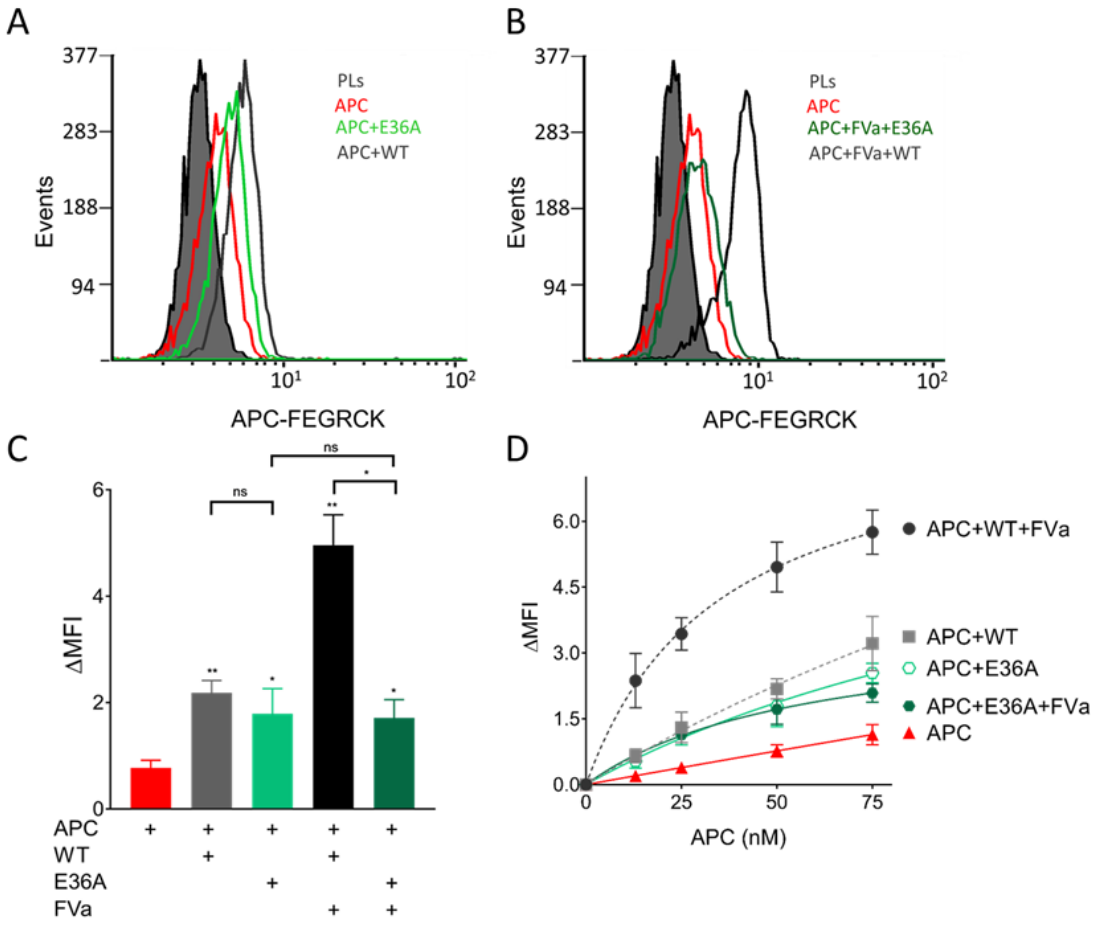


Figure 3.32. Enhancement of APC binding to phospholipids by protein S E36A
(A and B) Representative histograms and **(C)** quantification plot of binding of 50nM APC-FEGRCK to phospholipid-coated magnetic beads in the presence and absence of 25nM FVa and 100nM of protein S E36A using flow cytometry**. (D)** Binding of increasing concentrations (0-75nM) APC-FEGRCK to phospholipid-coated beads was measured in the presence and absence of 25nM FVa and 100nM protein S E36A MFI was measured 1 min after addition of APC. Results are plotted as mean ± SD (n≥3-7).

In the presence of FVa, the decrease in enhancement was pronounced for all three variants of protein S. These results suggest that protein S variants with impaired cofactor function (E36A, D95A, CHIII) have reduced abilities to assemble into the inactivation complex with APC and FVa. My results may therefore, for the first time, provide a potential explanation for their low anticoagulant activities.

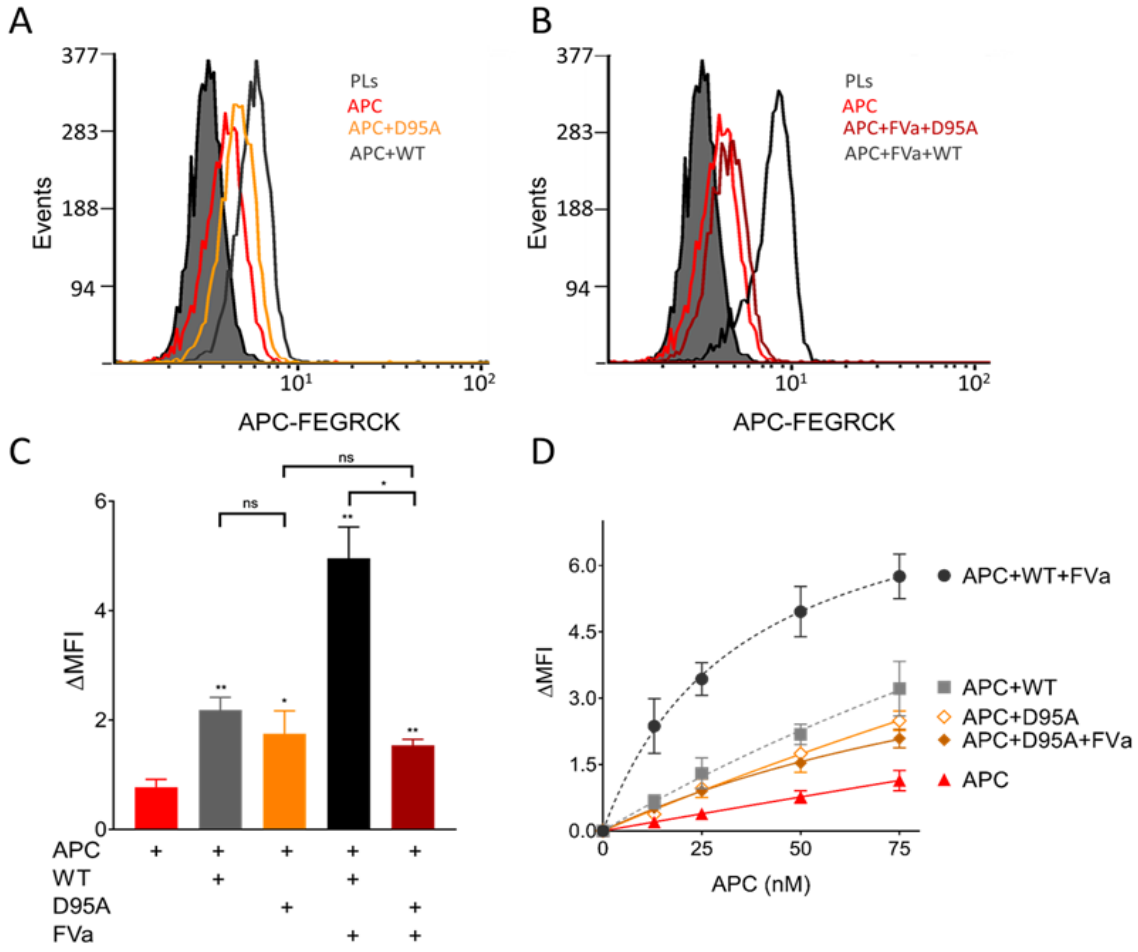


Figure 3.33. Enhancement of APC binding to phospholipids by protein S D95A
(A and B) Representative histograms and **(C)** quantification plot of binding of 50nM APC-FEGRCK to phospholipid-coated magnetic beads in the presence and absence of 25nM FVa and 100nM of protein S D95A using flow cytometry**. (D)** Binding of increasing concentrations (0-75nM) APC-FEGRCK to phospholipid-coated beads was measured in the presence and absence of 25nM FVa and 100nM protein S D95A MFI was measured 1 min after addition of APC. Results are plotted as mean ± SD (n≥3-7).

However, it is important to bear in mind that only interactions which influence phospholipids binding can be studied using this method. Since the reduction of the enhancement by protein S variants (E36A, D95A) was more apparent in the presence of FVa, these might be interpreted as these residues are involed in the FVa interaction rather than APC. And, similarly, the lack

of enhancement by protein S CHIII in the absence of FVa may suggest that protein S SHBG is involved in the interaction with APC. However these results have to be approached with caution. The presence of protein S, and thereby also an effective interaction between protein S and APC, is mandatory for the enhancement by FVa and protein S. Further study will therefore need to be conducted to determine whether the substituted residues of protein S are important for interaction with FVa or APC.

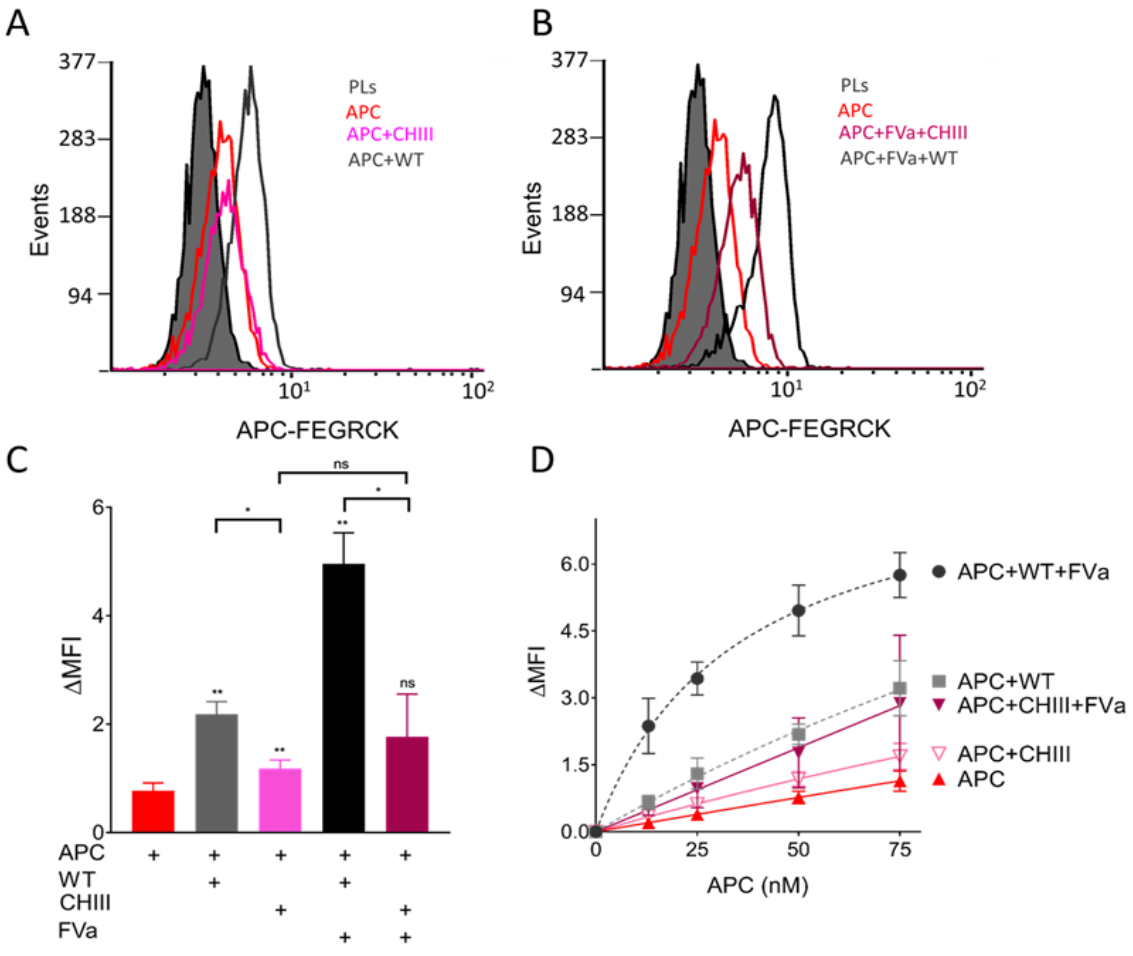


Figure 3.34. Enhancement of APC binding to phospholipids by protein S CHIII
(A and B) Representative histograms and **(C)** quantification plot of binding of 50nM APC-FEGRCK to phospholipid-coated magnetic beads in the presence and absence of 25nM FVa and 100nM of protein S CHIII using flow cytometry**. (D)** Binding of increasing concentrations (0-75nM) APC-FEGRCK to phospholipid-coated beads was measured in the presence and absence of 25nM FVa and 100nM protein S CHIII MFI was measured 1 min after addition of APC. Results are plotted as mean ± SD (n≥3-7).

