CHARACTERISATION OF THE MOLECULAR BASIS OF PROTEIN S ANTICOAGULANT FUNCTION

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by

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ABSTRACT

Protein S has an established role in the protein C anticoagulant pathway as a cofactor for anticoagulant protein C (APC) and has also recently been shown to serve as a cofactor enhancing the anticoagulant activity of tissue factor pathway inhibitor (TFPI). Despite its physiological role and clinical importance, the molecular bases of its functions are not fully understood. The aim of my thesis was to clarify the molecular mechanisms involved in the protein S interaction with APC and TFPI. More than 30 point or composite protein S variants were constructed and analysed during this project. These variants spanned the Gla, thrombin sensitive region (TSR), epidermal growth-factor1 (EGF1) and EGF2 domains of protein S. Protein S was expressed in mammalian cells and was purified by chromatography, as required. Protein S was characterised by size, cleavage, multimerisation, γ-carboxylation of the Gla domain, binding to phospholipids and to domain specific monoclonal antibodies. Variants were evaluated for their APC and TFPI cofactor activities both by calibrated automated thrombography and in purified FVa inactivation or FXa inhibition assays, respectively. The protein S variant, protein S D95A, with substitution in EGF1 was found to be largely devoid of functional APC cofactor activity and I believe that this residue plays an important role in protein S anticoagulant function. It was γ-carboxylated and bound phospholipids and domain specific monoclonal antibodies with an apparent dissociation constant similar to that of wild type protein S. Importantly, protein S D95A enhanced the anticoagulant activity of TFPI, suggesting that distinct residues in protein S mediate its APC and TFPI cofactor activity. Two composite mutants in the protein S EGF1 domain had partially reduced TFPI cofactor activity in plasma. However, none of the more than 30 variants spanning the Gla-TSR-EGF1-EGF2 domain of protein S completely disrupted the protein S cofactor activity towards TFPI. Collectively, these results shed light on the molecular basis of protein S cofactor function and suggest distinct residues in protein S are involved in the binding to APC and to TFPI.

DECLARATION OF ORIGINALITY

I, Helena Maria Andersson, 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.

Helena Maria Andersson

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ABBREVIATIONS

APC	activated protein C
AT	antithromin
AP	activation peptide
Bp	base pair
BSA	bovine serum albumin
C4BP	C4b-binding protein
CAT	calibrated automated thrombography
cDNA	complimentary deoxyribonucleic acid
CAT	calibrated automated thrombography
CIAP	calf intestinal alkaline phosphatase
CMV	cytomegalovirus
CTI	corn trypsin inhibitor
Ctrl	control
CV	coefficient of variation
DEAE	diethylaminoethyl cellulose anion exchange
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
DOPC	1,2-Dioleoyl-*sn*-glycero-3-phosphocholine
DOPE	1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine
DOPS	1,2-Dioleoyl-*sn*-glycero-3-phosphoserine
ECM	subendothelial extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
EPCR	endothelial protein C receptor
ER	endoplasmic reticulum
ETP	endogenous thrombin potential
Eq	equation
FCS	foetal calf serum
FPLC	fast protein liquid chromatography
FRET	fluorescence resonance energy transfer
GAG	glycosaminoglycans
Gla	γ-carboxylated glutamic acid
GPI	glycosyl phosphatidylinositol

HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HEK	human embryonic kidney fibroblast
HN	25 mM HEPES pH 7.7, 150 mM NaCl
$HNBSACa^{2+}$	HN, 5 mM $CaCl_2$, 5 mg/ml BSA
HRP	horseradish peroxidise
Hyn	β-hydroxyasparagine
K	Kunitz
Kb	kilobase
kDa	kilodaltons
K_d	dissociation constant
$K_{d(app)}$	apparent dissociation constant
K_i	initial inhibition constant
K_i*	final inhibition constant
K_m	Michaelis-Menten constant
Kobs	apparent rate constant for transition from V_0 to V_s
LB	Luria-Bertani medium
LDL	low-density lipoproteins
LG	laminin G
mA	milli absorbance
mAU	milli absorbance unit
MEM	minimum essential media
mRNA	messanger ribonucleic acid
MW	molecular weight
OPD	o-phenylenediamine dihydrochloride
PAI	plasminogen activator inhibitor
PBS	phosphate buffered saline
PC	L-α-phosphatidylcholine
PCR	polymerase chain reaction
PE	L-α-phosphatidylethanolamine
PEI	linear polyethylenimine
PS	L-α-phosphatidylserine
QFF	Q sepharose fast flow
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SHBG	sex hormone binding globulin
SPR	surface plasmon resonance
SUV	small unilamellar vesicles

TAFI	thrombin activatable fibrinolysis inhibitor
TBE	tris borate EDTA
TF	tissue factor
TFF	tangential flow filtration
TFPI	tissue factor pathway inhibitor
TM	thrombomodulin
t-PA	tissue-type plasminogen activator
TSR	thrombin sensitive region
u-PA	urinary plasminogen activator
VWF	von Willebrand factor
V_0	initial velocity of S-2765 conversion
V_s	final velocity of S-2765 conversion
WT	wild type
Å	Ångström

Comon abbreviations not spelled out in the text

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

Power prefix abbreviations

m	milli
µ	micro
n	nano
p	pico
f	femto

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

The cardiovascular system has a vital importance in distributing nutrients and oxygen, while also, removing toxic metabolites. It maintains blood homeostasis by regulating variables such as body temperature, pH, pressure and the concentration of transported molecules.

The integrity of the cardiovascular system is preserved by a highly regulated process called haemostasis. Under normal circumstances, circulating blood is maintained fluid by the physiological anticoagulants acting at the surface of endothelial cells and by the lack of procoagulant stimuli. In case of injury of the blood vessel, there are three major phases that serve to prevent blood loss and enable vessel repair which are all regulated both spatially and temporally, primary haemostasis (or platelet plug formation), secondary haemostasis (or coagulation) and fibrinolysis. Balanced haemostasis results in rapid occlusion of the damaged tissue avoiding bleeding, infiltration of pathogens in the blood and allowing enough time to the injured vasculature to repair. The main components regulating haemostasis are blood vessels, platelets and coagulation factors. These components cooperate, through multiple positive and negative feedback mechanisms, to maintain circulating blood fluid and to stop bleeding in case of injury.

1.1.1 Primary haemostasis

Primary haemostasis is the initial response to vascular injury. Following damage of the endothelial cell layer the subendothelial extracellular matrix (ECM) is exposed to the bloodstream. This results in the lack of the anticoagulant endothelial surface and exposure of the adhesive macromolecules of the ECM such as collagen, fibronectin, laminin, thrombospondin and vitronectin. While platelets are able to interact directly with the ECM, this interaction is greatly enhanced by von Willebrand Factor (VWF).[1] VWF is a large multimeric adhesive glycoprotein found in plasma and is secreted by endothelial cells and activated platelets.[2-4] When collagen is exposed to the bloodstream VWF binds to it, and

shear stress induces unravelling of the globular VWF, making its A1 domain accessible to platelets.[5] Circulating platelets bind loosely to VWF through the receptor complex GPIb-V-IX, causing the platelets to roll along the VWF multimer.[6] This rolling enables platelets to come into contact with exposed collagen and to bind to it through their GPVI receptor. Binding of collagen induces intracellular signalling that augments the affinity of cell surface receptors termed integrins for their ligands, causing adherence, aggregation and activation of platelets.[7] Activated platelets release additional VWF resulting in a positive feedback loop that leads ultimately to the formation of a primary loose platelet plug.[8] During activation, the platelet membrane composition is altered exposing negatively charged phospholipids, such as phosphatidylserine, that are confined to the inner phospholipid layer in the inactivated cell.[9] This serves as a surface for the activation and assembly of coagulation factors.

1.1.2 Secondary haemostasis

Secondary haemostasis involves coagulation and requires activation of multiple zymogens to active serine proteases, as well as activation of cofactors. The coagulation cascade can be initiated through two different pathways, the contact activation pathway and the tissue factor (TF) pathway. [10] While the physiological relevance of the contact pathway is still debated, the TF pathway is now recognised as the principal physiological initiator of coagulation. The following paragraphs will only consider the TF pathway. Once coagulation is triggered there is a first stage of initiation of coagulation producing only traces of thrombin, which through positive feedback activation leads to propagation of coagulation resulting in more thrombin and the formation of an insoluble fibrin clot.[10]

1.1.2.1 Initiation of coagulation

The major initiator of coagulation is TF, a transmembrane protein expressed on the surface of cells at extravascular sites. Following vessel damage, TF is exposed to the blood.[10] While clotting factors with an enzymatic activity usually circulate as inactive zymogens, a small amount (~1%) of factor VII (FVII) circulates in its partially active form, FVIIa.[11] Both FVII and FVIIa are able to bind

to TF, but only the TF/FVIIa complex activates FIX and FX (Figure 1.1). In the absence of its cofactor, FVa, FXa converts only a small amount of prothrombin into thrombin.

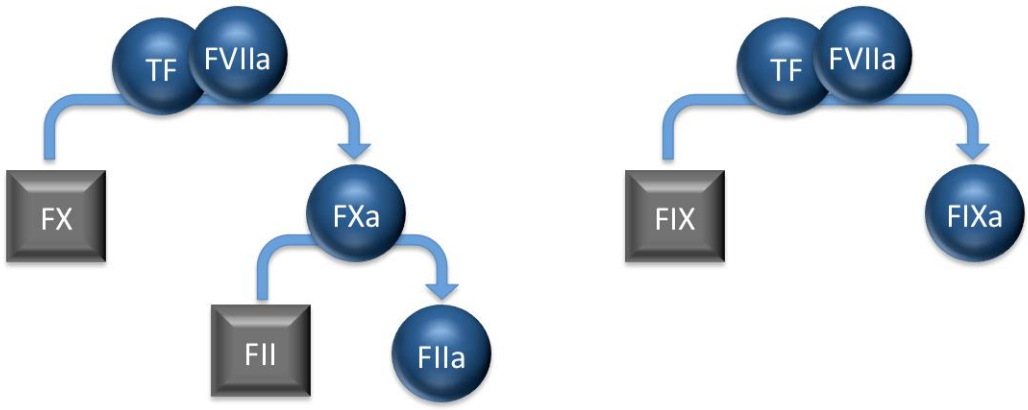

Figure 1.1 Initiation of the coagulation cascade.
Circulating FVIIa binds to TF exposed to the bloodstream. The TF/FVIIa complex activates FX and FIX. FXa generates trace amounts of thrombin (FIIa).

1.1.2.2 Feedback activation

The small amount of thrombin that is generated during the initiation phase produces a positive feedback reaction by activating FVIII and FV[12], the cofactors of FIXa and FXa, respectively (Figure 1.2). The FIXa/FVIIIa complex assembled upon phospholipids is also termed the tenase complex as it activates FX. This leads to an amplification of FXa production bypassing the requirement for TF/FVIIa activity. The FXa/FVa complex on phospholipids, or the prothrombinase complex, converts prothrombin into thrombin up to 278000 times more efficiently than FXa alone.[13-15] Once the damaged tissue and subendothelial cells start to be covered by the platelet plug, it has been proposed that secondary haemostasis is allowed to continue by the adherence of alternatively spliced soluble TF or circulating microparticles containing TF to the surface of activated platelets.[16-18] This results in the propagation of both primary and secondary haemostasis. Assembly of the functional tenase and prothrombinase complex upon negatively charged phospholipids (such as those exposed on activated platelets) depends on domain-specific phospholipid interaction sites such as those provided by the Gla domain, of prothrombin, FVII, FIX, and FX or the C domains of FV and

FVIII.[19-22] In addition to spatially confining these complexes, it also results in accelerated formation of the proteases FXa and thrombin.

Once feedback activation of coagulation has taken place, sufficient thrombin is generated for efficiently conversion of fibrinogen into fibrin, (Figure 1.2) which is cross-linked by FXIIIa to form an insoluble fibrin clot.[23] The fibrin clot prevents the loss of blood, the entrance of pathogens into the blood stream and allows the damaged endothelium and subendothelial layers to repair.

Thrombin plays a central role in regulating haemostasis, acting both as a procoagulant enzyme by generating a feedback activation of itself, and as an anticoagulant enzyme by proteolytically activating protein C.[24]

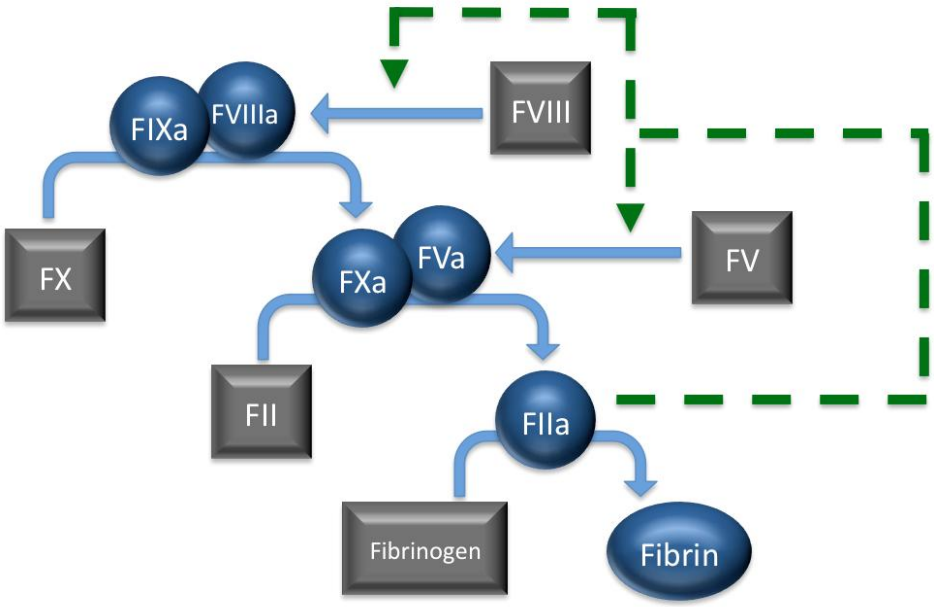

Figure 1.2 Propagation of the coagulation cascade.
Thrombin (FIIa) generated during the initiation stage excerts a feedback mechanism activating FVa and FVIIIa, the cofactors of FXa and FIXa respectively. This leads to a massive generation of thrombin which converts fibrinogen into fibrin.

1.1.3 Regulation of coagulation by anticoagulant pathways

The procoagulant pathway is tightly regulated by three major anticoagulant pathways, the tissue factor pathway inhibitor (TFPI) pathway, the protein C pathway and the antithrombin (AT) pathway (Figure 1.3).[25] These three pathways are each critical for the regulation of the haemostatic plug. Each pathway targets quite different components of the coagulation cascade regulating haemostasis

both temporally and spatially.

TFPI is a Kunitz type serine protease inhibitor. TFPI inhibits the extrinsic coagulation pathway by inhibiting TF/FVIIa and FXa on the subendothelial cells and on the growing platelet plug. TFPI targets the initiation of coagulation, as once enough thrombin is formed the necessity of TF/FVIIa for generating FXa is by-passed by the FIXa/FVIIIa complex.[26]

The activation of protein C by thrombin complexed to thrombomodulin (TM) on surrounding intact endothelial cells enables activated protein C (APC) to act at the edge of the plug formation inhibiting expansion of fibrin deposition, by inactivating FVa and FVIIIa. As activation of protein C requires the formation of thrombin, APC is probably only generated during the propagation phase.[27] The anticoagulant activity of APC is greatly enhanced by a cofactor, protein S, the central focus of this thesis.

AT is a serine protease inhibitor circulating at a plasma concentration of ~2-5 μM.[28] It inhibits multiple serine proteases in the TF pathway (thrombin, FXa, FIXa and FVIIa) by forming a stable 1:1 stoichiometric complex, trapping them in a form in which their active site is not accessible to their usual substrate.[29] The inhibitory activity of AT is greatly enhanced by its binding to heparan sulphate on intact endothelial cells surrounding the tissue damage. Binding of AT to heparan sulphates also results in a conformational change of the former, increasing greatly the rate of inactivation of FXa, FIXa and FVIIa.[30,31] AT efficiently inhibits all of the free serine proteases that escape the immediate proximity of plug formation, assuring the latter remains localised throughout the haemostatic response.[32]

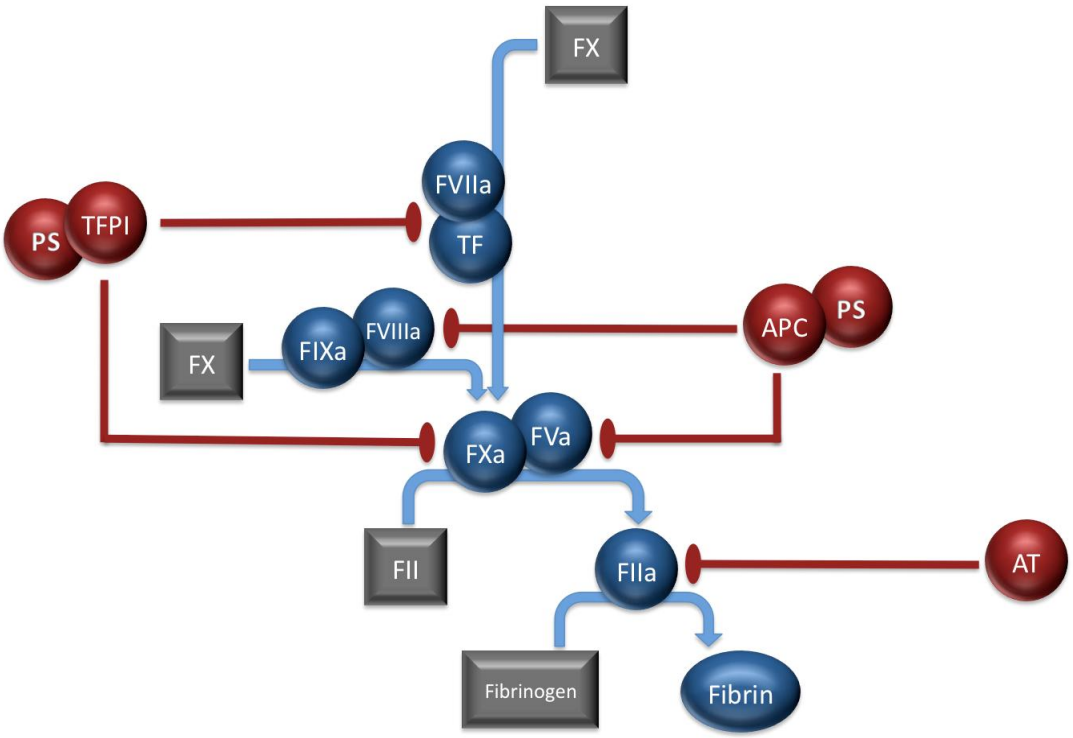

Figure 1.3 Regulation of the coagulation cascade by the anticoagulant pathways.
The coagulation cascade is tightly regulated by the three main anticoagulant pathways, TFPI, APC and AT. Protein S (PS) acts as a cofactor both for TFPI and APC. Protein S/TFPI inhibits TF/FVIIa and FXa. The protein S/APC complex inactivates FVa and FVIIIa. AT inhibits multiple serine proteases. For simplicity in this diagram only inhibition of thrombin (FIIa) is shown.

1.1.4 Fibrinolysis

Fibrinolysis, the final phase of haemostasis, is the process by which the fibrin clot is remodelled and dissolved once the damaged blood vessel has been repaired. Tissue-type plasminogen activator (t-PA) and urinary plasminogen activator (u-PA) are the two major activators of plasminogen.[33,34] Plasminogen is activated to plasmin, the enzyme which degrades fibrin. Under normal circumstances where there is no fibrin, t-PA and plasminogen circulate in the blood. As soon as fibrin is formed, it acts as a template bringing t-PA and plasminogen into proximity, enhancing plasminogen activation ~1000-fold. These proteases are counterbalanced by fibrinolysis inhibitors such as thrombin activatable fibrinolysis inhibitor (TAFI), plasminogen activator inhibitor (PAI)-1 and PAI-2.[33,34]

1.1.5 The haemostatic balance

Haemostasis represents a fine balance with respect to procoagulant and anticoagulant factors. Coagulation disorders result either in haemorrhage or in thrombosis. Inherited deficiencies in procoagulant factors result in bleeding disorders, the most commonly known are von Willebrand Disease (VWF deficiency), haemophilia A (FVIII deficiency) and haemophilia B (FIX deficiency).[35,36] In contrast, inherited deficiencies in anticoagulant factors result in an increased risk of thrombosis, the most commonly known are AT, protein C and protein S deficiencies as well as FV Leiden (FV resistance to inactivation by activated protein C).[37] Because my thesis is primarily concerned with anticoagulant pathways, I will describe these in more detail in what follows.

1.2 TFPI

1.2.1 TFPI gene

TFPI is expressed mainly by endothelial cells, but also by vascular smooth muscle cells, megakaryocytes, monocytes, mesangial cells, fibroblasts and cardiomyocytes.[38-40] The human *TFPI* gene is localized on chromosome 2 where it spans approximately 70 kilobase (kb) and consists of 9 exons.[41] The TFPI transcript is alternatively spliced giving origin to two isoforms, TFPI-α and TFPI-β. The physiological role of TFPI-β has not yet been determined and most results have been derived from *in vitro* experiments. It is, however, known that TFPI-β contains an alternatively spliced C-terminal domain and associates to endothelial cells through glycosyl phosphatidylinositol (GPI)-anchors, possibly within caveolae.[42] Interestingly, it has been shown to still exert an anticoagulant function by inhibiting FXa and TF/FVIIa.[43] TFPI-α is the most abundant transcript and the one on which my project will focus.[24] TFPI-α is translated into an unprocessed precursor of 304 amino acids with a predicted molecular weight (MW) of 35 kilodaltons (kDa). Cleavage of the 28 amino acid signal peptide and post-translational modifications, result in mature full-length TFPI protein of 276 amino acids and a MW of 43 kDa. If not indicated otherwise in this thesis the term TFPI will hereafter refer to the α-isoform.

1.2.2 TFPI structure

The full-length TFPI protein derived from the TFPI- α transcript has a negatively charged N-terminal region (amino acids 1-12), 3 Kunitz domains (K1, amino acids 13-78; K2, amino acids 92-150; K3, amino acids 182-241) separated by 2 linker regions and a positively charged C-terminal region (amino acids 242-273) (Figure 1.4).[44] It is N-linked glycosylated at Asn117 and Asn167 and O-linked glycosylated at Ser174 and Thr175. Truncated forms of TFPI derived from the TFPI- α transcript are also present in plasma. Truncation usually results in TFPI variants lacking their C-terminal tail and often also part of their K3 domain.[45] The physiological origin of these truncated forms is not known but it has been shown *in vitro* that TFPI can be cleaved by proteases that TFPI may encounter *in vivo,* such as thrombin, plasmin, neutrophil elastase and some matrix metalloproteinases.[46-49]

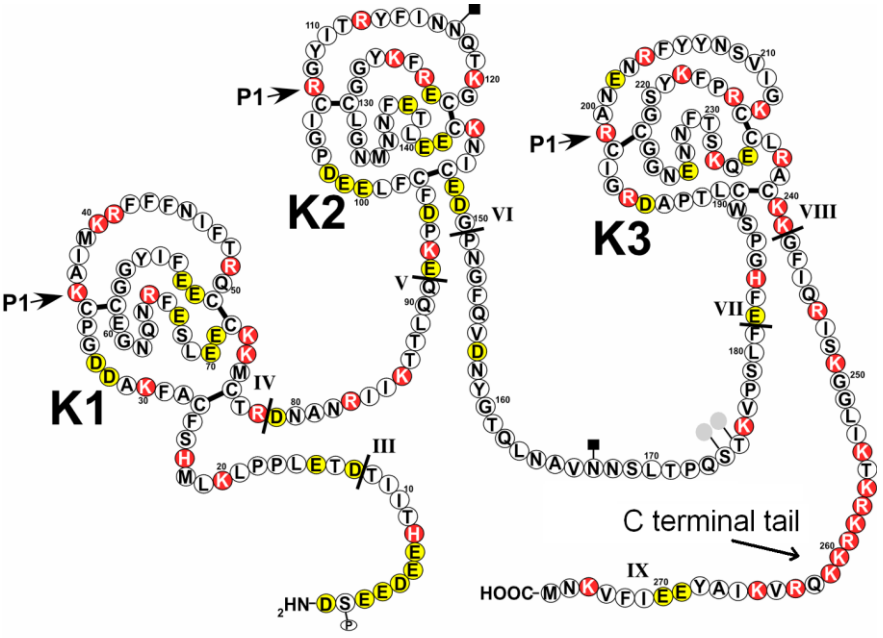

Figure 1.4 Schematic representation of full-length TFPI.
The N-terminal region contains negatively charged residues (yellow), while the C-terminal region contains positively charged residues (red). N-linked glycosylations are shown as black squares, O-linked glycosylations as gray circles. P1 residues in the respective Kunitz domains are indicated by arrows (Figure from Crawley and Lane, 2008).[26] The usually reported sequence contains a Y in position 115, G in position 121 and L in position 197.

The majority of TFPI (~80%) is associated with the endothelial cell surface (Figure 1.5). It has been reported that the mechanism of this association is mainly

(~75%) through an indirect binding to GPI in the caveolae.[42,50-53] As TFPI does not contain a GPI anchor sequence it has been hypothesised that TFPI is associated to the cell surface indirectly through a GPI binding protein.[54] The remaining amount (~25%) of TFPI associated to the endothelial cell surface does so through a direct interaction with cell surface glycosaminoglycans (GAG) mediated by its C-terminal tail.[55] This pool of TFPI is released following heparin injection augmenting 2-4-fold the amount of circulating full-length TFPI.[40,56-59]

The remaining TFPI (~20%) is either located inside platelets or circulates at a plasma concentration of ~2.5 nM (Figure 1.5).[55] The small amount (~5%) of plasma TFPI localised inside platelets is in storage granules that are distinct from α-granules.[60] This TFPI can be released following dual stimulation of platelets with collagen and thrombin which leads to platelet activation.[61-63] Of plasma TFPI, only ~10% is in its full-length 43 kDa form and represents the fraction of TFPI immediately available to counterbalance the procoagulant response. A small amount (~5%) of TFPI circulates as free truncated TFPI. The remaining plasma TFPI (~80%) is bound to lipoproteins.[64,65]

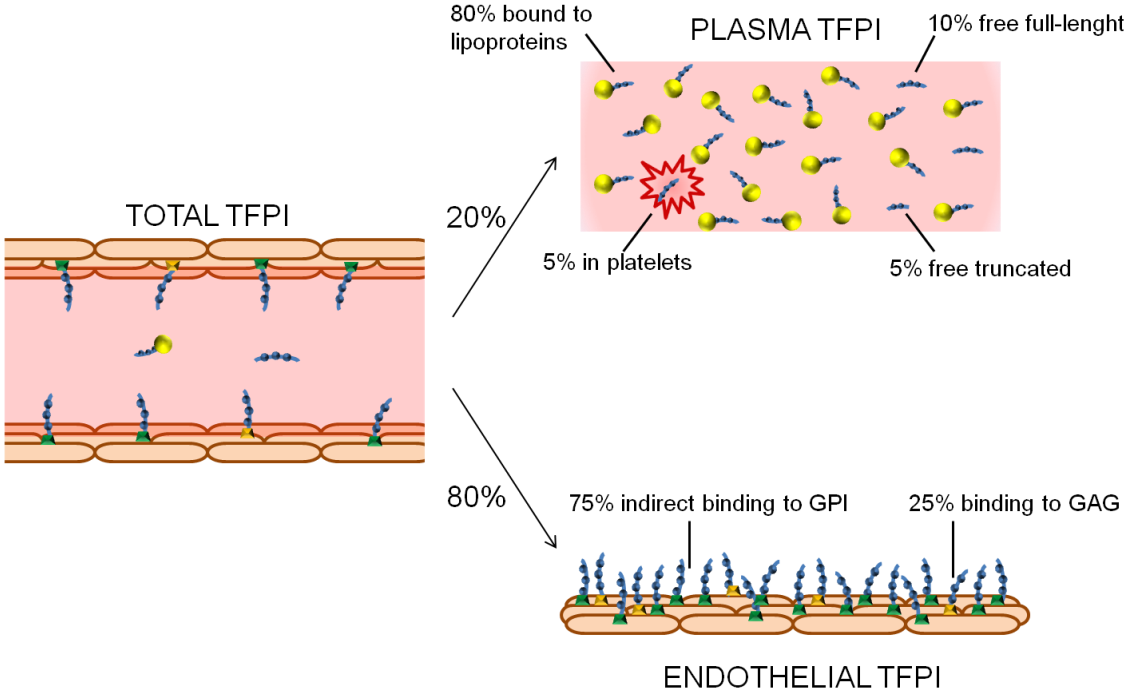

Figure 1.5 Distribution of TFPI.
Schematic representation of the distribution of TFPI in plasma and on the endothelial cell surface.

1.2.3 TFPI function

TFPI binds to and directly inhibits FXa and, in a FXa-dependent way, inhibits the TF/FVIIa complex by forming a quaternary, inactive FXa/TFPI/TF/FVIIa complex.[66-68] By inhibiting TF/FVIIa, TFPI inhibits the initiation phase of coagulation. The need for TFPI to bind and to inhibit FXa to effectively inhibit TF/FVIIa ensures that the TFPI pathway only regulates coagulation once it has started.[69]

1.2.3.1 TFPI mediated inhibition of FXa

FX is composed of a Gla domain responsible for its binding to phospholipids, two epidermal growth factor (EGF) domains required for protein-protein interaction and a serine protease domain responsible for the enzymatic activity of FXa. FX circulates as an inactive zymogen at a plasma concentration of ~136 nM and is activated following proteolysis by TF/FVIIa or by FVIIIa/FIXa.[70]

TFPI is a slow, tight-binding competitive inhibitor. TFPI binds FXa in a 1:1 stoichiometry, occupying its active site through its P1 residue, Arg107, in the K2 domain.[66] TFPI reversibly inhibits FXa by binding FXa in its active site. TFPI acts as a competitive inhibitor preventing FXa from binding to and activating prothrombin. It has been shown that FXa is able to hydrolyse TFPI after its P1 residue in the K3 domain, however, the physiological significance of this still needs to be evaluated.[71]

The kinetic mechanism of TFPI mediated inhibition of FXa is described as a two step reaction (Equation (Eq) 1).[72]

Eq1. $FXa + TFPI \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} FXa/TFPI \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} FXa/TFPI^*$

K_i

K_i^*

The first step describes the rapid formation of a loose initial FXa/TFPI complex. This is followed by a slower step resulting in formation of a final tight-binding of the enzyme to the inhibitor (FXa/TFPI* complex). Tight-binding is characterised by inhibition occuring at concentrations of inhibitor close to that of the enzyme.

The first step is described by the inhibition constant K_i (K_i=[FXa]*[TFPI]/[FXa/TFPI]) and the overall reaction by the inhibition constant K_i* (K_i*=[FXa]*[TFPI]/([FXa/TFPI]+[FXa/TFPI*])).

It has been shown that both phospholipids and FVa can enhance TFPI mediated inhibition of FXa.[72,73] Phospholipids are likely to co-localise FXa and TFPI on its surface, as FXa binds phospholipids through its Gla domain and it has been suggested that the positively charged C-terminal tail of TFPI can interact with negatively charged phospholipid surfaces and cell surfaces.[74-76] TFPI inhibition of FXa in the prothrombinase complex is, however, inhibited by the presence of sub-physiological concentrations of prothrombin.[73,77] It is likely that this is a result of prothrombin competing for the active site of FXa.

It has been shown that TFPI truncated, either after its K3 domain (lacking the K3 domain and the C-terminal tail) or lacking the C-terminal tail, is not as potent inhibitor of FXa as full-length TFPI.[78-80] It has been suggested that the K3 domain and C-terminal tail of TFPI might interact with phospholipids and cell surfaces,[74-76] and that the C-terminal tail of TFPI might interact with the Gla domain of FXa[79,80] possibly partially explaining the reduced activity of truncated TFPI. More recently, it was shown that protein S acts as a cofactor towards full-length TFPI mediated inhibition of FXa but not towards TFPI lacking its K3 and C-terminal domain.[81]

1.2.3.2 TFPI mediated inhibition of TF/FVIIa

TFPI inhibits TF/FVIIa in a FXa dependent way.[66-68] As described in section 1.2.3.1 TFPI is a slow, tight-binding competitive inhibitor. It reversibly inhibits TF/FVIIa by binding the active site of FVIIa through its P1 residue (Lys36) in the K1 domain.[66] It has been shown that the intact Gla domain of FXa is required for TFPI mediated inhibition of TF/FVIIa.[82,83] One study used a plate binding assay and showed that FXa enhanced the binding of TFPI to phospholipids.[76] It has been suggested that the Gla domain and EGF1 domain of FXa are important for interactions with the TF/FVIIa complex, potentially explaining its importance in TFPI mediated inhibition of TF/FVIIa.[84,85]

Inhibition of TF/FVIIa is usually described as a two-step process, in which TFPI first binds to FXa (this being the rate-limiting step) and then inhibits TF/FVIIa by binding to FVIIa (Figure 1.6 left). Kinetic studies, however, favour the binding of

TFPI to FXa that is already bound to TF/FVIIa (Figure 1.6 right).[68] The resulting inactive quaternary TFPI/FXa/TF/FVIIa complex leads to inhibition of the initial procoagulant stimuli. Although the K3 domain and C-terminal tail of TFPI are not absolute requirements for inhibition of FXa/TF/FVIIa, their presence appreciably enhances the TFPI anticoagulant activity.[78,79]

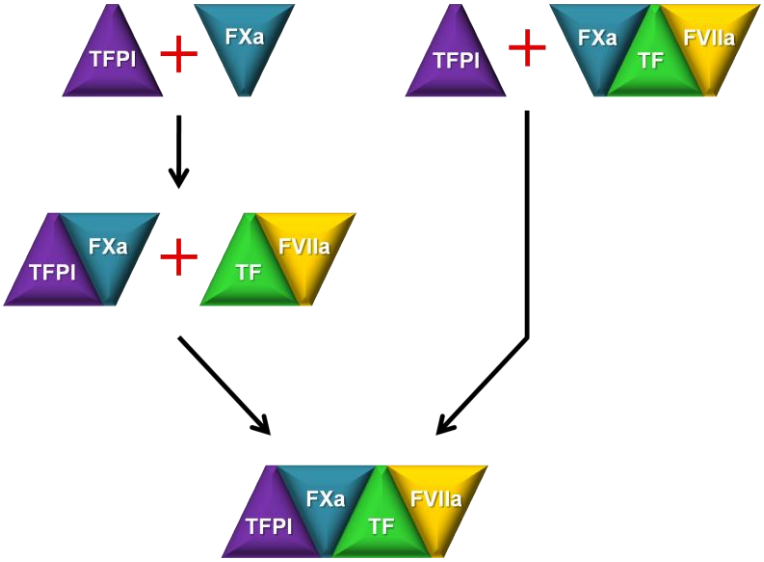

Figure 1.6 TFPI binds to and inhibits FXa and TF/FVIIa.
Inhibition of TF/FVIIa is frequently described as a two-stage process (left) in which TFPI first binds FXa and then the TFPI/FXa complex binds and inhibits TF/FVIIa. However, kinetic studies favour a model whereby TFPI binds to newly activated FX prior its release from the TF/FVIIa/FXa complex (right). In both cases the resulting inactive quaternary complex is TFPI/FXa/TF/FVIIa.

1.3 Protein C/APC

1.3.1 PROC

Protein C is a plasma protein that is synthesised and secreted by hepatocytes. *PROC*, the gene that encodes protein C, is localised on chromosome 2 and contains 9 exons.[86] Translation of the messanger ribonucleic acid (mRNA) results in an immature protein of 461 amino acids.[87] Post-transcriptional modifications include γ-carboxylation, β-hydroxylation, glycosylation and removal of the Lys156-Arg157 dipeptide by proteolysis which results in conversion of protein C from a single chain to a two-chain protein. Following removal of the pro- and pre-peptide protein C is secreted as a mature zymogen of 419 amino acids with a MW of 62 kDa.

1.3.2 Protein C structure

Protein C circulates as a zymogen at a plasma concentration of ~65 nM. It is composed of a Gla domain, two EGF domains, an activation peptide (AP) and a serine protease domain (Figure 1.7).[88] The Gla domain (amino acids 1-45) contains 9 γ-carboxylated Glu (Gla) residues (Gla6, 7, 14, 16, 19, 20, 25, 26, and 29).[87,89] These are essential for coordination of Ca^{2+} ions and binding to negatively charged phospholipids surfaces.[19] It has also been shown that Leu8, which is part of the hydrophobic ω-loop, is essential for binding to the endothelial protein C receptor (EPCR).[90] In addition, substitution of Asp36/Leu38/Ala39 results in a APC variant that can not be enhanced by protein S. The two EGF domains are predicted to be important for protein-protein interactions but no definitive role has so far been reported in the literature. EGF1 (amino acids 46-92) is β-hydroxylated at Asp71 and this residue in conjunction with Asp46 and Asp48 coordinates a Ca^{2+} ion.[91] A study using a Fab fragment against this region suggested that the EGF1 of protein C might be important for interactions with protein S.[91] EGF2 (amino acids 93-137) is glycosylated at Asn97 and this has been reported to be important for effective secretion of protein C.[92] The serine protease domain (amino acids 138-419) is composed by a catalytic triad (His211, Asp257 and Ser360), and contains loop 225 and loop 70 involved in Na^{+} and Ca^{2+} binding, respectively.[93] It has been shown that binding of one ion to its loop increases the affinity of the other ion.[93] The serine protease domain also contains loop 37, loop 60 and loop 148. Basic residues in loop 37, loop 70 and loop 148 are believed to form an exosite for binding to FVa and FVIIIa.[94-98]

Figure 1.7 Schematic structure of protein C.
The complete model of zymogen protein C was made by combining the crystal structure of the Gla-domainless APC with the Gla domain modelled from the crystal structure of the TF/FVIIa complex.[88] The AP was introduced by homology modelling. The serine protease domain is coloured in red, with the catalytic triad showed by the darker red spheres. The EGF2 is colured in dark blue, the EGF1 in cyan and the Gla domain in orange. The 9 Gla residues in the Gla domain are shown as sticks and coordinate calcium ions (yellow spheres). The Leu8 residue is shown in olive green and the Asp36/Leu38/Ala39 residues in the bright orange spheres.

1.3.3 Protein C activation

Cleavage of the negatively charged AP (Asp158-Arg169) of protein C by thrombin results in activation of protein C. Activated protein C is a two chain protein composed of a light (Gla-EGF1-EGF2) and heavy (AP and serine protease domain) chain connected through a disulphide bond (Cys141 and Cys277). Protein C is activated only once a sufficient amount of thrombin is generated. Thrombin that escapes the site of injury can bind to TM on intact endothelial cells adjacent to the site of vascular injury. TM is composed of a C-terminal cytoplasmic tail, a transmembrane region, a serine-threonine rich domain, 6 EGF domains and an N-terminal type C lectin-like domain.[99] Thrombin binds TM in the EGF5 and EGF6 domains through its exosite I.[100,101] It has also been shown that a chondroitin sulfate moiety of TM can interact with the exosite II of thrombin.[102]

As exosites I and II of thrombin are required for the activation of procoagulant factors FVa and FVIIIa,[12,103-106] binding of thrombin to TM results in a loss of its procoagulant functions. Instead, the specificity of thrombin is altered and the anticoagulant properties of thrombin are enhanced. The ability of thrombin to activate protein C is enhanced by 2000-fold following binding to TM.[107] Thrombin activates protein C by cleaving after Arg169 removing its AP. TM also binds the serine protease domain in the loops 37, 60, 70 and 148 of protein C through its EGF4 domain (Figure 1.8).[107-109] The activation of protein C is appreciably enhanced (more than 5-fold) by the presence of EPCR which binds the Leu8 in the Gla domain of protein C localising protein C to the endothelial surface close to the thrombin/TM complex.[90,110-113] This increases the local concentration of protein C available for activation.

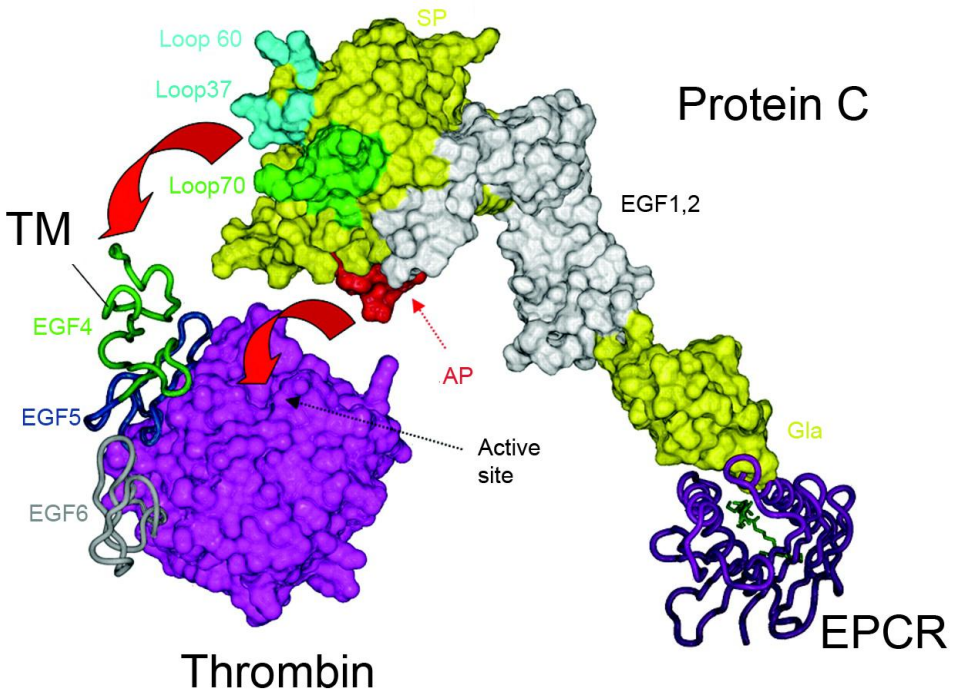

Figure 1.8 Activation of protein C.
Thrombin (light purple) binds to EGF5 and EGF6 of TM through its excosite I domain. EPCR (dark purple) binds the Gla domain of protein C resulting in the alignment of protein C with the thrombin/TM complex. EGF4 of TM is able to interact with loops 37, 60, 70 and 148 (not shown) of protein C bringing the AP of protein C in proximity of the active site of thrombin. Cleavage after Arg169 of protein C by thrombin activates protein C. Figure modified from Dahlbäck and Villoutreix.[114]

1.3.4 APC function

APC downregulates coagulation by proteolytical inactivation of FVa and FVIIIa, the non-enzymatic cofactors of FXa and FIXa, respectively.[27] FV and FVIII have a highly homologous domain structure containing an N-terminal A1 and A2 domain, a B domain, and a C-terminal A3 domain followed by a C1 and C2 domain. The C2 domains possibly also the C1 domains are responsible for FV and FVIII binding to phospholipids.[20-22] The A2 and A3 domains of FVa and FVIIIa are important for the interaction with FXa and FIXa respectively.[115-118]

1.3.4.1 APC mediated inactivation of FVa

Activation of FV occurs by the cleavage of peptide bonds in the B domain by either thrombin or FXa (Figure 1.9). This leads to dissociation of the B domain resulting in a two-chain molecule. The heavy chain (A1-A2 amino acids 1-709) and the light chain (A3-C1-C2 amino acids 1546-2196) remain associated by a non covalent complex between the A1 and A3 domains. While the association between the domains was believed to be through a high affinity Ca^{2+} interaction this has recently been questioned.[20] FVa can be inactivated by APC by proteolytic cleavage. APC can cleave FVa after Arg306, Arg506 and Arg679 (Figure 1.9).[119]

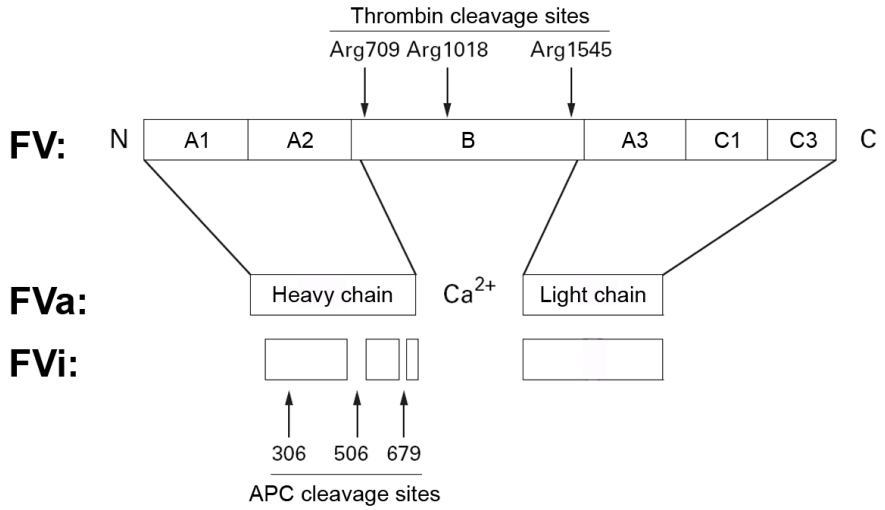

Figure 1.9 Activation of FV by thrombin and inactivation of FVa by APC.
Thrombin activates FV by proteolytic cleavage in its B domain. The heavy and light chains of FVa are associated through a high affinity binding to a calcium ion. APC inactivates FVa by proteolytic cleavage after Arg306, Arg506 and Arg679. Structure/figure of FV based on Kane *et al.* 1986, Kane *et al.* 1987, Jenny *et al.* and Laffan.[120-123]

While the physiological relevance of Arg679 cleavage is still being debated it is widely accepted that APC inactivates FVa in plasma by cleavage at Arg506 and Arg306. Cleavage at Arg506 is enhanced and cleavage at Arg306 dependent upon the presence of phospholipids, with phosphatidylserine and phosphatidylethanolamine both being important for the stimulation of APC mediated inactivation of FVa.[124-128] In FVa variants created for *in vitro* studies additional cleavage sites (Lys309, Arg313, Arg316, Arg317 and Arg505) have been identified.[129,130] However, the physiological role of these cleavage sites is questionable. Following cleavage after Arg506 and Arg306 the A2 domain of FVa dissociates resulting in complete inactivation of FVa. Inactivation of FVa is biphasic.[128,131,132] This is a consequence of the different rates of APC cleavage after Arg506 and Arg306. Arg506 is the first and kinetically most favoured cleavage site of APC and results in an intermediate FVa form which have been reported to have approximately 40% of intact FVa cofactor activity.[128] The second cleavage of FVa by APC occurs after Arg306 but is ~20-fold slower than the first cleavage at Arg506.[128] It has been shown that cleavage after Arg506, but to a minor extent also cleavage after Arg306, results in a significant decrease of FVa affinity for FXa and prothrombin.[128,133,134] It is unresolved whether complete loss of FVa activity occurs following Arg506 and Arg306 cleavage (before dissociation of the A2 domain) or only after dissociation of the A2 domain.[133,135]

It is known that FVa is protected from APC mediated proteolytic inactivation following binding to FXa[136-139] and prothrombin.[140-142] It has been shown that binding of FXa to FVa specifically inhibits APC cleavage after Arg506 by approximately 20-fold[137,138] and it has been recently suggested that FXa can stimulate APC cleavage at Arg306.[137] Binding of prothrombin to FVa has been shown to inhibit both APC mediated cleavage at Arg306 and Arg506 of FVa, however prothrombin did not inhibit APC mediated inactivation of FVIIIa.[141,142]

1.3.4.2 APC mediated inactivation of FVIIIa

FVIII circulates in plasma bound to VWF which act as a carrier protein increasing the half life of FVIII.[143-145] FVIII is activated following proteolytical cleavage by thrombin or FXa in the B domain. Only after activation FVIIIa dissociates from VWF.[146,147] Similarly to FV activation, activation of FVIII results in a two chain

molecule with a light and heavy chain. However, an additional cleavage between the A1 and A2 domains by thrombin leads to a weak association between the A1 and A3 domain which are linked by a Cu^{2+} ion.[148-150] Binding to Ca^{2+} or Mn^{2+} is also required to yield active FVIIIa.[150-152] FVIIIa is unstable and spontaneous dissociation of the A2 domain results in loss of activity.[153,154] Incorporation of FVIIIa into the tenase complex stabilises FVIIIa and also protects it from APC mediated inactivation.[155,156] APC inactivates FVIIIa by proteolytical cleavage after Arg336 and Arg562. Cleavage occurs preferentially at Arg336 and thereafter at Arg562, resulting in dissociation of the A2 domain and complete loss of function of FVIIIa.[157,158]

1.4 Protein S

1.4.1 PROS1

Protein S is encoded by the *PROS1* gene on chromosome 3 (p11.1), which spans more than 100 kb and gives rise to an mRNA of 3292 bases.[159,160] Following transcription the 676 amino acid long protein is directed to the endoplasmic reticulum (ER) by its hydrophobic signal peptide. Here, protein S goes through several post-transcriptional modifications, including γ-carboxylation, glycosylation and β-hydroxylation. γ-carboxylation is carried out by the vitamin K-dependent γ-carboxylase which recognises and binds to the propeptide of protein S. Prior to secretion both the signal peptide and propeptide are proteolytically removed generating a mature protein of 635 amino acids with a reported molecular weight of 69-77 kDa.[161,162]

Protein S is synthesised and secreted into the blood stream mainly by hepatocytes but also by endothelial cells, megakaryocytes and vascular smooth muscle cells.[163-166] The resulting concentration of protein S in plasma is ~0.35 µM.[167] Of plasma protein S, 60% circulates as a non-covalent, high-affinity complex with C4b-binding protein (C4BP) while the remaining 40% is free.[168]

1.4.2 Protein S structure

The mature protein S is composed of an N-terminal Gla domain (amino acids 1-46), a thrombin sensitive region (TSR, amino acids 47-75), four EGF domains (EGF1, amino acids 76-116; EGF2 amino acids 117-160; EGF3, amino acids 161-202; EGF4, amino acids 203-242) and a C-terminal Sex Hormone Binding Globulin-like domain (SHBG) (amino acids 243-635) containing two laminin G (LG)-type domains (Figure 1.10).[169,170]

The N-terminal Gla domain of protein S contains one disulphide bond (Cys17-Cys22) and 11 glutamic acid residues (Glu 6, 7, 14, 16, 19, 20, 25, 26, 29, 32 and 36) that are γ-carboxylated by the vitamin K-dependent γ-carboxylase in the ER.[171] The resulting N-terminal Gla residues coordinate 8 calcium ions which induce an appropriate conformational change in protein S for activity.[19,171,172] Folding of the Gla domain enables protein S to bind to phospholipids through the insertion of its hydrophobic ω-loop into the membrane and ionic interactions between positively charged calcium ions and the negatively charged phospholipid surface.[19,171,172] Binding of protein S to phospholipid surfaces localises protein S to activated endothelial cells and activated platelet surfaces and is required for both its anticoagulant and non-anticoagulant activity (see section 1.4.3).

The TSR is formed by two α-helixes brought in close contact through a disulphide bond between Cys47 and Cys72 and is a domain unique to protein S. Its name derives from the observed cleavage of this region by thrombin. While cleavage at Arg49 and Arg70 by thrombin has been observed *in vitro,* this only occurs in the absence of Ca^{2+} and phospholipids, suggesting that this is not of physiological importance.[173] FXa has also been shown to be able to cleave the TSR at Arg60. Recently, it was shown that the TSR domain of protein S could bind to FXa in the presence of phospholipids.[174] Although FXa cleavage is both Ca^{2+} and phospholipid dependent, it is unlikely to occur *in vivo* due to its slow kinetics and the inability of FXa to cleave protein S when bound to its cofactor FVa or when in presence of prothrombin.[173] It is, however, interesting to notice that cleavage of the TSR has been observed to inhibit the APC-dependent cofactor activity of protein S.[175-177] The effect of cleavage upon the protein S APC-independent activity is still not clear, but it has been suggested that it is abolished only

following cleavage after both Arg49 and Arg70.[176] It has been reported that approximately 10% of plasma protein S circulate in its cleaved form.[178]

The EGF1 domain of protein S contains 3 disulphide bonds and is β-hydroxylated at Asp95.[171] This domain has to date not been found to bind Ca^{2+} and β-hydroxylation is thought not to be a requisite for protein S function.[179]

The EGF2-4 domains all contain 3 disulphide bonds each and a β-hydroxyasparagine residue (Hyn 136, Hyn 178 and Hyn 217). Each domain also contains a high affinity Ca^{2+} binding site[180] (dissociation constant (K_d) nM range), which is thought to include the modified Asn residue. Binding of Ca^{2+} to these domains have been shown to be important for the function of protein S. In 2006 Rezende and colleagues demonstrated that the abolition of binding of Ca^{2+} to EGF4 impaired both the structure and function of EGF1.[181] It has also been suggested that a naturally occurring deletion mutation in EGF4 (deletion I203D204) alters the conformation and function of the Gla domain,[182] while deletion or replacement of the entire EGF2 domain results in a significant loss of activity.[183]

The C-terminal SHBG domain of protein S represents more than half of its amino acids and is kept in its globular state through 3 disulphide bonds. It contains 3 potential N-linked glycans at Asn458, Asn468 and Asn489 and is also predicted to contain Ca^{2+} binding sites.[184,185] As anticipated, 60% of protein S circulates as a non-covalent, high-affinity complex with C4BP. This interaction is mediated through the SHBG domain of protein S.[184,186] C4BP is a 570 kDa multimeric protein consisting of 6 or 7 identical α-chains with one or no β-chain held together by disulphide bonds. It is this β-chain which contains the binding site for protein S.[187] The net plasma concentration of C4BP is ~260 nM. Of this only ~80% (~208 nM) has the β-chain and is able to bind to protein S with a 1:1 stoichiometry.[187] The amount of unbound or free circulating protein S is equal to the molar excess of protein S over β-chain containing C4BP (~145 nM) and represents the pool of protein S with major anticoagulant function.[188]

Multimerisation of protein S has been documented in the literature. Protein S is generally thought to exist in plasma in its monomeric form (although some have reported multimeric forms of protein S in plasma) and multimerisation has been attributed at least in part to *in vitro* manipulation.[189-192]

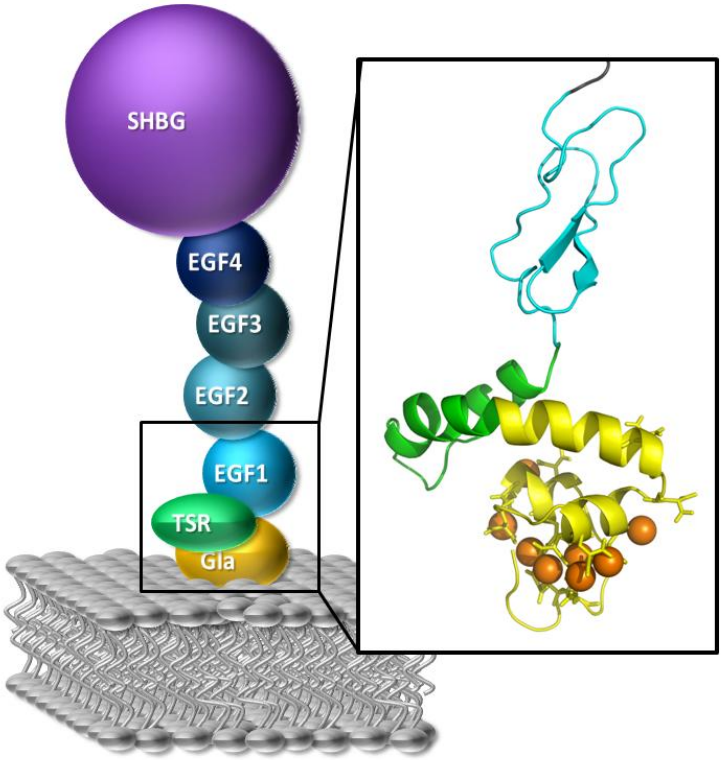

Figure 1.10 Schematic representation of protein S and its domains.
Protein S contains a N-terminal Gla domain that is responsible for binding to phospholipid surfaces. This is followed by a TSR domain, 4 EGF domains and a SHBG domain. The figure in the box has been created from the Gla-TSR-EGF1 model of protein S.[193] The Gla domain is coloured in yellow, with the 11 Gla residues shown as sticks and the 8 calcium ions shown as orange spheres in the box at the right. The ω-loop of protein S can be seen underneath the calcium ions. The TSR is shown in green and the EGF1 domain in cyan.

1.4.3 Protein S functions

Protein S is a multifunctional protein involved in coagulation, the classical complement pathway and apoptotic clearance.[194-196] It is also thought to stimulate vascular injury repair by acting as a mitogen towards smooth muscle cells and has recently been found to have a neuroprotective effect during ischemic brain injury in mice.[197-200]

The most studied protein S function is its anticoagulant activity, which is also the focus of my thesis. Protein S has an important role *in vivo*, as is shown clinically by infants with complete deficiency, who suffer purpura fulminans despite treatment of anticoagulants, and by heterozygous carriers of *PROS1* gene deletions and point mutations, who are at enhanced risk of venous thromboembolism.[201-203] Hereditary protein S deficiency is an uncommon autosomal dominant disorder. It is classified into type I, type II and type III

deficiencies. Type I and type III deficiencies are defined as quantitative deficiencies of total and free protein S, respectively, and correspond to ~96% of all protein S deficiencies.[202] The remaining ~4% are represented by type II deficiencies. This subtype is classified as a qualitative deficiency.[202] Acquired protein S deficiencies are more common and occur as a consequence of oral anticoagulant therapy, oral contraception, liver disease, nephritic syndrome and pregnancy.[202,204] Protein S deficiencies, inherited or acquired, both lead to an increased risk of thrombotic events.[205] The importance of protein S has also been demonstrated in murine knockouts, which fail to survive development.[206,207]

Protein S is best known for its anticoagulant activity towards APC. Protein S was discovered in 1977 by Richard DiScipio and was 3 years later shown to be the cofactor of APC.[208] Protein S was, however, also suggested to have an APC-independent activity in 1988.[209] In the early 1990's it was demonstrated that protein S could bind to FVa,[116,210-212] FXa[174,213,214] and FVIIIa[215] inhibiting both the tenase and prothrombinase complex in an APC-independent manner. It was shown that the observed inhibition of the tenase and prothrombinase complex was due to competition of protein S multimers for binding to limiting phospholipid surfaces.[191,192,216,217] However, it was later shown that protein S had an APC-independent activity in plasma also when saturating amounts of phospholipids were present, but which could be demonstrated only at low concentrations of TF.[218]

1.4.3.1 Protein S enhances the anticoagulant activity of APC

1.4.3.1.1 Protein S enhancement of APC mediated inactivation of FVa

Protein S enhances the APC mediated inactivation of FVa in a phospholipid dependent manner.[124,208,219] It does so by selectively enhancing by approximately 20-fold the slow cleavage at Arg306 of FVa.[125,138] The use of FV variants FV R306Q/R679Q and FV R506Q/R679Q, that can be cleaved only in Arg506 and Arg306, respectively, has been useful to characterise the effect of protein S and other components on the individual cleavage sites.[125,137,220] Through the use of such variants it has recently been suggested that protein S can also enhance ~5-fold APC mediated cleavage at Arg506.[125]

Protein S has been proposed to enhance APC mediated inactivation of FVa in 3 distinct ways. It has been shown to enhance APC binding to phospholipid surfaces, platelets and endothelial cells,[127,221,222] consequently localising APC in close proximity to the membrane bound FVa and FVIIIa. This proposal is supported by the low affinity of APC for phospholipids. While the apparent dissociation constant ($K_{d(app)}$) of protein S for phospholipids is between 4-15 nM[182,223-225] the $K_{d(app)}$ of APC for phospholipids is around 2-7 µM,[226,227] which is well outside the range of the physiological plasma concentration of APC (~65 nM). The second mechanism by which protein S is thought to enhance APC anticoagulant activity is through inducing an allosteric conformational change in APC.[228,229] Yegneswaran *et al.* used fluorescence resonance energy transfer (FRET) and showed that the active site of APC was localised 94 Ångström (Å) above the membrane surface. However, in the presence of protein S the active site of APC was relocated to 84 Å from the membrane surface.[229] It has been suggested that this shift in the active site of APC in the presence of protein S facilitates the preference of APC cleavage from Arg506 to Arg306. As described in section 1.3.4.1, FXa and prothrombin are able to protect FVa from APC mediated cleavage. The third mechanism by which protein S is thought to enhance APC mediated inactivation of FVa is through overcoming the protective effect of FVa.[137,219] It is still being debated whether protein S competes with the binding of FXa to FVa or not.[116,137,138] Depending on this, protein S could either act by competing with FXa binding[116] to FVa. Alternatively, protein S could enhance cleavage of free FVa resulting in depletion of free FVa and dissociation of FXa/FVa complexes.[137] An other study showed that the binding sites of protein S and FXa on FVa are distinct and that protein S is likely to enhance APC mediated cleavage at Arg306 of FVa even when the latter is bound to FXa.[138] A recent paper suggests that not only protein S but also FV can act as a cofactor for APC mediated inactivation of FVa.[230]

While it was previously thought that only free protein S could act as a cofactor for APC, recent work shows that protein S bound to C4BP is able to enhance cleavage of FVa.[231] However, while cleavage after Arg306 by this complex is enhanced 10-fold (compared to ~20-fold by free protein S), cleavage after Arg506 is inhibited 3-4-fold (compared to no or little influence of free protein S).[231] Consequently, bound protein S enhances APC cleavage of FVa, but is 6-8-fold

less effective that free protein S, potentially explaining why in prior experiments the addition of C4BP resulted in a decreased APC activity.

1.4.3.1.2 Protein S enhancement of APC mediated inactivation of FVIIIa

It has been shown that protein S can enhance both the slow cleavage at Arg562 by ~3-fold and cleavage at Arg336 by ~2-fold. [232,233] However, FV cleaved at Arg506 also acts in synergy with protein S as a cofactor enhancing ~10-fold APC mediated inactivation of FVIIIa.[157,233,234] Both free protein S and protein S bound to C4BP act as cofactors for APC mediated inactivation of FVIIIa.[235] However, it has been reported that while free protein S acts in synergy with FV in the APC mediated inactivation of FVIIIa, bound protein S does not.[235]

It is believed that protein S exerts its cofactor activity through increasing the binding of APC to phospholipid surfaces, platelets and endothelial cells, as described in section 1.4.3.1.1. [127,221,222]

Similar to FVa in the prothrombinase complex, when FVIIIa is assembled in the tenase complex cleavage is protected by FIXa. Protein S can overcome the protective action of FIXa by competing with its binding to FVIIIa.[236]

1.4.3.2 Protein S enhances the anticoagulant activity of TFPI

1.4.3.2.1 Protein S enhancement of TFPI mediated inactivation of FXa

In 2006 Hackeng *et al.* showed that protein S enhances the anticoagulant activity of TFPI, potentially providing a molecular explanation behind the APC-independent activity observed in plasma.[81] Since it had been shown that the APC-independent activity of protein S was visualised only at low concentrations of TF,[218] their investigation hypothesised that protein S had an effect on TF mediated activation of FX. Hackeng *et al.* analysed thrombin generation by calibrated automated thrombography (CAT). They showed that in normal plasma, in the presence of polyclonal antibodies against APC, antibodies against protein S increased thrombin generation. The same effect was, however, not observed in TFPI depleted plasma, suggesting the anticoagulant activity of protein S was dependent upon the presence of TFPI. This was confirmed by supplementing the plasma with TFPI. They also performed a FXa inhibition assay. This was a

purified assay in which FXa hydrolysis of a chromogenic substrate was measured over time. It was observed that protein S could potentiate the TFPI mediated inhibition of FXa by enhancing the formation of the initial FXa/TFPI complex (see section 1.2.3.1). Little effect was seen on the transition from the loose FXa/TFPI complex to the tightly bound FXa/TFPI* complex. The effect of protein S was phospholipid dependent and resulted in a 10-fold reduction of the K_i (see section 1.2.3.1), from 4.4 nM to 0.5 nM, with little effect on the K_i*.[81] Ndonwi and Broze confirmed that protein S enhances TFPI inhibition of FXa.[237] Hackeng *et al.* also showed that protein S was not able to enhance the activity of TFPI lacking its K3 domain and C-terminal tail, and that protein S bound to C4BP only exerted 60% of the activity of free protein S.[81] Ndonwi *et al.* recently showed that the K3 domain of TFPI and in particular its P1 residue were necessary for protein S binding and cofactor activity towards TFPI.[238]

Rosendaal and colleagues observed that higher plasma concentrations of protein S resulted in a higher anticoagulant activity of TFPI.[239] Recently, it has been shown that the impaired protein S enhancement of TFPI is potentially an important component in patients with protein S deficiency.[240] Interestingly, it has been suggested that protein S binds to TFPI in plasma and that this might account for the observed decrease in TFPI antigen levels in protein S deficient patients.[241] The detailed molecular mechanisms of the TFPI-dependent protein S anticoagulant activity were unknown and form an important part of the investigation carried out in my thesis.

Even though the APC-independent activity of protein S now has a plausible explanation (it acts as a cofactor of TFPI), Heeb and colleagues have reported that protein S also has a “direct” activity, independent of both APC and TFPI, exerted by direct binding to and inhibition of FXa and TF/FVIIa.[189,190,242]

1.4.3.2.2 Protein S enhancement of TFPI mediated inactivation of TF/FVIIa

Studying the protein S cofactor activity on TFPI mediated inactivation of TF/FVIIa is complicated by the difficulty in distinguishing between enhanced TF/FVIIa inhibition and enhanced inhibition of FXa. While it was initially suggested that protein S could enhance TFPI inhibition of TF/FVIIa activation of FX,[81] Ndonwi and Broze suggested that this was not the case.[237] The latter support the

proposal that TFPI predominantly binds to FXa already bound to TF/FVIIa, rather than the previously suggested two-step mechanism by which TFPI first binds FXa and FXa/TFPI subsequently binds TF/FVIIa (see section 1.2.3.2). Consequently, Ndonwi and Broze suggested the major role of protein S enhancement of TFPI was to down-regulate any FXa that escapes TF/FVIIa/FXa inhibition by TFPI.

1.5 Domains of protein S which mediate functional interactions

While the functions of the domains of protein S have been extensively studied, the APC cofactor function and the recently discovered TFPI cofactor activity of protein S remain poorly understood at the molecular level. A number of basic and clinical studies have confirmed that alteration of protein S can disrupt phospholipid binding and thereby impair function of protein S.[182,223] Consequently, variants with impaired cofactor function need to be evaluated for their ability to bind to phospholipids and those that clearly alter phospholipid binding can be discounted from further investigation. A search of the literature has suggested the domains of protein S that are most likely involved in APC and TFPI cofactor activity (see sections 1.5.1 and 1.5.2 below).

1.5.1 Domains of protein S reported to be important for APC cofactor activity

Saller *et al.* prepared protein S/prothrombin chimeras and on the basis of functional studies supported by molecular modelling, identified a cluster of protein S Gla domain residues (termed Face2) which they proposed would provide an APC interaction site.[224] Additional studies have focused on the possible role of TSR and EGF domains. Several studies have reported that cleavage in the TSR of protein S affects its APC-dependent and APC-independent cofactor activity.[175-177] Dahlbäck *et al.* used domain specific monoclonal antibodies directed against protein S and their Fab fragments and suggested that the TSR and EGF1 domains may be involved in APC cofactor activity.[243] The TSR and EGF1 domain of protein S have also been reported to be responsible for APC species specificity.[244] By using EGF-like modules Stenberg *et al.* concluded the EGF1 module to be crucial for interaction with APC.[245] Hackeng *et al.* demonstrated that

the isolated EGF1 domain of protein S could inhibit full-length protein S anticoagulant enhancing function for APC: direct binding of the domain to APC was also demonstrated.[246] Mille-Baker *et al.* prepared EGF2 domain deletion and substitution variants of protein S and demonstrated reduced APC anticoagulant cofactor activity, in the presence of normal phospholipid binding.[183] Collectively, these investigators have suggested Gla, TSR, EGF1 and EGF2 domains may each contain recognition elements on protein S needed for APC cofactor function.

1.5.2 Domains of protein S that are potentially important for TFPI cofactor activity

Work presented in preliminary form at the ISTH congress in Geneva by Hackeng *et al.*, who had carryied out an *in silico* molecular docking experiments of protein S and TFPI, suggested that the TSR of protein S might be involved in the interaction with TFPI. Recent reports suggest that free protein S, but not protein S bound to C4BP, form a reversible interaction with free full-length TFPI in plasma.[241] In addition, free protein S seems to act more efficiently as a cofactor for TFPI than protein S in complex with C4BP.[81] Collectively, these results would suggest that the N-terminal domains of protein S are more likely to be involved in an interaction with TFPI than the C-terminal domains, which can be bound to C4BP.

1.6 Aims of my thesis

The aims of my project were to determine the domains and residues of protein S involved in the interaction with both APC and TFPI. Following selection of the domains, over 30 point or composite variants in these domains were made. Wild type (WT) protein S and protein S variants were expressed in human embryonic kidney fibroblast (HEK) cells and purified as required. The functional cofactor activity of protein S variants towards APC and TFPI anticoagulant activities was evaluated and compared to that of WT protein S in various functional assays including calibrated automated thrombography (CAT), FVa inactivation assay and FXa inhibition assay.

2 METHODOLOGY

A schematic overview of the sequence of methods used during this project and the sections in which they are described is shown in Figure 2.1.

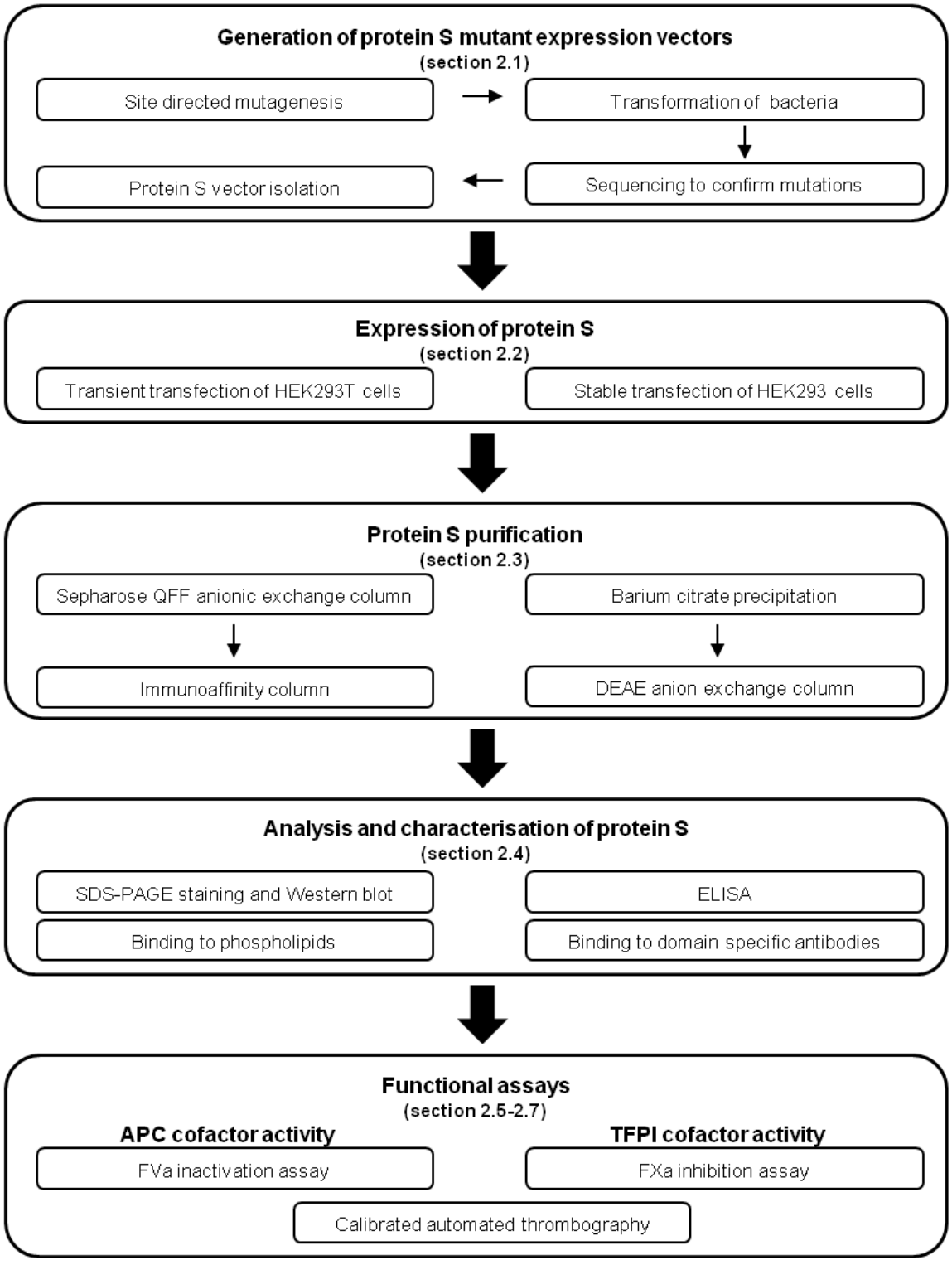

Figure 2.1 Overview of the Methodology of this thesis.

2.1 Generation of protein S mutant expression vectors

2.1.1 Mammalian protein S expression vector and composite mutants generated

A pcDNA6/protein S vector (Figure 2.2) containing the complimentary deoxyribonucleic acid (cDNA) for the WT protein S sequence had previously been generated in our lab by Dr S.M. Rezende (Universidade Federal de Minas Gerais, Brazil).[247] The V5 epitope and the polyhistidine tag were excised from the pcDNA6/V5-HisB vector (Invitrogen) by digestion with the NheI restriction enzyme (New England Biolabs). The cDNA of protein S, amplified by polymerase chain reaction (PCR) with primers containing the restriction site for NheI, was digested with the restriction enzyme and ligated into the vector. The resulting pcDNA6/protein S vector contains the SV40 origin of replication, cytomegalovirus (CMV) and T7 promoters, and resistance to ampicillin and blasticidine. The plasmid was used as a template to produce single point mutations by PCR mediated site-directed mutagenesis. Newly produced cDNA with point mutations were subsequently used as a template to introduce additional mutations resulting in single and composite mutants.

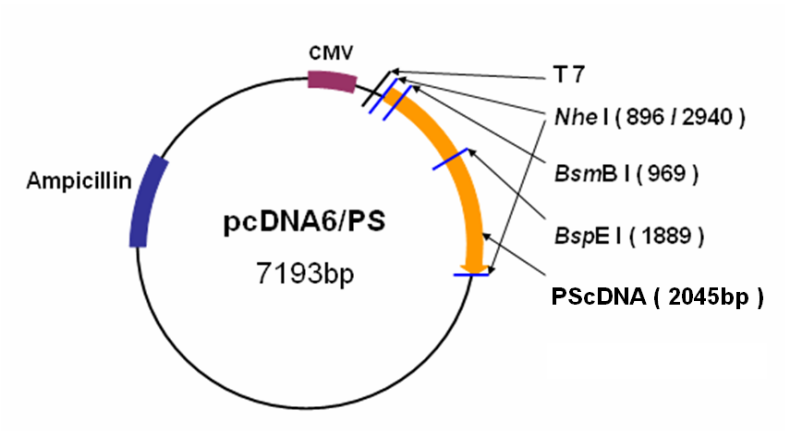

Figure 2.2 Protein S expression vector.
The cDNA for protein S has been inserted into the vector through ligation of the NheI digested ends of the insert to the vector. The vector contains resistance to ampicillin for selection of transformed bacteria and the CMV promoter for expression in mammalian cells. The restriction sites BsmBI and BspEI are used for subcloning of the mutants generated (see section 2.1.6). bp; base pair.

2.1.2 Generation of protein S mutants by site-directed mutagenesis

Complementary primer pairs with the desired base pair substitutions (for amino acid residues mutated see Table 3.1, for primer sequences see Appendix) were manufactured by Thermo Scientific. PCR reactions were performed using Stratagene's QuickChange XL Site-Directed Mutagenesis Kit. In detail, final concentrations of 100 ng of protein S vector, 65 ng of forward primer, 65 ng of reverse primer, 0.5 µl deoxynucleoside triphosphate (dNTP) mix, 1X reaction buffer, 1.5 µl of QuikSolution and 1.25 U of high fidelity *Pfu ultra* deoxyribonucleic acid (DNA) polymerase were used in a final volume of 25 µl.

Alternatively, mutagenesis was performed with the KOD hot start DNA polymerase (Novagen). In detail, final concentrations of 100 ng of dsDNA template, 0.3 µM of forward primer, 0.3 µM of reverse primer, 0.2 mM dNTPs, 0.5 µl DMSO, 1.5 mM $MgSO_4$, 1X reaction buffer and 0.5 U KOD hot start DNA polymerase were used in a final volume of 25 µl.

Cycling parameters for *Pfu ultra* and KOD hot start DNA polymerase are outlined in Table 2.1 and Table 2.2 respectively.

After PCR, parental dsDNA template was digested with 5 U of DpnI at 37°C for 1 hour before transforming bacteria.

Stage	Phase	Cycles	Temperature (°C)	Time
Denaturation		1	95	2 minutes
Amplification	Denaturation	18	95	50 seconds
	Annealing		60	50 seconds
	Extension		68	1 min/kb plasmid
Final extension		**1**	**68**	7 minutes

Table 2.1 PCR cycle parameters for *Pfu ultra* DNA polymerase.
PCR cycle parameters used to generate protein S mutants by site-directed mutagenesis. Min; minute.

Stage	Phase	Cycles	Temperature (°C)	Time
Denaturation		1	95	5 minutes
Amplification	Denaturation	18	94	15 seconds
	Annealing		60	30 seconds
	Extension		72	20 sec/kb plasmid
Final extension		1	72	5 minutes

Table 2.2 PCR cycle parameters for KOD hot start DNA polymerase.
PCR cycle parameters used to generate protein S mutants by site-directed mutagenesis. Sec; seconds.

2.1.3 Transformation of competent cells

Competent cells (XL-10 gold ultracompetent cells from Stratagene, One shot TOP10 competent cells from Invitrogen or NEB Turbo competent cells from New England Biolabs) were transformed with the mutated protein S vector according to the manufacturer's instructions. In detail, 45 µl of cells were thawed on ice. XL-10 gold ultracompetent cells were incubated with 2 µl of β-mercaptoethanol for 10 minutes, gently swirling the tube every 2 minutes. Competent cells were incubated with 2 µl of DpnI treated DNA; the tube was swirled and incubated on ice for 30 minutes. Cells were heat-pulsed in a water-bath at 42°C for 30 seconds and placed on ice for an additional 2 minutes. Transformed bacteria were grown in 0.25-0.5 ml of preheated S.O.C. medium at 37°C for 1 hour with shaking. Bacteria were subsequently plated out on an LB-agar (Invitrogen) plate containing 100 µg/ml ampicillin and incubated at 37°C over night. Ampicillin-resistant clones were picked and grown in 5 ml LB-broth (Invitrogen) with 100 µg/ml ampicillin over night at 37°C with shaking.

2.1.4 Generation of glycerol stocks

Of the 5 ml of bacteria in section 2.1.3, 700 µl were mixed with 300 µl of autoclaved 50% glycerol solution. The resulting mix was vortexed and stored at -80°C. This could be partially thawed when additional transformed bacteria were

required. The remaining 4.3 ml of bacteria were used to extract plasmid DNA by miniprep.

2.1.5 Plasmid miniprep and sequencing

Plasmid DNA was extracted with the Qiagen Miniprep kit according to instructions. Mutations were confirmed by sequencing the protein S vector with the T7 primer 5'-TAA TAC GAC TCA CTA TAG GG-3' (MRC Genomics Core Facility).

2.1.6 Subcloning of the protein S mutants into the pcDNA6/Protein S vector

Prior to mammalian expression, the mutated cDNA was sequenced with additional primers spanning the whole cDNA of protein S to make sure no unwanted mutations had been introduced (see Appendix for sequencing primers). Alternatively, the cDNA region containing the mutation was subcloned into the original WT vector. To perform such subcloning, the vector with the protein S mutation (40 µl, ~16 µg) and the WT vector (1 µg) were digested with BsmBI (New England Biolabs) at 55°C for 2 hours and subsequently with BspEI (New England Biolabs) at 37°C for 2 hours 30 minutes in buffer 3 (New England Biolabs) in a total volume of respectively 50 and 25 µl (Table 2.3).

	Digestion of:	
	Protein S mutant vector	**WT vector**
DNA	40 µl (16 µg)	0.77 µl (1µg)
BsmBI (10 U/µl)	1 µl	0.2 µl
BspEI (10 U/µl)	1 µl	1 µl
Buffer 3	5 µl	2.5 µl
Water	3 µl	20.53 µl
Total volume	50 µl	25 µl

Table 2.3 Enzymatic digestion of WT and mutated pcDNA6/PS vector.

Digested DNA products were run on a 1% agarose gel containing SYBRSafe in tris borate ethylenediaminetetraacetic acid (TBE) buffer. Samples and marker (1 kb or 100 bp) were electrophoresed for 20-40 minutes at 80 V. The DNA bands of interest were excised and purified using the QIAquick Gel extraction kit (QIAGEN) according to the manufacturer's instructions.

The linearised pcDNA6/Protein S vector was dephosphorylated with 0.01 U of Calf Intestinal Alkaline Phosphatase (CIAP) as described in Table 2.4, according to the manufacturer's instructions (Invitrogen). The reaction was performed at 37°C for at least 1 hour and the dephosphorylated vector was subsequently purified using QIAquick PCR purification Kit protocol (QIAGEN).

Dephosphorylation	
DNA (~0.48 pmol)	25 µl
10X dephosphorylation buffer	4 µl
Water	10 µl
CIAP (0.01 U/µl)	1 µl
Total volume	40 µl

Table 2.4 Dephosphorylation of the linearised WT vector.

2.1.7 DNA ligation

Approximately 60 fmol of the DNA fragment containing the mutation were ligated into 20 fmol of the dephosphorylated vector (molar ratio insert:vector, 3:1) with 0.1 U of T4 DNA ligase in a total volume of 20 µl, at 23-26°C for 1 hour as described in Table 2.5.

Ligation reaction	
Vector	3 µl (20 fmol)
Insert	0.5 µl (60 fmol)
5X ligase buffer	4 µl
T4 ligase (0.1 U/µl)	1 µl
Water	11.5 µl
Total volume	20 µl

Table 2.5 Ligation of the mutated protein S region into the WT protein S vector.

2.1.8 Transformation of competent cells with subcloned mutants

The ligation product was used to transform One shot TOP10 (Invitrogen) or NEB turbo chemically competent cells (New England Biolabs) according to the manufacturer's instructions. Transformed bacteria were plated out on LB-agar/ampicillin plates over night at 37°C. Ampicillin resistant-colonies were picked and grown in 5 ml Luria-Bertani medium (LB) broth over night. The plasmid was extracted with the Qiagen Miniprep kit and sequenced (for primers see Appendix) as outlined previously (MRC Genomics Core Facility). Clones were sequenced across both restriction sites to confirm correct ligation.

2.1.9 DNA maxiprep/megaprep and quantification

Bacteria containing the successfully mutated protein S vector were cultured on a large scale (200-500 ml) to obtain sufficient amounts of plasmid (extracted with a Qiagen Maxiprep or Megaprep kit) for transfection of mammalian cells.
The DNA was quantified with the Quant-IT™ nucleic acid Assay Kit (Invitrogen) according to manufacturer's instructions or with a NanoDrop spectrophotometer by measuring the absorbance at 260 nm.

2.2 Expression of protein S

2.2.1 Mammalian cell culture

HEK293T and HEK293 cells (ATCC) were used respectively for transient and stable transfections. Cells were cultured at 37°C, 5% CO_2, in complete media; minimum essential media (MEM, Invitrogen) supplemented with 10% foetal calf serum (FCS) (Biosera), 2 mM L-glutamine (Invitrogen), 50000 U penicillin/ 50000 µg streptomycin and 1X non-essential amino acids (Invitrogen), and were generally grown in T175 flasks or T175 triple flasks. When confluent, cells were washed with phosphate buffered saline (PBS; 10 mM phosphate buffer, 2.7 mM potassium chloride, 137 mM sodium chloride) (5 ml/T175 flask) split 1:3 using 1 ml trypsin/T175 flask to detach cells and grown to confluence again in 3 days. Vitamin K (10 µg/ml) was added to complete MEM media, at least 24 hours prior

expression of protein S. This allows cells to take up vitamin K which is essential for the γ-carboxylation of protein S. Protein S was expressed in OptiMEM I (Invitrogen) reduced serum-medium supplemented with 10 µg/ml vitamin K and 100 mg/L $CaCl_2$ for 3 days.

2.2.2 Transient transfection of HEK293T cells

Protein S was transiently expressed in HEK293T cells. Cells were cultured in T175 triple flasks and grown in complete media. When cells reached ~60-70% confluency, the media was changed and supplemented with 10 µg/ml vitamin K. Cells were grown in the vitamin K containing media for at least 24 hours before being transfected. On the day of transfection, for one T175-triple flask, 257 µl of linear Polyethylenimine (PEI) (Polysciences,Inc) (1 mg/ml) were diluted into 3.75 ml of 0.15 M autoclaved NaCl. The resulting PEI solution was added drop wise to 3.75 ml of 0.15 M autoclaved NaCl containing 114 µg DNA and the mix was incubated at room temperature for 15-20 minutes. The DNA-PEI solution was added to 75 ml OptiMEM supplemented with 10 µg/ml vitamin K and 100 mg/L $CaCl_2$. Each T175-triple flask was washed with PBS and cells were grown in the 82.5 ml of OptiMEM containing the transfection solution. Media containing the secreted protein S was harvested after 3 days.

2.2.3 Stable transfection of HEK293 cells

A heterogeneous stable HEK293 cell line expressing WT protein S had already been generated in our lab by Dr S.M. Rezende (Universidade Federal de Minas Gerais, Brazil). Heterogeneous stable cell lines expressing protein S variants of interest were produced during this project. HEK293 cells were seeded in a 6 well plate in complete media and were allowed to reach 85-95% confluence before being transfected. Ten µl of Lipofectamine 2000 (Invitrogen) was mixed with 250 µl OptiMEM (GIBCO) and incubated for 5 minutes at room temperature. Following incubation, 4 µg of DNA pre-diluted into 250 µl OptiMEM were added drop-wise to the Lipofectamine 2000 solution. The resulting DNA-Lipofectamine 2000 mix was incubated for 5 minutes at room temperature before being added to the cells previously washed with PBS. Cells were incubated at 37°C, 5% CO_2 for 4-6

hours, after which the media was replaced by complete MEM media. After 24-48 hours cells were split 1:10 and seeded in complete MEM media containing 5 µg/ml Blasticidine-HCl (Invitrogen). Stably transfected HEK293 cells were selected by culturing the cells in selective media at a confluence below 60% for 4 weeks. A stable cell line expressing full-length TFPI was also set up in HEK293 cells. These stably transfected cells were selected for with 500 µg/ml G418 as the expression vector for TFPI contained the neomycin resistance cassette. TFPI in conditioned media was concentrated, quantified (by my colleague Verity Hockey) and used in the plasma assay (see section 2.5.3).

2.2.4 Cryopreservation of mammalian cells

HEK293T, HEK293 and protein S stable cell lines were cryopreserved in liquid nitrogen. Once cells reached confluence in a T175 flask they were washed with PBS and trypsinised with 1 ml of trypsin. Trypsin was neutralised by adding 10-20 ml complete media and cells were centrifuged at 1200 rpm for 5-10 minutes. The supernatant was discarded and cells were resuspended in 2 ml of cold complete media. Two ml of cold complete media with 10% dimethyl sulfoxide (DMSO) was added dropwise to the cells and the solution was mixed. Cells were aliquoted in 1 ml fractions in cryovials and stored in a cryo freezing container (Nalgene) at -80°C for a minimum of 3 hours and maximum over night prior to being transferred into liquid nitrogen.

To recover cryopreserved cells, vials were transferred from the liquid nitrogen to ice and then thawed at 37°C. Cells were seeded into a T75 flask with 20-30 ml of complete media to dilute out the amount of DMSO.

2.2.5 Expression and harvesting of WT and variant protein S

Both stable and transiently transfected cells were cultured in triple T175 flasks containing 75 ml of OptiMEM (GIBCO) supplemented with 10 µg/ml vitamin K and 100 mg/L $CaCl_2$. After 3-4 days the media was harvested, centrifuged and filtered to remove cell debris, and concentrated using a labscale tangential flow filtration (TFF) system (Millipore) with 10 kDa cut off membrane. The same procedure was

repeated for non-transfected HEK293T cells to obtain media, not containing protein S, to be used as a negative control in functional assays.