3.6 FV Nara

Recently, Nogami *et al.* identified a naturally occurring FV mutation (W1920R; FV Nara) which causes APC resistance and is associated with reocurring DVT.[118] An interesting aspect of FV Nara is that the mutation is localised in the FV C1 domain, far from the APC cleavage sites. Although residue W1920 is close to proposed phospholipid binding sites, the authors claimed that the variant has normal phospholipid binding. Furthermore, the authors suggested that APC resistance of FV Nara arises from a functional impairment of an APC binding site on FVa Nara. I was intrigued to investigate this variant to see whether it might shed light on the nature of FVa inactivation by APC.

3.6.1 Evaluation of FV Nara in the prothrombinase assay

I first investigated the ability of FV Nara to function in prothrombinase assays. While there are properties required of FVa that are distinct from the formation of the inactivation complex, there are also common requirements, such as phospholipid binding, which is why I performed these assays. In my prothrombinase assays, thrombin generation was initiated with increasing concentrations of prothrombin (0-600nM) in the presence of 8pM WT FVa by 5nM FXa.

Thrombin formation was followed over time through cleavage of a specific chromogenic substrate (S2238), as can be seen in Figure 3.35A. A dose dependent relationship between prothrombin concentration and amount of generated thrombin was observed. In order to quantify generated thrombin, a standard curve with known concentration of thrombin was prepared. For this, the rate of the substrate hydrolysis was plotted against thrombin concentration as shown in Figure 3.35B. The rate of thrombin generation was shown as a function of prothrombin concentration, see Figure 3.35C. The conditions were optimised to ensure that the assay was completely FVa dependent and sensitive to any decrease in FVa function. Under these conditions, FVa Nara showed reduced ability to enhance thrombin generation (Figure 3.35C), with a 2.2-fold reduction in Vmax (0.14 ± 0.012 nM/s vs 0.06± 0.01 nM/s) and a 2.3-fold increase in Km (118.2 ± 57.2nM vs 51.81±12.91nM), compared to WT FVa. As can be seen in Figure 3.35C, FXa is essentially an inefficient enzyme in the absence of its cofactor. I also calculated kcat for my reactions, using the FXa/FVa concentration as the

enzyme concentration. The k_{cat} for FVa Nara was reduced by 2.2-fold in comparison to WT FVa (17.8 ± 1.53 s^{-1} vs 8.0 ± 1.78 s^{-1}). My results for FVa are similar to those previously published in similar assay.[56] However, more importantly, my results for FVa Nara are in agreement with those previously described by Nogami *et al.* since they also detected a similar reduction in kcat for FV Nara.[118] Since formation of prothrombinase complex is phospholipid dependent, it is possible that the reduced ability of FVa Nara to enhance thrombin generation arises from an impaired phospholipid interaction. However, the possibility that the FVa W1920R mutation affects FXa or prothrombin binding sites cannot be excluded.

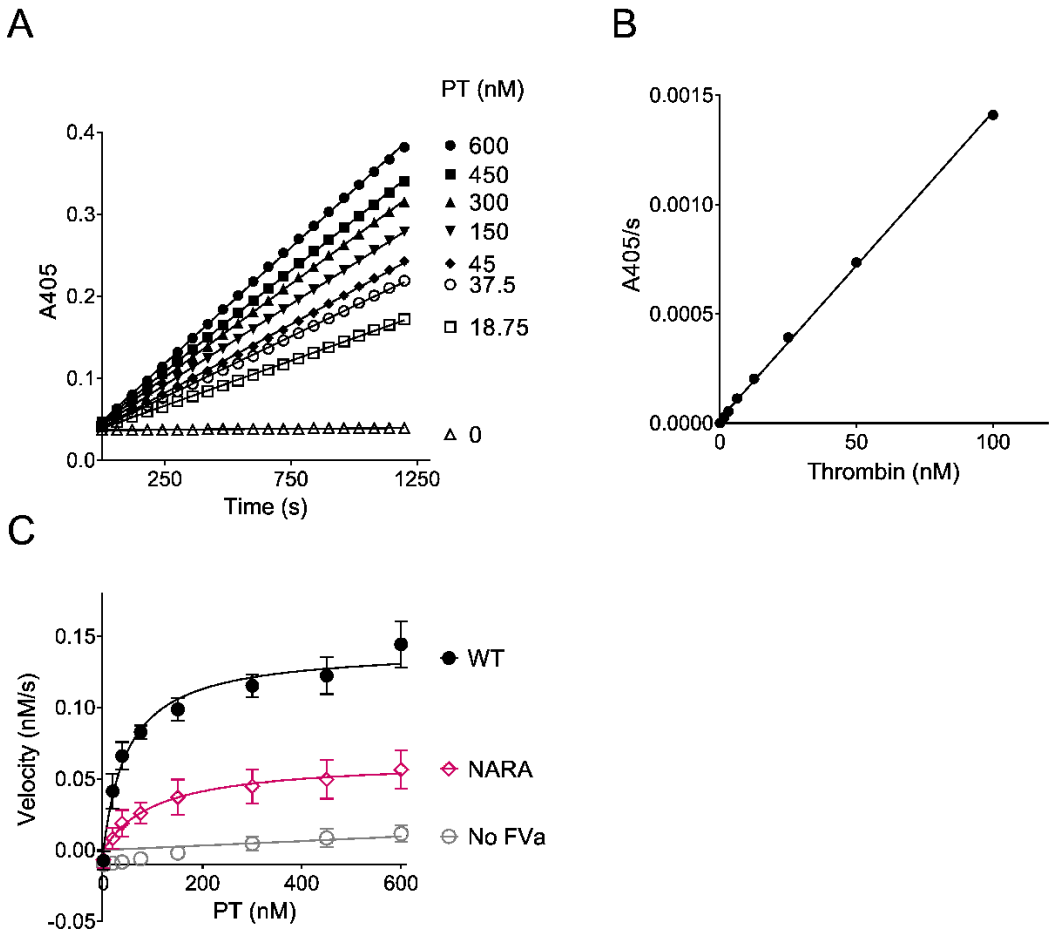


Figure 3.35. The cofactor function of FVa Nara in a Prothrombinase assay.
The ability of WT FVa and FVa Nara to enhance thrombin generation was compared in prothrombinase assays. Thrombin generation was measured in the presence of WT FVa or FVaNara (8pM), 5nM FXa and 50 µM phospholipid vesicles. The reaction was initiated by the addition of increasing concentrations of prothrombin (0-600nM). **(A)** The rate of thrombin generation was measured by cleavage of the thrombin specific chromogenic substrate (S2238) at 405 nm for 20 minutes. **(B)** The amount of thrombin generated over time was quantified using a standard curve with known amounts of thrombin. **(C)** The velocity of thrombin generation was plotted against prothrombin concentration. Km and kcat were derived from these experiments. Results are plotted as mean ± SD, (n=3).

3.6.2 Evaluation of FV Nara in the FVa inactivation assay

To test the resistance of FV Nara to inactivation by APC and thereby further characterise FV Nara, FVa inactivation assays were performed. FVa and FVa Nara were inactivated in the presence (Figure 3.36A) and absence (Figure 3.36B) of 100nM protein S with increasing concentrations of APC. The remaining FVa activity was measured in prothrombinase assays, where 100% activity corresponds to the activity measured at 0nM APC. In the presence of protein S, FVa Nara inactivation was around 8-fold less efficient than the inactivation of WT FVa (Figure 3.36A). While as little as 7.8pM APC was needed to achieve 50% reduction of FVa WT activity, the same level of inhibition for FVa Nara was obtained with 62pM APC. Interestingly, inactivation of FV Nara in the absence of protein S was very similar to that of WT FVa (Figure 3.36B). While the enhancement by protein S and proteolysis of FVa Arg306 were completely phospholipid dependent, FVa Arg506 could be cleaved also in the absence of phospholipids. The conditions of the assay were therefore optimised to evaluate FVa inactivation in the absence of phospholipids. For this, APC was titrated up to 20nM in the presence and absence of protein S (Figure 3.36C and D). Under these conditions, there was no difference in the efficiency of inactivation of WT FVa and FV Nara, either in the presence and absence of protein S. Taken together, these results suggest that APC resistance of FV Nara could be caused by a decreased ability to bind phospholipids.