Stable cell lines were, in addition to being cultured in triple flasks, also cultured in CELLine adhere 1000 two-compartment bioreactors (IBS integra biosciences) according to the manufacturer's instructions. The cell compartment contained 15 ml OptiMEM, 10 µg/ml vitamin K, 100 mg/L $CaCl_2$ and the media compartment contained 1 L complete MEM media supplemented with vitamin K. Media was changed weekly and OptiMEM from the cell compartment, containing protein S, was harvested.

Media samples to be used in functional assays were dialysed with at least 3 changes of buffer in 3-5 L of 20 mM Tris-HCl pH 7.5, 140 mM NaCl, for 1 hour each time at room temperature. Later during my project media samples (protein S variants 74A, KTK, NEDM and NNEDM) were dialysed in 20 mM Tris-HCl pH 7.5, 140 mM NaCl, 3 mM $CaCl_2$. Samples were concentrated by Amicon Ultra centrifugal filter devices with a cut off of 50 kDa (Millipore), aliquoted and stored at -80°C.

2.3 Protein S purification

2.3.1 Protein S purification by FPLC on an anion exchange QFF column and by immunoaffinity chromatography

Protein S was purified by a two step method. Fast protein liquid chromatography (FPLC) was performed using an ÄKTApurifier UPC-10 (GE healthcare) and the Unicorn 5.1 (Build 407) software, strategy version 1.00 and consisted of a passage over an anion exchange Q Sepharose Fast Flow (QFF) column (section 2.3.1.1) followed by a second passage over an immunoimmunoaffinity column (section 2.3.1.3). This purification procedure had previously been optimised by Sofia Carlsson (in Professor Björn Dahlbäck's lab, Lund University, Malmö, Sweden).

2.3.1.1 Partial purification of protein S by FPLC on an anion exchange QFF column

Concentrated and dialysed conditioned media containing protein S supplemented with 4 mM ethylenediaminetetraacetic acid (EDTA) was applied to an anionic 5 mL of HiTrap QFF column (GE Healthcare) equilibrated with 20 mM Tris-HCl (pH 7.4), 100 mM NaCl, 4 mM EDTA, and 5 mM benzamidine-HCl (Sigma-Aldrich). The column was washed with 20 mM Tris-HCl (pH 7.4), 100 mM NaCl, and 5 mM benzamidine-HCl to first elute weakly bound proteins. Protein S was eluted from the column with 20 mM Tris-HCl (pH 7.4), 0.5 M NaCl, and 5 mM benzamidine-HCl. The Sepharose HiTrap™ QFF anion exchange column was stripped of more tightly bound proteins with 20 mM Tris-HCl, pH 7.4, 1 M NaCl and 5 mM Benzamidine-HCl.

To prevent cross-contamination between protein S preparations, the column was cleaned with 2 M NaCl in a reversed flow direction for 15 minutes and 1 M NaOH for 1-2 hours. The column was then stored in 20% ethanol.

2.3.1.2 Preparation of the immunoaffinity column

An immunoaffinity column for protein S was made with 2.5 mg of monoclonal mouse anti-human protein S MK21 antibody.[243] All monoclonal domain specific antibodies described in this thesis were kindly provided by Professor Björn Dahlbäck and Sofia Carlsson, Lund University, Malmö, Sweden and have been described in the literature.[243] The MK21 antibody is a calcium dependent antibody specific for the Gla domain of protein S. The antibody was thoroughly dialysed in the coupling buffer (0.2 M $NaHCO_3$, 0.5 M NaCl, pH 8.3). The antibody was coupled to a 1 ml HiTrap NHS-activated HP column (GE healthcare) according to manufacturer's instructions and any excess active groups were deactivated with ethanolamine and acetate. The immunoaffinity column was stored in 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% sodium azide.

2.3.1.3 Purification of protein S by FPLC on an immunoaffinity column

To lower the ionic strength (from 500 mM NaCl to ~166 mM NaCl) and add $CaCl_2$, two volumes of 7.5 mM $CaCl_2$ were added to the elution fraction from the anion

exchange QFF column, before injecting the sample on the immunoaffinity column. The fraction eluted from the QFF column containing protein S was applied to the equilibrated column (equilibration buffer: 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM $CaCl_2$). After washing with 1 M NaCl, the ionic strength was lowered by passing 3 column volumes of equilibration buffer over the column prior to elution of protein S with 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 10 mM EDTA. An excess of $CaCl_2$ was present in the tubes collecting the eluted protein S to neutralise the EDTA. The column was regenerated by stripping with 0.1 M glycine-HCl pH 2.7.

Samples to be used in functional assays were dialysed and concentrated as described in section 2.2.5.

2.3.2 Protein S purification by barium citrate precipitation and by FPLC on an anion exchange DEAE column

2.3.2.1 Barium citrate precipitation of protein S

Protein S was partially purified by barium citrate precipitation as previously described.[248] The conditioned media containing protein S that had previously been concentrated to 50 ml in the TFF (see section 2.2.5) was divided into two 50 ml tubes, each containing 25 ml media, and incubated on ice. 1.13 ml of a 0.5 M stock of trisodium citrate was added to each tube containing concentrated conditioned media and incubated on ice for 10 minutes. 1.13 ml of a 1 M stock $BaCl_2$ was added to each tube and the media was vortexed and incubated on ice for 1 hour. The sample was centrifuged at 5000 g for 30 minutes and the pellet that formed was resuspended in 25 ml of 100 mM $BaCl_2$, 100 mM NaCl and incubated on ice for 1 hour. The sample was spun as before and the pellet was resuspended in 15-50 ml of 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 150 mM trisodium citrate.

Barium citrate precipitated protein S was dialysed in 20 mM Tris-HCl pH 7.5, 140 mM NaCl for at least 1 hour at room temperature before dialysing it at 4°C.

2.3.2.2 Purification of protein S by FPLC on an anion exchange DEAE column

The purification of protein S on a diethylaminoethyl cellulose anion exchange (DEAE) column was optimised by my colleague Dr Josefin Ahnström. Briefly, a 5 ml DEAE column (GE healthcare) was equilibrated with 20 mM Tris-HCl pH 7.5, 150 mM NaCl. Barium citrate precipitated protein S was loaded onto the column at 1-1.5 ml/minute and protein S was eluted with a $CaCl_2$ gradient from 0-20 mM. The anion exchange DEAE column was stripped of more tightly bound proteins with 20 mM Tris-HCl pH 7.5, 50 mM $CaCl_2$, 1 M NaCl.
The column was cleaned and stored as described for the QFF column in section 2.3.1.1.

2.4 Analysis and characterisation of protein S

2.4.1 PNGase digestion

To determine whether the double band observed when running protein S on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was due to different glycosylation or cleavage, protein S was diluted to 8 ng/ul and a total of 10 µl were digested with 250 U of PNGase at 37°C over night.

2.4.2 SDS-PAGE and Native PAGE

SDS-PAGE under reducing (30% β-mercaptoethanol) and non-reducing conditions was performed to assess the purity of the different protein S preparations. Samples were heated at 70-90°C for 5-10 minutes. Electrophoresis was performed on 10% polyacrylamide cast gels, 4-12% or 10% precasted NuPAGE® Novex® Bis-Tris gels (Invitrogen) at 200 V for 30-40 minutes.
Samples in a native state were run on NativePAGE™ Novex® 3-12% precast Bis-Tris gels (Invitrogen), at 150 V for 90 minutes. Anode and cathode buffer were supplied by Invitrogen.
Gels were washed 3 x 5 minutes with water, stained for 1 hour with blue safe protein stain (Thermo scientific) and then destained in ddH_2O, stained by silver staining (see section 2.4.3) or used for Western blot analysis (see section 2.4.4).

2.4.3 Silver staining

Electrophoresis was performed on SDS-PAGE gels as described in section 2.4.2. During all steps in the silver staining procedure (Table 2.6) volumes of 50 ml/gel were used and incubations were performed at RT with shaking.

Proteins were fixed by incubating the gel for 30 minutes in 50% methanol, 10% glacial acetic acid (buffer A). An additional incubation in 5% methanol (buffer B) was performed for 15 minutes. The gel was washed with water 3 x 5 minutes and incubated with sodium thiosulphate (buffer C) for 2 minutes. After a second wash, 3 x 30 seconds, the gel was stained with Silver Nitrate (buffer D). The gel was washed 3 x 1 minute and developed with buffer E for up to 10 minutes. The colour development was stopped with EDTA (buffer F).

Buffer	Composition
A	50% CH_3OH, 10% CH_3COOH
B	5% CH_3OH
C (made up fresh)	1.26 mM $Na_2S_2O_3$*$5H_2O$
D	169.9 mM $AgNO_3$
E (per 50 ml, made up fresh)	283 mM Na_2CO_3, 25 µl of 37% HCOH, 1 ml solution C
F	37.6 mM Na_2EDTA

Table 2.6 Composition of silver staining buffers.

2.4.4 Western Blot

Western blots were performed by transferring proteins separated in unstained polyacrylamide gels (section 2.4.2) to a Hybond-ECL nitrocellulose membrane (Amersham Biosciences) or a PVDF-plus membrane (Osmonics INC) for SDS-PAGE and Native PAGE, respectively. Proteins were transferred to the hybond-ECL nitrocellulose membrane in a transfer buffer composed of 25 mM tris base, 190 mM glycine, 20% methanol. The PVDF-plus membrane was preincubated in methanol for 30 seconds, washed in water and immerged in the supplied transfer buffer (Invitrogen).

The transfer, for both types of membranes, was performed at 30 V for 60-75 minutes. Membranes were blocked with 4% milk for 40-60 minutes. Protein S was

detected with 20 ml of 0.588 µg/ml polyclonal rabbit anti-protein S antibody (DAKO) for 40-60 minutes. Following a 3 x 5 minutes wash with PBS, 0.1% Tween, membranes were incubated with 20 ml of 0.175 µg/ml peroxidise conjugated polyclonal goat anti-rabbit antibody (DAKO or Sigma Aldrich) for 40-60 minutes. The membrane was washed 4 x 5 minutes and developed with the chemiluminescent horseradish peroxidise (HRP) substrate Immobilon (Millipore) and Amersham Hyperfilm ECL (GE healthcare). Alternatively, to detect only γ-carboxylated protein S, 5 µg/ml (20 ml) of a monoclonal mouse antibody directed against γ-carboxyglutamic residues (American Diagnostica INC) was used as a primary antibody and 20 ml of 2.352 µg/ml polyclonal goat anti-mouse IgG HRP labelled antibody as a secondary antibody (DAKO).

2.4.5 Quantification of total protein

To quantify the total protein concentration in protein S preparations the absorbance at 280 nm was read in a NanoDrop spectrophotometer. Beer-Lambert law for concentration c=A/ ε L with an extinction coefficient ε 1% 9.5 was used.[161] The total protein concentration was then compared with the Enzyme-linked immunosorbent assay (ELISA) results to determine the % purity of protein S.

2.4.6 In-house ELISA with an in-house detection antibody

Protein S concentrations in conditioned media were determined using an in-house ELISA. A polyclonal rabbit anti-protein S antibody (1 µg/ml, DAKO) was immobilised onto 96-well Nunc Maxisorp microplates in 50 mM sodium carbonate buffer pH 9.6 at 4°C over night. All incubations were carried out in a plate shaker at 37°C for 1 hour unless stated otherwise. Washing steps were performed in triplicate with 250 µl PBS 0.1% Tween between each step. Wells were blocked with PBS containing 3% bovine serum albumin (BSA, Sigma or Santa Cruz) for 2 hours. A standard curve, 0-2 nM protein S, was made by either diluting normal plasma or plasma purified protein S (Enzyme Research Laboratories). Normal plasma is known to contain 25 µg/ml protein S. The reported MW of protein S ranges from 69-77 kDa.[161,162] In this thesis a MW of 69000 Da was assumed and

the concentration of protein S in normal plasma was calculated to be 362 nM. Protein S samples, 100 µl, were incubated in the wells for 1 hour. Bound protein S was detected by 4 nM of a mouse monoclonal antibody directed against the EGF3-4 domain of protein S (MK55) followed by 8 nM of an HRP-conjugated goat anti-mouse antibody (DAKO). PBS 0.5% BSA was used as buffer in which to dilute protein S and the antibodies. The plate was developed with 100 µl/well chromogenic substrate o-phenylenediamine dihydrochloride (OPD, Sigma), the enzymatic reaction was stopped with 50 µl/well 2 M H_2SO_4 and the absorbance was read at 492 nm. Data was analysed with GraphPad Prism 4.03 and data points were fitted to a sigmoidal dose response curve. Intra ELISA variability was determined by calculating the coefficient of variation (CV) between the same sample diluted to 2-3 different final concentrations in the same ELISA. Inter ELISA variability was determined by calculating the CV obtained for the same sample in (3-10) different ELISA.

2.4.7 In-house ELISA with commercially available antibodies

An in-house ELISA with commercially available antibodies was also developed to avoid dependence upon the availability of the MK55 antibody generously supplied by Professor Björn Dahlbäck. The assay was set up as described for the in-house ELISA in section 2.4.6 with the exception of 20 mM Tris-HCl pH 7.5, 140 nM NaCl, 3 mM $CaCl_2$ being used as a buffer and that protein S was detected with 4 nM of the mouse monoclonal antibody AHPS-5092 (Haematologic Technologies Inc).

2.4.8 Preparation of phospholipid vescicles

Activated platelets or cells exposing negatively charged phospholipids such as phosphatidylserine are required *in vivo* for coagulation to occur. In *in vitro* assays these are often replaced by phospholipid vescicles. Phospholipid vescicles were prepared during this project to be used as a procoagulant surface in functional assays and to assess protein S binding to phospholipids.

All phospholipids were from Avanti Polar Lipids Inc. Phospholipid mixtures in chloroform were prepared and the chloroform evaporated under a nitrogen

stream. The phospholipids were resuspended in 20 mM Tris-HCl pH 7.5, 140 mM NaCl for the plasma and FXa inhibition assay or in 25 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) pH 7.7, 150 mM NaCl (HN) for the FVa inactivation assays. Unilamellar phospholipids vesicles were obtained either by sonicating the phospholipids in an ice container at amplitude 22% for 7 minutes or by extruding them as previously described.[141] Briefly, extrusion was performed by passing the phospholipid mixture through a 100 nm polycarbonate membrane 19 times. Synthetic phospholipids 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) were used in the plasma assay, the plate binding assay and the FXa inhibition assay. Natural phospholipids L-α-phosphatidylserine (PS, brain extract), L-α-phosphatidylethanolamine (PE, egg extract) and L-α-phosphatidylcholine (PC, egg extract) were used in the FVa inactivation and prothrombinase assays.

2.4.9 Binding of protein S to phospholipids

As binding of protein S is a prerequisite for its function, binding of protein S and protein S variants to phospholipids was assessed.

Phospholipids vesicles (DOPS/DOPC/DOPE, 20:60:20), 25 µg/ml, were coated onto 96-well Nunc Maxisorp microplates in 50 mM sodium carbonate buffer pH 9.6 at 4°C over night. Washing steps were performed in triplicate with 250 µl/well 20 mM Tris-HCl pH 7.5, 140 mM NaCl, 5 mM $CaCl_2$, 0.3% BSA between each step and incubations were carried out in a plate shaker at 37°C. The same buffer was used to dilute protein S and the antibody in. Wells were blocked with 20 mM Tris-HCl pH 7.5, 140 mM NaCl, 5 mM $CaCl_2$, 3% BSA for 2 hours. Protein S or protein S variants, 0-120 nM, partially purified by barium citrate precipitation, were incubated in the wells in duplicate for 2 hours and were detected with an HRP-conjugated anti-protein S antibody (Affinity Biologicals) for 45 minutes. Controls were performed by assessing binding of protein S to phospholipids in the presence of 10 mM EDTA and by assessing aspecific binding to the plate in the absence of phospholipids and presence of $CaCl_2$. The plate was developed, stopped and read as previously described for the ELISA. Data was analysed with GraphPad Prism 4.03 and curves were fitted to a one site binding hyperbola. The

$K_{d(app)}$ for protein S binding to the phospholipids were derived and that of protein S variants was compared with that of WT protein S.

2.4.10 Binding of protein S to domain specific monoclonal antibodies

To assess the correct folding of protein S variants, their binding affinity to domain specific monoclonal antibodies was compared to that of WT protein S.
Domain specific monoclonal antibodies MK21 (Gla domain), MK54 (EGF1 domain) and MK61 (SHBG domain), 1 µg/ml, were coated on 96-well Nunc Maxisorp microplates as described for the ELISA. Washing steps were performed in triplicate with 250 µl 20 mM Tris-HCl pH 7.5, 140 mM NaCl, 3 mM $CaCl_2$, 0.1% Tween between each step. Incubations were carried out in a plate shaker at 37°C for 1 hour unless stated otherwise. Wells were blocked with 200 µl 20 mM Tris-HCl pH 7.5, 140 mM NaCl, 3 mM $CaCl_2$, 3% BSA for 2 hours. Protein S, 0-160 nM was incubated in the plate for 1 hour and was detected by a rabbit polyclonal antibody against protein S followed by a goat anti-rabbit HRP-conjugated antibody. Protein S and the antibody were diluted in a Tris-HCl pH 7.5, 140 mM NaCl, 3 mM $CaCl_2$, 0.5% BSA buffer. The plate was developed, stopped and read as previously described for the ELISA. Data was analysed with GraphPad Prism 4.03 and data was fitted to a one site binding hyperbola. The $K_{d(app)}$ for protein S binding to the monoclonal antibodies were derived and that of protein S variants was compared with that of WT protein S.

2.5 CAT assay

2.5.1 Overview of CAT

CAT is an assay that allows measurement of thrombin generation. It was used during this project to evaluate the different cofactor activity of protein S and protein S variants toward APC and TFPI. It is a plasma-based assay in which a coagulant response is initiated by the addition of TF, $CaCl_2$ and phospholipids and the amount of thrombin generated is measured through the cleavage of its fluorogenic substrate over time and quantified by reference to a standard calibrator. Phospholipids are added into the reaction to provide a surface on which coagulation can occur once triggered by TF. As the plasma is citrated and

most coagulation factors are calcium dependent the reaction does not start until calcium is added into the reaction. Calcium is added together with Z-Gly-Gly-Arg-AMC (Bachem), a fluorogenic substrate of thrombin. Thrombin generation is described and quantified by the lag time (which approximates the clotting time), peak height (maximal velocity of thrombin generation) and the endogenous thrombin potential (ETP) which is the total amount of thrombin generated over time and corresponds to the area under the curve.

2.5.2 Evaluation of the protein S-APC cofactor activity

Protein S deficient plasma (Affinity Biologicals), 80 µL, was incubated with 65 µg of corn trypsin inhibitor (CTI, Haematologic Technologies Inc) per ml of plasma to inhibit contact activation, 50 µM phospholipid vesicles (DOPS/DOPC/DOPE, 20:60:20), 1 pM TF (Dade Innovin; Dade Behring), 4 to 16 nM APC (Enzyme Research Laboratories) with 0-120 nM protein S, in a final volume of 100 µL (all concentrations are final). A polyclonal anti-TFPI antibody (Haematologic Technologies Inc), 100 nM, was used to inhibit any protein S cofactor activity toward TFPI. Polyclonal antibodies against protein S (1500 nM, DAKO) and protein C (130 nM, Sigma-Aldrich or Haematologic Technologies Inc) were used as further controls. All conditions were performed in duplicate and assays were performed at least twice. Thrombin generation was assessed using a Fluoroscan Ascent FL Plate Reader (Thermo Lab System) in combination with the Thrombinoscope software (SYNAPSE BV).

Thrombin generation was initiated by automatic dispensation of 20 µL of 2.5 mM Z-GlyArg-AMC-HCl (Bachem), 2.5% Me_2SO, 20 mM Tris-HCl (pH 7.5), 60 mg/mL BSA, 100 mM $CaCl_2$ into each well. The reaction was performed at 37°C and measurements were performed with an excitation and emission wavelength of 390 nm and 460 nm, respectively. To compare the cofactor activity of protein S and protein S variants towards APC, the changes in peak height and ETP were evaluated.

2.5.3 Evaluation of the protein S-TFPI cofactor activity

To evaluate the ability of protein S to enhance the anticoagulant activity of TFPI, protein S deficient plasma (Affinity Biologicals, Hyphen Biomed or Enzyme Research Laboratories) was incubated with CTI, phospholipids, TF (as described in section 2.5.2), 0-10 nM TFPI (either purified, kind gift of the Chemoserotheraputic Institute Kaketsuken, or in concentrated conditioned media) and 0-120 nM protein S or protein S variants in a final volume of 100 µL (all concentrations are final). A polyclonal antibody against protein C (130 nM, Sigma-Aldrich or Haematologic Technologies Inc) was included, as required, to inhibit protein S cofactor activity towards any protein C activated during the assay. To show the specificity of the system polyclonal antibodies against protein S (1500 nM, DAKO) or polyclonal antibodies against TFPI (100 nM, Haematologic Technologies Inc) were used. All conditions were performed in duplicate and assays were performed at least twice. Thrombin generation was assessed as described in section 2.5.2. To compare the cofactor activity of protein S and protein S variants towards TFPI, the changes in peak and lag time were evaluated.

2.6 FVa inactivation assay

2.6.1 Overview of the FVa inactivation assay

The FVa inactivation assay is an assay with pure components that was used during this project to assess the ability of protein S and protein S variants to enhance APC mediated FVa inactivation. FVa inactivation by APC is indirectly measured by performing a prothrombinase assay. The prothrombinase assay relies on the amount of FVa that has not been inactivated by APC and its ability to form a complex with FXa and activate prothrombin. The amount of thrombin generated is then measured by the rate at which it cleaves its chromogenic substrate S-2238 (Chromogenix).

2.6.2 FV activation

To obtain a source of FVa to assess APC mediated FVa inactivation, FV was first activated. A previously prepared and reported[125] double variant of FV, FV R506Q/R679Q, was activated with 0.5 U/ml human thrombin at 37°C for 10 minutes. This variant can not be cleaved in Arg506 and Arg679 allowing evaluation of cleavage at Arg306 by APC which is the site mainly enhanced by protein S (see section 1.4.3.1.1). The reaction was stopped by the addition of hirudin (5 U/ml, final concentration). The activated FVa variant was used as a substrate for APC to determine the ability of protein S to enhance cleavage of Arg306 of FVa by APC.

2.6.3 FVa inactivation

The FVa inactivation was performed either by evaluating the cofactor activity of increasing amounts of protein S (0-100 nM) at 10 minutes or by evaluating the cofactor activity of a fixed concentration (50 nM) of protein S at different timepoints (0-20 minutes).

The highly purified protein S preparations, 0-100 nM, were incubated with 0.5 nM APC, 25 µM phospholipids vesicles (PS/PC/PE, 10:70:20) and 0.8 nM FVa R506Q/R679Q in HN, 5 mM $CaCl_2$, 5 mg/ml BSA (HNBSACa^{2+}, only high purity grade BSA from Sigma A7030 was used) in a total volume of 50 µl (all concentrations are final). The solution was incubated at 37°C for 10 minutes and the reaction was stopped by performing a 1:25-fold dilution in ice cold HNBSACa^{2+}. The remaining FVa activity was measured in a prothrombinase assay.

Time course experiments were performed to derive the rate constant of FVa cleavage. APC, 0.5 and 3 nM, and 50 nM protein S were used and aliquots were quenched at intervals between 0-20 minutes. The remaining FVa activity was measured in a prothrombinase assay. Controls containing FVa R506Q/R679Q in the absence of APC either in the presence or absence of protein S and protein S variants were performed. To calculate the pseudo-first order rate constant for

APC mediated cleavage at Arg306, the previously reported equation (Eq2)[141] was used:

Eq2. $Va_t = Va_0 * e^{-k306*t} + C * Va_0 * (1 - e^{-k306*t})$

Va_t represents the cofactor activity determined at time *t*, Va_0 is the cofactor activity determined at time point 0, *C* is the remaining procoagulant cofactor activity of FVa after cleavage at position 306, and *k*306 is the rate constant of cleavage at position 306.

2.6.4 Prothrombinase assay

The amount of FVa inactivated was indirectly measured by performing a prothrombinase assay. An aliquot, 25 µl, of the FVa inactivation reaction was incubated with phospholipid vesicles (PS/PC 10:90) and FXa in the presence of $CaCl_2$. The reaction was initiated by the addition of prothrombin, in a final volume of 125 µl. The buffer contained HN and 0.5 mg/ml ovalbumin. Final concentrations were 50 µM PS/PC, 5 nM FXa and 0.5 µM prothrombin. The solution was incubated at 37°C for 2 minutes and the reaction was terminated by an 8-fold dilution in 50 mM Tris-HCl pH 7.9, 100 mM NaCl, 20 mM EDTA, 1% PEG 6000. The amount of thrombin generated was measured by cleavage of its chromogenic substrate S-2338 (Chromogenix) at 405 nm for 15 minutes at 30 seconds intervals. The cofactor activity of protein S and protein S variants towards APC was compared.

2.7 FXa inhibition assay

2.7.1 Overview of the FXa inhibition assay

The FXa inhibition assay was performed to assess the different cofactor activity of protein S and protein S variants towards TFPI mediated inhibition of FXa. In the FXa inhibition assay the ability of FXa to cleave its chromogenic substrate S-2765 is measured over time. The ability of TFPI to inhibit FXa and of protein S to enhance the TFPI anticoagulant activity is measured as a change in the rate of

substrate proteolysis. The cleavage of the chromogenic substrate S-2765 was measured for up to 1 hour at 1 minute intervals.

2.7.2 FXa inhibition assay

Purified human FXa (final concentration of 0.5 nM; Hyphen Biomed) was added to wells already containing the chromogenic substrate S-2765 (200 µM; Chromogenix), phospholipids (10 µM), $CaCl_2$ (5 mM), in the presence or absence of varying concentrations of purified TFPI (0-10 nM, kind gift of the Chemoserotheraputic Institute Kaketsuken) and protein S or protein S variants (0-160 nM). The reactions were set up in a buffer containing 20 mM Tris-HCl buffer pH 7.5, 140 mM NaCl, 5 mg/ml BSA (only high purity grade BSA from Sigma A7030 was used), with a final volume/well of 100 µl. The hydrolysis of S-2765 by FXa was followed over time by measuring the A_{405} at regular intervals for up to 1 hour at room temperature. A control was obtained by incubating FXa and S-2765 in the absence of TFPI, in the presence or absence of WT or variant protein S. A blank was obtained by including TFPI and S-2765 but not FXa. All conditions were performed in duplicate and assays were repeated at least twice.

2.7.3 Kinetic data analysis of FXa inhibition by TFPI in the presence and absence of protein S

To calculate the inhibition constant of TFPI for FXa in the presence and absence of protein S, 0-8 nM TFPI ± 100 nM protein S were used. The assay was either set up as described in section 2.7.2 by starting the assay with the addition of FXa or, alternatively, FXa was present in the wells and the reaction was started with the addition of TFPI and S-2765.
Data was fitted with the previously described equation (Eq3):[249]

Eq3. $A_t = A_0 + V_s t + (V_0 - V_s)\{1-\exp(-k_{obs}t)\}/k_{obs}$

Where t represents time, A_t represents the absorbance at time point t, A_0 the absorbance at time zero, V_0 the initial velocity, V_s the final steady state velocity of

S-2765 conversion and k_{obs} the apparent rate constant for the transition from V_0 to V_s.

The maximum initial velocity V_{max} (FXa conversion of S-2765 in the absence of TFPI) was divided by the initial velocities at each time point and the resulting V_{max}/V_0 value was plotted against the concentration of TFPI. Values were fitted with a linear regression and the x intercept was used to calculate the K_i value with the following formula (Eq4):

Eq4. $K_i = -x_{intercept}/(1+[S]/K_m)$

Where [S] represent the concentration of the chromogenic substrate and K_m, the Michaelis-Menten constant, the concentration of substrate that results in half maximal cleavage by FXa.

3 RESULTS

The results of this thesis are divided into 4 sections. The first (section 3.1) describes the mutagenesis, expression and purification of protein S. The protein S obtained either in concentrated conditioned media or purified was then quantified by ELISA and analysed for multimeric content and cleavage (section 3.2). In the final two sections, 3.3 and 3.4, the cofactor activity of protein S and protein S variants towards APC and TFPI respectively are evaluated.

3.1 Mutagenesis, expression and purification of protein S

3.1.1 Selection and generation of protein S variants

Based on the domains of protein S known from the literature to be important for APC and TFPI cofactor activity (outlined in section 1.5.1 and 1.5.2) protein S variants with substitutions in the Gla, TSR, EGF1 and EGF2 were produced. Composite variants in these N-terminal domains produced during this thesis were; protein S Face1, Face2, Gla1, TSR, NEDM, NNEDM and KTK. Twelve additional alanine point variants in the EGF1 domain and 12 alanine point variants in the EGF2 domain of protein S were made available through collaboration with Dr S.M. Rezende (Universidade Federal de Minas Gerais, Brazil). Additional point variants expressed during this project are protein S N74A, D78A, Q79A and D95N resulting in a total of 35 variants covering 49 amino acid residues of protein S.

Table 3.1 lists the variants produced during this project and indicates in which domain the substitutions have been introduced. Single point variants were named according to the residue that was mutated. The name given to the composite variant is shown in Table 3.1.

Table 3.2 shows the N-terminal amino acid residues of protein S highlighting those that have been mutated during this project. Variants were tested both for their APC cofactor activity and their TFPI cofactor activity.

Domain	Name of variant	Residues mutated
Gla	Face1	N33S/P35T/E36A/Y39V
Gla	Face2	L21T/ N23S/K24Y/R28F/D34S/Y41W/L45T
Gla/EGF1	Gla1	D38N/V46G/K97Q
TSR/EGF1	TSR	R49Q/R60Q/D68N/R70Q/D78N
TSR		N74A
EGF1	NEDM	N86A/E87Q/D88A/M91A
EGF1	NNEDM	N74A/N86A/E87Q/D88A/M91A
EGF1	KTK	K94A/T103A/K105A
EGF1 (from Dr S.M. Rezende)		K94,D95,K97,S99,T101,T103, K105,P106,W108,Q109,E111,K112
EGF1		D78A,Q79A,D95N
EGF2 (from Dr S.M. Rezende)		D135,N136,T137,Y141,H142,S144, K146,S153,N154,K155,K156,D157

Table 3.1 Protein S variants produced during this thesis.
Single or point variants of protein S were produced by site-directed mutagenesis targeting residues located in the Gla domain, TSR domain, EGF1 or EGF2 domain.

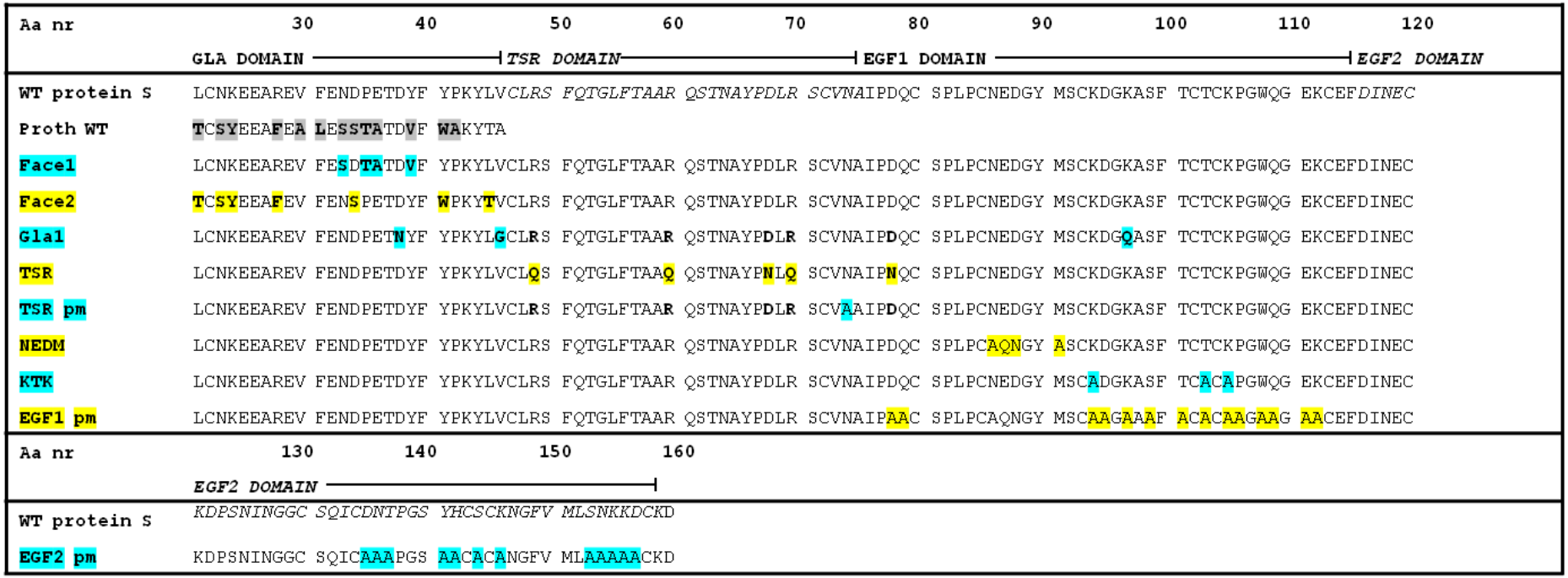

Table 3.2 N-terminal amino acid sequence of protein S, prothrombin and protein S variants.
Amino acid residues of the Gla domain of protein S that are not conserved in prothrombin were swapped for those of prothrombin in the composite variants Face1 and Face2. No mutations in the first 20 amino acid residues of protein S were made in an attempt to not disrupt phospholipids binding. Gla1 and NEDM were made by mutating residues into similar non charged residues. TSR was created by substituting charged residues in the TSR domain with polar residues. KTK and all EGF1 and 2 point variants were mutated to alanine. NNEDM (not shown) were made by introducing the N74A substitution into NEDM. Domains are labelled below the Aa nr. Amino acids in the TSR and EGF2 domain are in italics in WT protein S. Aa nr, amino acid number; Proth, prothrombin; pm, point variants.

Protein S Face1 and Face2 variants, previously evaluated for their APC cofactor activity by Saller *et al.*[224] are chimeras of the human protein S Gla domain and the human prothrombin Gla domain. These were produced in our lab by Ms Y. Yu by inverse PCR (KOD Hot Start DNA Polymerase Kit, Novagen) and site-directed mutagenesis (QuikChange site-directed mutagenesis Kit, Stratagene).

To identify residues in the Gla and EGF1 domain that might interact with APC or TFPI, these domains were aligned with homologous domains of other coagulation factors. Non conserved amino acids were selected and clusters of amino acids with surface exposed R groups were selected. This was done by using the structural protein S model spanning the Gla domain to the EGF1 domain created by Villoutreix *et al.*.[193]

As the TSR is unique to protein S, no swap substitutions could be made. Hence, charged residues in the TSR were substituted with the most similar non-charged amino acid (R to Q and D to N), to minimize the risk of adverse conformational changes, resulting in the generation of the TSR variant.

Dr S.M. Rezende (Universidade Federal de Minas Gerais, Brazil) mutated residues between the third/fourth and fifth/sixth Cys residues of EGF1 and EGF2 as these regions on the EGF-like domains are likely to be involved in protein interaction as suggested by (i) the highly divergent sequence of the B region; (ii) the association of dysfunctional proteins with natural *PROS1* mutations in these regions and (iii) the location of many mutations in these regions associated with dysfunctional proteins in genes coding for other EGF-containing coagulation proteins, such as FVII, FIX and protein C.[202,250]

All mutations were confirmed by sequencing. Subcloned fragments were also sequenced across the ligation sites to confirm correct insertion.

3.1.2 Expression of protein S

Protein S was expressed by transient or stable transfection of HEK293T or HEK293 cells, respectively. The amount of protein S harvested from HEK293 cells over time did not result in a decreased expression according to ELISA results, suggesting that all cells were stably transfected after the initial 4 weeks selection with blasticidine.

WT protein S expressed approximately at 1.5-2 µg/ml, as determined by ELISA, in triple flasks and on average at 60 µg/ml in the CELLine adhere 1000 two-compartment bioreactor, with expression levels increasing over time in the latter. The high concentration obtained in the bioreactor is a consequence of harvesting the media after a week rather than after 3 days and of the bioreactor concentrating protein S in the cell compartment. Expression levels of the variants were not formally assessed against that of WT protein S in a dedicated experiment where number of cells and transfection efficiency were taken into account. However, the great majority of variant protein S preparations expressed well (1-2 µg/ml), with the notable exceptions of protein S T108A and protein S N136A, which expressed at lower than 0.5 µg/ml. Protein S expressed from transient transfections yielded up to 10 times less than protein S expressed from a stable cell line. Harvested protein S media was either dialysed, concentrated and assayed directly, or purified by FPLC.

3.1.3 Purification of protein S by FPLC on an anion exchange QFF column and immunoaffinity column

Harvested and concentrated media was supplemented with EDTA and protein S was partially purified on a Sepharose HiTrap QFF anion exchange column (elution buffer: 20 mM Tris-HCl pH 7.4, 0.5 M NaCl, 5 mM Benzamidine). Figure 3.1A (left chromatogram) illustrates this purification step on WT protein S with the milli absorbance unit (mAU) at 280 nm displayed against the volume of liquid flowing through the column. As expected, it shows that large amounts of protein (mAU values of ~260) are present in the flowthrough, although these are diluted in a large volume (~20 ml in this representative chromatogram). A high and narrow elution peak was obtained, while very small washing and stripping peaks could be observed. Fractions of the flowthrough, washing, elution and stripping peaks from the anion exchange chromatography were analysed by SDS-PAGE. Proteins in the respective fractions were stained with Blue Safe protein stain (Figure 3.1B). Protein S was specifically detected by an anti-protein S antibody on a Western blot (Figure 3.1C). The stained SDS-PAGE gel shows a large amount of protein is present both in the flowthrough and elution fraction (Figure 3.1, Panel B, first 4 lanes after the marker). The bands of protein in the flowthrough fraction

are quite faint as proteins were diluted into a large volume (flowthrough volume of ~20 ml). A large protein band is present in the elution fraction that migrates with the same mobility as commercially available plasma purified protein S (last lane of gel, labelled as Ctrl). Western blot analysis (Figure 3.1, Panel C, first 4 lanes) confirmed that protein S was present in the elution fraction. A faint band of higher molecular weight was also present in the elution fractions. A higher MW band was sometimes visualised on gels when high amounts of purified protein S was loaded and is likely to correspond to aspecific binding of the secondary antibody. Protein S was also detected in the flowthrough of the QFF anion exchange column using Western Blot (Figure 3.1C). According to ELISA results, the amount of protein S lost in the flowthrough and washing peak varied from batch to batch but was generally between 5-15%, while the majority of protein S (~80%) was found in the elution fraction. This is compatible with what can be seen in the elution fraction of the Western blot (Figure 3.1C). Protein S content and total amount of protein in the elution peak were measured, respectively, by ELISA and total protein assay and suggested a mean purity of ~25%.

To obtain high purity protein S the elution fraction from the Sepharose HiTrap QFF anion exchange column was applied on an immunoaffinity column (elution buffer: 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 mM EDTA). A representative chromatogram is shown in Figure 3.1A (right chromatogram). Fractions were collected and the flowthrough, washing peak, elution peak and stripping peak were analysed by SDS-PAGE. The proteins were visualised by Blue Safe protein staining (Figure 3.1B, lane 5-8 after the marker); protein S was specifically detected by polyclonal anti-protein S antibodies following Western blot (Figure 3.1C, last four lanes). The elution fraction from the immunoaffinity column in the stained SDS-PAGE gel (Figure 3.1B) shows a single band migrating with the same mobility as plasma purified protein S (last lane of gel, labelled as Ctrl). Western blot analysis (Figure 3.1C) confirms that this is protein S, suggesting pure protein S could successfully be isolated following this two step purification procedure. It is observed that the majority of contaminants visualised in the elution fraction of the QFF anion exchange column (Figure 3.1B) are present in the flowthrough of the immunoaffinity column. Protein S was, however, also present in the flowthrough fraction and washing fraction of the immunoaffinity column (Figure 3.1C). It should be noted that ten fold more in terms of volume of

the washing fraction was loaded in comparison with the other fractions to determine whether this contained any protein S. The high amount of protein S in the flowthrough fraction partially explains why the absorbance on the chromatogram of this fraction is so high in comparison with that of the elution fraction (considering ~25% of the proteins loaded onto the immunoaffinity column should be protein S). However, the high absorbance in the flowthrough in comparison to the elution peak is also due to the presence of Benzamidine in the loaded sample (see section 2.3.1.1) as this absorbs at 280 nm. Benzamidine is, on the other hand, not present in the elution buffer.

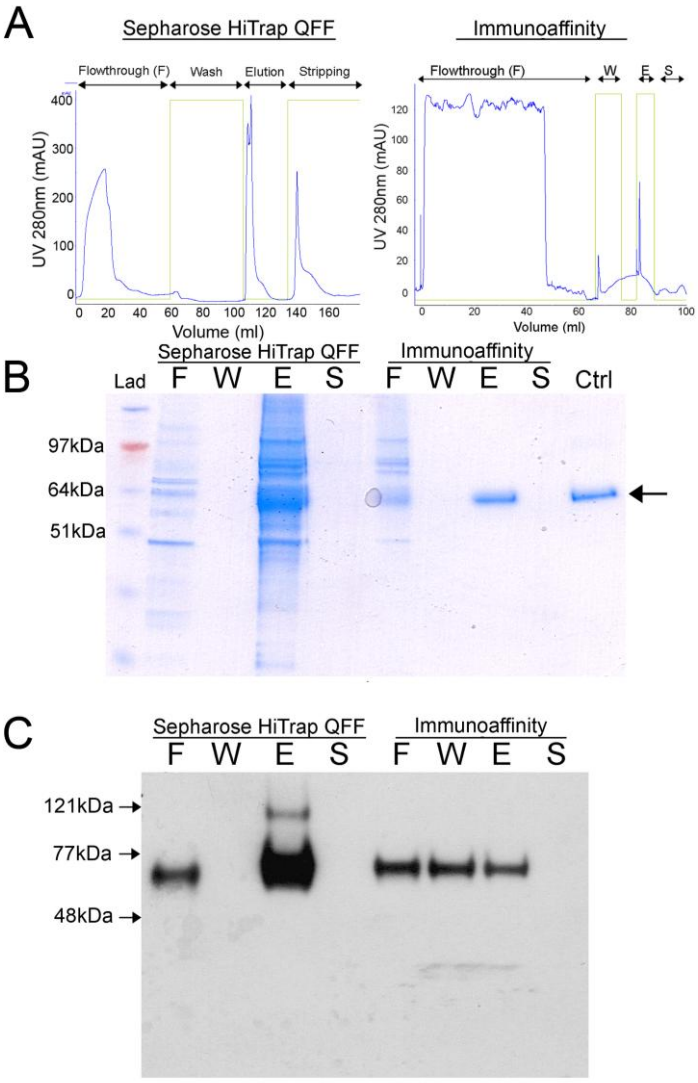

Figure 3.1 Purification of WT protein S by FPLC on a Sepharose HiTrap QFF anion exchange column followed by an immunoaffinity column and analysis of elution fractions. (A) Purification of protein S on sepharose QFF anion exchange purification (left chromatogram) followed by immunoaffinity purification (right chromatogram). The 280 nm absorbance (blue line) is plotted against the elution volume. The vertical green lines represent a change of buffer. Total protein content in the fractions was detected by Blue safe protein stain of an SDS-PAGE (B) and protein S was specifically detected with a polyclonal antibody against protein S (C). F, flowthrough; W, washing; E, elution; S, stripping.

To evaluate why so much protein S was present in the flowthrough fraction from the immunoaffinity column, γ-carboxylation of protein S was firstly assessed as the monoclonal MK21 antibody used for the purification recognises the Gla domain of protein S. Importantly, when fractions from the Sepharose HiTrap QFF anion exchange column were analysed by Western blot with a monoclonal anti-Gla antibody (recognising only γ-carboxylated protein S), only protein S in the elution fraction was recognised by both antibodies (compare Figure 3.2, detection with monoclonal anti-Gla antibody with Figure 3.1C, detection with polyclonal antibody). This suggests that, non γ-carboxylated protein S is in the flowthrough, indicating that this method selectively purifies γ-carboxylated protein S. This was thought to be of considerable importance, as only γ-carboxylated protein S is functional. A consequence of this is that all protein S loaded onto the immunoaffinity column was γ-carboxylated. The finding that protein S in the elution and flowthrough fractions from the immunoaffinity column was γ-carboxylated was confirmed by recognition by the monoclonal anti-Gla antibody (Figure 3.1C, detection with polyclonal antibody and Figure 3.2, detection with monoclonal anti-Gla antibody). Ten fold more of the washing fraction was loaded in the gel where protein S was detected with the polyclonal antibody in comparison with the one in which it was detected with the monoclonal antibody recognising only γ-carboxylated Gla domain. However, when ten fold more of the washing fraction was loaded (not shown) the washing fraction was recognised by the anti-Gla antibody.

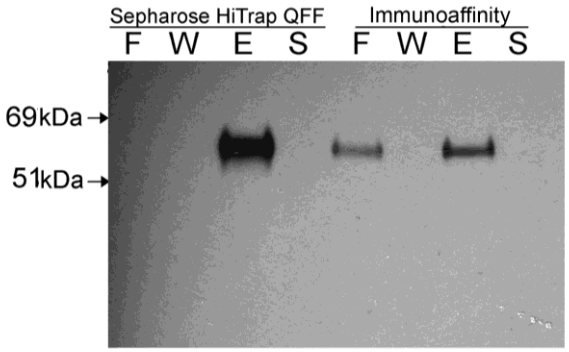

Figure 3.2 Western blot analysis of protein S in the fractions obtained during purification on the Sepharose HiTrap QFF and on the immunoaffinity column.
Flowthrough (F), washing (W), elution (E) and stripping (S) peaks of, respectively, Sepharose HiTrap™ QFF purification (lane 1-4) and affinity purification (lane 5-8) were blotted with a monoclonal anti-Gla antibody recognising only γ-carboxylated Gla domains.

The finding of a single band in the elution fraction of the immunoaffinity column (Figure 3.1B), together with its identification as protein S by Western blot (Figure 3.1C), demonstrates that pure protein S could successfully be isolated using this two-step purification method. The results of Figure 3.2 indicate that this purified protein S was γ-carboxylated. However, a large part of γ-carboxylated protein S was also present in the flowthrough of the immunoaffinity column. To investigate this further, a similar sample containing protein S was applied to the immunoaffinity column continuously over night at a greatly reduced flow rate of 0.5 ml/minute to increase contact time. However, this did not appreciably increase the amount of protein S in the elution fraction. When the flowthrough fraction was reapplied to the immunoaffinity column, additional protein S could be purified and eluted with EDTA (results not shown), suggesting the protein S in the first flowthrough was more likely associated with low binding capacity of the immunoaffinity column. Consequently, the flowthrough was reapplied to the column until all protein S was purified and was no longer in the flowthrough. All fractions from the elution peak from the immunoaffinity column containing protein S were pooled, dialysed and concentrated on spin columns. This purification procedure was used during the early part of my work to purify WT protein S, protein S D95A, protein S Face2 and protein S TSR.

3.1.4 Purification of protein S by barium citrate precipitation and by FPLC on an anion exchange DEAE column

Due to the low binding capacity of the immunoaffinity column, an alternative purification method for protein S was developed. WT protein S and protein S N74A, protein S KTK, protein S NNEDM variants and some additional protein S D95A were purified according to this alternative protocol.

For this, protein S was first partially purified from concentrated conditioned media by barium citrate precipitation. This favours precipitation of protein S that is γ-carboxylated and allows removal of any non γ-carboxylated protein S.[248] Following barium citrate precipitation, protein S was then further purified by FPLC on a DEAE anion exchange column. This was optimised during the last year of my work by my colleague Dr Josefin Ahnström. A representative chromatogram is

shown in Figure 3.3A where WT protein S was eluted with a calcium gradient of 0-20 mM $CaCl_2$. Conditioned media, barium citrate precipitated protein S and the various fractions eluted from the DEAE anion exchange column were run on SDS-PAGE and analysed by silver staining or by Western blot (Figure 3.3B and C, respectively). The silver stained gel shows that barium citrate precipitation removes most of the contaminants present in the concentrated conditioned media (Figure 3.3B). The purity of the barium citrate precipitated protein S, however, varied. Protein S expressed in high amounts from stably expressed cells was relatively pure following barium citrate precipitation (as can be seen in Figure 3.3B), while barium citrate precipitated protein S from transiently transfected cells contained more contaminants (results not shown). The lower band contaminant seen in the silver stain in the barium citrate precipitated material is partially removed following passage on the DEAE anion exchange column and can be seen in the flowthrough fraction (Figure 3.3B). The higher band in the barium citrate precipitated sample and the main band in the elution fractions were identified as protein S on the Western blot (Figure 3.3C). Fractions from the elution peak that contained protein S and trace amounts of contaminants were pooled, concentrated and used in functional assays. An overall yield, as determined by ELISA, after barium citrate precipitation and purification on a DEAE anion exchange column, of 10-30% was obtained.

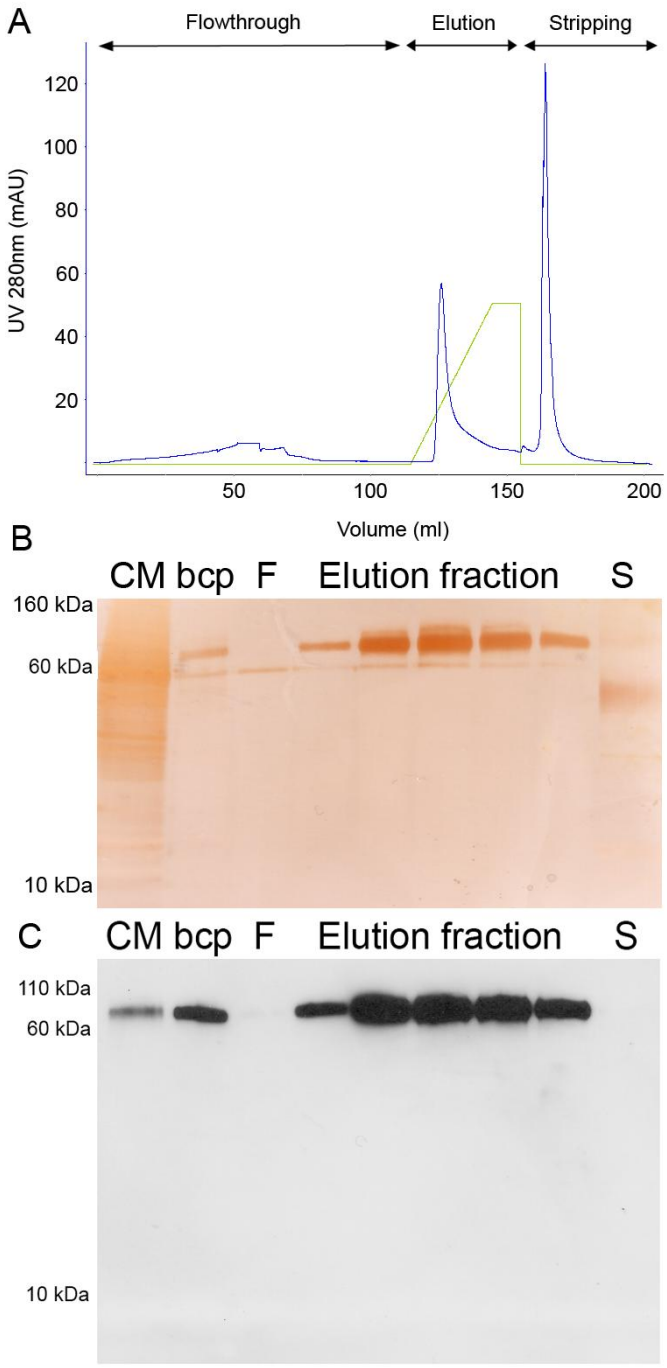

Figure 3.3 Purification of barium citrate precipitated WT protein S on an anion exchange DEAE column and analysis by SDS-PAGE.
(A) The 280 nm absorbance (blue line) is plotted against the ml of liquid passing through the column. The green lines represent a change of buffer; equilibration buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl), elution buffer (20 mM Tris HCl pH 7.5, 150 mM NaCl, 0-20 mM $CaCl_2$) and stripping buffer (20 mM Tris HCl pH 7.5, 1 M NaCl, 50 mM $CaCl_2$). Peaks were collected and analysed. Total protein was detected by silver staining (B) and protein S was detected by a polyclonal antibody (C). Small fractions from the elution peak were collected separately and run on the gel. CM; conditioned media, bcp; barium citrate precipitated protein S, F; flowthrough, S; stripping.

3.2 Characterisation of recombinant protein S

3.2.1 Quantification of protein S by ELISA

The concentrations of purified protein S and protein S in conditioned media were determined by ELISA. A standard curve of 0-2 nM protein S was fitted to a sigmoidal dose-response curve and samples were diluted to ensure they fell in the linear part of the curve (generally between 0.25-1.5 nM protein S). The plasma purified protein S gave a higher absorbance reading than the corresponding concentration of protein S in normal plasma when detected with the monoclonal antibodies (Figure 3.4). This could potentially be due to slightly different amounts of protein S in the two standard preparations, to plasma protein S being monomeric and purified protein S multimeric (see section 3.2.3) or due to the fact that ~60% of plasma protein S is bound to C4BP and is less recognised by the monoclonal antibody.

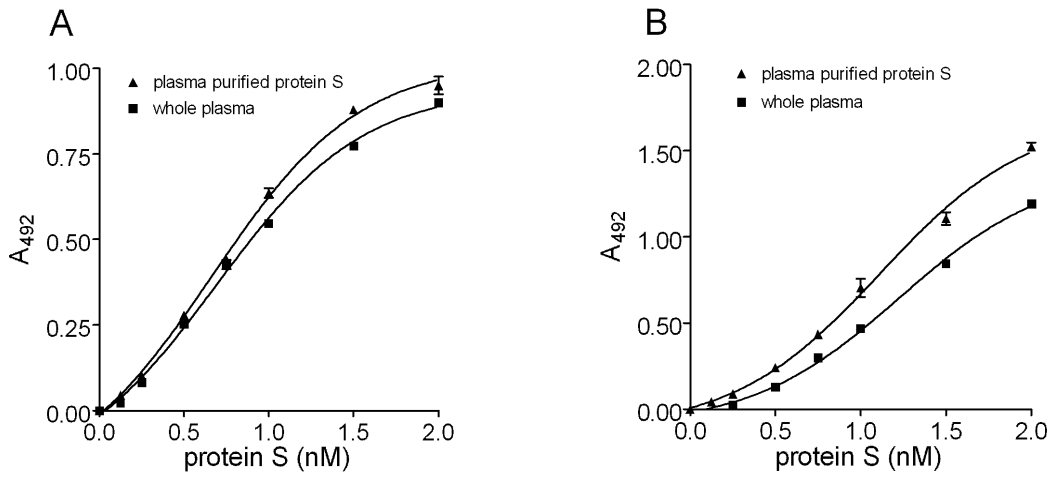

Figure 3.4 Standard curves of protein S used in the ELISA.
Plasma purified protein S or protein S in normal plasma were used as a standard curve (0-2 nM) and were detected either with the MK55 monoclonal antibody (A) or with the commercially available monoclonal antibody AHPS 9052 (B).

When protein S in normal plasma (362 nM) was analysed by Western blot alongside plasma purified protein S and detected by a polyclonal antibody, bands of similar intensity were obtained suggesting that the reason behind the difference in absorbance observed between plasma purified protein S and protein S in media was not due to different concentrations of protein S. When my quantified

recombinant protein S preparations (pure or in conditioned media) were analysed by Western blot, bands of equal intensity of purified protein S and protein S in conditioned media were only obtained if the concentration of pure protein S was determined with the plasma purified protein S standard and if protein S in conditioned media was determined with the normal plasma standard (results not shown). Since the monoclonal antibody MK55 recognises the EGF3-4 domains and the difference in detection of plasma protein S and purified plasma protein S by the monoclonal antibody AHPS 9052 is not 60% (difference between total and free protein S) it is unlikely that the differences in detection are due to the fraction of bound protein S in plasma. Multimerisation was analysed in section 3.2.3 and might account for the differences in detection between plasma protein S and purified protein S.

The concentration of purified protein S was, therefore, determined with the plasma purified protein S standard curve; that of protein S in conditioned media was determined with the normal plasma standard curve. Intra and inter ELISA variability varied, with purified protein S preparations giving higher CV values than protein S in conditioned media. Generally, the intra-assay CV was less than 10% for purified protein S and less than 5% for protein S in media. The mean inter-assay CV was 11% with samples being assessed by ELISA a minimum of 3 times.

3.2.2 Analysis of the purity of protein S

SDS-PAGE and silver staining of purified protein S preparations were performed to assess purity. WT protein S and protein S variants analysed under non-reducing conditions, irrespective of the purification method, migrated predominantly as a single band (Figure 3.5) of identical size to commercially available plasma purified protein S (see Figure 3.1B). A band of lower molecular weight than protein S was observed in some gels. This was, however, very faint in comparison with the protein S band and could only be visualised following long development of the silver stain. The concentration of purified protein S samples was assessed by absorbance at 280 nm, which corroborated the concentrations obtained by ELISA, suggesting that the protein S preparations were suitably pure for the *in vitro* experiments used in this project.

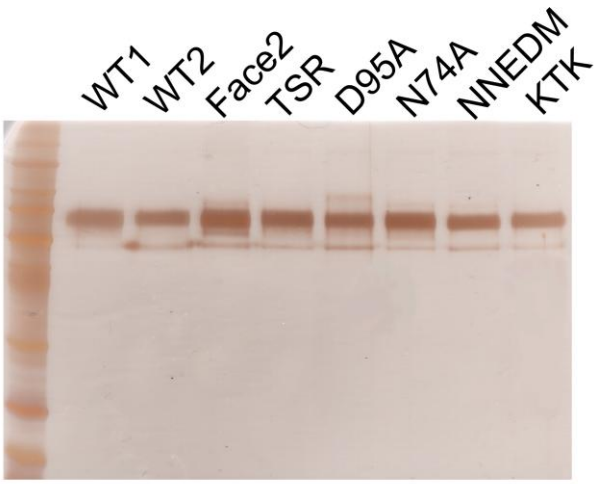

Figure 3.5 Silver staining of purified protein S samples.
WT and variant protein S samples (100 ng/well) were stained by silver staining. WT1; WT protein S purified by QFF anion exchange chromatography followed by immunoaffinity chromatography. WT2; WT protein S purified by barium citrate precipitation followed by DEAE anion exchange chromatography.

3.2.3 Multimerisation of protein S

Multimerisation of protein S has been well documented in the literature and it has been reported that multimeric protein S binds to phospholipids with higher affinity than monomeric protein S.[189-192,251]

To assess multimer formation of WT protein S, a Western blot was performed on a native PAGE gel. Protein S in dialysed concentrated conditioned media appeared to be monomeric (Figure 3.6A,B). Protein S partially purified by QFF anion exchange chromatography was mainly monomeric but also contained some dimers (Figure 3.6A). However, protein S partially purified by barium citrate precipitation (Figure 3.6B), fully purified protein S (Figure 3.6A) and plasma purified protein S (Figure 3.6B) all exhibited appreciable multimerisation. These results are consistent with previous reports.[191,192] It was difficult to obtain high quality Western blots showing protein S multimers. Nevertheless, no major difference in multimer formation between barium citrate precipitated protein S, fully purified protein S (independently of the purification protocol) and plasma purified protein S was observed.

To allow for potential differences in activity between monomeric and multimeric protein S, both protein S in concentrated conditioned media (monomeric) and purified protein S (multimeric) was used, where possible, in the functional assays.

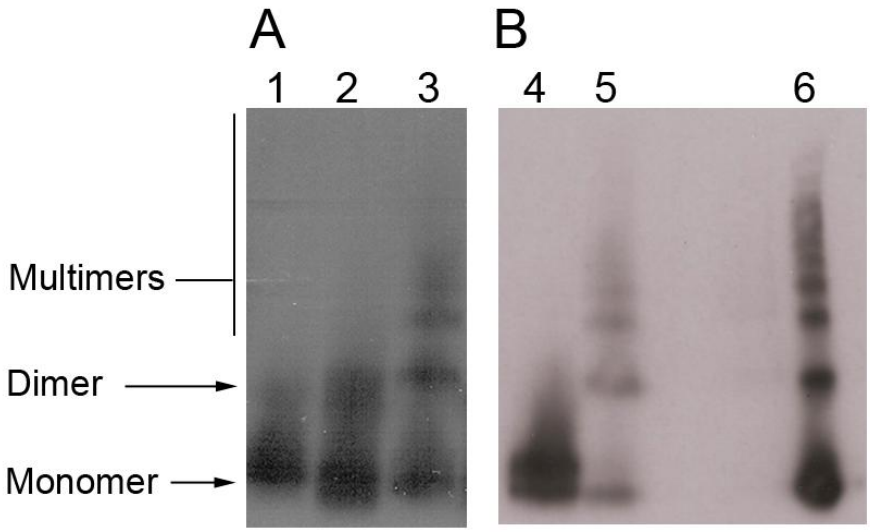

Figure 3.6 Western blot of a native PAGE analysis to identify multimer formation occurring during the purification.
Protein S in dialysed concentrated conditioned media (lanes 1 and 4) is predominantely monomeric (A,B). Protein S partially purified by QFF anion exchange column (2) exhibited some dimers while its subsequent passage over the immunoaffinity column lead to multimerisation (3) (A). Barium citrate precipitated protein S (5) and plasma purified protein S (6) were both multimeric (B).

3.2.4 Cleavage of protein S

Cleavage of protein S has previously been reported to occur after one or more of the 3 arginine residues (Arg49, Arg60, Arg70) in the TSR domain. Once cleaved, the disulphide bond between Cys47 and Cys72 keeps the Gla domain and the EGF1 domain together. However, under reducing conditions the disulphide bond is broken and the protein S fragment spanning from the cleavage site in the TSR to the SHBG domain is observed below the band of full-length protein S. The effect of protein S cleavage upon protein S function remains to be fully defined.[175-177]

When partially purified protein S was analysed by Western blot under reducing conditions a doublet was observed. To investigate whether the two closely migrating bands were due to variable glycosylation or cleavage of protein S, partially purified protein S samples were incubated with or without PNGase. Western blot analysis (Figure 3.7) was performed both with a polyclonal anti protein S antibody (Panel A) and a monoclonal anti-Gla antibody (Panel B) under reducing conditions. Results in Panel A show that the double band was conserved after WT protein S and protein S Face2 are digested with PNGase,

suggesting that the difference in size is not due to differences in glycosylation. Protein S TSR was present only as one band in the blot revealed using a polyclonal antibody. Protein S TSR has all the arginines (after which protein S is cleaved) substituted to Gln. When protein S was detected with the anti-Gla antibody (Panel B) only one band was present. According to the MW, this band corresponds to the higher band of the duplets detected for WT and Face2 with the polyclonal antibody. The isolated Gla domain is not detected by the antibodies, probably because too small (~4.5 kDa) and present in too low amounts. This is consistent with previously reported results in the literature.[252] Together, these results suggest the doublet band observed under reducing conditions is due to cleavage of protein S in the TSR domain rather than variable glycosylation. Blots of protein S in conditioned media suggests that cleavage occurs during the course of protein S expression (results not shown).

To account for any differences in activity due to cleavage of protein S, regular checks on every preparation of interest were made to ensure cleavage of the protein S variants was no more than that of WT protein S.

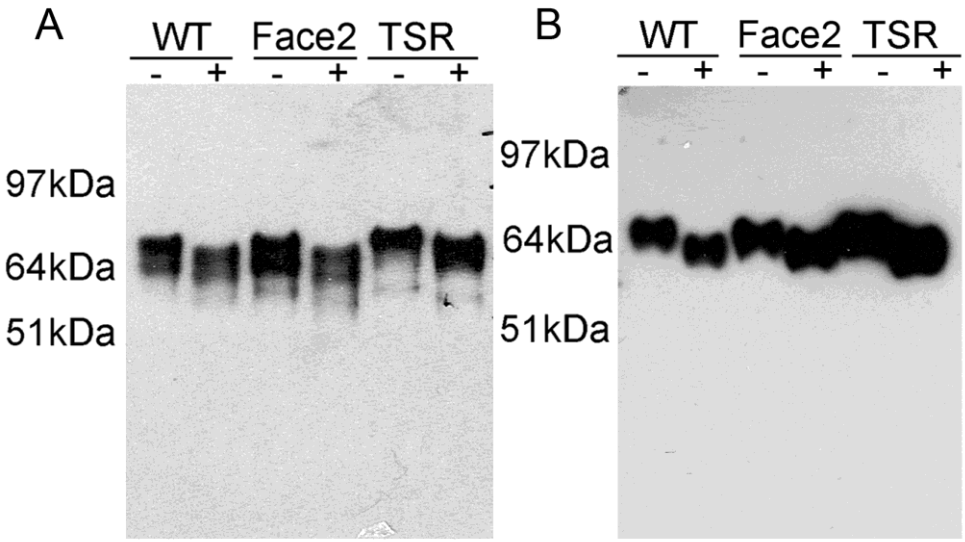

Figure 3.7 Analysis of protein S cleavage.
Protein S incubated (+) and not incubated (-) with PNGase was analysed by SDS-PAGE under reducing conditions and detected either by a polyclonal anti-protein S antibody (A) or a monoclonal anti-Gla antibody (B).

3.3 Evaluation of APC cofactor activity of protein S

3.3.1 Preliminary experiments in protein S deficient plasma

Before analysing the APC and TFPI dependent cofactor activity of protein S in plasma, TF concentration was titrated in the CAT assay for optimal analysis of the activity of endogenous protein C and TFPI in the plasma. Protein S deficient plasma is more procoagulant than normal plasma and it has been reported that only low amounts of TF (~1.4 pM) allow visualisation of the TFPI cofactor activity of protein S.[218] To establish the concentration of TF to use, I performed a titration of TF from 0.0625 to 1 pM (Figure 3.8). This resulted in a prolongation of the lag time but thrombin peak height and ETP did not vary significantly. However, variability between the replicates (not showed) increased at lower TF concentration. TF concentration at 1 pM was chosen to trigger coagulation in all my plasma assays (unless otherwise stated) as duplicates were good and this is compatible with what is currently in use in the literature to trigger coagulation in plasma.[240,241,253,254] The stock concentration of recombinant TF in Innovin was considered to be 6000 pM, as estimated by P. Giesen in the Thrombogram guide of Synapse b.v. Hence, a 1:6000 dilution of the TF stock was performed to obtain 1 pM final concentration in the assay.

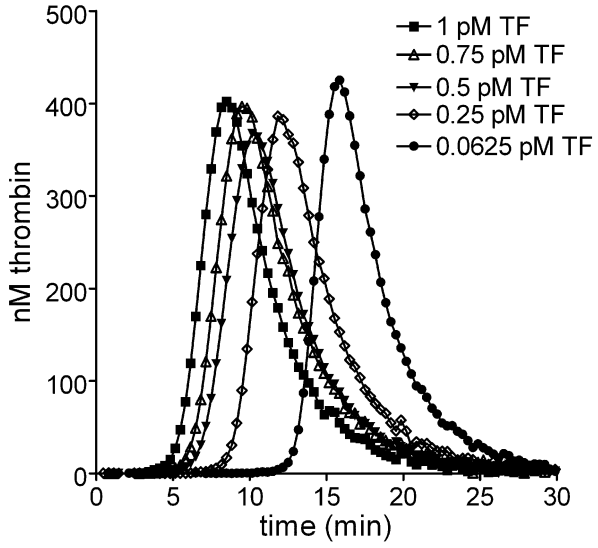

Figure 3.8 TF initiation of coagulation in protein S deficient plasma.
A titration of TF (0.0625-1 pM) was performed in protein S deficient plasma from Affinity Biologicals supplemented with 50 µM phospholipids and 65 µg CTI per ml plasma in a final volume of 100 µl to assess the amount of thrombin generated.

To evaluate the activity of both endogenous protein C and TFPI in the protein S deficient plasma, polyclonal antibodies against APC (150 nM) and polyclonal antibodies against TFPI (100 nM) were used. When these were added to the plasma, no change in thrombin generation was observed (Figure 3.9). Addition of 120 nM plasma purified protein S to protein S deficient plasma had no effect on thrombin generation (Figure 3.9). Results suggest that APC and TFPI in protein S deficient plasma under these experimental conditions do not appreciably regulate thrombin generation, either in the presence or absence of protein S. The lack of APC influence is not surprising, considering that protein C does not circulate in its active form and that EPCR and TM (absent in these experiments) are important for physiological activation of protein C by thrombin. The lack of influence of TFPI was attributed to co-depletion of protein S and TFPI from the plasma during its preparation by the manufacturer (see section 3.4.1). Consequently, APC or TFPI were supplemented to the plasma preparations to evaluate cofactor activities of protein S. APC from Enzyme Research Laboratories was used unless otherwise stated.

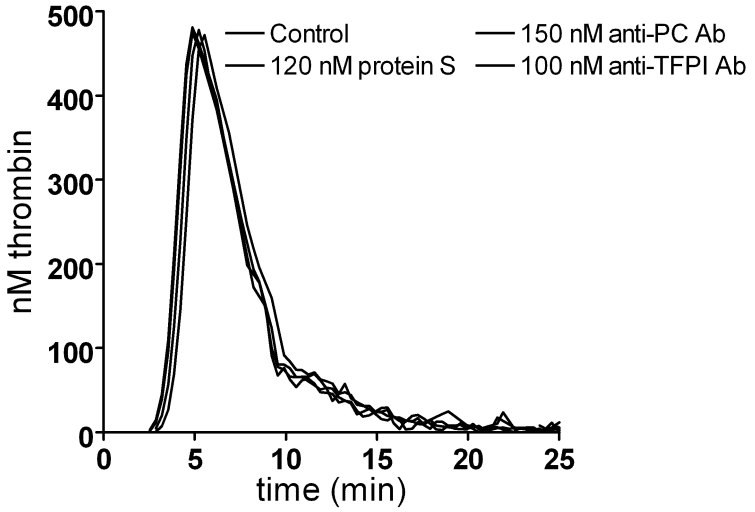

Figure 3.9 Activity of endogenous APC and TFPI in protein S deficient plasma.
Thrombin generation was performed in protein S deficient plasma in the presence and absence of 130 nM polyclonal antibodies against protein C (Sigma-Aldrich), 100 nM polyclonal antibodies against TFPI (Haematologic Technologies Inc) and in the presence of 120 nM protein S. Ab; antibody. PC, protein C.

3.3.2 Evaluation of APC cofactor activity of protein S by CAT

To assess the APC cofactor activity of protein S, thrombin generation was measured by CAT using protein S deficient plasma from Affinity Biologicals supplemented with recombinant WT protein S and its variants in conditioned media. Under the conditions of the assay, in the absence of added protein S, APC (0-10 nM) did not influence thrombin peak height, ETP, lag time or time to peak, in protein S deficient plasma (Figure 3.10, Panel A). When (0-120 nM) WT protein S was introduced into the assay in the presence of APC, dose-dependent changes were observed, primarily on reduction of the peak height and of ETP values (Figure 3.10, Panel B). The extent of inhibition of peak height and ETP was dependent both on the concentration of APC and protein S. For example, the higher the APC concentration, the less protein S was necessary to completely inhibit thrombin generation. Under these experimental conditions, 10 nM APC and 120 nM protein S reduced both the peak height and ETP by ~84%. To verify that those effects were a direct consequence of APC cofactor activity of protein S, polyclonal antibodies against either protein S (Figure 3.10, Panel C) or protein C (Figure 3.10, Panel D) were used. As seen in Figure 3.10 these completely inhibited the anticoagulant APC-protein S activity.

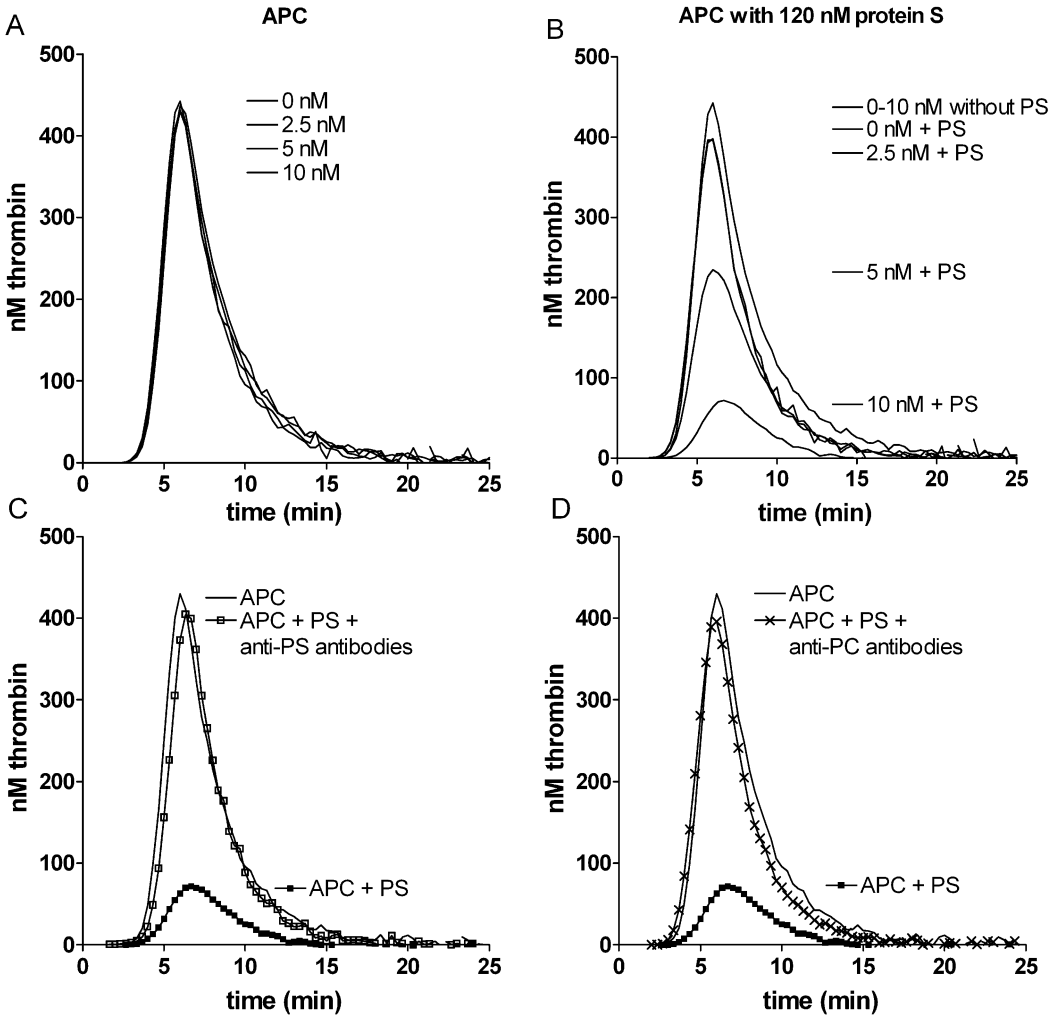

Figure 3.10 APC and protein S anticoagulant activity in plasma.
Thrombin generation was performed in protein S deficient plasma containing 100 nM inhibitory antibodies against TFPI. Up to 10 nM APC had no effect on thrombin generation in the absence of protein S; all concentrations generate lines that are superimposable (A). Following addition of 120 nM protein S (at 0-10 nM APC) an APC dose-dependent effect was observed (B). The upper single line represents 0-10 nM APC in the absence of protein S. Protein S in the presence of no or 2.5 nM APC generated lines that were superimposable. Conditions used are noted adjacent to the peak heights to which they refer. The anticoagulant activity of 10 nM APC and 120 nM protein S was inhibited by polyclonal antibodies against protein S (1.5 µM, DAKO) (□) (C) or against protein C (130 nM, Sigma-Aldrich) (x) (D). PS, protein S; PC, protein C. Representative experiments are shown (n=3).

To compare the anticoagulant activity of recombinant WT protein S in concentrated conditioned media with that of protein S purified by different techniques, the former was compared to protein S purified first by anion exchange chromatography and thereafter by immunoaffinity chromatography, or alternatively by barium citrate precipitation followed by anion exchange chromatography (see sections 2.3.1 and 2.3.2). All protein S preparations showed a similar inhibition of thrombin generation as can be seen in Figure 3.11. As protein S in concentrated conditioned media showed a similar activity to purified protein S, and furthermore also considering that protein S in media is essentially monomeric, conditioned media samples rather than fully purified protein S samples were preferably used to evaluate thrombin generation by CAT. In some experiments where purified protein S was used to assess APC cofactor activity, these preparations were purified by anion exchange chromatography followed by immunoaffinity chromatography.

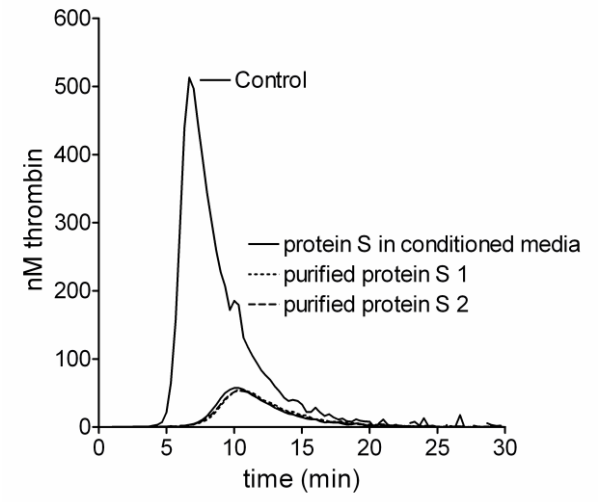

Figure 3.11 APC cofactor activity of different preparations of protein S in plasma.
The anticoagulant activity of protein S in conditioned media was compared with that of protein S purified either by anion exchange chromatography followed by affinity purification (purified protein S 1, dotted lines) or protein S purified by barium citrate precipitation followed by anion exchange chromatography (purified protein S 2, dashed lines), 90 nM, in the presence of 4 nM APC (Xigris). APC from Xigris was used in this experiment and was more active than the commercial APC preparation mostly used in this thesis (Enzyme Research Laboratories).

Having optimised the CAT assay for APC-dependent protein S cofactor activity, I then aimed to screen my panel of protein S variants to identify those with a functional deficit. The screening of the protein S variants for their APC cofactor activity was performed at a high concentration of APC (16 nM) to achieve almost complete inhibition of thrombin generation in the presence of 100 nM WT protein

S (Figure 3.12). Figure 3.12 shows the % peak height obtained for each protein S variant in comparison with the peak height obtained in the absence of protein S. When % ETP values were plotted instead of % peak height a very similar graph was obtained (result not shown). In total, 28 variants were screened for their APC cofactor activity. As previously reported,[224] the cofactor activities of Face1 and of Face2 for APC were reduced. Importantly, these screening experiments identified a single point variant in EGF1 of protein S, D95A, which had a severely impaired APC cofactor activity that had not previously been implicated in protein S function. Under all conditions used (4, 9 or 16 nM APC, 60, 90 or 100 nM protein S), the reduction in APC cofactor activity of protein S D95A was greater than that observed for the previously reported variant with 7 amino acids exchanged (prothrombin residue swap), Face2.

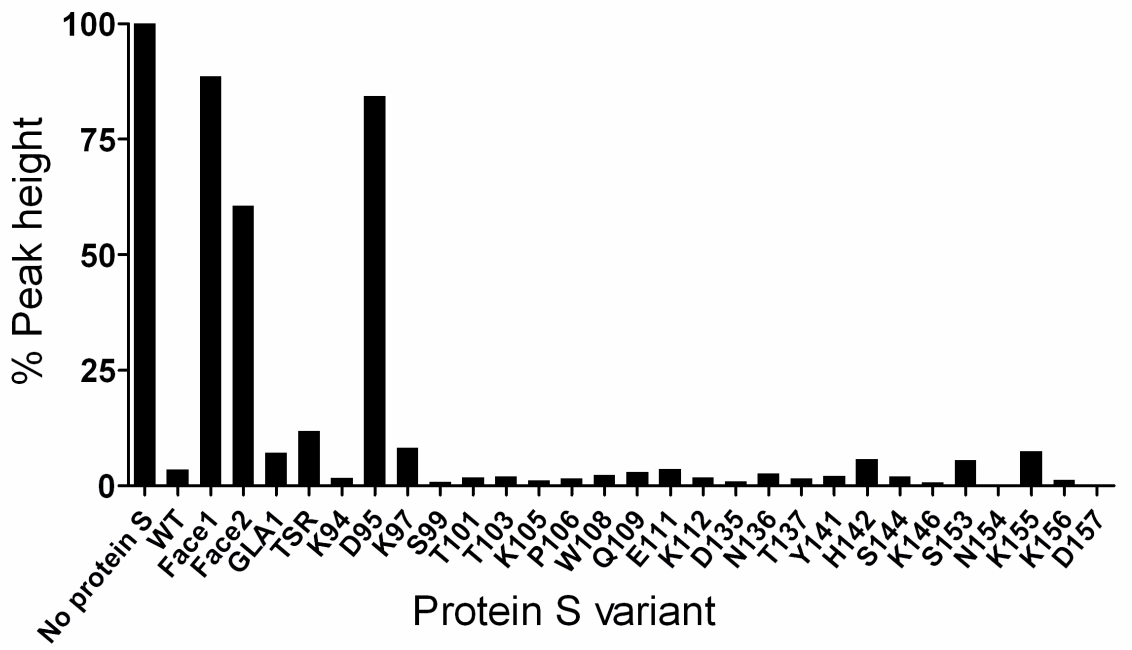

Figure 3.12 Screening of protein S variants for APC cofactor activity.
The APC cofactor activity of protein S was evaluated at 16 nM APC and 100 nM protein S by CAT. The peak height in the absence of protein S was set to 100%. A high concentration of APC, leading to almost complete inhibition of thrombin generation with 100 nM WT protein S, was chosen specifically for screening purposes as this allows widening of the assay window at which variants with reduced APC cofactor activity are visualised.

As Saller *et al.*[225] did not investigate the APC cofactor activity of protein S Face1 and Face2 variants in the CAT assay, I titrated both variants alongside WT protein S (0-120 nM) in the presence of 16 nM APC (Figure 3.13). Protein S Face1 (Panel B) had almost completely abolished APC cofactor activity while the

cofactor activity of Face2 (Panel C) was partially reduced (Figure 3.13). These results from the CAT assay, corroborate the results obtained by Saller *et al.*[225] using clotting assays. Saller *et al.*, however, showed a 9-fold reduction in protein S Face1 binding to phospholipids. They could therefore not conclude whether the residues comprising the variant were important or not for a cofactor interaction with APC. Protein S Face2, however, bound phospholipids and domain specific monoclonal antibodies with similar $K_{d(app)}$ values to those of WT protein S.[224]

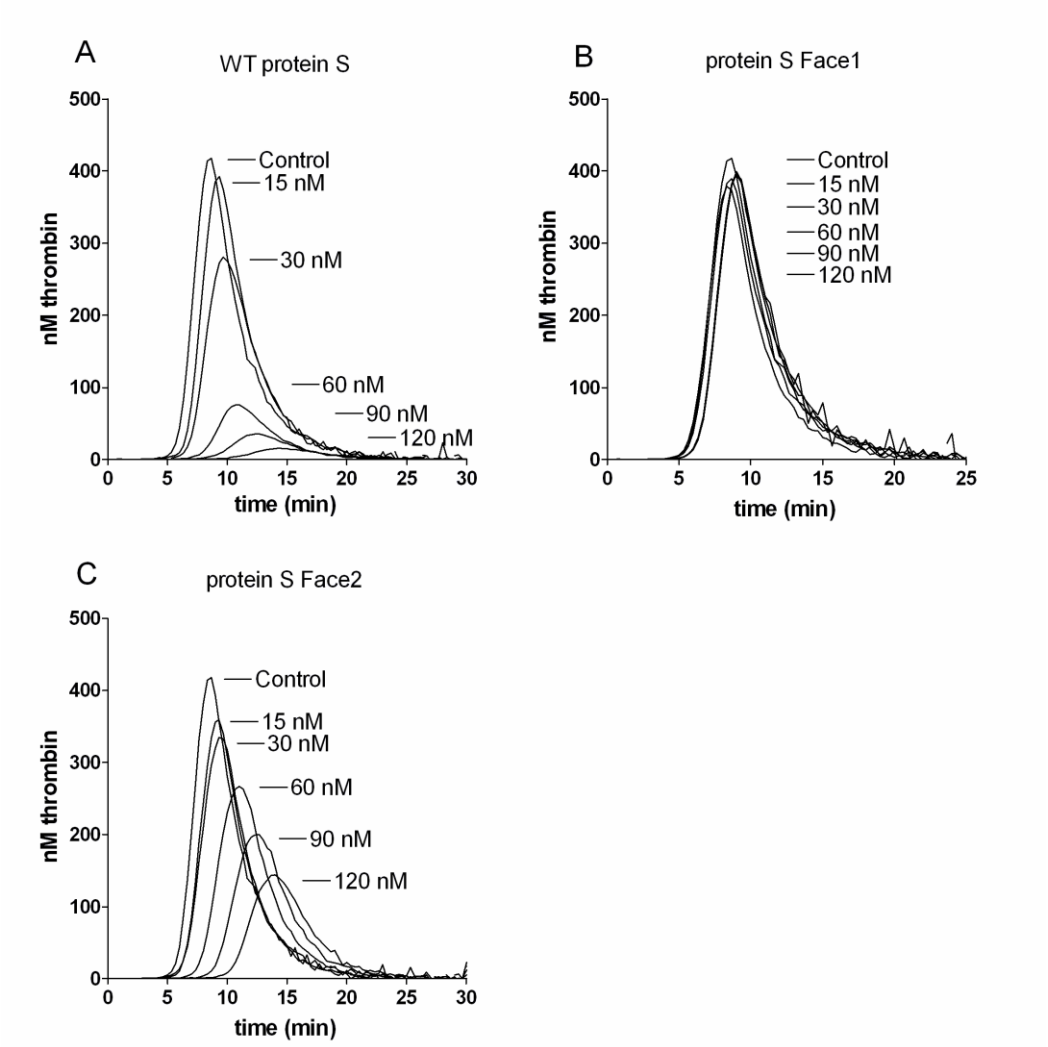

Figure 3.13 APC cofactor activity in plasma of WT protein S, Face1 and Face2 variants. Thrombin generation was measured in protein S deficient plasma supplemented with 16 nM APC, and 0-120 nM WT protein S (A), protein S Face1 (B) or protein S Face2 (C). Legends are positioned adjacent to the peak heights to which they refer.

To further evaluate the APC cofactor activity of protein S D95A, a titration (0-120 nM) of the variant (Figure 3.14, Panel B) was performed alongside WT protein S (Figure 3.14, Panel A), using 9 nM APC. Protein S D95A had a severely impaired APC cofactor activity, a finding replicated when a different concentration of APC

(4 nM) was used. The severely impaired APC cofactor function of protein S D95A was also confirmed in protein S deficient plasma supplied by Hyphen Biomed to ensure that these effects were not plasma specific (n=1, results not shown). While the cofactor activity of WT protein S is highly dependent upon the APC concentration used (see Figure 3.10, Panel B) that of protein S D95A is not, explaining the difference in fold activity between WT protein S and protein S D95A in Figure 3.12 and Figure 3.14.

Using 9 nM APC, (Figure 3.14, Panel A and B), 120 nM of WT protein S reduced peak height and ETP by ~80%, while 120 nM of protein S D95A only reduced peak height and ETP by ~10%.

Protein S is susceptible to proteolysis in the TSR domain and this may impair its APC cofactor function.[175-177] To ensure that any difference in activity between WT protein S and protein S D95A was not due to difference in TSR proteolytical cleavage, samples were analysed by Western blot under reducing conditions. No cleavage was observed either in WT protein S or in the D95A variant, showing that the different APC cofactor activity was not a result of different cleavage of the two preparations (results not shown).

Asp95 in protein S is β-hydroxylated and while it has not been shown to bind calcium, it is part of a partially conserved calcium binding motif. To ensure disruption of β-hydroxylation or loss of coordination of a calcium ion was not the reason behind the impaired APC cofactor activity of protein S D95A, Asp95 was also substituted with Asn, rather than Ala. Asn is structurally more similar to Asp than Ala and it can also be β-hydroxylated and potentially coordinate a calcium ion. When the protein S D95N variant was titrated, it was observed that it also had severely reduced APC cofactor activity (Figure 3.14, Panel C), with 120 nM of the D95N variant inhibiting peak height and ETP by only ~20%.