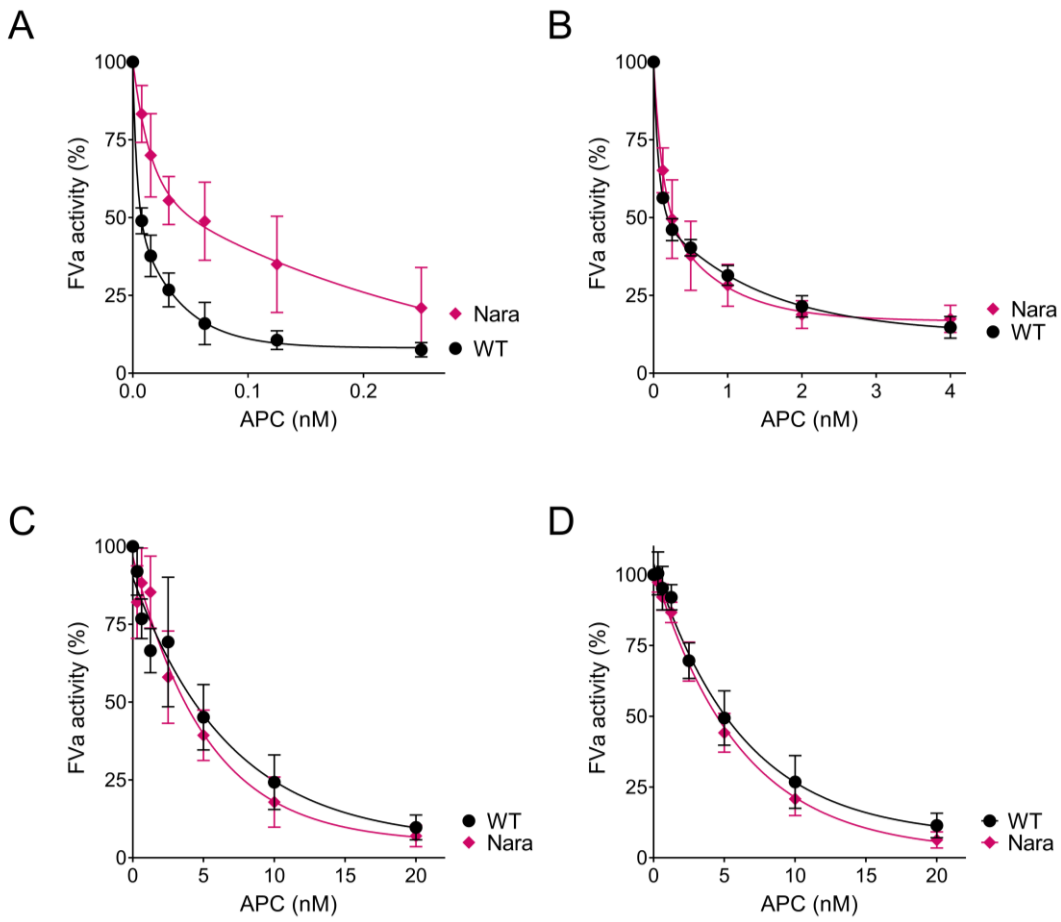


Figure 3.36. APC-mediated inactivation of FVa Nara.
Inactivation of FVa Nara by APC was assessed in FVa inactivation assays in the presence **(A and B)** and absence **(C and D)** of phospholipids. In the presence of phospholipids, FVa (0.8nM) was inactivated in the presence of 100nM protein S with increasing concentrations of APC (0-0.25nM) **(A),** or without protein S with 0-4nM APC for 10 minutes **(B).** In the absence of phospholipids, FVa was inactivated by 0-20nM APC in the absence **(C)** or presence **(D)** of 100nM protein S for 30 minutes. Remaining FVa activity was quantified using a prothrombinase assay. The results are plotted as a mean ± SD, (n=3)

3.6.3 Ability of FV Nara to bind to phospholipid vehicles

Since my results of FVa Nara inactivation suggested impaired phospholipid binding of this variant, I decided to explore this possibility using pull-down experiments (Figure 3.37). Phospholipid-coated beads were incubated with WT FVa or FVa Nara for 25 minutes. Bound and unbound FVa was analysed by Western blotting. The amount of WT FVa bound to the beads (Eluates) was substantially higher than of FVa Nara. This was confirmed by the high amount of FVa Nara remaining in the supernatant (Supernatants), compared to WT FVa. These results strongly suggest that FVa Nara may have reduced affinity for phospholipids.

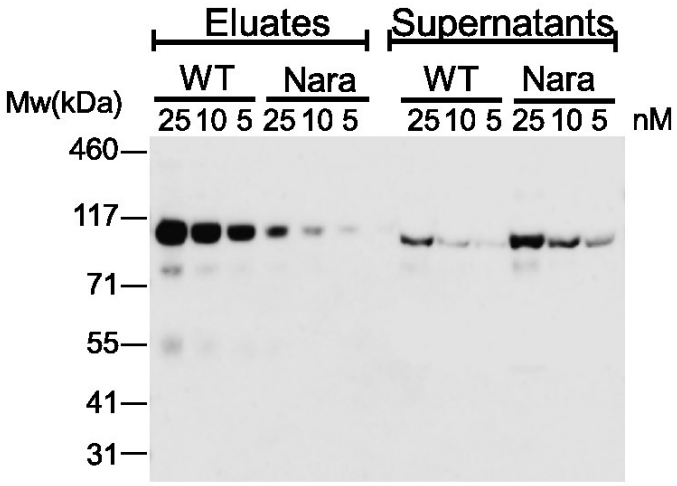


Figure 3.37. Phospholipid binding of FV Nara evaluated in pull-down experiment.
Phospholipid-coated magnetic beads were incubated with 5, 10, 25nM WT FVa or FVa Nara for 25 minutes. The FVa present in the bound fraction (30 µl) (Eluates) and in the supernatant (100 µl) (Supernatants) were analysed by Western blotting. 15 µl of each fraction was separated on a 4-12% SDS-PAGE, transferred to nitrocellulose membranes and detected using monoclonal anti-FV antibodies (AHV-5146; HTI). The blot shown is representative of n=2.

3.6.4 FV Nara binding to phospholipids examined by flow cytometry

To establish whether the FV W1920R mutation has any effect upon the ability of FVa to induce APC/protein S/FVa complex formation, I again employed my flow cytometry assay. The binding of 50nM APC-FEGRCK to phospholipid-coated beads was analysed in the presence of protein S and/or FVa WT alongside FVa Nara (25nM) (Figure 3.38). Compared to WT FVa, FVa Nara showed severely reduced ability to synergistically enhance association of APC to phospholipids. When I titrated FVa Nara (0-75nM) in the presence of 100nM protein S and 50nM APC, greatly reduced APC binding was observed. In fact, 50nM of FV Nara enhanced phospholipid binding of APC less effectively than 6nM of WT FVa (Figure 3.38D). These results demonstrate that FV residue Trp1920 is crucial for efficient formation of the APC/protein S/FVa inactivation complex.

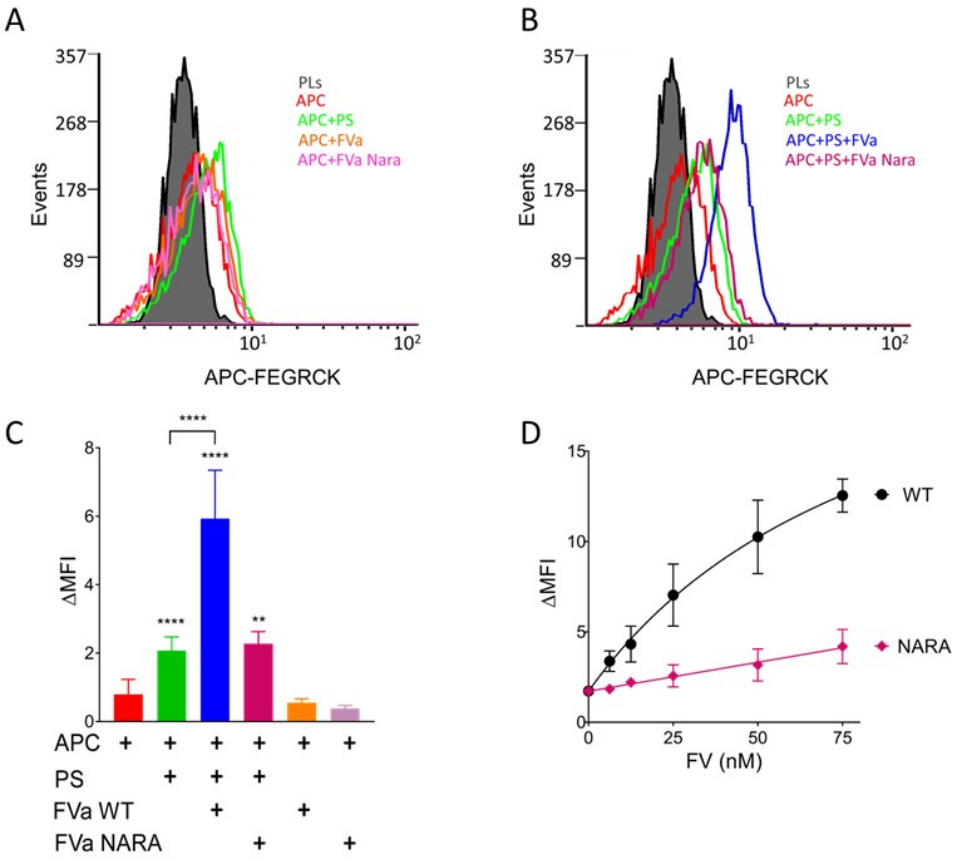


Figure 3.38. Reduced ability of FVa Nara to enhance the formation of the APC/protein S/FVa tri-molecular complex.

(A and B) Representative histograms and **(C)** quantification plot of binding of 50nM APC-FEGRCK to phospholipid-coated magnetic beads was determined in the presence and absence of 100nM protein S and/or 25nM WT FVa or FVa Nara or **(D)** with increasing concentration of WT FVa or FVa Nara (0-75nM) using flow cytometry. The MFI was measured at 1 minute after addition of APC. The results are presented as mean ± SD (n=3). ***p*<0.0021; *****p*<0.0001 according to Mann-Whitney tests compared to APC alone, unless otherwise stated.

Together, my results from prothrombinase assays, FVa inactivation assays and flow cytometry assays all show a reduction of functionality of FVa Nara. One aspect which all of these functions of FVa has in common is the need for phospholipid binding. It was therefore not surprising that my pull-down experiments showed more efficient binding of WT FVa to phospholipid-coated beads than of FV Nara (Figure 3.37). Importantly, a decrease in FVa-phospholipid binding can explain the lack of complex formation, which in turn can explain its resistance of APC proteolysis. However, it is also possible that W1920R mutation affects interaction with protein S.

3.7 Discussion: The inactivation complex

Protein S was identified as a cofactor for APC almost 40 years ago. Since then a large number of studies have been conducted where the molecular mechanisms behind this cofactor has been investigated. The extent of enhancement of APC activity by protein S was one of the first aspects of its cofactor functions to be investigated. Early studies, using pure-component FVa inactivation assays, showed only a weak, twofold stimulation.[47,211,244] Following adjustment and optimisations of the assay, published studies by Rosing and Norstrøm identified a higher degree of stimulation of APC by protein S (20-30 fold) on FVa proteolysis.[62,153] However, even this degree of enhancement does not seem to reflect the profound effect protein S has on APC catalysed FVa degradation in plasma. My results using plasma based CAT assay show that even 70nM of APC does not reduce et all thrombin generation, while 9nM APC in the presence of 80nM protein S inhibited thrombin generation by 70% (Figure 3.12). This result shows that APC, at physiological concentration, is not an effective anticoagulant in the absence of protein S.

Several different hypotheses have been presented in the literature regarding the precise nature of the APC cofactor function of protein S. Initially, Walker demonstrated that protein S exerts its cofactor function by increasing the affinity of APC to negatively charged phospholipid surfaces.[209] An alternative hypothesis, presented by Solymoss and colleagues, suggested that protein S reverses the protective effect of FXa on FVa against APC-mediated inactivation.[211] Yegneswaran *et al*. on the other hand, proposed that protein S relocates the active site of APC closer to phospholipid surface for optimal FVa proteolysis.[213] While the important role of protein S in FVa proteolysis has been established by many research groups, the precise mechanisms underlying the function of protein S action have yet to be fully defined.