All results in Figure 3.14A-C were conducted with protein S and its variants in concentrated conditioned media. Concentrated conditioned media from cells not expressing protein S was used as a control and had no influence on thrombin generation (results not shown). The % change in peak is represented in Figure 3.14, Panel F. To confirm that the differences in APC cofactor activity I observed arose from protein S rather than the media, WT protein S and the protein S D95A variant were purified to homogeneity using anion exchange and immunoaffinity chromatography (see section 2.3.1). Figure 3.14D shows SDS-PAGE gel

visualised with silver staining which suggests that the protein S preparations were essentialy pure. Addition of 90 nM of purified WT protein S to protein S deficient plasma in the presence of 9 nM of APC, appreciably attenuated thrombin generation (Figure 3.14, Panel D), replicating the findings with WT protein S in conditioned media. When 90 nM purified protein S D95A was used, only a minimal effect on thrombin generation was seen (Figure 3.14, Panel D) corroborating the results from the experiments using concentrated conditioned media.

Inspection of a model of Gla-TSR-EGF1 domains of protein S[193] suggested that two residues with solvent exposed R groups, Asp78 and Gln79, are in close proximity to that of Asp95. These residues were not included in the first round of screening (shown in Figure 3.12). These were therefore mutated to alanine and expressed in HEK293T cells as described in sections 2.1.2 and 2.2.2. When protein S D78A and Q79A in concentrated conditioned media were analysed in the thrombin generation assay alongside WT protein S, a severely reduced APC cofactor activity was also observed for both (Figure 3.14, Panel E). The proximity of these residues to Asp95 suggests these three residues could form a possible cluster of residues involved in APC cofactor activity of protein S.

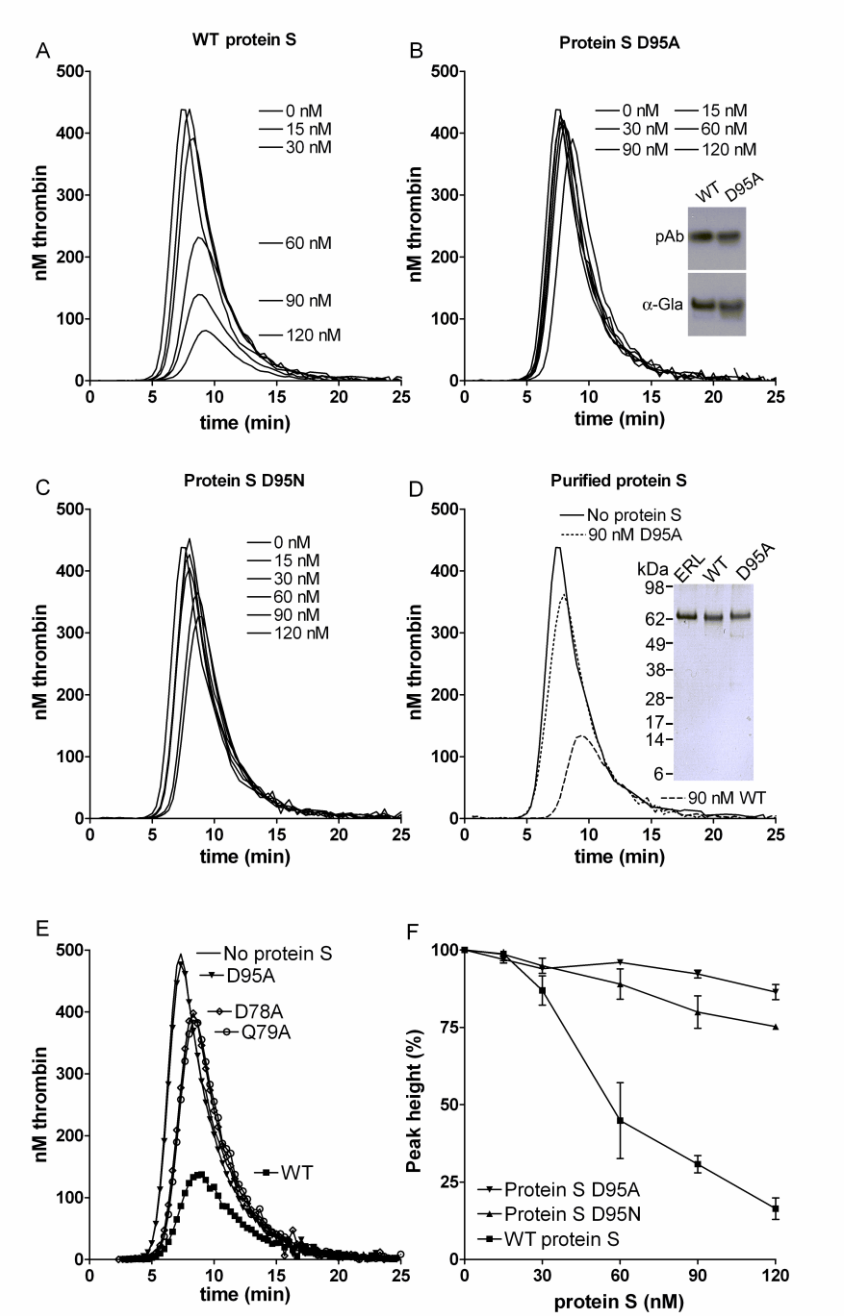

Figure 3.14 APC cofactor activity in plasma of WT protein S, protein S D95A, D95N, D78A and Q79A variants.

Thrombin generation was measured in protein S deficient plasma supplemented with 9 nM APC, 100 nM antibodies against TFPI and 0-120 nM WT protein S (A), protein S D95A (B), protein S D95N (C) or 90 nM purified WT (dashed line) or purified protein S D95A (dotted line) (D). Protein S concentrations are positioned adjacent to the peak heights they refer to. The cofactor activity of 60 nM WT protein S and protein S variants D95A (▼), D78A (◊) and Q79A (○) was compared at 9 nM APC (E). Typical experiments are shown (n=3). Dose response data from titrations with WT protein S, protein S D95A, and protein S D95N in the presence of 9 nM APC are shown in Panel F (data is expressed as mean ± SD of two independent experiments performed in duplicate). Inset in Panel B shows recognition of WT protein S and protein S D95A in media by polyclonal antibodies (pAb) and a monoclonal antibody recognising only γ-carboxylated Gla domains (α-Gla). Inset in Panel D shows the See Blue prestained marker, plasma purified protein S from Enzyme Research Laboratories (ERL, lane 1), purified recombinant WT protein S (lane 2) and purified protein S D95A (lane 3) visualised with silver staining.

3.3.3 Binding of protein S to phospholipid surfaces

As phospholipid binding is a prerequisite for protein S function, the binding of WT protein S and of protein S D95A to phospholipids was evaluated. Partially purified (using barium citrate precipitation) protein S, 0-120 nM, was incubated for 2 hours on a plate coated with 25 µg/ml phospholipids and bound protein S was detected by an HRP-conjugated antibody against protein S. As shown in Figure 3.15, both WT protein S and protein S D95A were able to bind phospholipids, with $K_{d(app)}$ of 5.7 ± 1.2 and 9.5 ± 2.3 nM (n=3), respectively (Table 3.3).

$K_{d(app)}$ values were analysed by the Mann Whitney test and were found not to be statistically different ($p>0.05$). Binding was observed in the presence of Ca^{2+}, but not in the presence of EDTA or in the absence of phospholipids, as expected, confirming the specificity of the assay. These findings are broadly consistent with previously reported $K_{d(app)}$ values for protein S binding to phospholipid surfaces.[182,190,223-225] My results suggest the loss of APC cofactor activity observed for protein S D95A is not due to reduction or loss of binding to phospholipid surfaces.

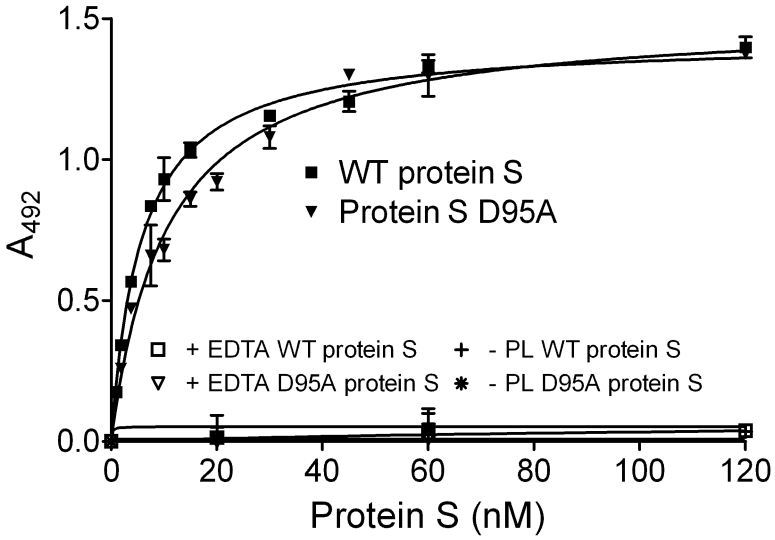

Figure 3.15 Binding of protein S to phospholipid surfaces.
Protein S (0-120 nM) was incubated in a plate coated with 25 µg/ml phospholipids. Bound protein S was detected with an HRP-conjugated polyclonal antibody against protein S. A representative experiment is shown. The $K_{d(app)}$ values, 5.69 ± 1.24 and 9.54 ± 2.26 nM for WT protein S and protein S D95A, respectively, were obtained by calculating the mean ± SD of three independent experiments performed in duplicate for each concentration of protein S.

3.3.4 Binding of protein S to domain specific monoclonal antibodies

Substituting residues in a domain can sometimes affect the overall folding of the protein, the structure of the domain in which the substitution has been introduced or the structure of adjacent domains. To assess the integrity of the domain structure and folding of protein S variants, binding of protein S to conformational domain specific antibodies was evaluated. Protein S in concentrated conditioned media was incubated in a plate coated with monoclonal antibodies recognising either the Gla domain (MK21), the EGF1 domain (MK54) or the C-terminal SHBG domain (MK61). Bound protein S was detected with polyclonal antibodies as described in the Methodology. Binding curves were fitted with a one site binding equation and the $K_{d(app)}$ values were obtained.

Table 3.3 represents the mean ± SD $K_{d(app)}$ values of three independent experiments performed in duplicate. $K_{d(app)}$ values were analysed by the Mann Whitney test and were found not to be statistically different ($p>0.05$). The results suggest that substitution of Asp95 does not result in a significant change in the domain structure of protein S.

	DOPS/DOPC/DOPE vesicles	MK21 (Gla)	MK54 (EGF1)	MK61 (SHBG)
WT protein S	5.69 ± 1.24	2.18 ± 0.97	0.81 ± 0.11	5.31 ± 1.11
Asp95 protein S variant	9.54 ± 2.26	2.53 ± 1.06	0.83 ± 0.03	5.39 ± 0.54

Table 3.3 Binding of protein S to phospholipids and domain specific monoclonal antibodies.
$K_{d(app)}$ values (nM) of WT protein S and the Asp95 protein S variant for phospholipid vesicles and domain specific monoclonal antibodies are expressed as mean ± SD of three independent experiments performed in duplicate. Binding of WT protein S and the Asp95 variant to phospholipids and domain specific monoclonal antibodies was analysed by Mann Whitney test and the differences were found not to be statistically significant ($p>0.05$).

3.3.5 Protein S enhancement of APC mediated cleavage of FVa at Arg306

Protein S cofactor function towards APC was initially assessed in the CAT assay (described above), which assesses thrombin generation in plasma. To more precisely measure and quantify the influence of protein S variants on APC cofactor function, a purified system was used that examines cleavage of FVa. The FVa variant, R506Q/R679Q, was used to evaluate protein S enhancement of APC mediated cleavage at Arg306 in FVa. FVa inactivation was performed in the presence or absence of 0.5 nM APC and a titration of purified protein S (0-100 nM) was performed as described in the Methodology (section 2.6.3). After 10 minutes the remaining FVa activity was measured using a prothrombinase assay. Using this approach, WT protein S efficiently enhanced APC mediated cleavage at Arg306 of FVa, in contrast to protein S D95A and protein S Face2 which showed almost no enhancement of APC activity (Figure 3.16, Panel A).

To quantify the rate of cleavage at FVa Arg306 in the presence and absence of protein S, time course experiments were performed. In the absence of protein S, or in the presence of protein S D95A or protein S Face2, 3 nM APC was used and aliquots were quenched at different time points (0-20 min). In the presence of WT protein S the APC concentration was lowered to 0.5 nM, as APC is efficiently enhanced by WT protein S. The remaining FVa activity at all time points was measured in the prothrombinase assay (Figure 3.16, Panel B). It is observed that approximately 6-fold more APC is needed in the presence of protein S D95A and protein S Face2 to obtain a similar amount of APC-mediated FVa R506Q/R679Q inactivation as with WT protein S. Using the FVa inactivation curves obtained, the apparent pseudo-first order rate constants were calculated (see section 2.6.3) and corrected for the APC concentrations used. Rate constants for APC, APC+WT protein S, APC+protein S D95A and APC+protein S Face2 were respectively $6.1\pm1.2\bullet10^5$, $8.3\pm1.6\bullet10^6$, $1.1\pm0.2\bullet10^6$ and $2.0\pm0.3\bullet10^6$ and were broadly consistent with those previously reported in the same laboratory.[141] Under these experimental conditions, APC mediated cleavage at FVa Arg306 was enhanced by WT protein S by 13.9 ± 3.6-fold, while protein S D95A was only able to enhance APC by 1.8 ± 0.4-fold and protein S Face2 by 3.3 ± 0.3-fold. Accordingly, protein S D95A had 12.9% and protein S Face2 30.3% of the activity

of WT protein S. Protein S had no effect on FVa activity in the absence of APC (not shown).

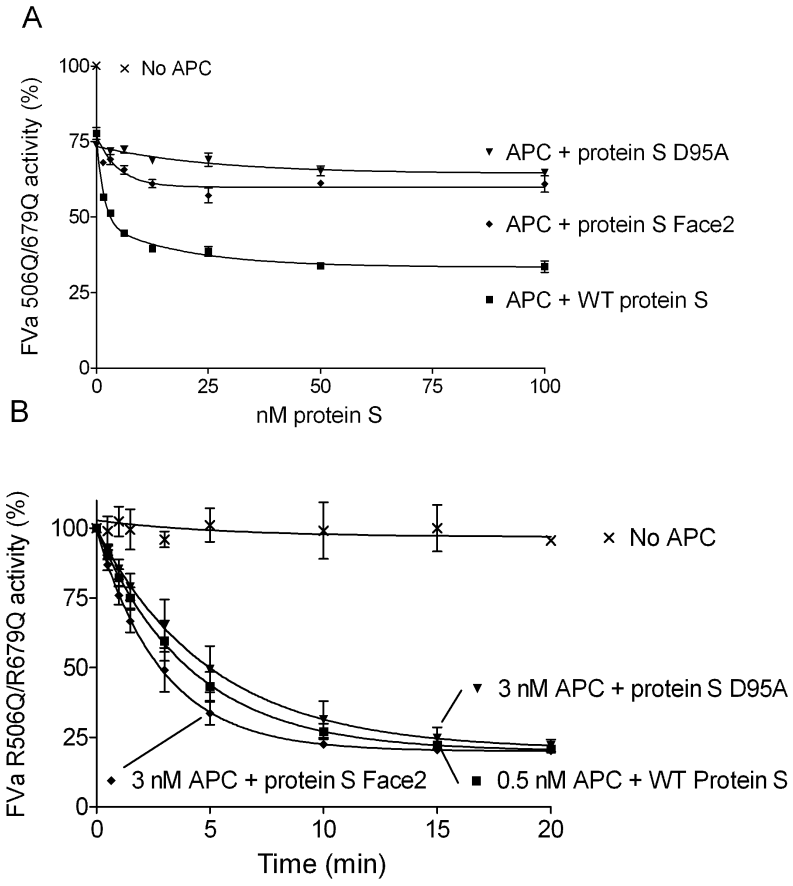

Figure 3.16 Protein S enhancement of APC mediated cleavage of FVa at Arg306.
Protein S (0-120 nM) in the presence of 0.5 nM APC was incubated with 0.8 nM FVa R306Q/R679Q in the presence of phospholipids for 10 minutes. The remaining FVa activity was measured with a prothrombinase assay. Results are plotted as mean ± SD from three independent experiments performed in duplicate (A). A time course experiment was performed to calculate the apparent pseudo-first-order rate constants of WT protein S, protein S D95A and protein S Face2 (B).

3.3.6 Discussion

Initially, 28 protein S variants with substitutions in the Gla, TSR, EGF1 and EGF2 domains were constructed and expressed. I evaluated their ability to enhance APC mediated anticoagulant activity in plasma. The anticoagulant function of these 28 variants, and the subsequently expressed variants protein S D78A, D79A and D95N, was assessed by a thrombin generation assay, the specificity of which was demonstrated by utilising polyclonal antibodies both against protein S and protein C that completely inhibited the anticoagulant response observed when adding protein S in the presence of APC. The advantage of the thrombin generation assay conducted in plasma is that it shows very clearly how APC is heavily dependent upon protein S.[218] In the absence of protein S, 10 nM APC has no effect on thrombin generation. In contrast, in the presence of protein S, maximal anticoagulant activities with near ablation of thrombin generation are obtained with APC concentrations of around 10 nM.

My initial screening results identified three protein S variants with appreciable reduction in APC cofactor function. These were the already reported protein S Face1 and Face2 variants (a 4 and a 7 residue composite variant in the Gla domain, respectively) and the novel protein S D95A point variant in the EGF1 domain. The Face2 variant, in the report by Saller *et al.*, retained binding to phospholipids and yet had appreciably reduced APC cofactor activity, while Face1 was shown to have an approximate 9-fold reduced phospholipid binding.[224] It is suggested that the 7 residues substituted in the protein S Face2 variant collectively present a face of the Gla domain to APC and form a potential contact region. This variant was selected for investigation here, because it is a well-characterised protein S variant with substantially reduced APC cofactor activity and could therefore act as a representative control for a dysfunctional protein S. Saller *et al.*, however, did not assess protein S Face2 and Face1 APC cofactor activity in the CAT assay. I confirmed a reduction in APC cofactor activity in plasma, assessed by CAT.

The natural protein S variants T103N[255] and K155E[256,257] have been reported to have reduced APC cofactor activity. I could not observe any significant reduction in APC cofactor activity of protein S T103A in the CAT assay. It is, however, important to point out that defective APC cofactor activity of protein S T103N was

not observed in the APC mediated inactivation of WT FVa.[255] In addition, at higher concentrations of APC (8 nM) no difference was observed between WT protein S and protein S T103N in the FVIIIa degradation assay.[255] Protein S Tokushima (K155E) has been described in the literature to have a cofactor activity towards APC of 31% [256] and 58% [257] of that of WT protein S. I did observe a decrease in protein S K155A cofactor activity towards APC, although this was quite moderate under conditions used in my screening assay. However, as this reduction was appreciably less than that for protein S D95A, it was not further analysis during my PhD project.

Considering pooled results from multiple assays (n=3), protein S S99A and N154A seem to be the variants that have most enhanced APC cofactor activity. It would also be interesting to further analyse these variants. A full titration in the CAT assay would allow an assessment of the extent of increased activity. It would be interesting to elucidate their importance in APC cofactor activity by assessing their activity in a purified assay and their ability to bind phospholipids.

The substitution of Asn136 in protein S abolishes the calcium binding site in the EGF2 domain of protein S.[258] I did not observe a reduction in APC cofactor activity. However, it was secreted in significantly lower levels than WT protein S and all other protein S variants from stably transfected cells (see section 3.2.1). As APC cofactor activity appeared normal I did not further investigate the expression of protein S N136A, but it is likely that mutating Asn136 to Ala influences the structure of protein S leading to intracellular retention of incorrectly folded protein S.

Protein S variants R49Q and Q52R in the TSR domain of protein S and protein S variants K97Q and P106S in the EGF1 domain were assessed in Dahlbäck's laboratory (He *et al.*),[244] and found to be important for species specificity towards APC. Following substitution of the human protein S residues into the corresponding residues of bovine protein S, an increased cofactor activity towards human APC was observed. They were also shown to specifically increase the activity of human protein S towards bovine APC. During my project I did not mutate Gln52 and as the substitution R49Q was part of the composite variant TSR it is not possible to directly compare my results with those previously reported. My protein S P106A variant had slightly increased activity in comparison with WT protein S, in accordance with previous results.[244] Protein S K97A,

however, had decreased activity in comparison with WT protein S. This is in discrepancy with the results of the K97Q variant of He *et al.* but is likely to depend on the different substitution, to alanine or glutamine. It is interesting that residues previously found to be important for species specificity (residues Arg49, Gln52, Lys97, Pro106) are spatially located in close proximity to residues Asp95, Asp78 and Gln79 here shown to be important for APC cofactor activity (Figure 3.17).

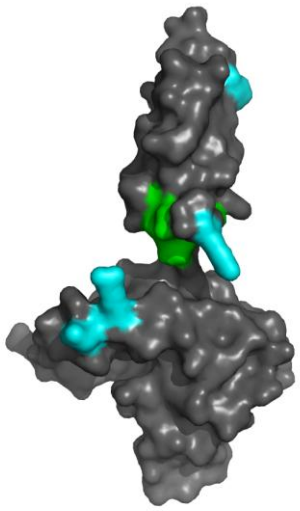

Figure 3.17 Location of residues important for species specificity in relation to Asp95, Asp78 and Gln79.
Residues Arg49, Gln52 in the TSR domain of protein S and residues Lys97 and Pro106 in the EGF1 domain of protein S have been reported to be important for species specificity[244] and are here highlighted in cyan. Residues Asp95, Asp78 and Gln79 important for APC cofactor activity are highlighted in green. This N-terminal model of the Gla-TSR-EGF1 domains of protein S is adapted from the model of Villoutreix *et al.*.[193]

Analysis of protein S function can be complicated due to its propensity to multimerise upon purification.[191,192] Accordingly, I have performed APC cofactor assays with unpurified protein S in concentrated conditioned media as well as with fully purified preparations. My results were consistent between protein S preparations. Both forms of the protein S D95A variant, purified or in concentrated conditioned media, had an appreciable reduction in APC cofactor activity when compared to the respective WT protein S preparation. In all comparison experiments carried out, the protein S D95A variant had less APC cofactor than the protein S Face2 variant. This suggests that Asp95 in protein S may occupy a pivotal position with respect to its interaction with APC. The importance of Asp95 in APC cofactor activity was further confirmed by the severely reduced activity

observed when mutating to alanine the two residues in close proximity to Asp95, Asp78 and Gln79. Asp78, Gln79 and Asp95 are conserved across species with the exception of Asp78 that is replaced by the structurally similar Asn78 in birds (Figure 3.18). Using a working model of the Gla-TSR-EGF1 domains of protein S, I demonstrate the likely proximal spatial location of these three residues, see Figure 3.19, and their relationship to protein S Face2 residues.

```
Homo sapiens                          IPDQCSPLPCNEDGYMSCKDGKASFTCTCKPGWQGEKCE
Chimpanzee (Pan troglodytes)          ************************************
Rhesus Macaque (Macaca mulatta)       ************************************R**
Cow (Bos Taurus)                      *S***N********F*T****Q*T***I**S********
Wild boar (Sus scrofa)                *****N********F*T****Q*M***I**S**E*****
Rabbit(Oryctolagus cuniculus)         *****N****SE***LN****Q*T***I**********
Dog (Canis lupus familiaris)          ***************KT*R**Q*T***I*******DR**
Rat (Rattus norvegicus)               *****D*M*******L*****QGA***I*******D**Q
Mouse(Mus musculus)                   *S***D*I*******LA*Q**Q*A***F*******DR*Q
Xenopus (Xenopus tropicali)           L****T*****RK**KE*I***GN***I***G***PL*D
Chicken (Gallus gallus)               *SN*******HR***KE*I**Q*KY**I********N**
Bird (Taeniopygia guttata)            *SN*******HK***KD*I**Q*KY**V**A**R**N**
Platypus(Ornithorhynchus anatinus)    *****F****H****LF****QGRY**V*R***H**R**
```

Figure 3.18 Conservation of Asp78, Gln79 and Asp95 across species.
The sequence of human protein S between amino acid residue 76 and 114 is shown. Asp95 (in red), and Gln79 (in purple) are conserved throughout species. Asp78 (in blue) is conserved across species with the exception of birds where Asp78 is replaced by the structurally similar Asn78.

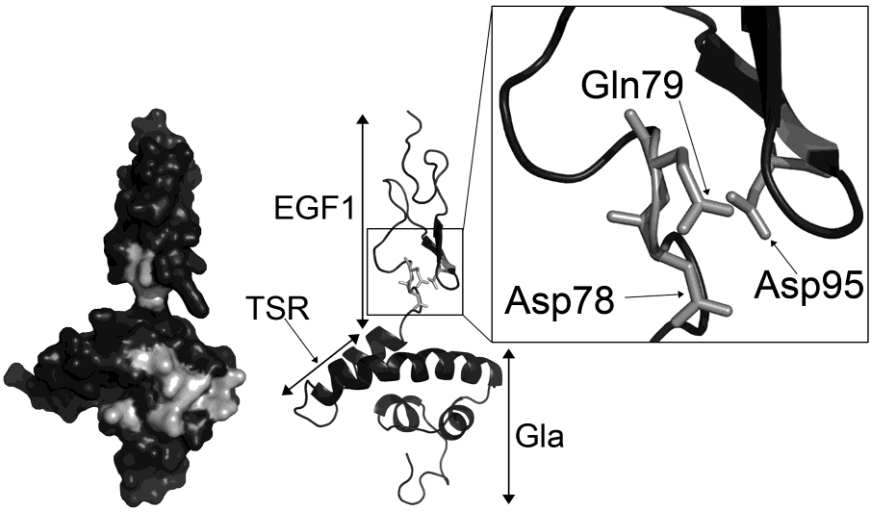

Figure 3.19 Location of Asp78, Gln79, Asp95 and Face2 within the protein S Gla-TSR-EGF1 model.
Domains are labelled on the right hand side cartoon model. Residues mutated in the protein S Face2 variant, Asp78, Gln79 and Asp95 are in light gray in the left hand side surface model. Residues Asp78, Gln79 and Asp95 are highlighted by the box to show their proximal spatial location. The model is that of Villoutreix *et al.*.[193]

I have investigated the mechanism of reduction of activity of protein S Asp95 variants. Asn136, in the EGF2 domain, Asn178 in the EGF3 domain and Asn217 in the EGF4 domain are all β-hydroxylated. These and other residues that are part of the calcium binding sequence motif in the respective domains have all been shown to coordinate a calcium ion with high affinity (Figure 3.20).[258] The EGF1 domain of protein S has not been shown to contain a calcium binding site.[258] The calcium binding motif in EGF1 is only partially conserved and according to the protein S Gla-TSR-EGF1 model of Villotreux *et al.*[193] residues of the calcium binding sequence motif before the first Cys residue and between the third and fourth Cys residues are localised on opposite sides of the EGF1 domain. However, to be sure that substitution of Asp95 to Ala did not result in disruption of a potential calcium binding site, I substituted Asp95 to Asn rather than Ala. Asn is structurally similar to Asp, can be β-hydroxylated and is able to coordinate a calcium ion (as has been observed in EGF2, 3 and 4).

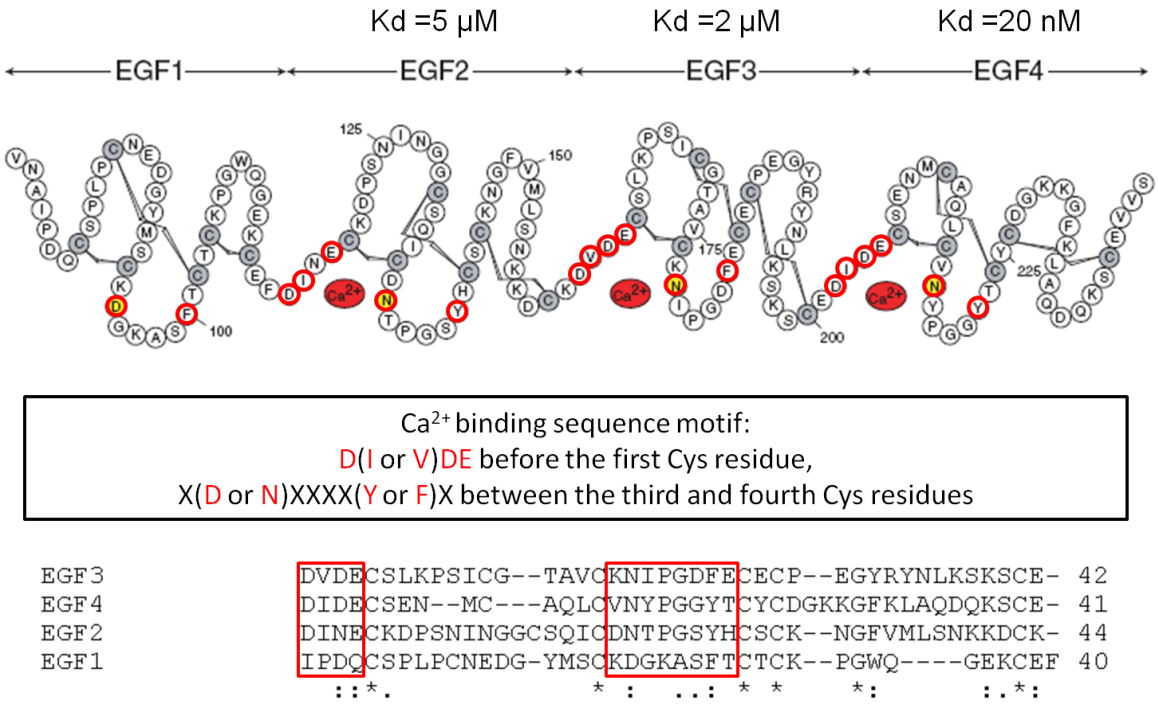

Figure 3.20 EGF domains of protein S and binding to calcium.
β-hydroxylated residues in the EGF domains of protein S are highlighted in yellow. The K_d values of each domain for calcium is shown above each domain. K_d values were taken and the EGF diagram modified from Stenflo *et al.*.[258] The calcium binding sequence motif is shown in the box, in the red squares in the sequence alignment of the EGF domains of protein S and the specific residues are circled in red in the EGF diagram.

However, as the protein S D95N variant also had reduced activity, it is unlikely that disruption of a calcium binding site is responsible for this effect as this variant would be predicted to bind calcium normally. Furthermore, while Asp95 is known to be β-hydroxylated it has previously been shown that β-hydroxylation itself is not a requirement for anticoagulant activity of protein S.[179] My work favours a direct effect of Asp95 in APC cofactor activity. However, I can not discount the possibility that the dramatic effect on protein S activity of the Asp95 residue substitution could be through a conformational repositioning of other functional domains. To assess this I performed binding to phospholipids, as this property underpins all protein S function. Plate binding assays, however, indicated no major functional defect on phospholipid binding. Furthermore, domain specific monoclonal antibody binding to WT and variant protein S (against the Gla domain, the EGF1 domain and the C-terminal SHBG domain) was also normal, suggesting that substitution of Asp95 does not reduced APC cofactor function by disrupting adjacent domain structure.

A current view of protein S enhanced APC cofactor activity suggests a functional repositioning of the APC cleavage site away from FVa Arg506 towards FVa Arg306.[228] I therefore performed a FVa inactivation assay using a FVa variant, FVa R506Q/R679Q, that cannot be cleaved at position 506 and 679. This allowed me to specifically analyse cleavage at Arg306 by APC. This is the APC cleavage site in FVa that is mainly enhanced by protein S. Using both concentration dependent and time course assays, I was able to confirm reduced cleavage of this variant by APC in the presence of protein S D95A or protein S Face2. Importantly, the single point variant protein S D95A had more impaired cofactor activity towards APC mediated cleavage of FVa R506Q/R679Q than the previously described 7 amino acid prothrombin swap variant Face2.

The available results therefore suggest that Asp95 of protein S may play an important and a direct role in APC recognition, resulting in enhanced APC function. This is compatible with the study performed by Hackeng *et al.*,[246] who used the isolated EGF1 domain of protein S to functionally disrupt the protein S and APC interaction. They showed a direct binding of EGF1 to APC suggesting EGF1 as a potentially important APC contact site on protein S. My results suggest that Asp95 constitutes a critical residue within EGF1 mediating the APC

cofactor function. Together with Asp78 and Gln79, Asp95 could form an important functional interaction site for APC.

The importance of residues in protein S Face2 should be further investigated. Phospholipid binding and binding to monoclonal antibodies were not evaluated in my thesis but should also be reassessed. Saller *et al.* found no significant difference in binding to monoclonal antibodies, however, binding of protein S Face2 to the monoclonal antibody MK54 against the EGF1 domain had a slightly higher $K_{d(app)}$ value (5.0 ± 1.7 nM) in comparison with that of WT protein S (1.6 ± 0.3 nM).[224] It is possible that protein S Face2 has reduced APC cofactor activity primarily because so many residues have been substituted: this may cause a conformational change in regions important for direct interaction with APC. In all my experiments the APC cofactor activity of the single point variant protein S Asp95 was more pronounced than that of the 7 residue prothrombin swap in protein S Face2. Thus, to identify the mechanism of reduced APC cofactor activity of Face2 binding to monoclonal antibodies should be repeated and point variants of the 7 amino acids substituted in protein S Face2 should be made and evaluated in functional assays.

Face1 had previously been shown not to bind phospholipids.[224] Interestingly, when protein S Face1 was evaluated for its binding to phospholipids surfaces, I found that it was able to bind phospholipids with a similar affinity to WT protein S (n=1). Analysis of protein S Face1 to phospholipids (n=3) has been subsequently further evaluated by my colleague Dr Josefin Ahnstöm, who found no statistical difference between WT protein S and protein S Face1 in binding to phospholipids. To identify which residue(s) in the composite variant protein S Face1 is important for APC cofactor activity she made the individual point variants contained in protein S Face1; protein S N33S, protein S P35T, protein S E36A and protein S Y39V. Analysis by CAT showed that variants protein S P35T and E36A were those with most reduced APC cofactor activity (similar to the reduced activity of protein S Face1) (as these are results of Dr Josefin Ahnstöm, I have not shown them). As proline is known to be important for protein folding and stability, only protein S E36A was further investigated (Dr Josefin Ahnstöm). Protein S E36A bound phospholipids with a $K_{d(app)}$ similar to that of WT protein S. The binding of protein S Face1 and protein S E36A to domain specific monoclonal antibodies against the Gla domain (MK21 and MK47) and the TSR domain (MK67) was also

evaluated by my colleague Dr Josefin Ahnström. No difference between protein S Face1, protein S E36A and WT protein S was found. When she analysed the cofactor activity of protein S E36A in the FVa inactivation assay she found that it had only 11.3% of the cofactor activity of WT protein S (n=4). This reduction in protein S cofactor activity towards APC mediated cleavage in Arg306 of FVa was similar to that of protein S D95A (12.9%) and both were more pronounced than that of protein S Face2 (30.3%).

Residues Asp95, Asp78, Gln79 (red), Gla36 (green) and residues in Face2 (blue) are highlighted in the working model of the Gla-TSR-EGF1 domains of protein S showing their likely spatial location (Figure 3.21).

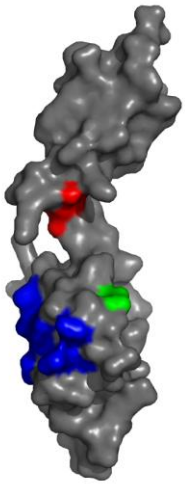

Figure 3.21 Location of residues found to have reduced APC cofactor activity.
The novel residues found to have reduced APC cofactor activity in my project Asp95, Asp78 and Gln79 in EGF1 are highlighted in red. Residues in the previously reported protein S Face2 variant are shown in blue. The novel residue identified by my colleague Dr Josefin Ahnström, Gla36, is highlighted in green. The model is taken from Villoutreix *et al.*.[193]

These results suggests that Gla36 of protein S seems to be equally important as is Asp95 for APC cofactor function, suggesting that possibly both the Gla and EGF1 domains of protein S are important for APC cofactor activity.

Collectively, our results suggest that Asp95 and Glu36 constitute critical residues within the EGF1 and Gla domain of protein S mediating the APC cofactor function.

3.4 Evaluation of TFPI cofactor activity of protein S

3.4.1 Evaluation of TFPI cofactor activity of protein S by CAT

From preliminary results described in section 3.3.1, I decided to use 1 pM TF to trigger coagulation in all my plasma assays and to supplement TFPI in the protein S deficient plasma. Hence, TFPI cofactor activity of protein S was assayed by CAT in protein S deficient plasma supplemented with recombinant TFPI and protein S. When 0-5 nM TFPI and 0-120 nM protein S were supplemented in protein S deficient plasma from Affinity Biologicals only a very small prolongation of lag time and reduction in peak height were observed. I therefore evaluated the protein S enhancement of TFPI in protein S deficient plasma supplied by two different companies, Affinity Biologicals and Hyphen Biomed. I observed that the effect on thrombin generation of TFPI (3 nM) with or without protein S (120 nM) varied depending on the source of plasma (Figure 3.22), with a more pronounced protein S/TFPI anticoagulant activity being observed in plasma supplied from Hyphen Biomed (Panel B) in comparison with that from Affinity Biologicals (Panel A). As can be seen in Figure 3.22, while the total protein S/TFPI anticoagulant activity in plasma from Affinity Biologicals prolonged the lag time by ~210% and reduced the peak height by ~20%, in plasma from Hyphen Biomed the lag time was prolonged by ~340% and the peak height reduced by ~65% (Figure 3.22). To investigate the difference between the two different sources of plasma my colleague, Verity Hockey, determined the amount of total TFPI in an in-house ELISA (n=1). This suggested that protein S deficient plasma from Affinity Biologicals contained approximately 25%, and Hyphen Biomed approximately 50%, of the amount of total TFPI present in normal plasma. During the course of this thesis it was shown by Castoldi *et al.* that TFPI can be co-depleted when protein S is immunodepleted from normal plasma in the production of protein S deficient plasma.[241] They also analysed the amount of TFPI in protein S deficient plasma from Affinity Biological and found it contained 25.5% of normal TFPI antigens levels which is in accordance with our findings. In subsequent experiments, protein S deficient plasma supplied by Hyphen Biomed was used, as it allowed better visualisation of the protein S/TFPI anticoagulant activity. However, to ensure that the effect observed in protein S deficient plasma from

Hyphen Biomed was not unique to this plasma, protein S deficient plasma supplied from Enzyme Research Laboratories was also tested. Both plasma from Hyphen Biomed and Enzyme Research Laboratories showed a similar prolongation of the lag time and reduction of the peak height following addition of TFPI in the presence and absence of protein S (results not shown).

In my initial experiments, I used TFPI in concentrated conditioned media that I had expressed from a stable cell line in our lab. This was quantified by a preliminary ELISA by my colleague Verity Hockey. Later in my project, purified TFPI (kind gift of the Chemoserotheraputic Institute Kaketsuken) was used. Unless specifically stated, protein S in concentrated conditioned media was used in all experiments.

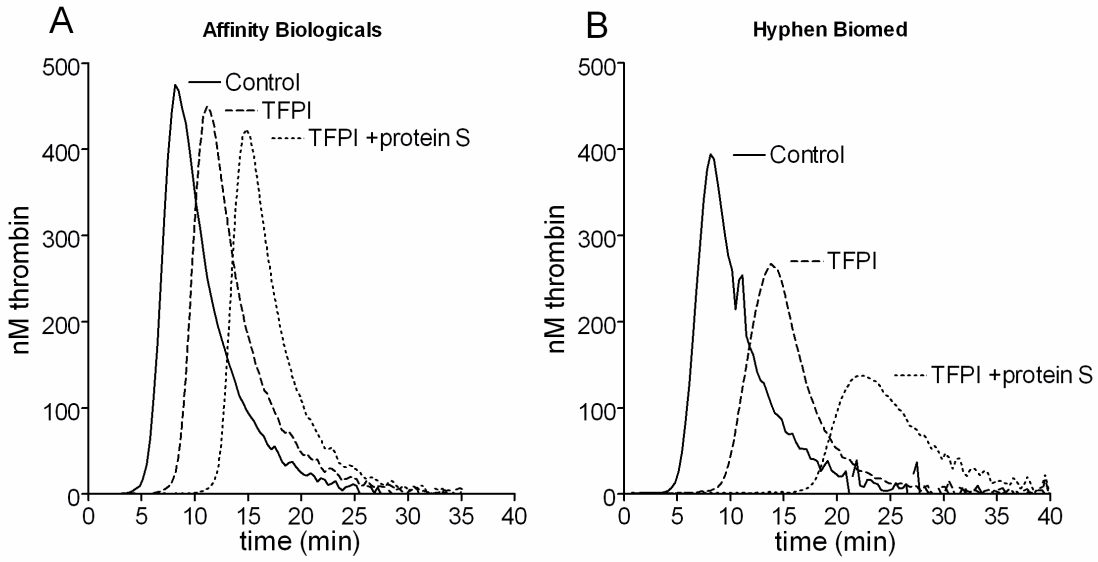

Figure 3.22 TFPI anticoagulant activity in the presence or absence of protein S in plasma. Purified TFPI (3 nM) was incubated in the presence or absence of protein S (120 nM) in protein S deficient plasma from Affinity Biologicals (A) and Hyphen Biomed (B), in the presence of 50 µM phospholipids, 65 µg CTI per ml plasma and 1 pM TF.

The specificity of the anticoagulant activity seen when supplementing TFPI in the plasma in the presence or absence of protein S was confirmed by performing CAT assays in presence or absence of inhibitory polyclonal antibodies. Polyclonal antibodies against TFPI (100 nM, Figure 3.23, Panel A) or polyclonal antibodies against protein S (1.5 µM, Figure 3.23, Panel B) were used. These completely inhibited the effect seen when supplementing TFPI and protein S in concentrated conditioned media to the plasma. Concentrated conditioned mock media not containing protein S and TFPI had no effect on thrombin generation (results not shown).

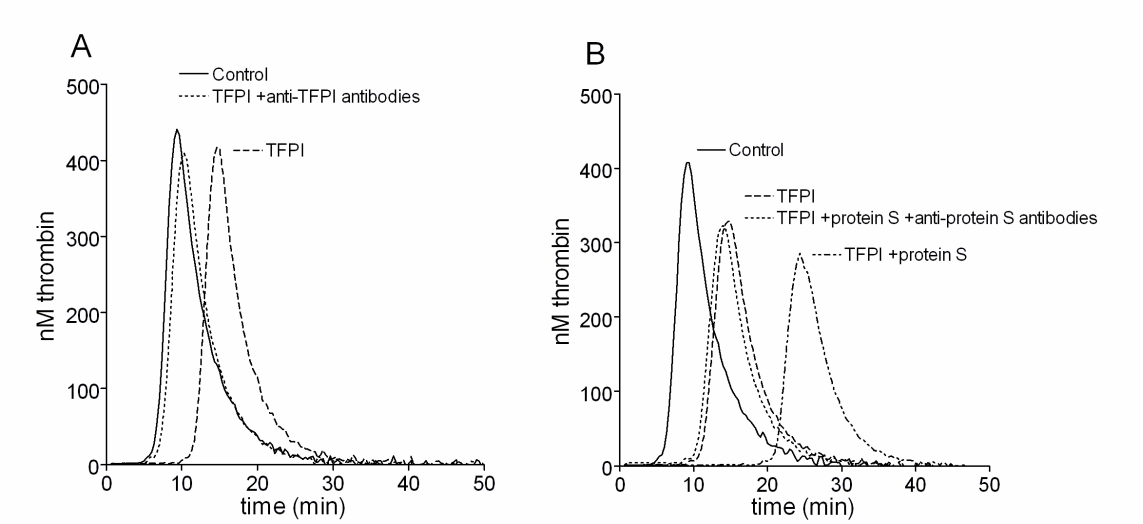

Figure 3.23 Influence on thrombin generation of polyclonal antibodies against TFPI and against protein S.
TFPI (2.5 nM) in concentrated conditioned media was incubated in the presence or absence of protein S (60 nM) in protein S deficient plasma from Hyphen Biomed. The activity observed by supplementing the plasma with TFPI and protein S was reversed by use of 100 nM polyclonal antibodies against TFPI (A) or 1.5 µM polyclonal antibodies against protein S (B).

While protein C does not circulate in its activated form, there remained a possibility that endogenous protein C might be activated in low levels by thrombin generated during the assay. To eliminate this possibility, TFPI and protein S were supplemented in the protein S deficient plasma in the presence or absence of 130 nM polyclonal antibodies against protein C (Figure 3.24). The polyclonal antibodies had previously been shown to be inhibitory against APC (see Figure 3.10). No difference in thrombin generation was observed following addition of inhibitory antibodies against protein C. This demonstrated that the thrombin generated during these assay conditions (i.e. in the absence of TM and EPCR) was unable to significantly activate protein C. Any anticoagulant activity of protein S was therefore independent of its APC cofactor function.

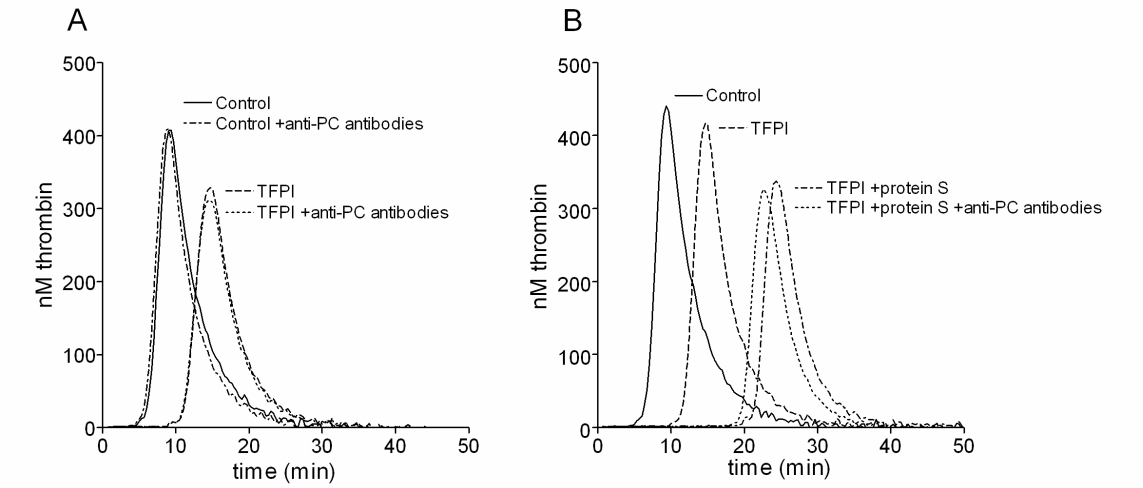

Figure 3.24 TFPI and protein S anticoagulant activity in plasma in the presence and absence of polyclonal antibodies against protein C.
The effect of TFPI in concentrated conditioned media (2.5 nM) in the absence (Panel A) and presence (Panel B) of protein S (60 nM) was assessed in the presence and absence of 130 nM polyclonal antibodies against protein C (PC). A representative experiment is shown. The results of addition of antibodies against protein C in the presence of TFPI and protein S was confirmed in additional experiments (n=3).

Once the assay conditions had been set up to guarantee the visualisation and specificity of the protein S enhancement of TFPI, I wanted to ensure that my different WT protein S preparations all enhanced TFPI to a similar extent. Thus, the activity of WT protein S in concentrated conditioned media in the presence of TFPI was compared with that of WT protein S purified by either purification protocol (see Methodology, section 2.3). Results (n=3) were analysed by a paired Wilcoxon test and no statistical difference in terms of thrombin peak height or lag

time was observed between the different preparations. Consequently, and because of its ready availability, monomeric protein S in concentrated conditioned media was preferentially used.

The TFPI cofactor activity of WT protein S in concentrated conditioned media was evaluated by titrating protein S (0-120 nM) in the presence of 3 nM TFPI. As can be seen in Panel A of Figure 3.25, WT protein S enhanced TFPI in a dose-dependent manner. To evaluate whether residues in protein S variants with reduced APC cofactor activity (see section 3.3.2) were also important for TFPI cofactor activity, protein S Face2 (Panel B), protein S D95A (Panel C), protein S D95N (Panel D), protein S D78A (Panel E) and protein S Q79A (Panel F) were titrated alongside WT protein S to evaluate their TFPI cofactor activity (Figure 3.25). To more easily visualise the difference in peak height and lag time between the variants and WT protein S, the results were plotted as % change in lag time and peak height in comparison with the control (Figure 3.26). Interestingly, Figure 3.25 and Figure 3.26 show that all variants were able to enhance TFPI anticoagulant function. However, protein S variants D95A, D78A and Q79A were somewhat less efficient than WT protein S. They all had a reduced lag time (by ~10-20%) and increased peak height (by ~5-20%) in comparison with WT protein S. Protein S D95N had a similar lag time to WT protein S, but an increased peak height (by ~10-25%). While protein S Face2 had a reduced effect on the lag time (by ~10-20%) compared to WT protein S, its effect on the peak height was enhanced (by up to 60%). The titration of WT protein S and protein S D95A was performed twice but the percentage effect on peak height and lag time could not be expressed as mean ± SD due to the different assay conditions (3 nM vs 2.5 nM TFPI). The TFPI cofactor activities of protein S variants D95A, D95N, D78A and Q79A were in addition evaluated using single protein S concentrations (n=3), which confirmed the results obtained from the titration. When the cofactor activity of protein S Face2 was reassessed, however, it was generally similar to that of WT protein S in terms of lag time, while the effect on peak height was more pronounced, suggesting that this variant has enhanced TFPI cofactor activity. To further assess the TFPI cofactor activity of protein S Face2 and protein S D95A these variants were purified and assessed in the FXa inhibition assay (see section 3.4.2.2).

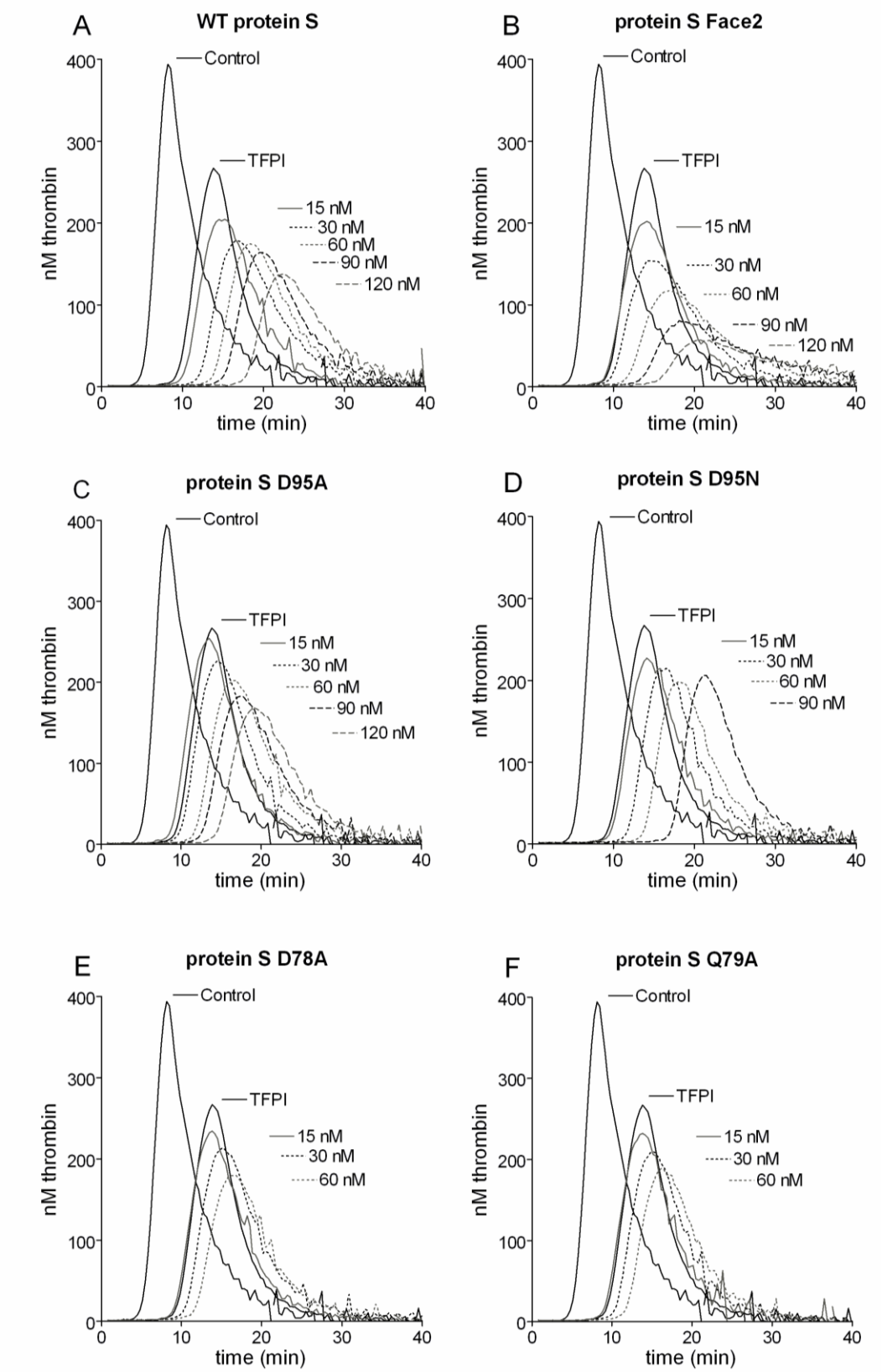

Figure 3.25 Thrombin generation in the presence of TFPI and WT protein S, protein S Face2, D95A, D95N, D78A or Q79A.

Thrombin generation was assessed at 3 nM TFPI in concentrated conditioned media in the presence of 0-120 nM WT protein S (A), protein S Face2 (B), protein S D95A (C), protein S D95N (D), protein S D78A (E) or protein S Q79A (F). Protein S D95N, protein S D78A and protein S Q79A could not be evaluated at higher concentrations than those described here due to their stock concentrations.

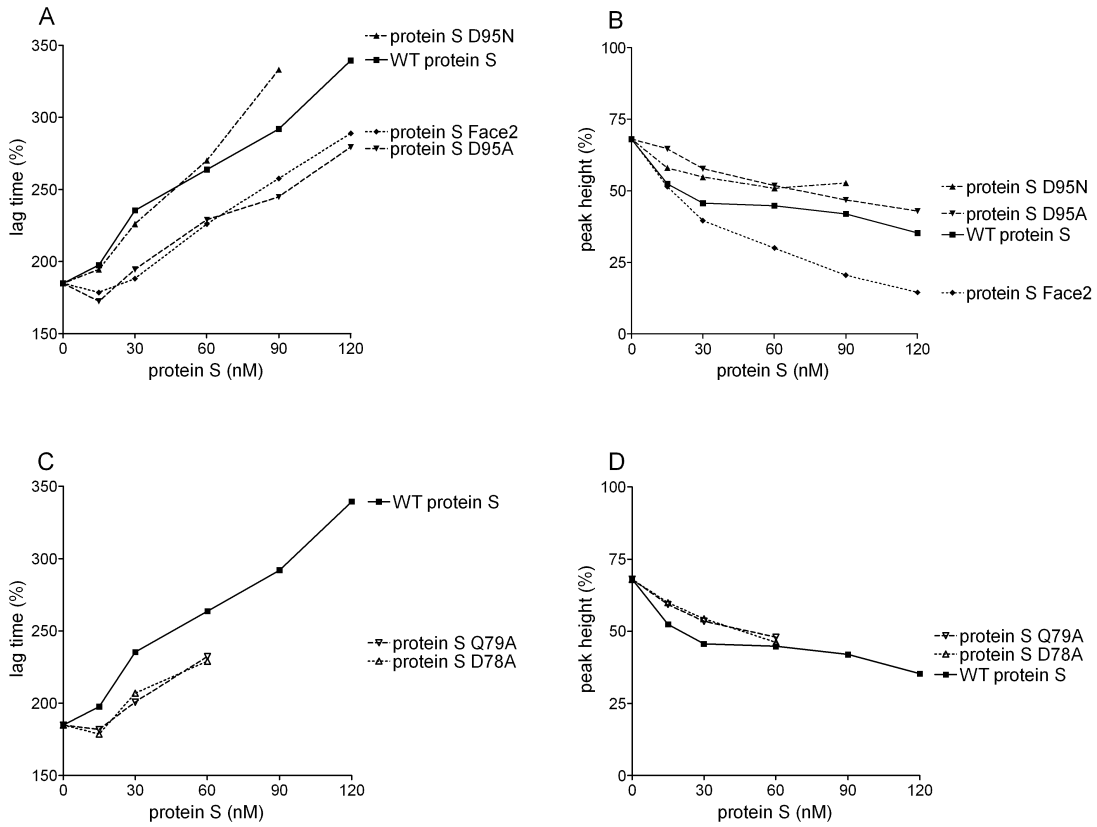

Figure 3.26 Change (%) in peak height and lag time of WT protein S, protein S Face2, D95A, D95N, D78A and Q79A in comparison with the control.
The effects of TFPI and protein S on peak height and lag time from Figure 3.26 were expressed as a % change in comparison with the control. The % change of WT protein S, protein S D95A, protein S D95N and protein S Face2 on lag time and peak height is shown in Panel A and B respectively. The % change of WT protein S, protein S D78A and protein S Q79N on lag time and peak height is shown in Panel C and D, respectively.

Additional experiments were performed with the EGF1 and EGF2 protein S point variants produced by my collaborator Dr S.M Rezende (Universidade Federal de Minas Gerais, Brazil). These were screened at 90 nM using 2.5 nM TFPI (Figure 3.27 and Figure 3.28). The TFPI cofactor activity of protein S T108A, protein S N136A, protein S S153A and protein S K155A could not be evaluated due to lack of material. As can be seen, observing the lag times and peak heights in Figure 3.27 and Figure 3.28, most protein S variants had a similar cofactor activity towards TFPI to that of WT protein S. Several protein S variants showed a slightly enhanced prolongation of the lag time and reduction in the peak height, however, none of the 19 variants tested, exhibited greatly reduced TFPI cofactor activity.

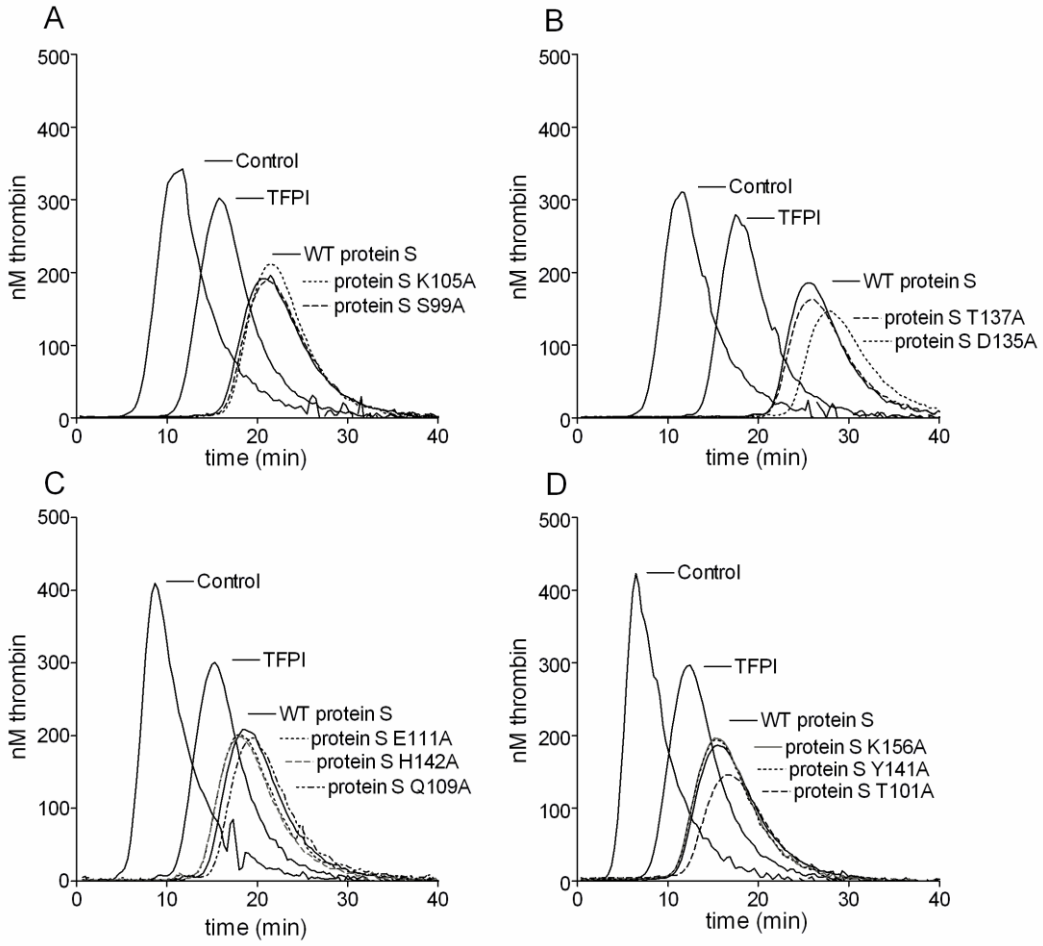

Figure 3.27 Thrombin generation in the presence of TFPI and WT protein S and protein S EGF1 and EGF2 point variants.
Thrombin generation was assessed in protein S deficient plasma supplemented with 2.5 nM TFPI in concentrated conditioned media. The cofactor activity of protein S variants, 90 nM, towards TFPI was assessed.

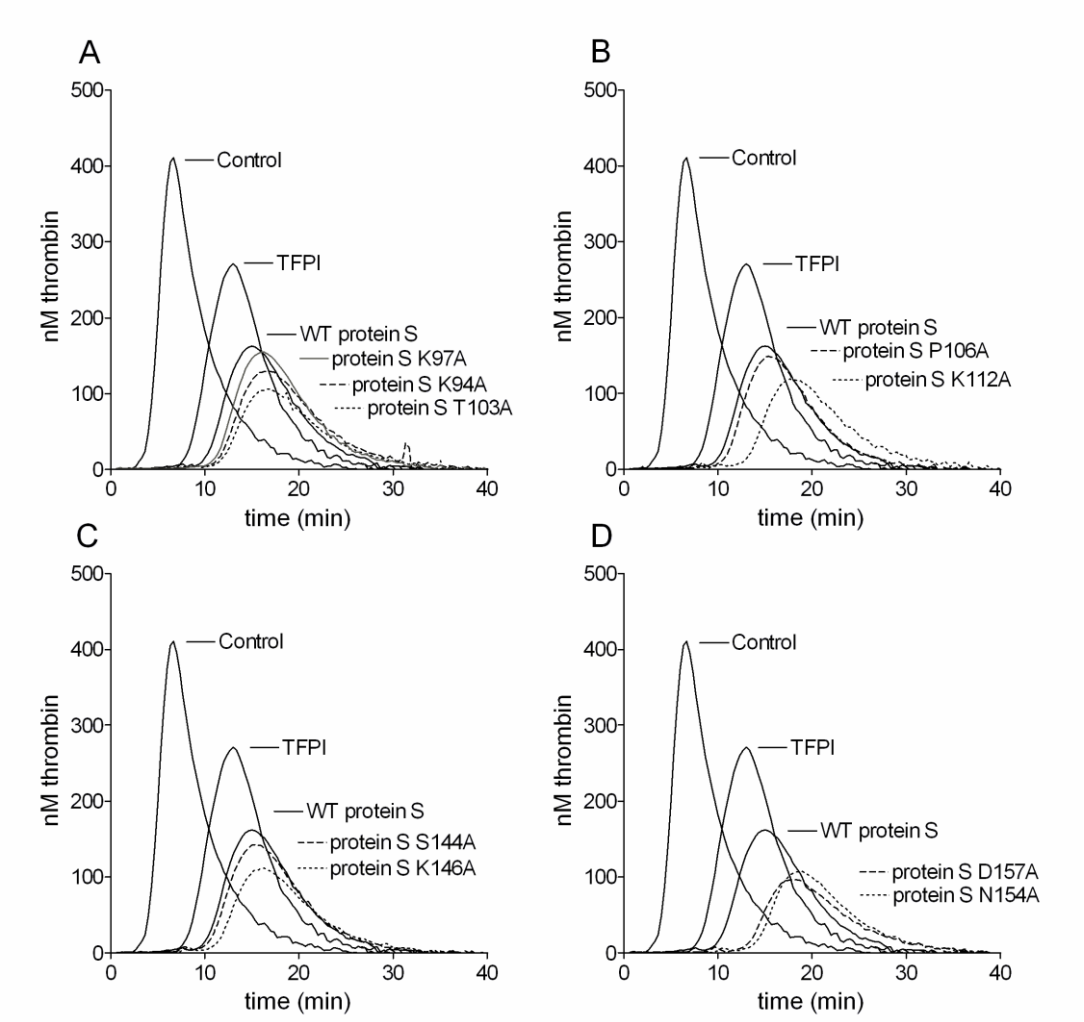

Figure 3.28 Thrombin generation in the presence of TFPI and WT protein S and protein S EGF1 and EGF2 point variants.
Thrombin generation was assessed in protein S deficient plasma supplemented with 2.5 nM TFPI in concentrated conditioned media. The cofactor activity of protein S variants, 90 nM, towards TFPI was assessed.

As stated in section 3.4.1, two different sources of TFPI were available, TFPI in concentrated conditioned media and purified TFPI. When 90 nM WT protein S was evaluated for its TFPI cofactor activity towards 2.5 nM purified TFPI (Figure 3.29, Panel A), a more pronounced anticoagulant activity was observed both in terms of prolongation of the lag time and reduction of the peak heights to that previously observed when using TFPI in concentrated conditioned media. When the anticoagulant activity of TFPI in concentrated conditioned media was compared with that of purified TFPI, a difference was observed both in the absence and presence of protein S (Figure 3.29, Panel B).

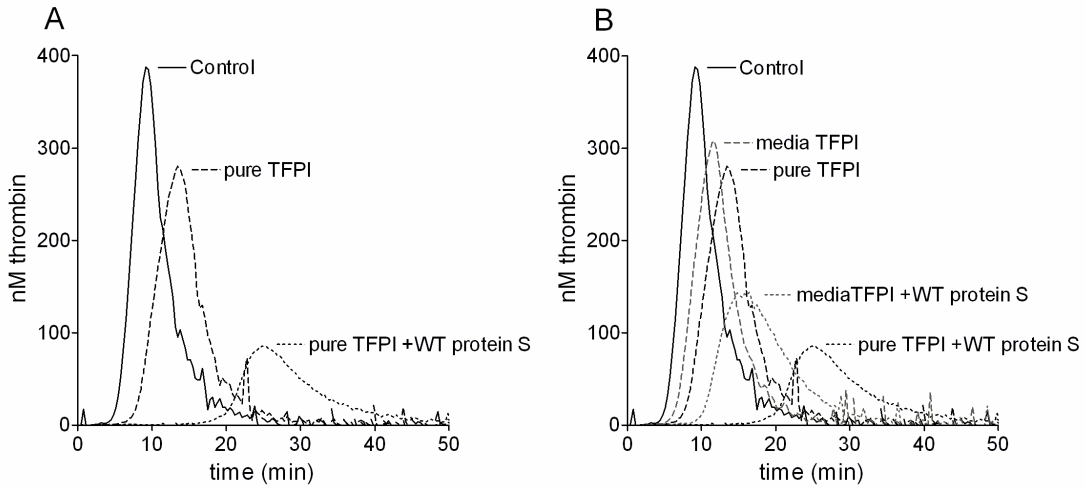

Figure 3.29 Thrombin generation in the presence of purified TFPI and TFPI in concentrated conditioned media in the presence and absence of protein S.
Thrombin generation in protein S deficient plasma was analysed in the presence of 2.5 nM purified TFPI in the presence or absence of 60 nM protein S (A). The anticoagulant activity of purified TFPI in the presence or absence of protein S was compared to that of TFPI in concentrated conditioned media (B).

To determine the reason for this discrepancy, I assessed how dependent the total anticoagulant activity of TFPI/protein S is upon the concentration of TFPI. Consequently, purified TFPI was titrated (2.5, 5 and 7.5 nM) in the presence or absence of 60 nM protein S (Figure 3.30). I observed that a small difference, in terms of anticoagulant activity, between 2.5, 5 and 7.5 nM TFPI in the absence of protein S translated into a large difference in the presence of protein S. Previous results (Figure 3.25), in which a titration of protein S was performed at a fixed concentration of TFPI, showed only small changes in cofactor activity between 30 nM increments in concentration of protein S. Taken together, these results (Figure 3.25 and Figure 3.30) suggest that the total TFPI anticoagulant activity in

the presence of protein S is more dependent upon the TFPI concentration than upon the protein S concentration.

The different anticoagulant activities of TFPI in concentrated conditioned media or in fully purified TFPI is likely attributable to inaccuracies in determination of the concentration of TFPI in concentrated conditioned media, as this and the initial experiments were performed prior to complete optimisation of our in-house TFPI ELISA. As TFPI in conditioned media was expressed in HEK293 cells and my purified TFPI was expressed in Chinese hamster ovary cells, the glycosylation will be different. However, this is unlikely to be the reason behind the discrepancy, as the purified TFPI from the Chemoserotheraputic Institute Kaketsuken was shown to have the same activity as plasma purified TFPI,[259] and the glycosylation from human HEK293 cells is likely to be broadly similar to that of plasma TFPI. In addition, Huang *et al.* evaluated the inactivation of FXa both by bacterial and mammalian expressed TFPI and found no significant difference, suggesting post-translational modifications do not play a major role.[72] To determine the reason behind the discrepancies between the two different sources of TFPI, I believe that the ELISA should be repeated in future studies and the TFPI from HEK293 cells purified and assessed alongside TFPI from the Chemoserotheraputic Institute Kaketsuken in functional assays.

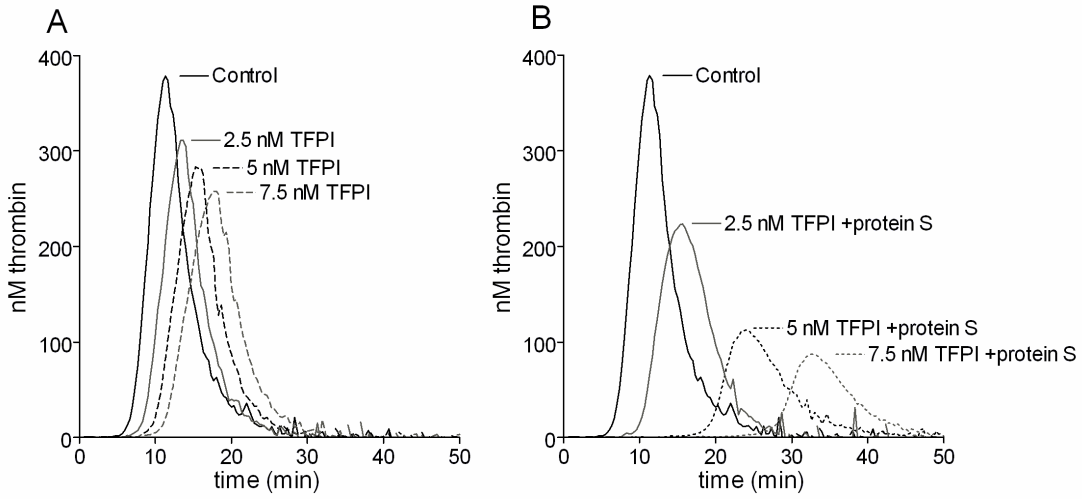

Figure 3.30 Protein S enhancement of TFPI at different concentrations of TFPI.
Thrombin generation in the presence of different concentrations of TFPI (2.5, 5 and 7.5 nM) was assessed in the absence (A) and presence (B) of 60 nM protein S.

To ascertain whether the different assay conditions (i.e. source of TFPI and TFPI concentration) influenced the outcome of the initial screenings (Figure 3.25, Figure 3.27 and Figure 3.28), the activity of some of the protein S variants (60 nM) was reassessed using 5 nM purified TFPI. Protein S variants Face2, D95N, Q79A, T103A and K112A, previously assayed in the presence of 2.5 nM TFPI in conditioned media (Figure 3.25 and Figure 3.28) were reassessed using 5 nM purified TFPI (Figure 3.31B,C,D). The results obtained with these protein S variants were consistent with earlier results, showing that protein S D95N and Q79A had somewhat reduced TFPI cofactor activity, while protein S Face2, T103A, K112A had slightly enhanced TFPI cofactor activity. This suggests that while the total effect of protein S in the presence of TFPI varies between different assay conditions, variants with reduced, normal or enhanced TFPI cofactor activity in comparison with WT protein S are reproducibly visualised in both assay conditions. Protein S variants Face1, Gla1 and TSR, that had not previously been assessed for TFPI cofactor activity, were also analysed (Figure 3.31A,C). Of the newly screened variants, protein S Gla1 had a similar TFPI cofactor activity to that of WT protein S, protein S Face1 had reduced cofactor activity and protein S TSR an increased TFPI cofactor activity (Figure 3.31). To further assess protein S variants with altered TFPI cofactor activity, protein S TSR, in addition to protein S D95A and protein S Face2, were purified and evaluated in the FXa inhibition assay. Protein S Face1 has been investigated by my colleague Dr Josefin Ahnström.

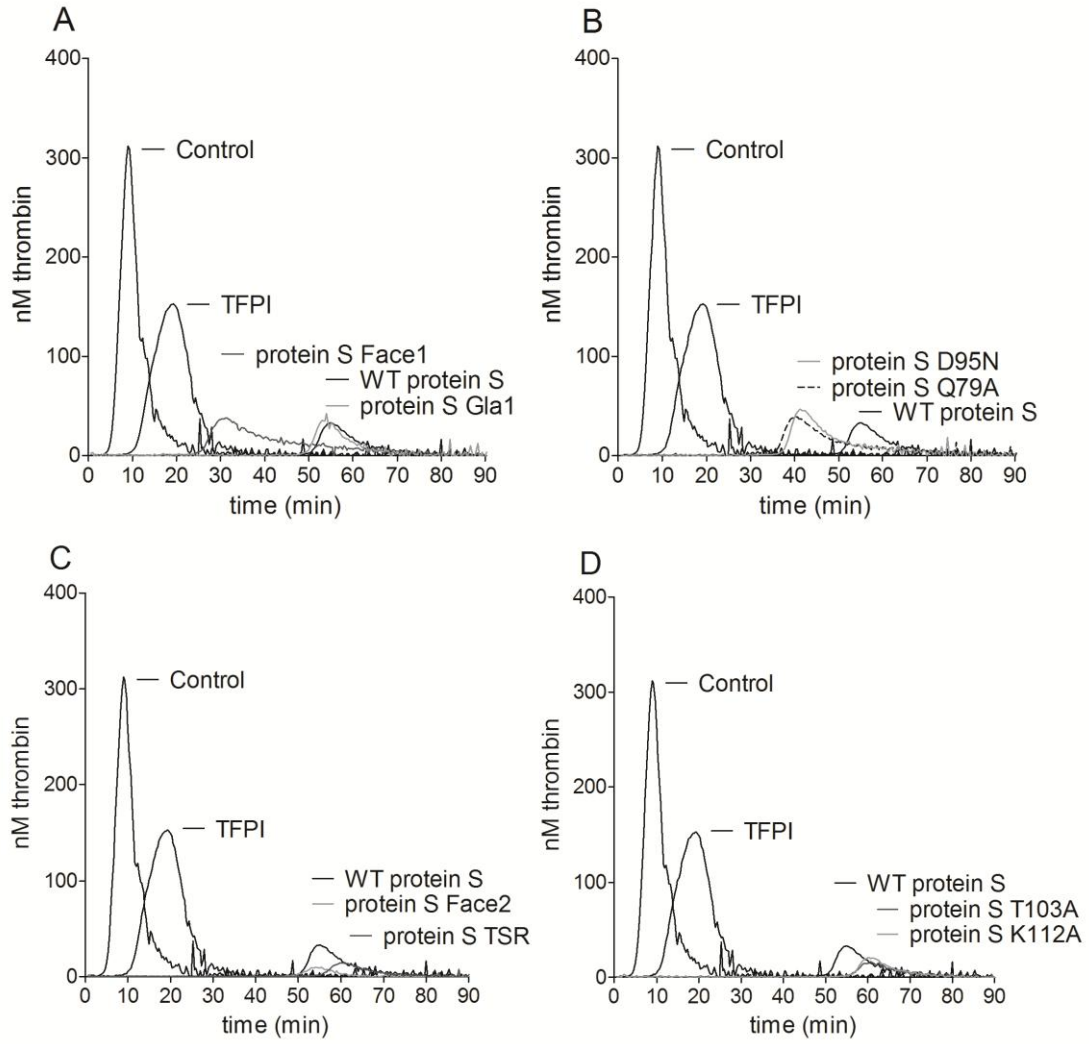

Figure 3.31 Thrombin generation in the presence of TFPI and WT protein S and protein S variants.
Thrombin generation was assessed in protein S deficient plasma supplemented with 5 nM purified TFPI. The cofactor activity of protein S variants towards TFPI was assessed at 60 nM.

Of the variants screened up to this point for their TFPI cofactor activity in the CAT assay, none had completely impaired cofactor activity, in terms of both impaired prolongation of the lag time and an impaired reduction of the peak height. In a further attempt to identify residues of protein S important for TFPI cofactor activity, additional variants in the Gla-TSR-EGF1 domains were made. The Gla-TSR-EGF1 model[193] was used to identify potential new residues to substitute. However, not many residues were left to mutate in the Gla domain once those close to the ω-loop, γ-carboxylated residues, alanine residues, cysteine residues, residues with a buried side chain and those already substituted, were excluded. All charged residues in the TSR had already been substituted and had been

incorporated into the protein S TSR variant. Polar residue Asn74 in the TSR was, however, chosen to create an additional point substitution, protein S N74A, as it is in close proximity of the partially conserved calcium binding motif in the EGF1. A small cluster of amino acid residues with an exposed R group, not conserved in the EGF1 domains of other coagulation factors, were identified on one side of EGF1. These were substituted to create the composite variant, protein S NEDM (N86A/E87Q/D88A/M91A). Asn74 was also incorporated in this composite variant creating composite variant protein S NNEDM. As single point variants might not be enough to disrupt protein S cofactor activity towards TFPI, a composite variant composed of the previously examined point substitutions K94A, T103A and K105A was also made, generating the variant protein S KTK. These residues were selected as T103N is a natural protein S variant in which reduction in APC cofactor activity could not be fully explained using purified FVa and FVIIIa inactivation assays.[255] Residues Lys94 and Lys105 were selected as they were charged and in close proximity to Thr103, and these three residues formed a cluster on a separate side to that of NEDM.

The new variants were screened by CAT at 60 nM in the presence of 2.5 nM purified TFPI (Figure 3.32, Panel A and B). The protein S variants were also simultaneously evaluated for their APC cofactor activity as this had not been done previously (Figure 3.32, Panel C and D). Protein S N74A had a normal APC cofactor activity (Panel C) and a slightly enhanced TFPI cofactor activity (Panel A). Composite variants protein S NEDM and protein S KTK had normal APC cofactor activity (Panel C), but reduced TFPI cofactor activity (Panel A). The addition of the N74A substitution to protein S NEDM, creating protein S NNEDM, resulted in a variant with both reduced APC cofactor activity (Panel D) and TFPI cofactor activity (Panel B). As can be seen in Figure 3.32 protein S NNEDM has reduced APC cofactor activity in comparison with protein S NEDM, however both variants have a similar TFPI cofactor activity. To further analyse these variants, protein S N74A, KTK, NEDM and NNEDM were purified to assess them in the FXa inhibition assay. Purification of protein S NEDM was not successful and it could not be re-expressed and purified, due to the time constraints of writing up this thesis. Protein S N74A, protein S KTK and protein S NNEDM were, however, successfully purified and further analysed in the FXa inhibition assay (see section 3.4.2.2).

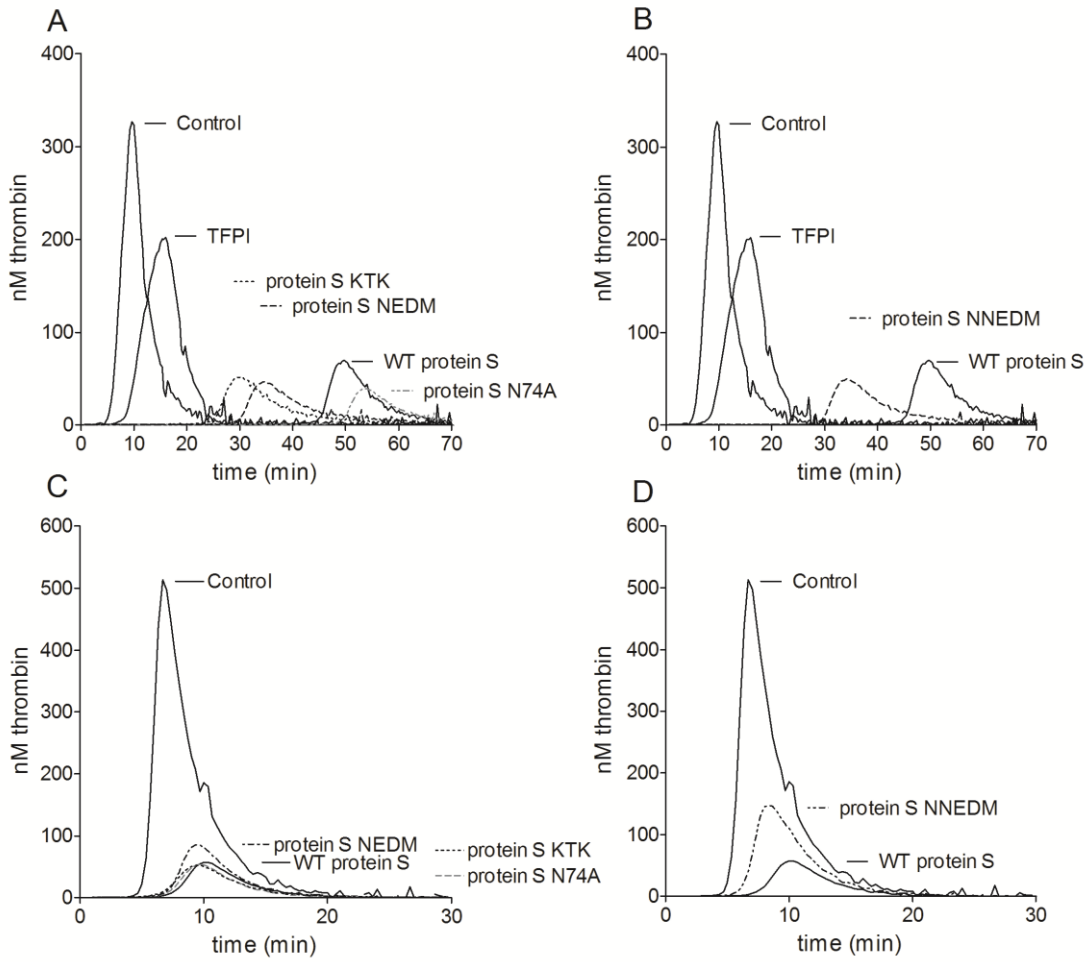

Figure 3.32 WT protein S, protein S N74A, KTK, NEDM and NNEDM studied by thrombin generation in the presence of TFPI or APC.
Thrombin generation was assessed in protein S deficient plasma supplemented with protein S either in the presence of 2.5 nM TFPI (A, B) or 4 nM APC (Xigris) (C, D). Protein S was used at 60 nM to evaluate TFPI cofactor activity and 90 nM to evaluate APC cofactor activity. Protein S N74A, KTK and NEDM all had normal APC cofactor activity (C). Addition of substitution N74A to protein S NEDM creating protein S NNEDM resulted in a partial loss of APC cofactor activity (D). Protein S N74A had an increased TFPI cofactor activity, while the cofactor activity of protein S KTK and NEDM towards TFPI was reduced (A). Addition of substitution N74A to protein S NEDM did not affect its cofactor activity towards TFPI (Panel B in comparison with Panel A). Note that APC from Xigris was used in this experiment and was more active than the commercial APC preparation mostly used in this thesis (Enzyme Research Laboratories).

3.4.2 Evaluation of TFPI cofactor activity of protein S in the FXa inhibition assays

3.4.2.1 Evaluation of FXa inhibition by WT purified TFPI and protein S

The FXa inhibition assay was optimised with purified TFPI (Chemoserotheraputic Institute, Kaketsuken) and purified plasma protein S (Enzyme Research Laboratories). Importantly, a recent paper by Mori *et al.*, (from the

Chemoserotheraputic Institute Kaketsuken) showed that the protease inhibitior activity of the recombinant purified TFPI towards FXa and TF/FVIIa was identical to that of plasma purified TFPI.[259] Protein S in media could not be used as an unknown protease in both this preparation and the mock media (without protein S) was able to cleave the FXa substrate in a dose-dependent manner (results not shown).

To assess the specificity of FXa inhibition by TFPI, purified TFPI (0-8 nM) was titrated and was shown to inhibit FXa in a dose-dependent manner (Figure 3.33). To assess the cofactor activity of protein S, low concentrations of TFPI (0.5-2 nM) were used to maximise the detection of the enhancing effect in the presence of protein S. Purified plasma protein S (0-160 nM) was titrated in the presence of 1.5 nM TFPI (Figure 3.34). A dose-dependent enhancement of TFPI was observed suggesting that the activity of TFPI was specifically enhanced by protein S. Importantly, protein S did not affect FXa activity in the absence of TFPI (as shown in subsequent figures), confirming the high specificity of the assay.

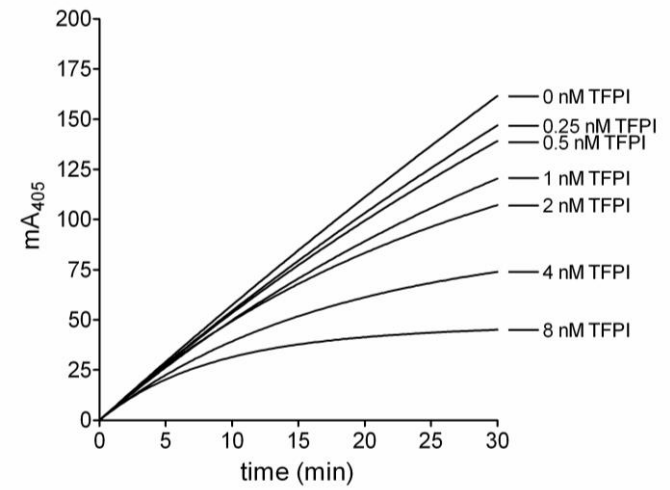

Figure 3.33 Dose-dependent inhibition of FXa by TFPI.
TFPI (0-8 nM) was incubated with phospholipids (10 µM), $CaCl_2$ (5 mM) and the FXa chromogenic substrate S-2765 (200 µM) in a 20 mM Tris-HCl, 140 mM NaCl, 5 mg/ml BSA buffer. The assay was started by the addition of FXa (0.5 nM) and the absorbance at 405 nm was read over time.

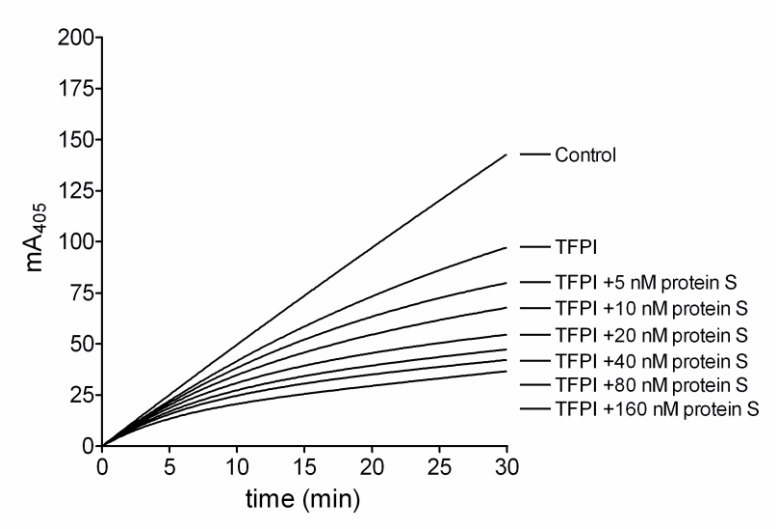

Figure 3.34 Dose-dependent enhancement of TFPI activity by protein S.
TFPI (1.5 nM) was incubated with 0-160 nM protein S, phospholipids, $CaCl_2$ and the FXa chromogenic substrate S-2765. The assay was started by the addition of FXa and the absorbance at 405 nm was read over time.