APC inhibits thrombin generation by degradation of FVa and FVIIIa.[99] While both factors, FVa and FVIIIa, share the same domain organization and 30-40 % of homology, the mechanisms of their inactivation are different. In the inactivation of FVa, protein S increases the rate of proteolysis at Arg306 by 20-30 fold and cleavage at Arg 506 by 1-5 fold.[153,245] While FVa is highly susceptible to proteolysis, making it an unstable protein, FVIIIa is even more so, making kinetic characterisation of its inactivation much more difficult. According to one study of

FVIIIa inactivation, protein S was shown to enhance cleavage at Arg562 ~7-fold whereas cleavage at Arg336 was stimulated only by 5-fold.[155] However, due to the very low stability of FVIIIa, the literature of protein S enhancement of APC-mediated FVIIIa enhancement is limited. Nevertheless, it is well known that the overall effect of protein S on FVIIIa proteolysis by APC is lower than that exerted on FVa inactivation, with some studies reporting only overall 1.5-3 fold enhancement of FVIIIa inactivation.[105 215]

APC-mediated inactivation of FVIIIa also depends upon FV as a synergistic cofactor, together with protein S.[49] Gale *et al.* demonstrated that both cofactors, in combination, stimulate proteolysis of FVIIIa at Arg 336 and Arg562 by 24 and 60-fold, respectively.[155] This finding made me wonder whether FV/FVa could play a similar role in its own degradation by APC. When APC cofactor function of protein S was studied in purified systems, a much lower enhancement was reported than what would be expected from the results seen in plasma.[47,211,244] Interestingly enough, among all components involved in the APC mediated FVa inactivation, FVa has the highest affinity to phospholipid membranes.[209] Moreover, it has been demonstrated that FV increases the affinity of APC to phospholipids by 5-fold while FVa enhanced the APC/phospholipids interaction by 10-fold.[246] Collectively, these findings brought me to the hypothesis that FVa together with protein S enhances the affinity of APC to phospholipid membranes, together forming a tri-molecular inactivation complex. Therefore, the main objective of my project was to clarify the nature of the interactions between FVa, APC and protein S.

In approaching this, I faced a big challenge. FVa inactivation, as well as the protein/protein interactions involved in this process are all phospholipid dependent, making individual reactions difficult to study. The methods available which can be used to investigate protein/protein interactions in the presence of phospholipids are limited. In addition, the proteolytic susceptibility of FVa, hinders the analysis of these interactions. Indeed, much of my PhD was spent expressing, re-expressing and purifying of FV to obtain good quality full length protein. Initially, I studied the influence of protein S and FVa on the interaction between APC and phospholipids using a pull down method. For this, I used an inhibited, active site labelled APC (APC-FEGRCK) (Figure 3.15). I observed that preincubation of the phospholipid coated magnetic beads with protein S, prior to addition of APC, increased the amount of APC which bound to them. Intriguingly, this amount was even higher when the

beads were preincubated with protein S and FVa together. In contrast, addition of FVa to the beads in the absence of protein S did not have any effect on APC binding to phospholipids. These results strongly suggested that FVa together with protein S increased binding of APC to negatively charged phospholipid membranes. This observation supported my hypothesis about the formation of an APC/protein S/FVa inactivation complex.

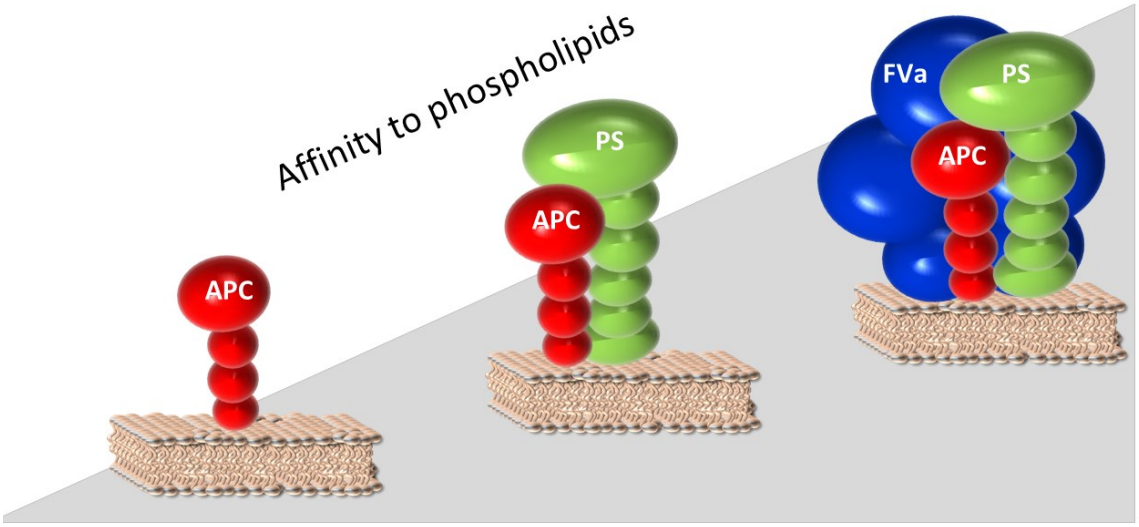


Figure 3.39. Proposed model of the inactivation complex between APC protein S and FVa.
Protein S on its own enhanced the APC-phospholipids associatiom moderetly. However Protein S together with FVa increases binding of APC to phospholipid membranes much further. The binding enhancement by protein S and FVa was reversed with specific anti-protein S and anti-FV antibodies. Taken together these finding suggest formation of the inactivation complex between APC, protein S and FVa.

Following my results from the pull-down experiments, I optimised a flow cytometry based method to investigate the interactions between FVa, protein S and APC on negatively charged phospholipid coated beads. In contrast to the pull down method, this technique allowed the study of the inactivation complex in a quantitative way. The use of inactivated, fluorescently labelled APC (APC-FEGRCK) facilitated the study of binding interactions between enzyme, cofactor and its substrate in the presence of phospholipids surfaces. One limitation of this method is its low sensitivity. In contrast to fluorescently labelled antibodies, with a number of fluorescence molecules attached, APC-FEGRCK only has one fluorophore at its active site. However, I avoided the use of an antibody method because the presence of a labelled antibody could perturb complex interaction between APC, protein S and FVa. To overcome the low level of fluorescent signal, I instead had to use high concentrations of APC-FEGRCK. Using flow cytometry technique, I confirmed that APC binds to phospholipids with low affinity

(K_d 0.07-17µM level).[61,239,240] Protein S (100nM) only moderately increased association of APC to phospholipids (Figure 3.17), which appers not to agree with the results from functional assays (Figures 3.11). In contrast, the binding enhancement induced by protein S and FVa in combination was considerably greater, while FVa, on its own did not stimulate association of APC to phospholipids (Figure 3.17). All of these results supported what I had observed in the pull-down experiments. I demonstrated specificity of the enhancement of APC association to phospholipids using antibodies. A combination of anti-protein S and anti-FV antibodies fully reversed the enhancement arising from protein S and FVa. Together, these findings strongly suggested formation of a tri-molecular inactivation complex between APC, protein S and FVa.

As mentioned earlier, APC also catalyses inactivation of FVIIIa and protein S serves as APC cofactor during this reaction. I was curious to see whether a similar multicomponent inactivating complex was formed during APC-mediated proteolysis of FVIIIa. To answer that question, I examined binding of APC-FEGRCK to phospholipids in the presence of FVIIIa and protein S. Contrary to FVa, FVIIIa did not enhance the association of APC to phospholipids. This result suggests that, unlike FVa, FVIIIa does not assemble into a detectable inactivation complex with APC and protein S. At least not through the same mechanisms as FVa. This observation supports the concept that the molecular mechanisms involved in the two APC catalysed inactivations are different. To date, it is well known that the sequence of APC-mediated cleavages, and the rates at which these occur, vary between the two substrates. During FVa degradation, the first cleavage to occur is the fast cleavage at Arg506, followed by slow proteolysis at Arg306. FVIIIa on the other hand, is first inactivated at Arg336 (homologous to Arg306), with subsequent cleavage at Arg562. Protein S serves as a cofactor in both protolytic reactions, albeit strength and specifity of its enhancements are different. Now I can confirm that also the mechanisms underlying both inactivations are different and that the mechanism, involved in the formation of the inactivation complex, appers to be specific to FVa proteolysis.

To study the affinity of APC to phospholipids in more detail, titration of APC-FEGRCK in my flow cytometry assay was performed. Titration of APC into the assay showed a dose dependent binding of APC to phospholipid surfaces. My results confirmed what was previously established through a number of studies, i.e. that APC binds to phospholipid membranes with low affinity.[61,239,240] Indeed, the affinity of APC-phospholipids association

was so low that it was not possible to determine dissociation constant of this interaction. Protein S provided same ~2-3 fold enhancement of APC-phospholipids association, FVa by itself did not enhance this interaction. However, the presence of both, protein S and FVa together, increased the binding of APC to phospholipids greatly. In the presence of protein S and FVa, the half-maximal binding of APC-FEGRCK was determined as 30nM. In order to estimate the affinity of APC interaction with phospholipids in the absence of protein S and FVa I constrained the level of maximum binding to the level observed in the presence of protein S and FVa. When calculated in this way, half-maximal binding of APC-FEGRCK was approximately 0.5 μM which was reduced to ~130nM in the presence of protein S.

A body of literature has defined a low affinity for the interaction between APC and phospholipid membranes. However, there is a large inconsistency between the results reported by different research groups. Reported affinities varies from 73nM, reported by Krishnaswamy, to up to 16-17 μM, demonstrated by Yegneswaran and Nelsestuen.[63,246,247] However, the majority of reported K_d values appear to cluster around a 0.5-3 μM range, which is in agreement with my results.[61,240] The discrepancy between published results could be attributed to the use of different techniques, as well as origin and composition of phospholipids. Many early studies used bovine protein C which later was found to bind weaker to phospholipid surfaces than human protein C.[240] Moreover, only a few studies were performed with the activated form of protein C. The study by Smirnov *et al*.[61] deserved particular attention as the authors assessed the affinity of the APC-phospholipid interaction using an approach similar to mine. Using fluorescently labelled APC and liposome-adsorbed latex beads, they found that binding of APC to phospholipids vesicles was strongly dependent on the presence of PE. The authors reported that APC bond to phospholipids composed of PS:PC (20:80) with K_d 500nM, while to vehicles with PE:PS:PC (50:20:30) with K_d 140nM. Interestingly, they also found that protein S on its own enhances the APC catalysed inactivation by 2-fold, whereas protein S and FVa together were reported to stimulate the proteolysis by 10-fold. It is worth noting that when I titrated APC in the flow cytometry assay at the concentration ranges reported in the Smirnov study I could not obtain saturation. This discrepancy is most likely due to differences in composition of phospholipids and incubation times. The detailed experimental design of the study by Smirnov was different from mine. Their experiments were designed to determine the influence of PE on binding of APC to

phospholipids. For that reason, in their study, the affinity of APC-phospholipids interaction was examined after 20 minutes incubation and with high percentage (50%) of PE in phospholipids preparation. In contrast, I aimed to prove formation of an inactivation complex between APC, protein S and FVa. The results from functional assays suggest that FVa is proteolysed by APC in the presence of protein S within 5 minutes. The time frame of observation is therefore crucial. The inactivation complex will only be functional and relevant until FVa is proteolysed. Therefore, in contrast to Smirnov and colleagues, I looked at binding of APC to phospholipid membranes over time, starting from the moment when APC was added to the beads. Importantly, the highest effect induced by protein S and FVa together was observed immediately after the addition of APC to phospholipids vehicles. This finding implies that once APC, protein S and FVa are in close proximity of each other, they rapidly form the inactivation complex. Considering the high affinity of FVa to phospholipids, compared to that of APC and protein S, my data suggests that FVa, the substrate, plays an active role in the formation of its own inactivation complex and helps to localize the other components to the phospholipid membrane.