As previously reported, the TFPI cofactor activity of protein S was phospholipid dependent (Figure 3.35).[81] In accordance with the literature,[72] TFPI mediated inhibition of FXa did not require phospholipids although its activity was moderately enhanced by their presence (see effect of TFPI on FXa inhibition in Panel A and B of Figure 3.35). This could be due to co-localisation of FXa and TFPI on the phospholipid surfaces. FXa binds phospholipids through its Gla domain and it has been suggested that the positively charged C-terminal tail of TFPI may interact with negatively charged phospholipid surfaces and cell surfaces[74-76] and FXa[79,80]. No difference in TFPI inhibition of FXa and protein S cofactor activity towards TFPI was observed between 10 and 50 µM phospholipids suggesting that saturating amounts of phospholipids were present at 10 µM phospholipids. This is potentially important, as protein S has previously been shown to have a protein S direct activity (independent of APC and TFPI) due to competition for phospholipid surfaces when these are present in limiting amounts.[189,190,242]

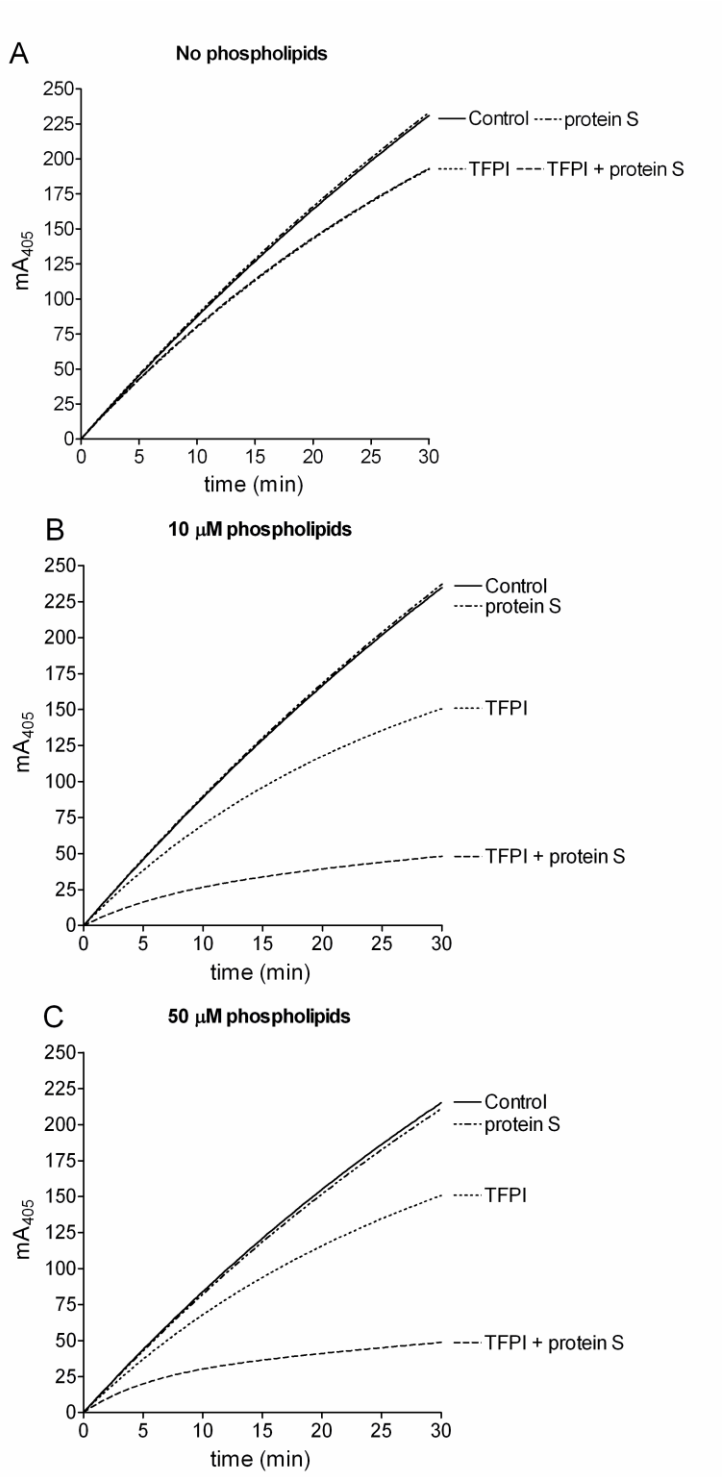

Figure 3.35 Inhibition of FXa by TFPI and protein S in the absence and presence of phospholipids.
TFPI (1.5 nM) and protein S (20 nM) were incubated with $CaCl_2$, S-2765 with no phospholipids (A), 10 μM phospholipid vescicles (B) or 50 μM phospholipid vescicles (C). The assay was started by the addition of FXa and the absorbance at 405 nm was measured over time. TFPI inhibition of FXa cleavage of S-2765 was observed in the absence of phospholipids (Panel A) but was enhanced by their presence (Panel B and C). Protein S enhanced the TFPI mediated inhibiton of FXa in the presence (Panel B and C) but not in the absence (Panel A) of phospholipids. Protein S did not affect FXa cleavage of S-2765 in the absence of TFPI in any of the experimental conditions.

3.4.2.2 Evaluation of domains and amino acids of protein S involved in TFPI cofactor activity by use of domain specific monoclonal antibodies and protein S variants

In an attempt to identify the domains of protein S important for TFPI cofactor activity, monoclonal domain specific antibodies against protein S (kind gift of Professor Björn Dahlbäck and Sofia Carlsson, University of Lund, Sweden) were used to inhibit protein S enhancement of TFPI. Protein S (20 nM) was preincubated in the presence or absence of monoclonal antibody (200-400 nM) in the presence of $CaCl_2$ (as some of the antibodies are calcium dependent, the final concentration of $CaCl_2$ in the well was 5 mM) for up to 30 minutes. Phospholipids and the FXa chromogenic substrate were subsequently added and the reaction was started by addition of 0.5 nM FXa (Figure 3.36). None of the monoclonal antibodies tested completely inhibited protein S enhancement of TFPI. The antibodies against the Gla domain of protein S, MK21 and MK47, showed the most inhibition of protein S cofactor activity. However, these monoclonal antibodies have previously been reported to inhibit phospholipid binding, which is a prerequisite for protein S function.[243] It was therefore not possible to conclude whether the Gla domain of protein S is involved in an interaction with TFPI. Monoclonal antibodies against the TSR domain, the EGF1 domain, the EGF3-4 domain and the SHBG domain had no or little effect upon protein S enhancement of TFPI (Figure 3.36). While this would suggest that the epitopes recognised by these antibodies are not involved in an interaction with TFPI, it is possible that other amino acid residues within the respective domains may be involved and remain accessible. Interestingly, the epitope of the monoclonal antibody MK54 against the EGF1 domain of protein S includes residue Lys97.[260] It is therefore likely that this antibody would inhibit a potential interaction involving Asp95 in protein S with TFPI. No monoclonal antibody directed against the EGF2 domain was available.

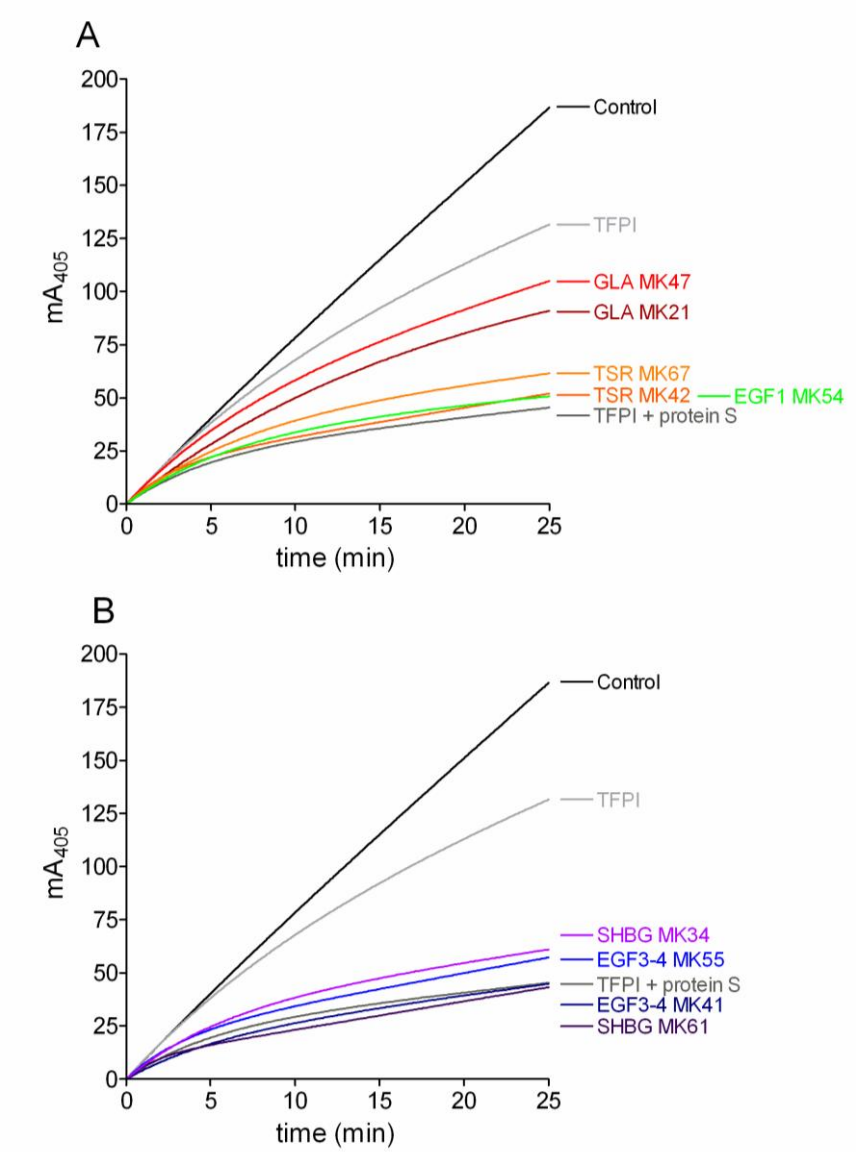

Figure 3.36 Inhibition of protein S cofactor activity towards TFPI by monoclonal domain specific antibodies.
Protein S (20 nM) was preincubated in the presence or absence of 200-400 nM of the domain specific monoclonal antibody against protein S in the presence of $CaCl_2$ for up to 30 minutes. TFPI (1.5 nM), S-2765 and phospholipids were subsequently added and the reaction was started by addition of FXa. Monoclonal antibodies against the Gla domain, TSR domain and the EGF1 domain are shown in Panel A. Monoclonal antibodies directed against the EGF3-4 domain and the SHBG domain are shown in Panel B. All data point were normalised against the control. Results represents the mean from two different experiments performed in duplicate. The labelling TFPI + protein S has been omitted in the presence of the antibody. The domain against which the antibody is directed precedes the name of each antibody.

Of the over 30 protein S variants screened for their TFPI cofactor activity in plasma (section 3.4.1), those of interest were purified and assessed in the FXa inhibition assay. Protein S Face2 and protein S TSR had been purified by anion exchange chromatography followed by immunoaffinity chromatography and were compared with WT protein S purified using the same method. Figure 3.37 shows

the protein S Face2 and protein S TSR cofactor enhancing activity in the FXa assay compared with that of WT protein S (20 nM) using 1.5 nM TFPI. Both protein S Face2 and protein S TSR showed a similar TFPI cofactor activity to that of WT protein S. The same results were observed over a range of TFPI (0.75-2 nM) and protein S (5-40 nM) concentrations (not shown) suggesting residues mutated in these variants are not critical for TFPI cofactor activity. This is in agreement with the results obtained in the plasma assay.

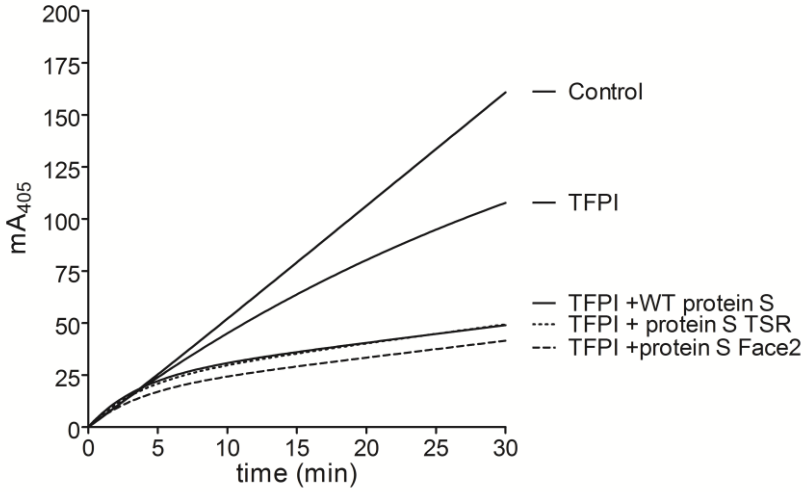

Figure 3.37 TFPI inhibition of FXa in the presence and absence of WT protein S, protein S Face2 and protein S TSR.
TFPI (1.5 nM) was incubated with 20 nM protein S, phospholipids, $CaCl_2$ and the FXa chromogenic substrate S-2765. The assay was started by the addition of FXa and the absorbance at 405 nm was read over time.

When protein S D95A (purified by anion exchange chromatography followed by immunoaffinity chromatography) was tested in the FXa inhibition assay, it appeared to have a reduced cofactor activity for TFPI. Due to lack of material, protein S D95A in conditioned media was re-purified by barium citrate precipitation followed by anion exchange chromatography alongside WT protein S, protein S N74A, KTK and NNEDM. Protein S variants (20 nM) were screened for their TFPI cofactor activity at 1.5 nM TFPI and were compared with WT protein S purified in the same way. Protein S KTK and protein S N74A had TFPI cofactor activities similar to that of WT protein S, as can be seen in Figure 3.38. The cofactor activities of protein S D95A and protein S NNEDM were, however, reduced in comparison with that of WT protein S (Figure 3.38). These results are

in agreement with those obtained in plasma, with the exception of protein S KTK which exhibited reduced cofactor activity in plasma.

None of the protein S preparations affected FXa cleavage of S-2765 in the absence of TFPI (results not shown). To further investigate the protein S cofactor activity for TFPI, kinetic assays were performed to allow determination of the K_i values and, consequently, to quantify the difference between WT protein S and protein S variants.

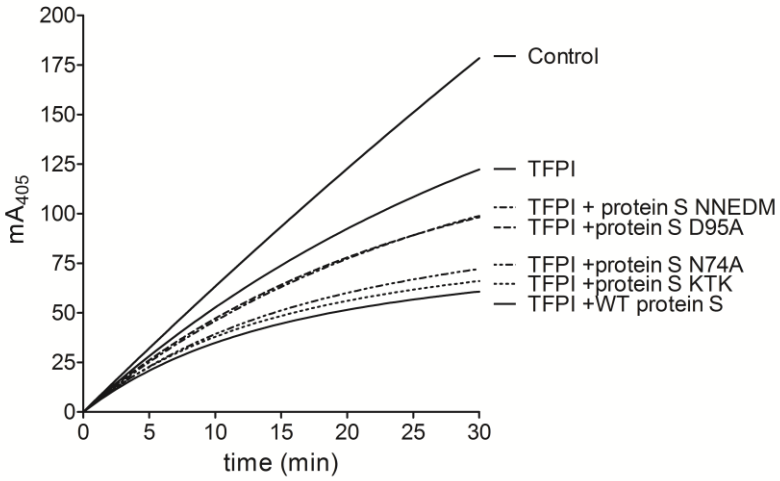

Figure 3.38 TFPI inhibition of FXa in the presence and absence of WT protein S, protein S D95A, protein S N74A, protein S KTK and protein S NNEDM.
TFPI (1.5 nM) was incubated with 20 nM protein S, phospholipids, $CaCl_2$ and the FXa chromogenic substrate S-2765. The assay was started by the addition of FXa and the absorbance at 405 nm was read over time.

3.4.2.3 Kinetic analysis of TFPI mediated inhibition of FXa in the presence and absence of protein S

To calculate the inhibition constant, K_i, of TFPI for FXa, the K_m for the hydrolysis of the chromogenic substrate S-2765 by FXa was first determined. The FXa inhibition assay was performed as described in section 2.7 but in the absence of TFPI and protein S and at a fixed concentration of FXa (0.5 nM). The substrate was titrated (0-1200 µM) and the initial velocity V_0 was plotted against the substrate concentration (Figure 3.39). Curve fitting of the initial velocities allowed determination of the K_m. A K_m of 46.3 ± 11.7 µM was obtained from four different experiments performed in duplicate. This is compatible with the K_m of 42.6 ± 2.3 µM reported by Broze and colleagues.[72]

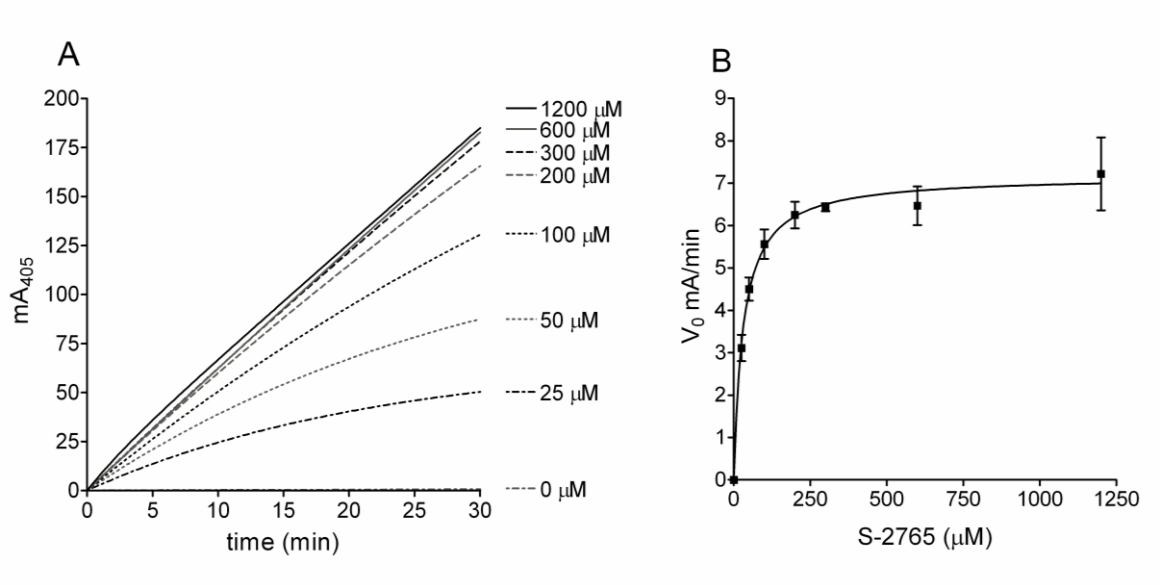

Figure 3.39 Determination of the K_m of FXa for the chromogenic substrate S-2765.
The K_m of the hydrolysis of S-2765 by FXa was determined by titrating the substrate over the range 0-1200 µM in the presence of phospholipids and $CaCl_2$ and in the absence of TFPI and protein S. A representative experiment is shown (A). The mean ± SD of the V_0 from four independent experiments performed in duplicate was plotted against the concentration of the substrate (B). A K_m of 46.3 ± 11.7 µM was obtained.

When the FXa inactivation assay was started either by the addition of FXa (as described by Ndonwi *et al.*[238]) or substrate and TFPI (similar to what was described by Hackeng *et al.*[81]), a difference in the V_0 was observed in the presence of TFPI and protein S. While the respective absorbance values at 30 minutes were similar, the shapes of the curves were appreciably different. When the assay was started with FXa, the data points describing the protein S enhancement of TFPI formed a distinctive curve with a high V_0 (Figure 3.40, Panel A). However, when the assay was started with the addition of substrate and TFPI, the protein S enhancement of TFPI was more linear and resulted in a lower V_0 (Figure 3.40, Panel B). These differences can be more easily visualised by looking at the first 5 minutes of the assay (Figure 3.40 C and D). An explanation for these discrepancies is not yet available.

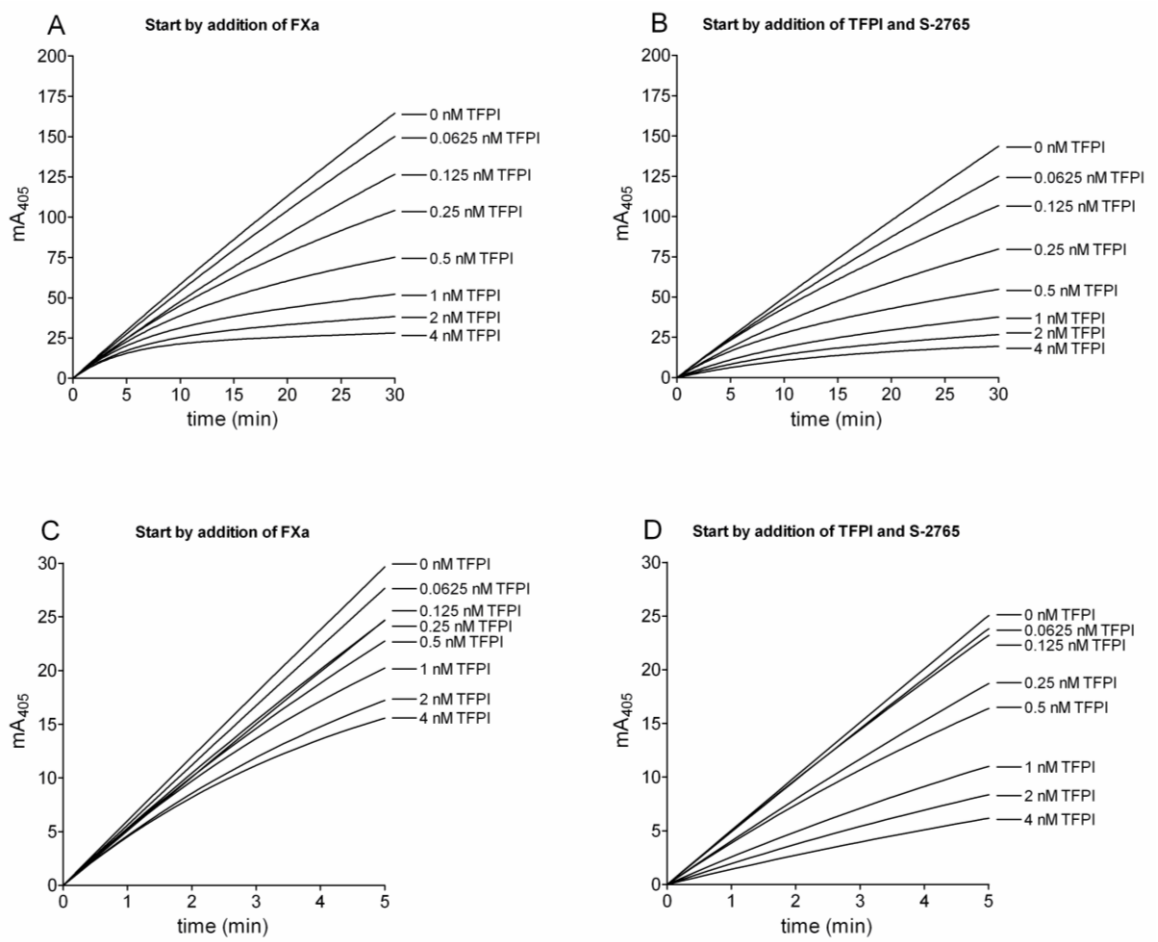

Figure 3.40 FXa inhibition assay started by the addition of FXa or by the addition of TFPI and S-2765.
Protein S (100 nM) enhancement of TFPI (0-4 nM) was evaluated. The assay was started either by addition of FXa (A) or addition of TFPI and S-2765 (B). Panels C and D shows the first 5 minutes of the results shown in Panel, A and B, respectively. Representative graphs are shown.

As the K_i of TFPI for FXa is dependent upon calculation of the V_0, the FXa inhibition assay was started with TFPI and S-2765 where a change in V_0 is more readily seen. The K_i values of TFPI in the presence or absence of protein S determined in the literature were derived by starting the FXa inhibition assay with FXa and then, after approximately 5 minutes, dispensing TFPI.[81] To determine the K_i, the timepoint at which TFPI was added was considered to be timepoint zero. As no dispenser was available for my experiments, the K_i values of TFPI in the presence or absence of protein S were determined by starting the FXa inhibition assay with a mix of substrate and TFPI. Titration of 0-8 nM TFPI was performed in the absence of protein S. As protein S efficiently enhances TFPI, the concentration of TFPI was lowered to 0-4 nM when using 100 nM protein S. Figure 3.41 shows the activity of TFPI in the absence (Panel A) and presence of

WT protein S purified by anion exchange chromatography followed by immunoaffinity chromatography (Panel B) or of WT protein S purified by barium citrate precipitation followed by anion exchange chromatography (Panel C). This is expressed in Figure 3.41, Panel D as a change in V_0 at increasing concentrations of TFPI in the presence or absence of protein S. The V_0, derived from the fitted equations in PanelA-C, has been expressed as a percentage of V_{max} (which corresponds to the V_0 obtained in the absence of TFPI). The results in Panel D represent the mean ± SD of three independent experiments. As can be seen in Panel D of Figure 3.41 both WT preparations enhanced TFPI to a similar extent.

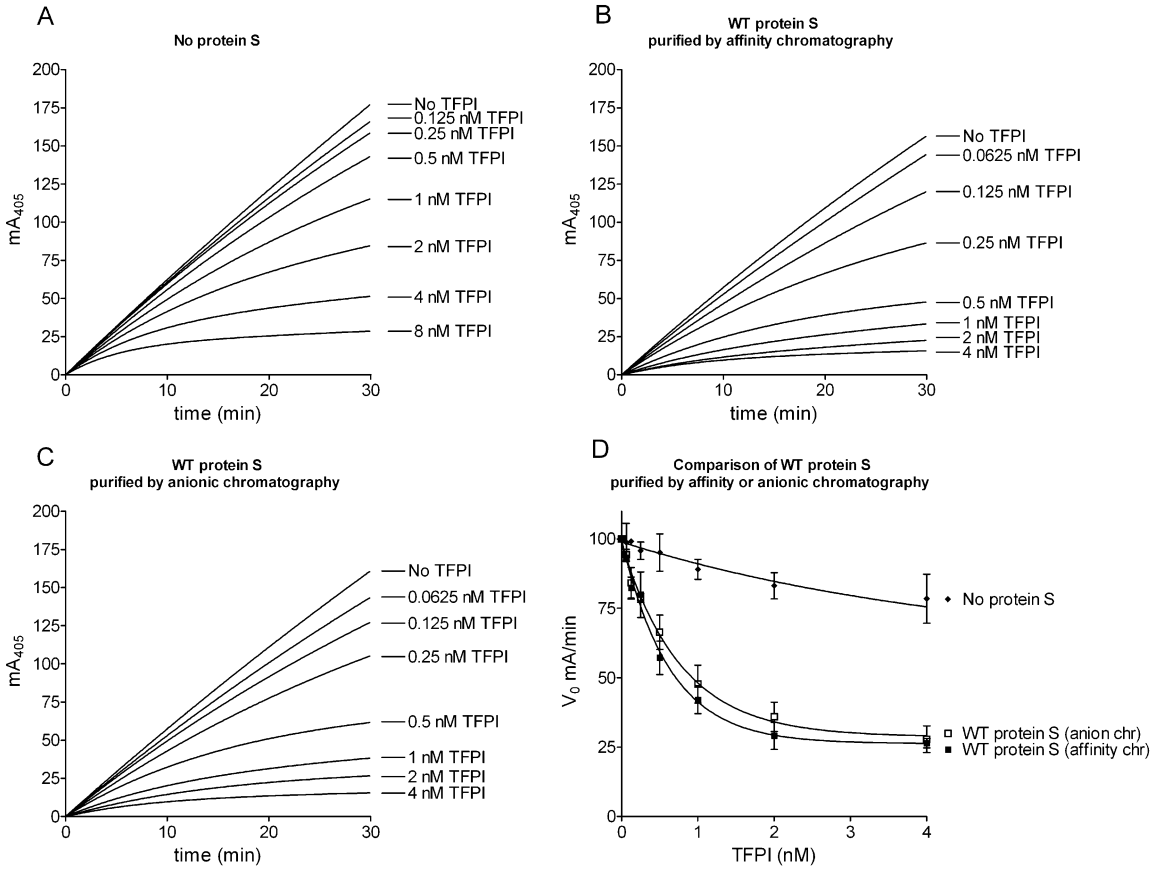

Figure 3.41 FXa inhibition by TFPI in the presence and absence of WT protein S.
TFPI (0-8 nM) was titrated in the absence (A) or presence of 100 nM WT protein S purified by anion exchange chromatography followed by immunoaffinity chromatography (B), or by barium citrate precipitation followed by anion exchange chromatography (C). The change in V_0 at increasing concentrations of TFPI in the presence or absence of WT protein S is shown in Panel D as mean ± SD from three independent assays. Anion chr, purified by barium citrate precipitation followed by anion chromatography; Affinity chr, purified by anion exchange chromatography followed by immunoaffinity chromatography.

Protein S D95A and protein S Face2 previously found to be critical for APC cofactor activity and protein S NNEDM, which had reduced TFPI and APC cofactor activity in the plasma assay, were assessed at 100 nM, at increasing concentrations of TFPI (0-4 nM). Protein S KTK was also assessed. Protein S KTK had normal APC cofactor activity. While plasma assays suggested it had reduced TFPI cofactor activity, its activity in the FXa inhibition assay seemed normal. The change in V_0 in the presence or absence of the protein S variants in comparison with WT protein S purified in the same way is shown in Figure 3.42. Protein S Face2 (Panel A), protein S KTK (Panel B) and protein S D95A (Panel C) all showed similar dose-dependent changes in V_0 to that of WT protein S. Protein S NNEDM (Panel D), however, had a reduced cofactor activity towards TFPI, visualised as a smaller reduction in V_0.

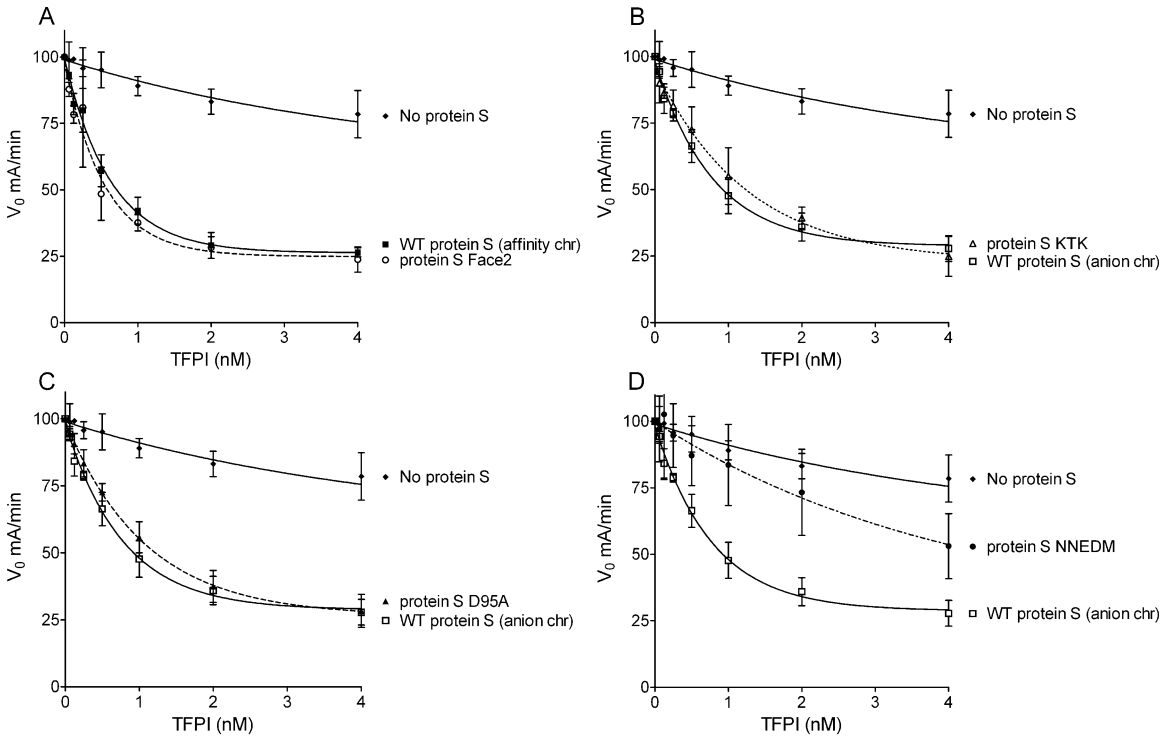

Figure 3.42 Change in V_0 at increasing concentrations of TFPI in the presence or absence of protein S.
TFPI (0-8 nM) was titrated in the presence or absence of 100 nM protein S. The graphs show the change in V_0 at increasing concentrations of TFPI in the presence or absence of protein S Face2 (A), protein S KTK (B), protein S D95A (C) and protein S NNEDM (D). Data points are expressed as the mean ± SD from three independent experiments. Anion chr, purified by barium citrate precipitation followed by anion exchange chromatography; Affinity chr, purified by anion exchange chromatography followed by immunoaffinity chromatography.

To quantify the difference between WT protein S and the protein S variants, the K_i values were determined. This was done by plotting the V_{max}/V_0 against the concentration of TFPI. The x intercept was then used to calculate the K_i, as described in section 2.7.3. Differences in K_i values obtained were analysed using an unpaired t-test.

There was no significant difference between the K_i values obtained with WT protein S purified by anion exchange chromatography followed by immunoaffinity chromatography (K_i= 0.13 ± 0.03 nM) or WT protein S purified by barium citrate precipitation followed by anion exchange chromatography (K_i= 0.19 ± 0.04 nM) (Table 3.4).

When protein S variants were compared to WT protein S purified using the same method, there was no significant difference between protein S Face2 and WT protein S in terms of K_i values (Table 3.4). This is in agreement with what I had seen in the single concentration screening assay (Figure 3.37). Confirming what was observed in the single concentration screening assay (Figure 3.38), protein S KTK enhancement of TFPI inhibition of FXa was not significantly different from WT protein S (Table 3.4). Interestingly, while protein S D95A seemed to have reduced cofactor activity towards TFPI at 20 nM in the screening assays (Figure 3.38), no difference was observed at 100 nM protein S which was used to determine the K_i of TFPI activity (Table 3.4). Importantly, my experiments with protein S NNEDM confirmed results obtained in the screening assay (Table 3.4). Protein S NNEDM had reduced TFPI cofactor activity (K_i = 0.91 ± 0.35 nM) in comparison with WT protein S (K_i = 0.19 ± 0.04 nM). While some of the residues in protein S NNEDM might be important for cofactor activity towards TFPI mediated inhibition of FXa, this variant also had reduced APC cofactor activity. Evaluation of protein S NEDM, which had normal APC cofactor activity might therefore provide interesting results in future experiments.

	K_i (nM)
TFPI	3.49 ± 0.96
WT protein S (anion chr)	0.19 ± 0.04
Protein S 95A	0.21 ± 0.06
Protein S KTK	0.24 ± 0.05
Protein S NNEDM	0.91 ± 0.35
WT protein S (affinity chr)	0.13 ± 0.03
Protein S Face2	0.12 ± 0.02

Table 3.4 Inhibition constants for TFPI inhibition of FXa in the presence or absence of protein S.
K_i values (nM) for WT protein S and protein S variants were determined from the x intercept obtained by plotting the V_{max}/V_0 values against TFPI concentration. Results are expressed as the mean ± SD from three independent experiments. WT protein S (anion chr) is protein S purified by barium citrate precipitation followed by anion exchange chromatography. WT protein S (affinity chr) is protein S purified by anion exchange chromatography followed by immunoaffinity chromatography.

3.4.3 Discussion

The CAT assay to assess protein S cofactor activity towards TFPI was initially set up. This assay allows evaluation of the cofactor activity of protein S towards TFPI in a plasma milieu, potentially representing a more physiological environment than purified systems. When I began my thesis, a method to evaluate protein S cofactor activity towards TFPI by CAT in protein S deficient plasma had not been published. However, recently Maurissen *et al.* were able to reproducibly study the TFPI/protein S anticoagulant activity by CAT in normal plasma.[240] This was achieved by using inhibitory polyclonal antibodies against TFPI and protein S.

During my thesis, I noticed differences in the anticoagulant activity of TFPI and protein S supplemented to protein S deficient plasma from different suppliers. Generally, as can be seen by the different peak heights (Figure 3.22), the protein S deficient plasma from Hyphen Biomed was less procoagulant that that of Affinity Biologicals. This is likely to influence the total TFPI/protein S anticoagulant activity as the latter has been shown to be dependent upon TF concentrations, and therefore the procoagulant environment.[218] Alternatively, the reported

difference in the concentration of endogenous TFPI between the different plasma preparations might be important. Co-depletion of full-length TFPI following removal of protein S during the production of protein S deficient plasma has recently been shown by Castoldi *et al.*.[241] However, differences in endogenous TFPI concentrations alone, are unlikely to explain the differences I observed between the different plasma preparations. Firstly, because the exogenous full-length TFPI (~2.5 nM) supplemented to the protein S deficient plasma exceeds by far the endogenous full-length TFPI (~0.25 nM) the total amount of TFPI in the two different plasma preparations were fairly similar. Secondly, the anticoagulant activity of 5 nM TFPI and protein S in plasma from Affinity Biomed was less than that observed using 2.5 nM TFPI and protein S in plasma from Hyphen Biomed (results not shown). Therefore, it is possible that co-depletion of other coagulation factors might account for the differences observed between the two plasma preparations, Affinity Biological and Hyphen Biomed.

It is interesting to note that the protein S/TFPI anticoagulant activity influences both the peak height, ETP and the lag time. This is in contrast with what is observed in the presence of APC, where protein S cofactor enhancement results mainly in an effect on peak height and ETP. Compatibly with my results, Hackeng and Rosing observed both a reduction in peak height and prolongation of the lag time following addition of TFPI to TFPI depleted plasma.[261] Other studies evaluating protein S enhancement of TFPI anticoagulant activity have been carried out in normal plasma. When Brodin *et al.* evaluated the effect of addition of recombinant TFPI to normal plasma, they noticed a prolongation of the lag time but saw no effect on the ETP.[253] Similarly, when they inhibited endogenous TFPI with antibodies, a shortening in the lag time but no effect on the ETP was observed. Previous reports have also shown that protein S, in the absence of APC, affects the lag time but not the ETP.[262] This study, however, combined protein S deficient plasma with different percentages of normal plasma, thus potentially confounding any effect of differences in other coagulation factors between the two plasma preparations. In contrast, when I supplemented TFPI to normal plasma (results not shown) I observed both an effect on the peak height and a prolongation of the lag time. Maurissen *et al.* assessed TFPI anticoagulant activity and its enhancement by protein S in normal plasma using inhibitory antibodies and showed a change in peak height, but not in lag time.[240] The

discrepancies between results from different protein S deficient plasma preparations and between protein S deficient plasma and normal plasma do not have a coherent explanation. It is possible that co-depletion of other coagulation factors contribute to the different TFPI/protein S anticoagulant activity observed between different plasma preparations. Considering that plasma levels of FV have been shown to correlate with full-length TFPI plasma levels, that TFPI and FV potentially form a complex *in vivo*,[263] that depletion of full-length TFPI results in co-depletion of FVa,[241] together with the observation that FV levels have been shown to influence the lag time in the CAT assay,[262] it should be evaluated if differences in FV level could contribute to the differences observed between normal and protein S depleted plasma.

To decrease intra-assay variability, variability between different sources of plasma and variabilities between different laboratories, parameters such as supplier of the plasma, potential differences between lyophilised and frozen plasma, batch variability of the plasma, source of TF, source of phospholipids, preincubation of additional components to the plasma, plasma dilution and temperature should all be carefully considered.[218,262,264,265]

In this thesis, the TFPI anticoagulant activity in the presence or absence of protein S was evaluated in protein S deficient plasma from Hyphen Biomed and the specificity of the assay was demonstrated with inhibitory antibodies against TFPI, protein S or protein C. As observed for APC (section 3.3.2), no significant difference in TFPI cofactor activity was observed between protein S in concentrated conditioned media and purified protein S. The assay developed during this thesis allowed visualisation of a clear TFPI effect and its enhancement by protein S. I also showed that the protein S activity in the presence of TFPI is highly dependent on the TFPI concentration and its anticoagulant activity. This is compatible with results of Hackeng and Rosing in their recent review paper in which TFPI depleted plasma was supplemented with TFPI in the presence or absence of inhibitory antibodies against protein S.[261] The dependence of the protein S/TFPI anticoagulant activity upon the TFPI concentration might be partially explained by the fact that the normal concentration of protein S present in plasma (free ~145 nM, total ~362 nM) is in vast excess over that of free full-length TFPI (~0.25 nM).

The inter-assay variability was quite large making it difficult to pool data from multiple assays. The CAT assay used in this thesis was, however, an efficient screening tool to identify protein S variants with reduced, normal or enhanced TFPI cofactor activity in comparison to WT protein S. Protein S variants in concentrated conditioned media were evaluated both for their ability to reduce the peak height of thrombin generated and to prolong the lag time. Of the 31 protein S variants screened for their TFPI cofactor activity by CAT none had completely ablated activity. Twenty-three protein S variants had normal to enhanced activity in comparison with WT protein S (Table 3.5). The protein S variants previously found to have reduced APC cofactor activity protein S D95A, D95N, D78A and Q79A had approximately 80% of WT protein S cofactor activity towards TFPI (Figure 3.25). Interestingly, protein S Face2, which had decreased APC cofactor activity, reduced the peak height of thrombin to approximately half of that of WT protein S (at 90-120 nM) in the presence of TFPI (Figure 3.25 and Figure 3.31). Protein S KTK, NEDM, NNEDM and Face1 (Table 3.5) showed reduced cofactor activity towards TFPI in comparison with WT protein S when screened by CAT (Figure 3.31 and Figure 3.32). This was visualised mainly as a change in lag time rather than in a change of peak height in comparison to WT protein S.

TFPI cofactor activity compared to WT protein S	**Protein S variants**
Normal to enhanced	Gla1, Face2, TSR, N74A, K94A, K97A, S99A, T101A, T103A, K105A, P106A, Q109A, E111A, K112A, D135A, T137A, Y141A, H142A, S144A, K146A, N154A, K156A, D157A
Reduced	Face1, KTK, NEDM, NNEDM, D78A, Q79A, D95A, D95N

Table 3.5 TFPI cofactor activity of protein S variants in comparison with WT protein S in plasma studied by CAT assay.
Any variant which in the CAT assay had shorter lag time or increased peak in comparison with WT protein S was classified in this table as having reduced activity compared to WT protein S.

Protein S variants Face2 and D95A, found to be important for APC cofactor activity, were purified and evaluated for their TFPI cofactor activity in the FXa

inhibition assay. Protein S KTK and NNEDM which had reduced TFPI cofactor activity in plasma were also purified and assessed in the FXa inhibition assay. Protein S N74A and TSR which had normal APC cofactor activity and normal to enhanced TFPI cofactor activity in plasma were also analysed in the FXa inhibition assay.

The FXa inhibition assay has the advantage of being a well established assay and allows specific evaluation of TFPI mediated inhibition of FXa without the complexity associated with normal plasma assays.[68,72,78,81,237] Importantly, purified protein S alone does not affect FXa cleavage of the chromogenic substrate S-2765 in the absence of TFPI ensuring the assay specifically assesses TFPI cofactor activity of protein S. A screening assay using 1.5 nM purified TFPI and 20 nM purified protein S was performed. Results from the screening assay showed that protein S variants Face2, TSR, KTK and N74A all had similar TFPI cofactor activity to that of WT protein S. Protein S D95A and protein S NNEDM, however, had a reduced TFPI cofactor. My colleague Dr Josefin Anhnström evaluated the cofactor activity of protein S E36A (a substitution present in the Face1 variant that had reduced APC cofactor activity, see section 3.3.6) and found it enhanced TFPI mediated inhibition of FXa to the same extent as WT protein S.

To more accurately quantify the differences between WT protein S and protein S variants, I derived the K_i values for TFPI inhibition of FXa in the presence of protein S. WT protein S reduced the K_i of TFPI in the absence of protein S from 3.49 nM to 0.13-0.19 nM, enhancing TFPI inhibition of FXa by 18-27-fold. The K_i values of protein S Face2, D95A, KTK and NNEDM were determined. No statistical difference between the K_i obtained in the presence of protein S Face2, D95A, KTK with that of WT protein S was found. Protein S NNEDM, however, enhanced TFPI inhibition of FXa only by ~3.8-fold in contrast to the ~18-fold of WT protein S. These kinetic results were in agreement with results from the screening in the FXa inhibition assay, with the exception of protein S D95A. While protein S D95A had reduced TFPI cofactor activity in the screening experiment in the FXa inhibition assay, the K_i value obtained with 100 nM protein S was not statistically different from that of WT protein S. It is possible that while at lower protein S concentrations protein S D95A is not as efficient as WT protein S, this is overcome at saturating concentrations of protein S. Overall, results from the FXa

inhibition assay correlate relatively well with those from the plasma assay, considering the intrinsic differences of the two assays. The main exception is protein S KTK, which had reduced TFPI cofactor activity in plasma and normal TFPI cofactor activity for the inhibition of FXa. The reason for this discrepancy still needs to be evaluated.