As mentioned previously, around 60% of protein S in plasma forms a high affinity complex with C4BP.[227] Proposed binding sites for C4BP are localised on SHBG domain of protein S, in close proximity to suggested FVa binding sites.[199,203,212] Moreover, it has been demonstrated that C4BP-bound protein S has reduced APC cofactor function and does not bind to FVa as efficiently as free protein S.[203] For these reasons, I investigated the influence of C4BP on assembly of the inactivation complex in flow cytometry. C4BP-bound protein S showed reduced enhancement of APC-phospholipids association, both in the presence and absence of FVa. In agreement with previous reports, suggesting that C4BP blocks protein S-FVa interaction, my results indicate that C4BP-bound protein S has strongly reduced ability to assemble into a complex together with APC and FVa. This observation also provides an explanation for the impaired cofactor function of C4BP-bound protein S.

Several research groups have shown that the Gla-TSR-EGF1 region of protein S plays an essential role in the anticoagulant function of protein S. Two variants with greatly reduced APC cofactor function were previously identified in my research group (protein S E36A and D95A).[182,189] Unlike a naturally occurring variant of protein S (G11D) which demonstrated decreased APC-cofactor function due to impaired phospholipid binding, protein S E36A and

D95A were both found to have normal affinity to phospholipids surfaces. While the functional consequence of the mutations were established, the mechanistic basis for their impared functions was not known. However, it is worth noting that residues 3-50 of protein S were suggested to contain a FVa interaction site and that the naturally occurring mutation T37M is associated with reoccurring DVT due to abnormal anticoagulant function.[64,183] I also included in my study a chimera of protein S, where the SHBG-like domain had been substituted for the corresponding domain of GAS6 (protein S CHIII).[207] Intriguingly, protein S CHIII had previously been shown to enhance APC-mediated inactivation of FVa equally well to WT protein S, but failed to promote FVIIIa proteolysis.[205,207]

I evaluated all three variants previously shown to have reduced APC cofactor function alongside variants of protein S containing substitutions spanning its EGF3-4 domain (protein S DEEE, REDD and DK). These variants were interesting to me since the importance of protein S EGF3-4 to function was uncertain. The variants were characterised in thrombin generation assays and FVa inactivation assays. Among the EGF3-4 variants only protein S REDD variant showed reduced APC cofactor function. My results from protein S E36A and D95A confirmed previous findings and they both showed greatly reduced ability to enhance APC. Protein S CHIII demonstrated strongly reduced APC cofactor function in thrombin generation assay (Figure 3.29) but not during APC-mediated inactivation of FVaR506Q using purified components (Figure3.30B). This agrees with earlier observations of Reglińska-Matveyev and Nyberg and can be possibly explained by the observation that protein S CHIII has been reported to have abolished APC cofactor function in FVIIIa degradation. In contrast to FVa inactivation assay, thrombin generation is performed in plasma where both APC substrates, FVa and FVIIIa, are present. In addition, protein S CHIII did not increase inactivation of WT FVa as efficiently as WT protein S. In this case, the protein S CHIII enhancement was moderately reduced what might suggest that SHBG-like domain might be of special importance for the first APC-mediated cleavage at Arg506. All protein S variants were then evaluated by flow cytometry. Protein S DEEE and DK enhanced APC-phospholipids association in the absence of FVa, similarly to WT protein S. Additionally only small reductions of the synergistic enhancement was observed for those variants. For these reasons, protein S DEEE and DK were not studied further. Contrary to the two other variants of EGF 3-4, protein S REDD failed to enhance APC both in the presence and absence of FVa. However, I realized that the REDD

mutation included a residue involved in a calcium binding site (Asp202). Since disrupted binding of calcium ions could have effects on structure and function of the protein, unambiguous interpretation of my results would therefore not be possible. It would be difficult to conclude whether the impaired cofactor function by protein S REDD arose from removal of a specific protein-protein interaction site or caused by a major structural change. Consequently, this variant was also excluded from further investigationlight

To better understand the role of substituted residues Gla36 , Asp95 as well as SHBG-like domain of protein S upon formation of the inactivation complex, these protein S variants were also evaluated in flow cytometry. In the absence of FVa, protein S variants, E36A and D95A were only slightly less effective than WT protein S in enhancing APC-phospholipids interaction (Figure 3.32-3.34). In contrast, protein S CHIII was more noticeable reduced under the same conditions. However, in the presence of FVa, the mutants had weak effect upon enhancement of APC binding. These results suggest that the studied variants of protein S are unable to assembly the inactivation complex, which could explain their low APC cofactor function, as observed in functional assays.

Since the enhancement of APC-phospholipids association by protein S variants (E36A, D95A, CHIII) was reduced both in the presence and absence of FVa, it is impossible to determine whether the substituted residue of protein S are selectively important for the interaction with either FVa or APC. However, as a reduction of the enhancement was minor in the absence of FVa, and no further enhancement was seen in its presence, it is tempting to speculate that protein S E36A and D95A have impaired interaction with FVa. It is somewhat surprising that no complex formation was observed in the presence of protein S CHIII, as its APC cofactor function in degradation of FVa R506QR679Q was only mildly reduced. Although further analysis of this variant needs to be done to explain this discrepancy, it seems possible that lack of formation of the inactivation of complex by this variant is compensated over time with another mechanism involved in protein S enhancement. As suggested earlier, assembly of the complex between APC, protein S and FVa seems to be important for localisation of the proteins on the membrane and the first APC cleavage at Arg506. Perhaps for that reason the enhancement of protein S CHIII was stronger reduced in inactivation of WT FVa in comparison to FVa R506QR679Q. It is important to bear in mind that the flow cytometry technique

demonstrates interactions which influence APC binding to phospholipids. Other possible mechanisms of protein S enhancement will not by detectable by this method.

Among all mutations of FV associated with APC resistance, FV Nara attracted my attention. Unlike the FV Leiden, FV Bonn and many others FV mutation localised close to APC cleavage sites, FV Nara is situated in the C1 domain of FV, close to proposed phospholipid binding sites.[118] FV Nara was initially identified as a cause of reoccurring DVT by Nogami *et al*. In their paper, Nogami *et al.* showed that recombinantly expressed FV Nara was more resistance to APC-mediated inactivation than FV Leiden, despite normal affinity to phospholipids. For that reason, the authors suggested that the APC resistance associated with the mutation could be attributed to an alteration of APC binding sites. I therefore wanted to investigate how the amino acid substation would affect the formation of the inactivation complex. I first aimed to assess whether this FV variant could function as a cofactor for FXa and thereby enhance thrombin generation, similarly to WT FV. Using prothrombinase assays, I identified a two-fold reduction in thrombin generation. It is well established that, assembly of the prothrombinase complex is dependent upon phospholipid interactions. Mutations localised in the FV C1 and C2 domains have previously been found to affect formation and function of prothrombinase complex.[57,248] Since the W1920R mutation is situated in the conserved hydrophobic core of C1 domain, it is likely that this observed reduction in FXa cofactor function can be caused by abnormal binding of FV Nara to phospholipids. However, at this point I could not exclude the possibility that the substitution could alter the interaction with FXa. I also tested the efficiency of APC-mediated inactivation of FVa Nara. In agreement with the previous study, APC-mediated inactivation of FV Nara was strongly reduced compared to WT FVa. Interestingly, however, my results from the FVa inactivation assays showed a reduction in inactivation only in the presence of protein S. In the absence of protein S, FV Nara was inactivated as efficiently as WT FVa. Importantly, inactivation of FV Nara was also comparable to that of WT FVa in the absence of phospholipids, strongly suggesting that the ability of FV Nara to bind phospholipids is disturbed.

Nogami and colleagues presented data which suggested a normal FV Nara-phospholipid interaction. Their conclusion was based on use of a plate binding assay. It is well known that the composition of phospholipids greatly affects the affinity of the coagulation factor-phospholipid interaction. In the majority of functional assays, PS is used at 5-40% percentage.

This concentration has been optimised to correspond to the proportion of exposed PS on activated platelets and endothelial cells. In the Nogami paper, the authors coated the plate with 100% PS. It is therefore possible that they may not have been able to see any abnormalities in phospholipids binding. To investigate the affinity of FVa Nara for phospholipid membranes compared to WT FVa, I instead chose to use my pull down method. In this method I used a universally accepted ratio of phospholipids, i.e. 20% PS, 20% PE and 60% PC. I also evaluated the levels of FVa bound after a quick wash, rather than relying on the affinity being strong enough to sustain lengthy antibody incubation steps. In my assay, substantially more of WT FVa bound to phospholipid-coated beads compared to FV Nara (Figure 3.37). While I unfortunately was unable to determine any binding affinity from this assay (for that a more comprehensive study has to be conducted, for example using SPR), this result clearly demonstrates that binding of FV Nara to phospholipid surfaces is greatly reduced.

The effect of the FV Nara mutation upon its ability to formation the inactivation complex was investigated using my flow cytometry method. As expected from the functional assays, FVa Nara did not show any ability to enhance binding of APC to phospholipids, neither in the presence or absence of protein S. My results suggest that FVa Try1920 is important for the formation of the inactivation complex. However, it is not clear at this time whether this is exclusively due to its decreased affinity to phospholipids or whether a combination of factors, such as impaired interaction with phospholipids and APC or protein S. To address this question, the affinity of FVa Nara towards phospholipid membranes would have to be studied using a more quantitative method. Additionally, assessing the binding between protein S and FVa Nara in the presence of phospholipids would also be beneficial. However, it is clear that the formation of the tri-molecular APC/protein S/FVa inactivation complex is strongly dependent on FVa (Figure 3.25) and that normal phospholipid interaction of all three proteins is essential.

Taken together, my findings confirm that protein S increases affinity of APC-phospholipid interaction. Moreover, binding of APC to phospholipid surfaces is further enhanced by protein S and FVa together, while FVa by itself does not stimulate this interaction. Using anti-protein S and anti-FV antibodies, I demonstrated specificity of the enhancement. These results strongly support existence of the inactivation complex between APC, protein S and FVa.

Investigation of FVIIIa proteolysis revealed that in contrast to FVa, it does not form a detectable inactivation complex with APC and protein S. Neither C4BP-bound protein S nor its variants with impaired APC cofactor function incorporate into the inactivation complex equally well to protein S. Lack of complex formation between APC, protein S and FV Nara can explain strong resistance of this variant to APC proteolysis. Overall, my findings provide evidence for a relative unexplored anticoagulant role of FVa, namely that it promotes its own degradation by forming the inactivation complex on the phospholipid surface.

3.8 Structural aspects of the FVa A-domains in its inactivation by APC

3.8.1 FV-810

When I started working on this project I was interested in FV-810 because full-length FV is very difficult to work with due to its instability. It had been suggested that FV-810 is a more stable alternative to FVa. I began my work in this thesis with FV-810 , but soon become aware that its inactivation by APC has quite different features to those of FVa. Due to these differences I optimised the FV expression and purification for my studies of the FVa inactivation complex. However, my findings on FV-810 are intriguing and can, instead of being used as an alternative to FVa, be used as a tool. Understanding the differences in APC-mediated inactivation of FVa and FV-810 may shed fresh light on the structural mechanisms involved in the inactivation of FVa by APC.

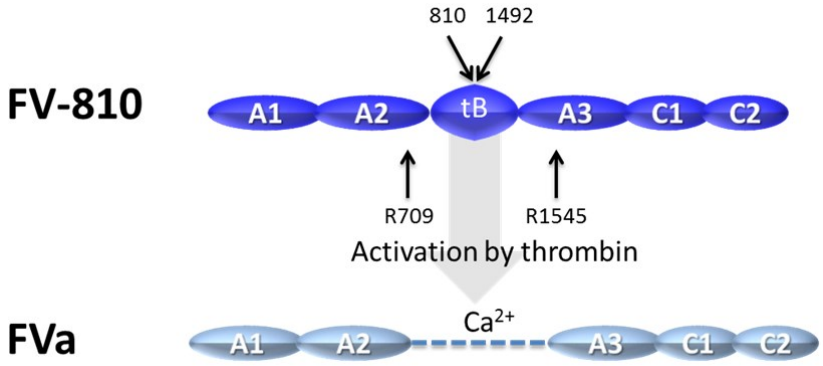


Figure 3.40 Schematic representation of FV-810.
FV-810 is B domain truncated FV derivative with deletion of residues 811-1491. This FV variant, expressed as a single chain protein without activation assembly prothrombinase complex as FVa. Once activated with thrombin FV-810 is indistinguishable from FVa.

3.8.1.1 Prothrombinase function of activated and non-activated FV-810

FV-810 has previously been described by Toso *et al.* as a more stable version of FVa in prothrombinase assays. To assess whether this is the case also in my hands, I studied whether FV-810 was able to be incorporated into a prothrombinase complex together with FXa, similarly to FVa, in prothrombinase assays. Initially, FV-810 activated by thrombin (FVa-810) was titrated (0-32pM) in these assays alongside rFVa and ppFVa. Thrombin generation was initiated by addition of 0.5µM prothrombin in the presence of 5nM FXa, and 25 µM

phospholipids. As shown in Figure 3.41A, activated FV-810 enhanced thrombin formation equally well as both types of FVa. This was expected since FVa-810 structurally should be identical to FVa. Thrombin generation was also analysed using increasing concentrations of non-activated FV-810 (0-8pM) (Figure 3.41B). Also in this case no difference in thrombin generation was detected. This result shows that FV-810 does not require activation to function as a cofactor for FXa in the prothrombinase complex.

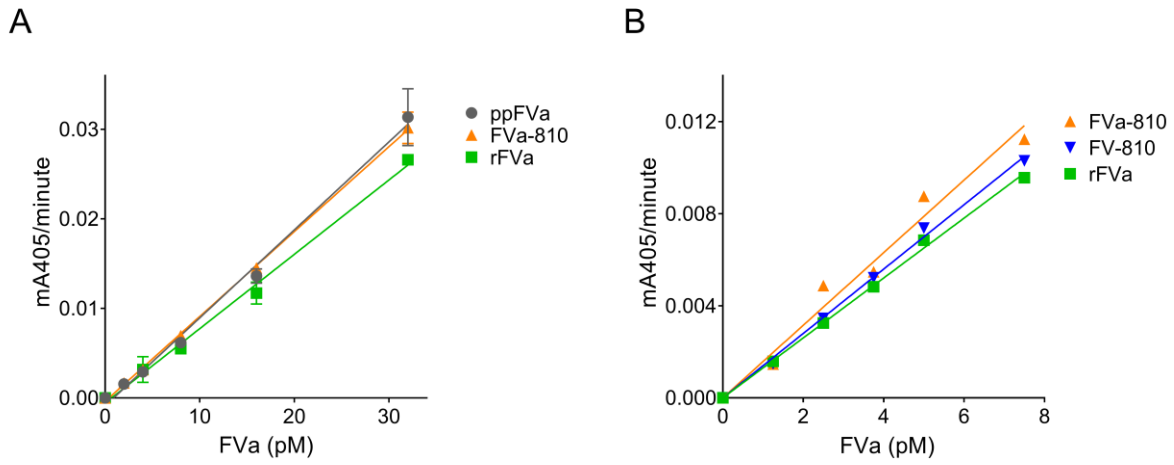


Figure 3.41. Functional properties of FVa-810 and FV-810 in a prothrombinase assay.
The ability of FV-810 to enhance thrombin generation was analysed in prothrombinase assays. 8pM of FVa, FV, FV-810a **(A)** or FV-810 **(B)** was incubated with FXa in the presence of phospholipid vesicles and 2mM $CaCl_2$. Thrombin generation was initiated by addition of prothrombin (0.5µM). After 2 minutes the reaction was stopped and thrombin generation was quantified using a chromogenic substrate (S2238).

3.8.1.2 Evaluation of APC-mediated inactivation of FV-810

To determine whether FV-810 can be inactivated by APC and protein S in the same manner as FVa, FVa inactivation assays were used. FVa and FV-810, (the latter activated and non-activated), were all inactivated with increasing concentrations of APC (0-0.5nM) (Figure 3.42). Somewhat surprisingly, APC-mediated inactivation of FV-810 was rapid and appreciably more efficient than FVa. In contrast, inactivation of the activated form of FV-810 was comparable to that of FVa, suggesting that the structural differences caused by thrombin cleavage decreases the efficiency in which FV-810 is inactivated, i.e. the change from a single chain to two chain molecule. To further investigate the differences in APC-mediated inactivation of FV-810 compared to FVa, time course experiments were performed. FV-810 and FVa were both inactivated by 50pM APC in the absence and presence of 100nM protein S (Figure 3.43A

and B). At defined time points, the inactivation reaction was stopped. Remaining FVa activity was quantified using prothrombinase assays. Figure 3.43B illustrates the efficiency of APC-mediated degradation of FV-810. The difference in the inactivation between FVa and FV-810 was most striking in the absence of protein S. While only 30% of FVa was inactivated in the absence of protein S, up to 70% of FV-810 was degraded under the same conditions. Importantly, to achieve the same rate of inactivation of WT FVa as that of FV-810, 40 times more of APC had to be used.

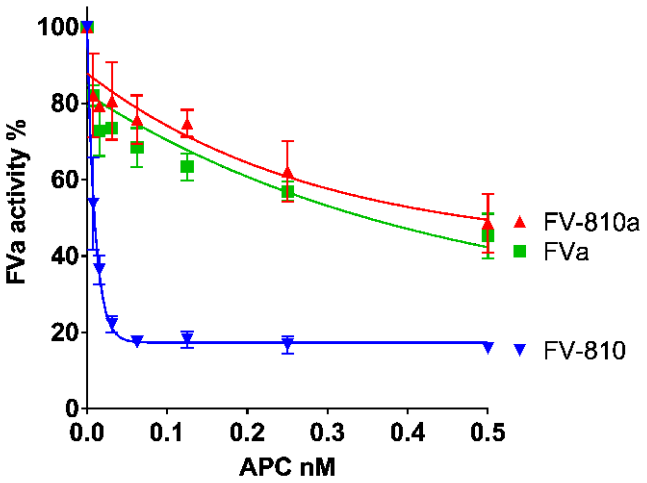


Figure 3.42. APC-dependent inactivation of recombinant FV-810, FVa-810 and FVa.
0.8nM of FV-810 and FVa-810 were inactivated with increasing concentrations of APC (0-0.5nM) in the absence of protein S. The reaction was allowed to proceed for 10 minutes. The remaining FVa activity was measured in the prothrombinase assay.

Interestingly, the inactivation curve of FV-810 by APC alone is comparable to that of WT FV by APC with protein S. In fact, when I repeated the assays in the presence of protein S, the inactivation of FVa was efficiently enhanced by protein S while the enhancement of APC-mediated inactivation of FV-810 was relatively moderate. These results suggest that, in contrast to WT FVa, FV-810 can be fully inactivated by APC alone and does not require protein S for enhanced cleavage at Arg 306 to occur.

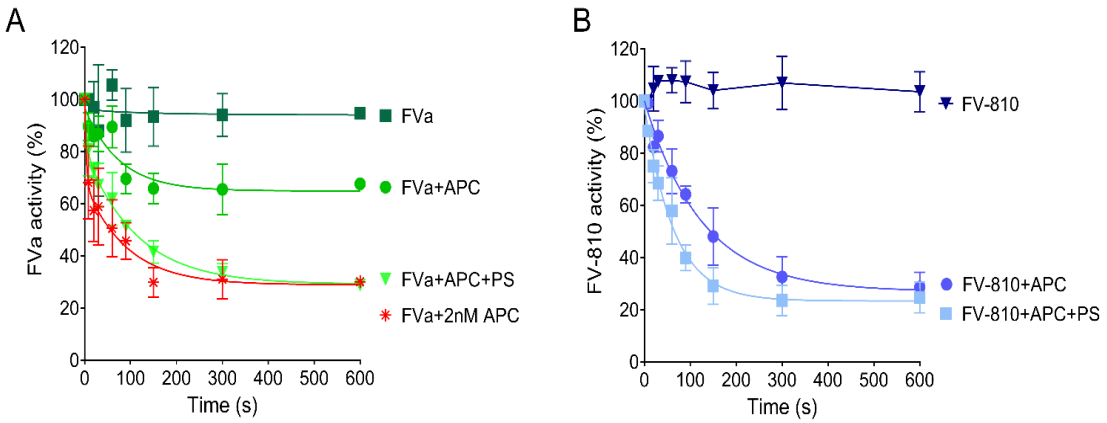


Figure 3.43. APC-dependent inactivation of FVa and FV-810.
Inactivation of FVa **(A)** and FV-810 **(B)** by APC was followed over time in FVa inactivation assays. FVa, and FV-810 (0.8nM) were inactivated in the presence and absence of 100nM protein S with 50 pM APC in the presence of phospholipids. FVa was also inactivated with 2nM APC in the absence of protein S **(A).** At indicated time points, the inactivation reactions were stopped and remaining FVa activity was quantified using prothrombinase assays. The results are plotted as a mean ± SD, (n=3).

3.8.1.3 Evaluation of FV-810 by flow cytometry

In my previous Result sections, I have described a new mechanism for APC-mediated inactivation of FVa, the enhancement of APC binding to phospholipid membranes by protein S and FVa acting together. Following the results described in Figure 3.43, it was important to determine whether FV-810 could be as efficiently, or even more efficiently, incorporated into the APC/protein S/FVa tri-molecular complex on phospholipids. To address this question, binding of APC-FEGRCK to phospholipid-coated magnetic beads was evaluated in the presence and absence of FV-810 or FVa-810 and in the presence and absence of 100nM protein S (Figure 3.44). Similarly to what I have previously shown for FVa, both FV-810 and FVa-810 enhanced APC binding to phospholipids, together with protein S. However, contrary to what could have been expected from the results presented in Figure 3.43, FV-810 did not have any effect on APC binding in the absence of protein S. These results do therefore not explain the increase in rate of FVa-810 inactivation observed in Figure 3.44. It appears that the mechanism of enhanced FV-810 proteolysis is not related to formation of APC/protein S/FVa complex upon phospholipids. This finding, while preliminary, suggests that there is another mechanism for protein S enhancement of FVa degradation.

The main difference between FV-810 and FVa-810 would most likely not be changes in affinity for phospholipids. If this was the case one would expect to see those differences in the

prothrombinase assays. However, there is one main structural difference between FV-810 and FVa, i.e. the change from a one-chain to two-chain molecule. Enhanced FV-810 proteolysis at Arg 306 can potentially be explained by the structure of this truncated variant. It is possible that cleavage sites Arg506 and Arg 306 of FV-810 are more easily accessible for APC.

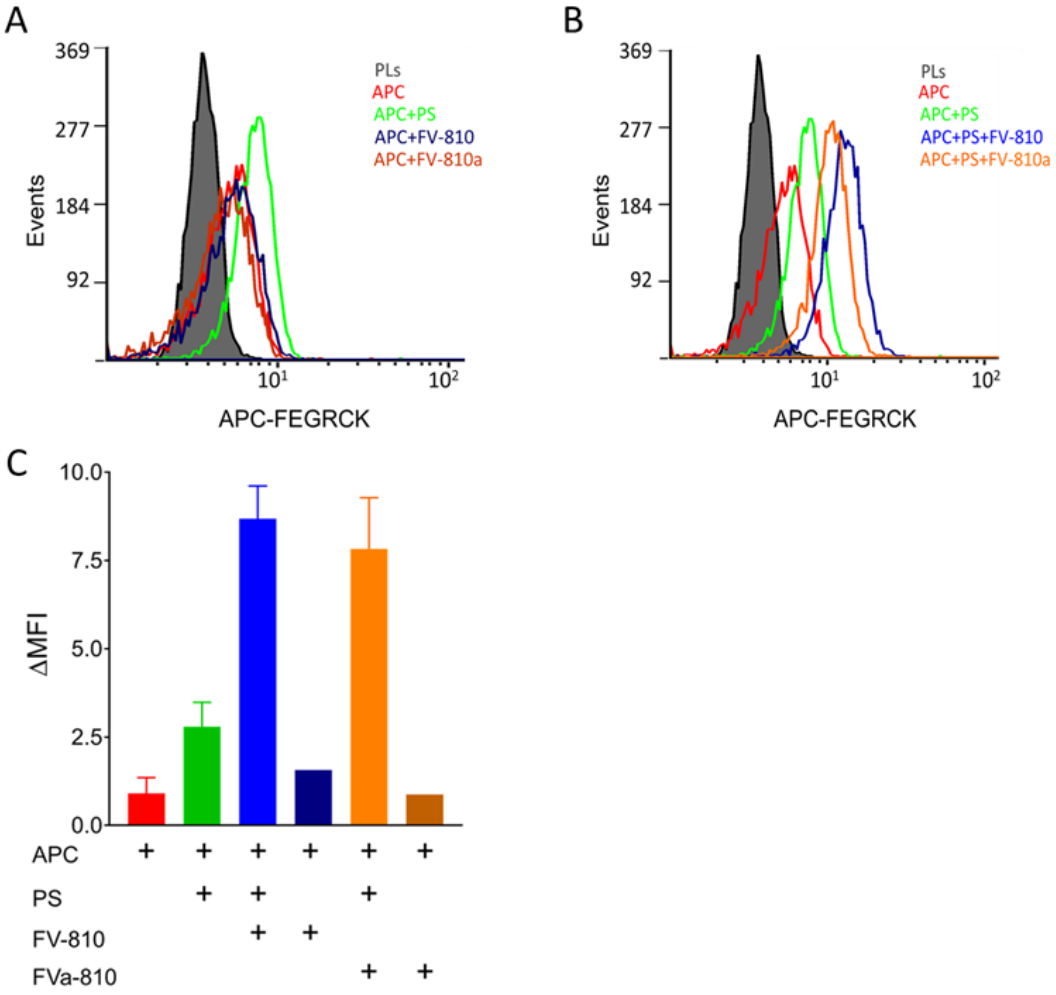


Figure 3.44. FV-810 and FVa-810 enhance the formation of the APC/protein S/FVa tri-molecular complex on phospholipids.

(A and B) Representative histograms and **(C)** quantification plot of binding of 50nM APC-FEGRCK to phospholipid-coated magnetic beads was studied in the presence and absence of 100nM protein S and/or 25nM WT FVa, FVa-810 or FV-810**.** The MFI was measured 1 minute after addition of APC. The results are presented as mean ± SD, (n=1-3)

3.9 Discussion: Further insights from my studies of FV-810

FV-810 was first developed and expressed by Kane *et al*., who showed that this B-domain truncated FV variant does not require activation to express procoagulant activity. The Kane *et al.* reported that FV-810 is expressed as a single chain and exhibits 38% activity of WT FVa. [91,249] This demonstrated that deletion of residues 811-1491 within the FV B-domain leads to exposure of important FVa cofactor function sites. Moreover, the expression levels of FV-810 was 2-5 fold higher than that of FV, due to the truncation of the highly glycosylated B domain.[91] Following on from the research by Kane *et al*., Toso and colleagues expressed and characterised FV-810 in a comprehensive study. Toso *et al*. demonstrated that the non-activated FV-810 can assemble into the prothrombinase complex and enhance thrombin generation as efficiently as WT FVa.[238] These discrepancies in the findings between the two groups were attributed to different expression systems and thereby differences in glysolytation pattern.[238] It has been suggested that FV-810 is easier to express and work with than FV and therefore can be used as its more stable alternative. During my PhD I initially aimed to employ FV-810 in my studies about the inactivation complex formation.

To avoid having problems of glycosylation mentioned above, I used the expression system used by Toso and expressed FV-810 in BHK cells. To verify whether FV-810 was as efficient as a cofactor for FXa in the prothrombinase complex as FVa, I initially ran prothrombinase assays. These assays would also indirectly help me answer whether FV-810 binds to FXa and forms the prothrombinase complex on a phospholipid surface equally well to FVa. My results confirmed those obtained by Toso *et al*. and showed that the FV-810 has normal FXa cofactor function and it is able to assemble into the prothrombinase complex on the phospholipid surface without prior activation (Figure 3.41).

Encouraged by this finding, I aimed to investigate the APC mediated inactivation of the FV-810. To address this question I used FVa inactivation assays. Unexpectedly, APC-catalysed inactivation of FV-810 in the absence of protein S was much more efficient than that of FVa. FV-810, while being truncated, still contains two thrombin cleavage sites and can be activated by thrombin into the same FVa molecule as FV. Interestingly, the activated form of FV-810 produced comparable results to FVa, showing that the increased sensitivity of FV-810 to APC-mediated proteolysis arise from the nature of this variant rather than anything innate in the

FV-810 preparation. While studying the efficiency of inactivation of FV-810 in more detail I found that it was so efficient that I had to reduce the concentration of APC in the assay from 0.25nM to 50pM to be able to study it further. Time course experiments of FV-810 inactivation in the presence and absence of protein S revealed that in contrast to FVa, FV-810 can be efficiently inactivated in the absence of protein S (Figure 3.43). In fact, in order to obtain the same rate of inactivation of FVa in the absence of protein S, 40 times more of APC had to be used.

It has been demonstrated, that APC mediated inactivation of FVa strongly depends on the presence and composition of phospholipids.[47,62] Therefore, improved inactivation of FV-810 could arise from enhanced interaction of the variant with phospholipids membranes. Many groups have demonstrated that the ability of FVa to enhance thrombin generation depends on its interaction with phospholipids.[56,250,251] It has been shown that the binding of FVa to phospholipids is facilitated by spikes of hydrophobic amino acids on the C1 and C2-domains, and mutations in that regions affect formation of prothrombinase complex and thrombin generation.[57,248] Together, these finding suggest that any increase or decrease in affinity for phospholipids of FVa would be directly reflected in its ability to enhance thrombin generation. However, FV-810 enhanced thrombin generation in the same manner as FVa, suggesting similar phospholipid binding.

If the affinity of FV-810 towards phospholipids vehicles is normal, its increased sensitivity to APC could be a result of enhanced interaction between those two proteins. I therefore investigated the ability of FV-810 as well as its activated form (FVa-810) to enhance APC-FEGRCK binding to phospholipid in the presence and absence of protein S in my flow cytometry assay. Both FV-810 and FVa-810 increased APC binding to phospholipids in the presence of protein S but not in its absence. It was somewhat surprising that FV-810 required the presence of protein S to enhance APC-phospholipid interaction since this did not reflect the findings from my functional assays. Moreover, there was no significant difference in the level of enhancement induced by the non-activated and activated forms of FV-810. As mentioned earlier, activated FV-810 is indistinguishable from FVa. Therefore, this result suggests that FV-810 derivative assembles into the inactivation complex with APC and protein S in the same manner as FVa. Consequently, this finding suggests that the increased susceptibility of FV-810 to inactivation by APC is not associated with the formation of the

APC/protein S/FVa complex on phospholipids surface. There must be another mechanism responsible for this phenomenon.

Unfortunately my flow cytometry method only allows me to study interactions which have a direct effect on APC binding to phospholipids. Different techniques will have to be employed to study proteolysis of FV-810 to follow up my observation. One important objective will be to determine which cleavage of FV-810, Arg306 or Arg506, has increased sensitivity to APC. I have already produced a FV-810 variant which only can be cleaved at Arg306. By running time course experiments in FVa inactivation assays using this variant, compared to the same variant of FVa, I can determine the kinetic constants of proteolysis at the different cleavage sites. This can be done both in the presence and in absence of protein S to increase our understanding of the molecular mechanisms behind the increased sensitivity towards APC. I am also planning to characterise the affinity of FV-810 towards phospholipids. Initially, I will adopt my pull down method which I successfully used to study the FV Nara-phospholipids interaction. Depending on the outcome, I will study binding of FV-810 to phospholipids using SPR.

The results I have obtained for FV-810 are preliminary and the variant has to be further assessed, however it is tempting to speculate about possible mechanisms of FV-810 inactivation. It is also important to note that while FV-810 is a somewhat artificial FV variant, my findings can be used to further understand the mechanisms underlying the protein S enhancement of APC in the inactivation FVa. A previous study by Sun *et al*.,[252] suggested that more than one mechanism is involved in the APC cofactor function by protein S. They generated a variant of APC (called QGNSEDY-APC) with strongly increased affinity to phospholipids and anticoagulant activity in comparison to WT APC. Interestingly the activity of the QGNSEDY-APC was further enhanced in the presence of protein S. This indicates that protein S enhancement not only arise from increased membrane interaction but also involves other mechanisms.

FVa cleavage sites must be exposed to the APC active site during the FVa inactivation. It has been suggested that binding of FVa to APC preceding proteolytic cleavages is sufficient to inhibit thrombin generation.[253] Moreover, much lower affinity of APC to the FV R506Q was observed, suggesting that the APC-FVa interaction is strongly dependend on the presence of

the Arg506 cleavage site. On the other hand, it has been shown that protein S predominantly stimulates cleavage at Arg306 while cleavage at Arg506 is only weakly stimulated.[62,153,254] It has been proposed that the selective enhancement by protein S for the Arg306 cleavage is caused by relocation of the APC cleavage site closer to the phospholipid membrane, thereby making the cleavage site more accessible,[254] or through improvement of the contact between FVa and APC.[214] It is, therefore, possible that with FV-810 one or both APC cleavages sites are in better orientation for binding to the catalytic site of APC. That FV-810 does not require protein S enhancement for efficient APC inactivation could suggest that the Arg306 cleavage site in FV-810 is localised in an optimal orientation for APC binding and cleavage also in the absence of protein S. Experiments using my FV R506Q variants will enable me to answer this question in future experiments.

CONCLUSIONS AND FUTURE PERSPECTIVES

The aim of my PhD project was to evaluate the mechanisms underlying APC-mediated inactivation of FVa. FVa is an important cofactor of FXa which enhances thrombin generation by several orders of magnitude. Since blood coagulation depends critically on the amount of generated thrombin, APC-catalysed FVa proteolysis is highly effective at shuting down the coagulation by dramatically reducing thrombin generation. FVa was initially known soley for its procoagulant function. However, as a consequence of the discovery of APC resistance a new anticoagulant role of FV was revealed. FV was found to act in synergy with protein S as an APC cofactor in FVIIIa degradation.[49] Since then, more anticoagulant functions of FV have been discovered. Recently our research group showed that FV, together with protein S, acts as a synergistic cofactor for TFPI during FXa inhibition.[39] During my PhD project I have identified a further novel anticoagulant role of FVa. My results show that FVa promotes its own proteolysis by enhancing binding of APC to negatively charged phospholipid membranes, together with protein S (See sections 3.3.1-3.3.2). my results also strongly suggest the formation of an inactivation complex between APC, protein S and FVa. In ongoing work, I am trying to provide additional evidence for the assembly of this complex using a cross linking method.

What remains to be established is how these three proteins interact with each other within this complex? Many findings indicate that there is a direct interaction between FV/FVa and protein S. As mentioned earlier, both proteins act together as synergistic APC and TFPI cofactors.[39,49] It has also been suggested that protein S can inhibit prothrombinase complex by interaction with FVa.[221] My results indicate that cross-talk between FVa, protein S, APC and phospholipids is required for efficient FVa inactivation.

Real-time visualization of thrombus formation in vivo shows that the first signs of a thrombus appears just few seconds after the injury and that thrombus is formed within few minutes after that time[255]. The role of APC is to restrict thrombus formation to the site of injury, therefore its action has to rapidly follow the generation of the thrombus. In line with this, I observed the highest enhancement of APC-phospholipid interaction by protein S and FVa immediately after addition of APC, which suggest rapid formation of the inactivation complex on the phospholipid surfaces. It will be a real challenge to develop tools that will be required

to visualise trimolecular complex formation in an in vivo setting, although the recent results from Ivaciu and colleagues, visualising the prothrombinase comples, suggest that it is possible.[256]

A number of studies have suggested that FVa present on the platelet surface is not readily susceptible to APC cleavage.[257-259] It has even been suggested that the presence of platelets protects FVa from APC-mediated inactivation.[260] In agreement with this findings, Oliver *et al*., demonstrated that FVa inactivation was more effective on the endothelium than on platelets.[24] Effective activation of protein C requires the presence of TM and is further enhanced by the presence of EPCR. Since both TM and EPCR are located on the endothelium, this is also the place where the physiological activation of protein C takes place. The endothelium surrounding the vascular injury therefore plays an important role in regulation of the blood coagulation. It seems plausible that it also is the primary biological surface for the formation of the inactivation complex between APC, protein S and FVa. While it is generally accepted that activated platelets are the predominant site for the assembly of the prothrombinase complex, more recent reports demonstrate an important role of the endothelium in supporting thrombin formation.[256,261] A study by Ivanciu *et al.*[256] investigated this using fluorescently labelled platelets, FVa and FXa together with a laser induced injury model in mice. They showed that FVa and FXa were not only co-localised with the platelet core, but were also distributed on the endothelium further from the site of injury. Interestingly, both proteins were also found upstream from the injured site, where there were no activated platelets. If the prothrombinase complex assembles on the endothelium outside of the main platelet core and thrombus, an important role of APC might be to prevent thrombin formation (and further blood coagulation) from spreading to more distal places on the endothelium. Protein S can inhibit the prothrombinase complex via direct interactions with FVa and FXa and reverses the protecting effect of FXa on FVa Arg506 cleavage.[221] It has also been suggested that FVa binding sites for protein S overlap with FXa binding sites and are located close to Arg506.[212] Protein S could therefore act as a switch for FVa, changing its functions from procoagulant to anticoagulant. Additional studies, using visualisation of APC, protein S and FVa under the flow, ideally *in vivo*, might provide further insights on physiological and pathological formation of the inactivation complex and the primary biological surface for its localisation.

APC is greatly enhanced by protein S and the function of protein S deserves particular attention as APC is an ineffective anticoagulant in its absence. Protein S also serves as an essential cofactor for two out of three endogenous anticoagulants (TFPI and APC). It has commonly been assumed that only free protein S exerts full anticoagulant activities. Maurissen *et al*., demonstrated that C4BP-bound protein S selectively inhibits APC-mediated cleavage of FVa at Arg506 by 3-4 fold and exerts approximately 2-fold reduced cofactor function during cleavage of Arg306.[216] In agreement with this observation, my results show that C4BP-bound protein S has strongly reduced ability to assemble into the inactivation complex together with APC and FVa. Interestingly, C4BP-bound protein S has previously been shown to stimulate APC-catalysed FVIIIa degradation, however it does not function in synergy with FV. Protein S is clearly an anticoagulant protein of great importance, yet no longer attracts widespread attention from researchers.

Protein S variants with limited APC cofactor function, namely protein S E36A, D95A and CHIII, did not incorporate into the inactivation complex. This result can explain their reduced ability to enhance APC function. However, it is still not clear whether the substituted protein S residues are important for the interaction with APC or FVa. I have tried to address this question evaluating direct interaction between FVa and protein S using plate binding assay which I optimised for another project.[241] However, the affinity I determined for the FVa-protein S interaction was very low (μM). There are several potential explanations for this. The binding of protein S to the immobilised FVa phospholipids could be restricted spatially and limited due to lack of appropriate interaction surface. It is also likely that the presence of phospholipids is needed for a more physiological interaction to occur. Further studies of the protein S-FVa interaction should be carried out in the presence of phospholipids and preferable be performed in solution. This is something I hope to tackle in the future. I aim to fluorescently label protein S and study its binding to phospholipids in the presence and absence of FVa using the flow cytometry approach I have developed in this thesis.

The results obtained for protein S CHIII were contradictory, making it difficult for me to draw any firm conclusions. This protein S variant showed completely abolished APC cofactor function in CAT assay and failed to form the inactivation complex in flow cytometry assay. However, its ability to enhance APC function in the inactivation of FVa was only slightly reduced and the inactivation through FVa cleavage at Arg306 was essentially normal.

Although further studies of this variant are needed to elucidate these differences, a recent study by Kamikubo *et al*., can potentially shed some light on this discrepancy.[262] They found that FVIII can be activated not only by thrombin, but also by the TF-FVIIa-FXa complex. This suggests that activation of the intrinsic pathway can be achieved by components of the extrinsic pathway, bypassing the activation of FXII. Therefore, despite the use of CTI in the CAT assay to prevent FXIIa activation, the contribution of FVIIIa to thrombin generation could be much higher than so far anticipated. That protein S CHIII previously has been found to enhance FVa degradation, but not inactivation of FVIIIa, can partially explain differences in results from my CAT and FVa inactivation assays. If this is the case, this would suggest that the importance of protein S as a cofactor for APC-mediated inactivation of FVIIIa is much higher than previously anticipated. I therefore plan to reinvestigate formation of the FVIIIa inactivation complex in the presence and absence of both cofactors, namely protein S and FV, even though I have so far failed to demonstrate it with the methods I have used so far. The finding that protein S CHIII is able to stimulate one site of APC-mediated proteolysis but not another (FVa Arg506 vs Arg306) is in itself interesting and deserves further investigation. This seems to suggest that the protein S SHBG-like domain might be important for targeting the first APC cleavage site at Arg506.

Proteolysis at Arg506 is required for FV to function as an APC cofactor during FVIIIa inactivation. It is possible that a direct interaction between FV/FVa, protein S, APC and the proteolysis at Arg506 has to occur before protein S exerts its full anticoagulant function. It would be interesting to study whether a FV/FVa R506Q variant can assemble into the inactivation complex together with APC and protein S.

Using the variant of FV, FV W1920R (FV Nara), I demonstrated that Trp1920 is needed for formation of the tri-molecular inactivation complex. Potentially, this could explain the predisposition to DVT in carriers. I showed reduced affinity of FV Nara to phospholipid vehicles, indirectly by prothrombinase assay and directly using a pull down method. These results show that the assembly of the inactivation complex is reliant on the high affinity of FVa for phospholipid surfaces. However, in order to quantify the affinity of the FV Nara-phospholipid interaction, more detailed studies are required. I plan to establish the K_d of the FV Nara-phospholipid association using SPR.

It is now generally accepted that protein S enhancement of APC action is complex and most likely involves more than one mechanism. My results obtained for FV-810 variant supports this view. In contrast to FVa, efficient APC-mediated proteolysis of this variant can occur also in the absence of protein S. However, the preliminary results from my flow cytometry assay do not reflect this finding. FV-810 does not form a complex with APC on the phospholipid membrane in the absence of protein S. Therefore, an alternative mechanism has to be involved, causing the increased susceptibility of this variant for APC-mediated proteolysis. The finding that protein S is not required for efficient FV-810 proteolysis might suggest that Arg 306 cleavage site of this FV derivative is in optimal orientation for the active site of APC. If so, the suggested re-positioning of APC active site by protein S would not be needed.[213] FV-810 may therefore be a useful tool to generate further insights into the enhancement of APC by protein S. As mentioned in Section 3.7.2, I have already generated WT FV and FV-810 which can be cleaved only at Arg306. Combined with the results from the time course experiments presented in Figure 3.43, experiments using these variants should help me determine whether the increase in APC-mediated proteolysis is specific for one of the two cleavage sites.

In my thesis I have demonstrated that FVa, protein S and APC interact together on the phospholipid surface. Until now, much focus has been on the APC-protein S interaction. However, the protein S-FVa interaction may be equally important. My results also points towards conformational changes occurring during the complex formation, which can explain the differences in preferential APC cleavage sites in FVa dependent on whether protein S is present or not. However, further studies are required to elucidate the mechanism that might be involved.

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PUBLICATIONS ARISING FROM THIS WORK

Magdalena Gierula, Isabelle I. Salles-Crawley, Salvatore Santamaria, Adrienn Teraz-Orosz, James T.B. Crawley, David A. Lane and Josefin Ahnström.
Activated factor V in synergy with protein S enhances APC association to phospholipids. Manuscript in preparation.

Salvatore Santamaria, Natalia Reglińska-Matveyev, **Magdalena Gierula**, Rodney M. Camire, James T. B. Crawley, David A. Lane and Josefin Ahnström
Factor V has an anticoagulant cofactor activity that targets the early phase of coagulation.
J Biol Chem. 2017 Jun 2; 292(22): 9335–9344.

Sofia Somajo, Josefin Ahnström, Juan Fernandez-Recio, **Magdalena Gierula**, Bruno O. Villoutreix and Björn Dahlbäck.
Amino acid residues in the laminin G domains of protein S involved in tissue factor pathway inhibitor interaction.
Thromb Haemost. 2015 May; 113(5):976-87.

ABSTRACTS LEADING TO POSTER PRESENTATION

Magdalena Gierula, Isabelle I. Salles-Crawley, James T.B. Crawley, David A. Lane and Josefin Ahnström
Factor Va in synergy with protein S enhances activated protein C binding to phospholipids.
ISTH 2015 congress 20-25th June 2015, Toronto, Canada.

Magdalena Gierula, Isabelle I. Salles-Crawley, James T.B. Crawley, David A. Lane and Josefin Ahnström
Synergistic enhancement of APC association to phospholipids by protein S and activated factor V.
ECTH meeting 28-30th September 2016, Hague, Netherlands. (oral poster presentation)

ABSTRACTS LEADING TO ORAL PRESENTATION

Magdalena Gierula, Isabelle I. Salles-Crawley, Santamaria S, James T.B. Crawley, David A. Lane and Josefin Ahnström.
Activated factor V in synergy with protein S enhances APC association to phospholipids.
Joint BSHT / AiP / UK Platelet Group meeting 9-11th November 2016, Leeds, UK.