Importantly, results from the plasma based assay and the purified FXa inhibition assay suggest that protein S D95A, protein S Face2 and protein S E36A (result of Dr Josefin Anhnström, not shown) were able to enhance TFPI mediated inhibition of FXa to a similar extent as WT protein S. This is of considerable importance as it suggests distinct residues in protein S interact with APC and TFPI.

Further work has to be done to identify residues in protein S involved in TFPI cofactor activity. Experiments with protein S KTK should be repeated to evaluate if there are any significant differences between the plasma based assay and the purified assay, and if so to determine the reason for any discrepancy. Protein S NNEDM had reduced TFPI cofactor but also reduced APC cofactor activity. Importantly, protein S NEDM had reduced TFPI cofactor activity and normal APC cofactor activity. Protein S NEDM should be purified and analysed in the FXa inhibition assay.

Further work also needs to be done in order to fully optimise the plasma based assay and potentially also the FXa inhibition assay. In the first instance the inter-assay variability of the plasma based assay has to be improved. Secondly, the different effect on V_0 in the FXa inhibition assay according to how the assay is started (Figure 3.40) has to be rationalised. While the reason for this is not clear, it might explain why Ndonwi *et al.*[238] quantifies the protein S enhancement of TFPI as a change in V_s determining the EC_{50} and Hackeng *et al.*[81] as a change in V_0 determining the K_i. To get accurate V_0 results a dispenser should also be used. Protein S enhancement of TFPI mediated inhibition of TF/FVIIa should also be assessed as this potentially represents a mechanism that occurs in plasma.

Results obtained during my thesis would suggest that substitution of a single residue of protein S is not enough to disrupt its cofactor activity towards TFPI. Even if protein S and TFPI interact through multiple interaction sites it is unlikely that residues substituted in my thesis and found to have normal to enhanced TFPI cofactor activity are involved in a critical way in the interaction with TFPI. Figure 3.43 shows the residues in protein S substituted in the Gla-TSR-EGF1

domain and analysed in this thesis (blue). Red residues in Figure 3.43 represent amino acids potentially involved in phospholipid binding, alanine residues or cysteine residues and are therefore not good candidates to substitute. Similarly, yellow residues represent residues which are predicted to have their R group buried inside the protein S structure. This leaves only few clusters of residues in these domains, of which only two charged residues, that have not been mutated during this thesis (gray) and could be evaluated alongside protein S KTK and NEDM. Alternatively, the interaction site between protein S and TFPI is not located at the sites and domains evaluated in this thesis.

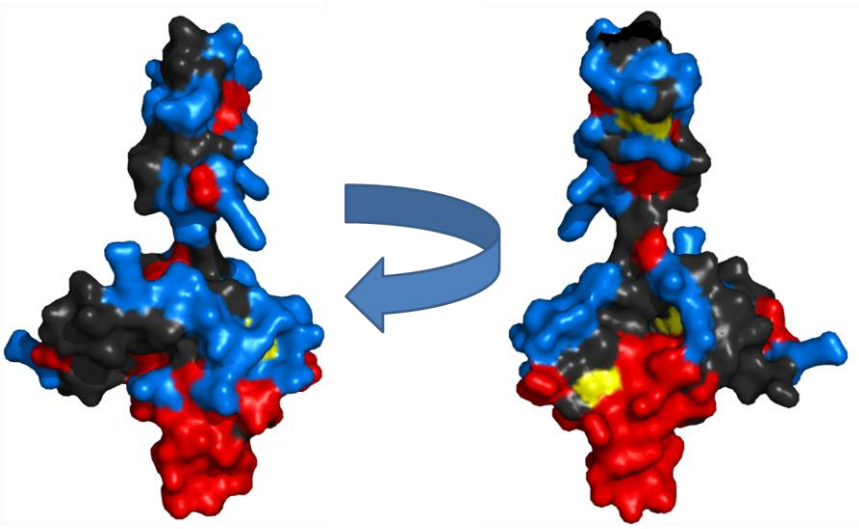

Figure 3.43 Residue substitutions in the Gla-TSR-EGF1 domains made during this project. Residues substituted during this project, either as single or composite variants, are coloured in blue. Residues in red and yellow were not mutated because their likely involvement in phospholipid binding, because they are alanine or cysteine residues or because their R group is buried within the protein S structure. Protein S model from Villoutreix *et al.*.[193]

4 CONCLUDING REMARKS

It is well established that protein S acts as a cofactor for APC. Recently, different groups have shown that protein S is also able to act as a cofactor for TFPI.[81,237] The aim of my project was to elucidate the molecular mechanism behind these interactions by identifying the residues in protein S involved in APC and TFPI cofactor activity. To address this aim, more than 30 protein S variants were created by site-directed mutagenesis, expressed and characterised for their cofactor activities towards both APC and TFPI. In this concluding chapter, I will attempt to put into context the results achieved during my PhD with what is already known in the literature. I will first address the issue of protein S cleavage, multimerisation and binding to phospholipids. I will postulate an interaction mechanism between protein S and APC, I will discuss the potential interaction and mechanisms regulating TFPI cofactor activity of protein S, and I will speculate on the regulation of protein S cofactor activity *in vivo* based on the novel results in this thesis and on recent results from the literature.

4.1 Protein S cleavage, multimerisation and its binding to phospholipids

It has been shown that 10-15% of plasma protein S circulates in a cleaved form.[178] The protease responsible for this cleavage *in vivo* is still not known and the effect of cleavage upon APC-dependent and APC-independent cofactor activity of protein S is still under debate.[175-177] It has been suggested that cleavage at both Arg49 and Arg70 affects protein S binding to phospholipids.[176] During this thesis cleavage of protein S was sometimes observed in processed concentrated conditioned media. Because of this, cleavage of protein S was minimised by keeping the time of processing of protein S as short as possible. Because of the issue of cleavage, I preferred using protein S in concentrated conditioned media rather than purified samples. I also aliquoted protein S to avoid extensive freeze thawing. Cleavage of protein S variants was formally assessed by performing SDS-PAGE under reducing conditions. WT protein S, protein S D95A and protein S Face2 in concentrated conditioned media were all analysed

by Western blot and this confirmed that no cleavage products were present. Purified WT protein S, protein S D95A and protein S Face2 were analysed by silver staining to make sure they were not more cleaved than WT protein S. These precautions suggest that reduced APC cofactor activity of these variants was not a consequence of increased cleavage.

The formation of protein S multimers is well documented in the literature.[189-192] It is not yet clear what causes multimer formation or how to prevent it. While some reports suggest that protein S multimers already exists in the blood,[189,190] others claim it is a consequence of *in vitro* manipulation.[191,192] It has been hypothesised that it is a result of 1) purification, 2) removal of Ca^{2+} ions, 3) concentration, or 4) spontaneously occurs over time. The effect of multimers on the ability of protein S to bind to phospholipids and to act as a cofactor for APC or TFPI is still being debated.[189-192] It has been shown that multimeric protein S is able to bind to phospholipids with a higher affinity than monomeric protein S.[191] APC- and TFPI-independent activities of purified multimeric protein S due to competition for phospholipids have also been documented at limiting amounts of phospholipids.[191,192,216,217]

Multimer formation was analysed during my work using Western blot of protein S from native gels. Protein S in concentrated conditioned media and plasma protein S both appeared monomeric. Partial purification on the anion exchange QFF column by FPLC resulted in protein S preparations that were largely monomeric with trace amounts of dimers being present. Purification by immunoaffinity chromatography or by barium citrate precipitation of protein S, however, definitely lead to multimer formation. Commercial plasma purified protein S from Enzyme Research Laboratories also appeared multimeric using this technique.

Accordingly, functional assays to assess protein S cofactor activity towards APC and TFPI were carried out both with monomeric protein S in concentrated conditioned media (where assay conditions permitted this) as well as with multimeric purified protein S. Saturating amounts of phospholipids are also used in all assays.

Protein S is a plasma protein whose functionality is dependent upon its ability to bind to phospholipid surfaces through its Gla domain. Binding to phospholipids requires post transcriptional γ-carboxylation of the glutamic acid residues in the Gla domain of protein S by the vitamin K-dependent γ-carboxylase.[171] Variations

in the efficiency of γ-carboxylation of protein S variants secreted from HEK293 cells can therefore potentially influence the cofactor activity of protein S measured in the functional assays. Variants of interest in concentrated conditioned media were analysed by Western blot and protein S was detected either by a polyclonal antibody or an antibody recognising only γ-carboxylated protein S. No difference in band intensity with the latter antibodies were observed between WT protein S and protein S D95A, suggesting both preparations contained a similar amount of γ-carboxylated protein S. γ-carboxylation was also controlled for by purifying WT protein S and the variants of interest by methods that selectively removes non γ-carboxylated protein S. Lastly, protein S was assessed for its ability to bind to phospholipids in a plate binding assay. My recombinant barium citrate precipitated WT protein S bound to phospholipids with a $K_{d(app)}$ similar to that reported in the literature.[182,223-225] Importantly, my colleague Dr Josefin Ahnström also confirmed that there was no difference in binding to phospholipid between partially and fully purified protein S (results not shown). Protein S D95A was assessed for its binding to phospholipid surfaces and there was no significant difference in $K_{d(app)}$ compared to WT protein S. While not formally assessed in this thesis, when protein S in concentrated conditioned media was evaluated for its ability to bind phospholipid surfaces, a much lower affinity was observed.

In summary, my results are compatible with those published studies showing that monomeric and multimeric protein S have different affinities for phospholipids, that protein S circulates in plasma as a monomer and that multimerisation is a result of *in vitro* manipulation.[191,192]

4.2 APC cofactor activity of protein S; interaction sites and a suggested mechanism

Protein S has been suggested to act as a cofactor for APC in three distinct ways; by increasing the affinity of APC to phospholipid surfaces, by relocating the active site of APC and by overcoming the protective effect of FVa following binding to FXa.[127,137,221,228,229]

Protein S circulates in plasma as a monomer and consequently its affinity for phospholipids is reduced compared to that reported in the literature for multimeric

protein S. The reported affinity of monomeric protein S for phospholipids ($K_{d(app)}$ =250 nM)[191] is, however, higher than that of APC (2-7 µM)[226,227] suggesting protein S enhancement of APC binding to phospholipids might have a physiological relevance.

It is well accepted that protein S enhances mainly the APC mediated cleavage at Arg306 of FVa. It has been shown that binding of protein S to APC results in relocalisation of the active site of APC closer to the membrane surface.[228,229,266] This relocalisation was proposed to be the mechanism behind protein S induced preferential cleavage at Arg306. Binding of protein S might cause a major conformational change in APC, resulting in the relocalisation of its active site. A recent paper using a FVa variant R306Q/R506Q/R679Q, identified additional APC cleavage sites in FVa (in close proximity to Arg306 or Arg506). Protein S equally enhanced the cleavage of all these residues (Lys309, Arg313, Arg316, Arg317 and Arg505).[130] While this does not contradict protein S mediated relocalisation of the active site of APC, it argues against the suggestion that this relocalisation is how protein S changes the preferential cleavage site of APC from Arg506 to Arg306. Tran *et al.* suggest their results argue against the hypothesis that Arg306 and Arg506 are located at different heights above the phospholipid surface. Relocalisation of the active site of APC closer to the membrane surface has also been observed following construction of APC chimeras containing the Gla domain of either FX or prothrombin.[228,267] In contrast to WT APC, protein S was not able to enhance the APC anticoagulant activity of these chimeras.

Because cleavage at Arg506 of FVa by APC does not require phospholipids, the affinity between the two proteins may be sufficiently high for them to bind to each other in the absence of membrane surfaces. It is possible that following cleavage at Arg506 the affinity of APC for FVa decreases. It has been shown that protein S binds with high affinity (K_d ~ 40 nM) to the 493-506 peptide of FV.[116] Consequently, protein S could also act as an APC cofactor by stabilising the APC/FVa complex on the phospholipid membrane (through binding to both APC and FVa), thereby allowing cleavage at Arg306.

The third mechanism by which protein S has been reported to enhance APC anticoagulant activity is by overcoming the protective effect of FXa on FVa cleavage.[219] One proposed hypothesis is that this is due to competitive binding of protein S and FXa for FVa.[116] If a competitive binding between protein S and FXa

for FVa exsists, I believe it is unlikely to play a physiological role *in vivo*, considering that binding of protein S to FVa is likely reduced once FVa is incorporated in the prothrombinase complex with its physiological substrate prothrombin. Prothrombin (1.39 μM) is also available in much higher concentrations than protein S (free protein S ~145 nM, total protein S ~362 nM). The alternative mechanism proposed was that protein S enhanced APC mediated cleavage of free FVa, thereby potentially affecting the equilibrium between free FXa and FVa, and the FXa/FVa complex.[137] This would cause FVa to dissociate from the complex, consequently being more susceptible to cleavage by APC. This proposed mechanism seems to me more likely to occur *in vivo*, as protein S is known to enhance APC mediated cleavage of FVa. In addition, considering that the plasma concentration of FV (~30 nM) is less than that of FX (~136 nM), the available FVa is likely to influence the equilibrium between free FVa and FVa bound to FXa. It has also been proposed that FXa only protects Arg506 cleavage and that protein S enhances APC mediated cleavage at Arg306 even when FVa is bound to FXa.[138] This proposed mechanism could occur *in vivo*, however, it is likely that it is reduced once the prothrombinase complex binds to its substrate prothrombin.

Three residues in the Gla domain of APC, Asp36/Leu38/Ala39, have been reported to be essential for cofactor activity of protein S.[90] When point substitutions of these residues in APC were analysed, Leu38 was shown to be the most critical residue for protein S cofactor activity.[268] One study used Fab fragments against APC and suggested the EGF1 domain of APC was important for protein S cofactor activity.[91] During this thesis, I identified Asp95, Asp78 and Gln79 in the EGF1 domain of protein S and my colleague Dr Josefin Ahnström identified Gla36 in the Gla domain of protein S as residues essential for APC cofactor activity. When residues Asn33, Pro35 and Tyr39 in protein S were substituted and evaluated by my colleague Dr Josefin Ahnström in the CAT assay, a reduced APC cofactor activity was also observed. This suggests amino acids in close proximity to Gla36 in protein S are also likely to contribute either directly or indirectly to APC cofactor function of protein S. I aligned the Gla and EGF1 domains of protein S and protein C (Figure 4.1A). Interestingly, residues of APC shown to be important for enhancement of its anticoagulant activity by protein S align with the residues of protein S identified as crucial for APC cofactor

activity. Similarly, when I highlighted these residues in the respective models of the proteins, they are predicted to be located at a similar distance from the N-terminal residues comprising the ω-loop involved in binding to the phospholipid membrane (Figure 4.1, B and C). It is likely that the pronounced APC cofactor activity of protein S depends on the formation of a functional complex between the two proteins. Protein interactions are usually mediated through multiple residue reciprocal contacts and can involve distinct interaction sites. However, it is unlikely, because of charge considerations, that the identified residues of negative charge in the respective domains (Asp95 and Gla36 in protein S and Asp71 and Asp36 in APC) interact directly with each other. The possibility that Leu38 of APC in conjunction with surrounding residues (such as Asp36 and Ala39), interact with a cluster of residues in protein S composed in part by residues such as Asn33, Pro35, Gla36 and Tyr39 (see arrow Figure 4.1) needs to be further investigated. While a monoclonal antibody directed against the EGF1 of APC, with Asp71 being part of its epitope, inhibited protein S enhancement of the anticoagulant activity of APC,[91] Asp71 is not necessarily involved in an interaction with protein S. However, it is interesting that Asp71 in APC coordinates a calcium ion[91] and that Asp95 in protein S is part of a partially conserved calcium binding site. It is possible that the calcium ion in EGF1 of protein C masks part of the negative charges in this region. It is difficult for me to predict how these residues (Asp95 in the EGF1 domain of protein S and Asp71 in the EGF1 domain of APC) might interact, and I suggest that further studies on these EGF domains of APC and protein S are required before a plausible model can be proposed. Nevertheless, it is worth noting that at least two distinct domains, Gla and EGF1, have been implicated in both APC and protein S mediated APC cofactor activity. Co-operative multidomain interactions could form the basis of further investigation.

To further assess protein S interaction with APC and relocation of the active site of APC by protein S, my colleague Dr Josefin Ahnström is planning to perform FRET analysis with WT protein S, protein S D95A, protein S E36A, WT APC and APC D36A/L38D/A39V.

A

```
PROTEIN S GLA DOMAN     ANSLLEETKQGNLERECIEELCNKEEAREVFENDPETDYFYPKYLV
PROTEIN C GLA DOMAN     ANSFLEELRHSSLERECIEEICDFEEAKEIFQNVDDTLAFWSKHVD
                        ***:*** ::..****** **:*: ***:*:*:*  :*  *:.*::

PROTEIN S EGF1 DOMAN    IPDQCSPLPCNEDGYMSCKDGKASFTCTCKPGWQGEKCEF
PROTEIN C EGF1 DOMAN    LEHPCASLCCGHG---TCIDGIGSFSCDCRSGWEGRFCQR
                        : . *:.* *...   :* ** .**:* *:.**:*. *:
```

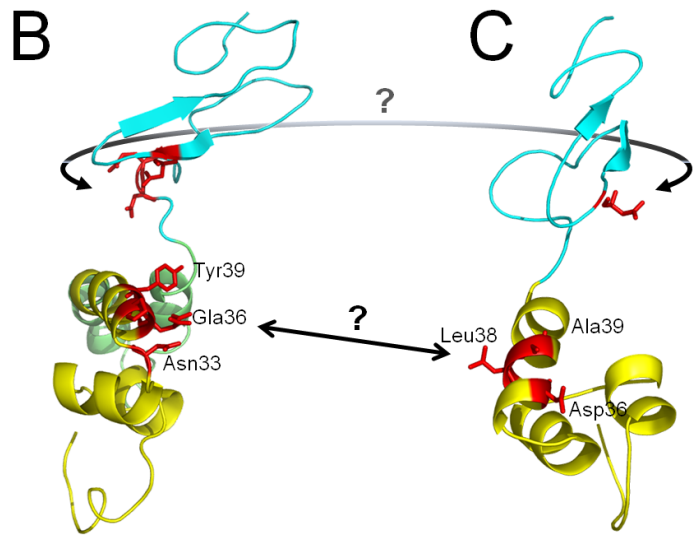

Figure 4.1 Location of residues in protein S and protein C shown to be important for APC cofactor activity of protein S.
The alignment of the Gla domains and EGF1 domains of protein S and protein C is shown in A. The model of protein S[193] (B) and APC[88] (C) show the Gla domains in yellow, the TSR domain of protein S in light green and the EGF1 domains in cyan. Residues found to be important for APC cofactor activity of protein S are shown as red sticks. Arrows indicate potential interaction sites between protein S and APC.

4.3 TFPI cofactor activity of protein S; possible interaction sites and mechanism

Hackeng *et al.* showed that the anticoagulant activity of truncated TFPI, lacking its K3 domain and its C-terminal tail, was not enhanced by protein S.[81] In our lab, we have shown that protein S is able to enhance TFPI lacking its C-terminal tail to the same extent as full-length TFPI (unpublished results of my colleague Verity Hockey) suggesting the C-terminal tail is not directly involved in the interaction with protein S. Recently, it was demonstrated that the K3 domain of TFPI is essential for protein S cofactor activity.[238] Within the K3 domain the P1 residue,

Arg199, was reported to play an important role.[238] Direct binding of protein S to the K3 domain was also shown.[238] Verity Hockey has constructed composite variants of charged residues in the K3 domain of full-length TFPI. These were shown inhibit FXa to the same extent as WT TFPI, however, the cofactor activity of protein S for E226Q/E234Q/R237Q and D194Q/R195Q/R199Q was reduced approximately 7 and 3-fold in comparison with WT TFPI (unpublished results). The TFPI variant K213Q/R215Q/K232Q was enhanced normally by protein S (unpublished results).

Protein S variants constructed during my thesis have failed to clearly identify residues in protein S critical for TFPI cofactor activity. However, I did identify a potentially interesting variant in the EGF1 domain, protein S NEDM, that needs to be further investigated. Considering the pronounced TFPI cofactor activity of protein S and the low concentrations of plasma full-length TFPI (~0.25 nM), their binding affinity *in vivo* must be much higher than those derived from surface plasmon resonance (SPR) experiments (K_d> 150 nM).[238,241] Binding experiments by blotting and SPR were performed in the absence of phospholipids. It is possible that the presence of phospholipids would increase the affinity of TFPI for protein S. Interestingly, co-immunoprecipitation studies have suggested full-length TFPI and free protein S do exist in plasma as a complex.[241] If protein S and TFPI interact *in vivo*, they are likely to do so through multiple sites, potentially explaining why substitution of single residues within protein S failed to completely disrupt protein S enhancement of TFPI. Alternatively, the interaction site on protein S for TFPI might be located in domains that were not evaluated during this thesis.

The mechanism by which protein S enhances TFPI is not yet completely understood. It is possible that, as for APC, protein S enhances the affinity of TFPI for negatively charged phospholipid surfaces. Protein S could also, by binding both TFPI and FXa, co-localise the proteins on the phospholipid surface and increase their affinity for each other by forming a trivalent complex. Alternatively, protein S could induce a conformational change and thereby facilitate TFPI binding and inhibition of FXa. I believe it is unlikely that protein S, by binding to the K3 domain of TFPI, induces a conformational change in the K2 domain of TFPI as the Kunitz domains of TFPI are connected through quite long (30 amino acid) linker regions. It is more plausible that protein S induces a conformational

change in FXa, as it has been suggested to do for APC. Interestingly, FXa and APC have a very similar domain structure and protein S is reported to bind FXa with a high affinity (~15 nM).[214] Conceivably, protein S binding to FXa induces a conformational change in its active site causing it to be more susceptible to inhibition by the K2 domain of TFPI.

While recent publications suggest a direct interaction of protein S with TFPI and possibly the K3 domain, the reported affinities are surprisingly low (K_d >150 nM) in comparison to those reported for protein S binding to FXa (K_d ~15 nM).[238,241] The interaction of the K3 domain of TFPI with protein S would represent an unusual interaction since Kunitz domains usually bind in active site clefts. It has been shown that FXa is able to cleave the K3 domain of TFPI after its P1 residue Arg199 *in vitro*.[71] While the physiological importance of this is questionable, it suggests that under certain conditions the K3 domain of TFPI is able to bind the active site of FXa. While not supported by current binding studies between protein S and TFPI, the possibility that protein S induces a conformational change in FXa that favours a stronger and quicker inhibition of FXa by the K3 domain of TFPI rather than a weaker and slower inhibition by the K2 domain of TFPI, needs to be examined further. If this is the case, the deletion or substitutions in the K3 domain of TFPI would result in loss of protein S cofactor activity, not because of lack of binding to protein S but because of lack of binding to the active site of FXa. This hypothesis could easily be tested by evaluating FXa inhibition by synthetic K2 and K3 domains in the presence and absence of protein S.

4.4 Protein S direct activity

It was initially thought that protein S could directly inhibit the prothrombinase complex by direct binding to FVa and FXa. This APC-independent anticoagulant activity of protein S was termed “protein S direct”. Later, it was shown that this activity of protein S in purified systems was due to competition of protein S multimers for limiting amounts of phospholipids.[191,192,216,217] However, an APC-independent activity of protein S was observed in plasma also at saturating amounts of phospholipids.[218] This was identified as protein S cofactor activity towards TFPI in 2006.[81] However, Heeb and colleagues have observed an APC- and TFPI-independent activity of protein S also at saturating amounts of

phospholipids.[189,190,242] They claim that protein S can directly inhibit FXa and TF/FVIIa through direct binding. That protein S direct has not been observed in many other laboratories has been potentially explained by Heeb and colleagues by showing that purification of protein S often lead to loss of binding to a zinc ion, which correlates with loss of protein S direct activity.[242] The low IC_{50} reported (~120 nM)[190] for protein S inhibition of the prothrombinase complex contrasts with the huge cofactor activity observed for APC and TFPI suggesting that while protein S direct might in some instances be evident *in vitro*, it has little *in vivo* relevance.

4.5 *In vivo* regulation of coagulation by protein S

Protein S plays a central role in regulating haemostasis and its importance is underpinned by the severe outcome of patients with protein S deficiencies, with homozygous deficiencies generally being incompatible with life.[201-203]

The recent finding that protein S acts as a cofactor not only for APC but also for TFPI raises the question whether APC and TFPI compete for binding to protein S *in vivo*. While it is yet too early to give a definitive answer, a first indication is given by the results obtained in this thesis. Both protein S Asp95 and protein S Gla36 had almost no APC cofactor activity but were still able to enhance the anticoagulant activity of TFPI. While this does not rule out a competition between APC and TFPI for protein S, it suggests that APC and TFPI interact with distinct residues of protein S. It has been shown that addition of APC to plasma increases the anticoagulant activity of TFPI/ protein S.[241] The reason behind this is probably twofold. Firstly, APC reduces the procoagulant response and delays the start of massive thrombin generation. This allows time for the slow binding and inhibition of FXa by TFPI to occur. Secondly, inactivation of FVIIIa and FVa by APC results in a reduced rate of activation of FX and could result in a shift in the equilibrium from FXa/FVa towards free FXa. This overcomes the protective effect of prothrombin on TFPI inhibition of FXa when FXa is incorporated in the prothrombinase complex.[77] It would therefore, seem that the APC/protein S and the TFPI/protein S anticoagulant activities act in synergy rather than the two pathways competing for protein S. Considering also the excess plasma concentration of protein S (free protein S ~145 nM, total protein S ~362 nM) over

that of protein C (~65 nM) and TFPI (full-length ~0.25 nM, total ~2.5 nM) and the different phases of regulation by APC and TFPI (propagation phase and initiation phase, respectively), I believe that it is unlikely that these two proteins compete for binding to protein S *in vivo*.

While *in vitro* assays represent valuable and powerful tools to analyse the biochemical interactions and processes that occur *in vivo* they inevitably exclude many of the variables present *in vivo*. In my plasma based assay, performed by CAT, phospholipids were used to provide a surface for coagulation factors on which to interact. While this simplifies the assay and decreases inter-assay variability, it represents a simplified model for platelets, platelet activation and its components such as platelet protein S, TFPI and FV. The procoagulant and anticoagulant activity of coagulation factors also seem to be differently regulated by the membrane composition and distribution.[125,269] In addition, anticoagulant coagulation factors seem more susceptible to plasma dilution than procoagulant factors.[264] Cell-free plasma based assays differs from *in vivo* assays as they lead to the formation of coagulation in suspension rather than on a surface. In my assays I have used 1 pM TF. However, it is unclear how this relates to the local concentrations of TF *in vivo*. The concentration of TF is also likely to vary during the course of the haemostatic response *in vivo*, following platelet plug formation above the subendothelial cells. A limiting factor of the *in vitro* plasma assays is that it inevitably leads to depletion of coagulation factors during its course. This is likely to be of particular importance for those factors that are present in small amounts such as TFPI. Based upon the vast amount of results derived from *in vitro* assays and those from *in vivo* assays it is, however, reasonable to speculate how coagulation is likely to occur *in vivo*.

Following vessel wall injury, the ECM and TF are exposed to the blood.[10] VWF binds to exposed collagen in the ECM allowing platelet adherance, aggregation and activation.[1,5] This leads to exposure of phosphatidylserine on the outer leflet of the platelet membrane which provides a surface for the binding of coagulation factors.[9] Simultaneusly, as the primary platelet plug is forming, TF triggers coagulation through binding to and activation of FVIIa. TF/FVIIa activates FIX and FX. TFPI is the main regulator of the initiation of coagulation as it inhibits FXa and, in a FXa dependent manner, inhibits TF/FVIIa on the subendothelial cells.[66-68] The finding that protein S enhances TFPI may be physiologically fundamental.

In the presence of protein S, the K_i for TFPI inhibition of FXa falls within the physiological plasma concentration of TFPI, allowing the TFPI pathway to play a potentially major anticoagulant role. While protein S only enhances free TFPI containing its K3 domain,[81] truncated forms of TFPI have been shown to be able to inhibit FXa and TF/FVIIa.[78-80] These free truncated TFPI variants in plasma (~5% of plasma TFPI) are unlikely to play a major role in regulating initiation of coagulation as they represent half the amount of free full-length plasma TFPI (~10% of plasma TFPI) and are not enhanced by protein S. They could, however, have a role in regulating the haemostatic balance under condition in which no tissue damage occurs. The majority (~80%) of plasma TFPI is, however, bound to lipoproteins and their anticoagulant role is still not clear.[64,65] Cell suface bound TFPI (~80% of total TFPI) has been shown to have some anticoagulant activity.[43] Considering vascular smooth muscle cells express TFPI in addition to TF, this pool of TFPI is likely to regulate the local initiation of coagulation on subendothelial cells exposed to the blood. TFPI on intact endothelial cells surrounding the tissue damage might also play a significant role in regulating coagulation by inhibiting FXa that escapes the site of vascular injury, therefore by confining the haemostatic plug. It is still not clear if protein S is able to act as a cofactor for endothelial bound TFPI. Free full-length TFPI circulates in plasma in low concentrations (~0.25 nM). This does exceed the predicted amount of TF (pM range) that triggers coagulation. However, it is far lower than the amount of FXa predicted to be generated from FX (total plasma concentration of FX ~136 nM) potentially explaining why TFPI is only able to inhibit initiation of coagulation. FXa that escapes inhibition by TFPI/protein S generates trace amount of thrombin. Thrombin produces a feedback mechanism by activating FVIII and FV.[12] This leads to massive generation of FXa which can no longer be significantly inhibited by TFPI. The feedback mechanism of thrombin finally results in a massive amplification of itself and is usually refered to as propagation of the coagulation cascade. Thrombin that escapes the immediate site of injury binds to TM on the endothelial cells surrounding the vessel damage. This leads to activation of protein C which acts as a potent anticoagulant together with its cofactor protein S.[114] Results in my thesis and previous reports show that the APC anticoagulant activity is highly dependent upon its cofactor protein S.[218] As protein C is activated at the edge of the vessel injury it is likely to play a role in spatially

confining the haemostatic plug. APC/protein S are also able to bind to the activated platelets through their Gla domains and inactivate FVa and FVIIIa on the growing haemostatic plug. Following the initial formation of the loose platelet plug over the subendothelial cells, it has been suggested that soluble TF or TF in microparticles continue to trigger coagulation on the surface of the growing platelet plug.[16-18] While this requires further investigation, it is seems plausable that TFPI secreted by platelets is an important regulator of this source of TF. As TFPI in platelets exists in its full-length form it is likely that even the activity of platelet TFPI is dependent upon its cofactor protein S.[63]

To conclude, protein S is crucial in regulating haemostasis. It does so by substancially enhancing the enzymatic activity of both TFPI and APC, thereby regulating both initiation and propagation of the coagulation cascade. Further work is necessary to unravel the complex molecular mechanism behind protein S function including, elaboration of the potential molecular interactions between residues found to be crucial in APC and protein S, the interacting residues between protein S and TFPI, and the molecular mechanism governing protein S enhancement of the anticoagulant activity of TFPI.

APPENDIX

Sequencing primers

	Nucleotide sequence	Area of protein S covered
T7 primer	5' taatacgactcactataggg 3'	ATG-middle EGF4
Nt456 primer	5' tattcactgctgcacgtcag 3'	TSR- beginning SHBG
Nt769 primer	5' gatgtggatgaatgctctttg 3'	EGF3- middle SHBG
BGH reverse	5' tagaaggcacagtcgagg 3'	SHBG-stop

Mutagenesis primers

All mutants were made by using the pcDNA6/protein S vector containing the WT protein S cDNA sequence, with the exception of NNEDM which was made by introducing the N74A substitution in the vector containing the NEDM substitutions and KTK which was made by introducing the T103A/K105A substitution in the vector already containing the K94A mutation (made by Dr S.M. Rezende). Please note that sequencing primers designed to introduce subsequent mutations in a region already mutated are designed to be complementary to the mutated template cDNA (with the exception of the new mutation to introduce) and not to the WT protein S template. All sequencing primers are from 5' to 3'. The name of each sequencing primer corresponds to the amino acid mutated, its numerical location in the mature protein S, followed by the residue into which it was mutated. f; foreward, r; reverse.

Gla1 composite mutants	
fD38N	cccggaaacgaattatttttatcc
rD38N	ggataaaaataattcgtttccggg
fV46G	ccaaaatacttaggttgtcttcgctc
rV46G	gagcgaagacaacctaagtattttgg
fK97Q	gcaaagatggacaagcttcttttacttgcac
rK97Q	gtgcaagtaaaagaagcttgtccatctttgc

TSR composite mutant	
fR49Q	ccaaaatacttagtttgtcttcagtcttttcaaactgggttattcac
rR49Q	gtgaataacccagtttgaaaagactgaagacaaactaagtattttgg
fR60Q	tgggttattcactgctgcacagcagtcaactaatgcttatcc
rR60Q	ggataagcattagttgactgctgtgcagcagtgaataaccca
fD68N R70Q	cagtcaactaatgcttatcctaacctacaaagctgtgtcaatgcc
rD68N R70Q	ggcattgacacagctttgtaggttaggataagcattagttgactg
fD78N	gctgtgtcaatgccattccaaaccagtgtagtcct
rD78N	aggactacactggtttggaatggcattgacacagc

NEDM composite mutant	
fN86A E87Q D88N M91A	cctctgccatgcgctcaaaatggatatgcgagctgcaaag
rN86A E87Q D88N M91A	ctttgcagctcgcatatccattttgagcgcatggcagagg

KTK composite mutant	
fT103A K105A	gcttcttttacttgcgcttgtgcaccaggttggcaaggag
rT103A K105A	ctccttgccaacctggtgcacaagcgcaagtaaaagaagc

Point mutants	
fN74A	cctgacctaagaagctgtgtcgctgccattccagaccagtg
rN74A	cactggtctggaatggcagcgacacagcttcttaggtcagg
fD78A	gtcaatgccattccagcccagtgtagtcctctgccatgc
rD78A	gcatggcagaggactacactgggctggaatggcattgac
fQ79A	gtgtcaatgccattccagacgcgtgtagtcctctgccatgc
rQ79A	gcatggcagaggactacacgcgtctggaatggcattgacac
fD95N	ggatatatgagctgcaaaaatggaaaagcttcttttacttg
rD95N	cctatatactcgacgtttttacctttcgaagaaaatgaac

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PUBLICATIONS ARISING FROM THIS WORK

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Josefin Ahnström, **Helena M Andersson**, Kevin Canis, Eva Norstrøm, Professor Björn Dahlbäck, Maria Panico, Howard R Morris, James TB Crawley and David A Lane.
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ABSTRACTS LEADING TO ORAL PRESENTATION

Helena Andersson, Verity Hockey, Yao Yu, S Mapara, Brenda M Luken, James TB Crawley, David A Lane.
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Helena Andersson, Márcia J Arantes, James TB Crawley, Brenda M Luken, David A Lane, Suely M Rezende.
Activated protein C cofactor function of protein S: a critical role for the EGF1-like domain
Awarded the young investigator award, XXII congress of the ISTH meeting 11-16th July 2009. Boston.

Helena M Andersson, Márcia J Arantes, James TB Crawley, Brenda M Luken, Suely M Rezende and David A Lane.
EGF1 residue Asp95 in protein S is essential for APC cofactor activity.
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Josefin Ahnström, **Helena M Andersson**, Kevin Canis, Yao Yu, Maria Panico, Howard R Morris, James TB Crawley and David A Lane.
Glutamic acid residue 36 of protein S and activated protein C cofactor function
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Activated protein C cofactor function of protein S: a critical role for Asp95 in the EGF1-like domain

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Protein S has an established role in the protein C anticoagulant pathway, where it enhances the factor Va (FVa) and factor VIIIa (FVIIIa) inactivating property of activated protein C (APC). Despite its physiological role and clinical importance, the molecular basis of its action is not fully understood. To clarify the mechanism of the protein S interaction with APC, we have constructed and expressed a library of composite or point variants of human protein S, with residue substitutions introduced into the Gla, thrombin-sensitive region (TSR), epidermal growth factor 1 (EGF1), and EGF2 domains. Cofactor activity for APC was evaluated by calibrated automated thrombography (CAT) using protein S–deficient plasma. Of 27 variants tested initially, only one, protein S D95A (within the EGF1 domain), was largely devoid of functional APC cofactor activity. Protein S D95A was, however, γ-carboxylated and bound phospholipids with an apparent dissociation constant (Kd_{app}) similar to that of wild-type (WT) protein S. In a purified assay using FVa R506Q/R679Q, purified protein S D95A was shown to have greatly reduced ability to enhance APC-induced cleavage of FVa Arg306. It is concluded that residue Asp95 within EGF1 is critical for APC cofactor function of protein S and could define a principal functional interaction site for APC. (*Blood.* 2010;115(23): 4878-4885)

Introduction

Protein S, a vitamin K–dependent plasma anticoagulant protein, functions as an enhancing cofactor to activated protein C (APC) in the inactivation of activated factors V (FVa) and VIII (FVIIIa).[1] Protein S also has an APC-independent activity that has recently been attributed to its ability to enhance tissue factor pathway inhibitor (TFPI).[2,3] Protein S has an important role in vivo, as is shown clinically by infants with complete deficiency, who suffer purpura fulminans, and by heterozygous carriers of *PROS1* gene deletions and point mutations, who are at enhanced risk of venous thromboembolism.[4,5] The importance of protein S has also been demonstrated in murine knockouts, which fail to survive development.[6,7]

Protein S binds phospholipids and helps to localize APC to the membrane surface, in proximity to FVa and FVIIIa. In doing so, rather than the preferential cleavage at Arg506 of FVa by APC, Arg306 is then favored, with cleavage enhanced approximately 20-fold.[8] Although this effect has generally been accepted to be due to protein S repositioning of the active site of APC,[9] this has recently been questioned.[10] Cleavage at Arg506 and Arg306 fully inactivates FVa, thereby efficiently down-regulating coagulation.[11] APC also inactivates FVIIIa by cleaving first at Arg336 and thereafter at Arg562. In the presence of protein S, it has been shown that the efficiency of cleavage after Arg336 by APC is enhanced approximately 3-fold.[12]

Protein S is a 635–amino acid glycoprotein, circulating at a plasma concentration of approximately 350nM. It is composed of an N-terminal Gla domain (amino acids 1-45), a thrombin-sensitive region (TSR; amino acids 46-75), 4 epidermal growth factor (EGF)–like domains (EGF1, amino acids 76-116; EGF2, amino acids 117-160; EGF3, amino acids 161-202; EGF4, amino acids 203-242), and a C-terminal sex hormone–binding globulin (SHBG)–like domain (amino acids 243-635).[13,14] Approximately 60% of protein S circulates as a noncovalent, high-affinity complex with β-chain containing C4b binding protein.[15] This interaction is mediated through the SHBG domain of protein S. Although it has been generally understood that the free form (~ 40%) of protein S alone is important in anticoagulant function, this has recently been questioned.[16]

Whereas the functions of the domains of protein S have been extensively studied, the APC cofactor function of protein S remains poorly understood at the molecular level. Several basic and clinical studies have confirmed that alteration of protein S can disrupt phospholipid binding and thereby impair function of protein S.[17,18] Saller et al prepared protein S/prothrombin chimeras and, on the basis of functional studies supported by molecular modeling, identified a cluster of protein S Gla domain residues (termed Face2), which may provide an APC interaction site.[19]

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Table 1. Protein S variants generated by site-directed mutagenesis

GLA1	D38N/V46G/K97Q
GLA2/Face2	L21T/N23S/K24Y/R28F/D34S/Y41W/L45T
TSR1	R49Q/R60Q/D68N/R70Q/D78N
EGF1 single-residue variants	K94A, D95A, K97A, S99A, T101A, T103A, K105A, P106A, W108A, Q109A, E111A, K112A
EGF2 single-residue variants	D135A, N136A, T137A, Y141A, H142A, S144A, K146A, S153A, N154A, K155A, K156A, D157A

Additional studies have focused on the possible role of TSR and EGF domains. Several studies have reported that cleavage in the TSR of protein S affects its APC cofactor activity.[20-22] Dahlbäck et al[23] and Giri et al[24] used domain-specific monoclonal antibodies directed against protein S and their Fab fragments and suggested that the TSR and EGF1 domains may be involved in APC cofactor activity. The TSR and EGF1 domains of protein S have also been reported to be responsible for species specificity.[25] Using EGF-like, modules Stenberg et al concluded the EGF1 module to be crucial for interaction with APC.[26] Hackeng et al demonstrated that the isolated EGF1 domain of protein S could inhibit full-length protein S anticoagulant enhancing function against APC: direct binding of the domain to APC was also demonstrated.[27] Mille-Baker et al prepared EGF2 domain deletion and substitution mutants of protein S and demonstrated reduced anticoagulant activity, in the presence of normal phospholipid binding.[28] Collectively, these investigators have suggested Gla, TSR, EGF1, and EGF2 domains may each contain recognition elements on protein S needed for APC cofactor function.

Because these diverse approaches have not yet fully defined the functional interaction site(s) on protein S for APC, we have investigated the role of individual and groups of residue substitutions in Gla, TSR, EGF1, and EGF2 domains of protein S. Using a library of 27 protein S variants, we performed thrombin generation assays in protein S–deficient plasma to which APC and these protein S variants were added. We report here that EGF1 has a critical role in anticoagulant function of protein S, provided specifically by Asp95, the mutation of which almost completely abolishes APC cofactor activity.

Methods

Generation and expression of protein S and its variants

A previous reported pcDNA6/protein S vector containing the cDNA for wild-type (WT) protein S[29] was used to generate recombinant full-length WT protein S and as a template to produce protein S mutants by polymerase chain reaction site-directed mutagenesis (QuikChange XL Site-Directed Mutagenesis Kit; Stratagene) using mutagenic oligonucleotide primers (Thermo Scientific). Single and composite residue variants produced are shown in Table 1.

Residues to mutate were selected based on nonconserved residues in domains of other human coagulation factors (Gla mutants) and charge (TSR mutant). Residues in the B and C regions (between the third/fourth and fifth/sixth Cys residues, respectively) of both EGFs were mutated, as these regions on the EGF-like domains are likely to be involved in protein interaction as suggested by (1) the highly divergent sequence of the B region,[30] (2) the association of dysfunctional proteins with natural *PROS1* mutations in these regions,[4] and (3) the location in these regions of many mutations associated with dysfunctional proteins in the other coagulation genes coding for EGF domains, such as FVII, FIX, and protein C.

WT and mutant protein S expression vectors were either transiently or stably transfected into HEK293T or HEK293 cells (ATCC), respectively. Stably transfected cells were selected with 5 μg/mL Blasticidin-HCl (Invitrogen) for 4 weeks. Protein S was expressed in OptiMem I (Invitrogen) with 10 μg/mL vitamin K (Roche Products Ltd) for 3 days. Media containing protein S was harvested, centrifuged, filtered, dialyzed in 20 mM Tris (tris(hydroxymethyl)aminomethane)–HCl (pH 7.5), 140 mM NaCl (TBS), and concentrated, as required. All variants were successfully expressed into the media and could be readily detected by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western blotting and by enzyme-linked immunosorbent assay (ELISA). We have previously reported that the protein S expressed in this way is functionally active in APC cofactor assays and can be fully γ-carboxylated.[17,31]

Purification of protein S

WT protein S and selected variants were either partially purified by barium citrate precipitation as previously described[32] for use in phospholipid binding analysis or extensively purified by chromatography for use in APC-dependent functional assays. Chromatography was performed using an ÄKTA purifier UPC-10 and the Unicorn 5.11 (Build 407) software, strategy Version 1.00 (both from GE Healthcare). Concentrated and dialyzed conditioned media containing protein S supplemented with 4 mM EDTA (ethylenediaminetetraacetic acid) was applied to an anionic Q Sepharose Fast Flow (5 mL of HiTrap QFF) column (GE Healthcare) equilibrated with 20mM Tris-HCl (pH 7.4), 100mM NaCl, 4mM EDTA, and 5mM benzamidine-HCl (Sigma-Aldrich). The column was washed with 20mM Tris-HCl (pH 7.4), 100mM NaCl, and 5mM benzamidine-HCl. Protein S was eluted from the column with 20mM Tris-HCl (pH 7.4), 0.5M NaCl, and 5mM benzamidine-HCl.

Two volumes of 7.5mM $CaCl_2$ were added to the elution fraction from the anionic QFF column to lower the ionic strength, before injecting the fraction onto an affinity column prepared by coupling 2.5 mg of monoclonal antibody against protein S, MK21, to a 1-mL HiTrap NHS HP column (GE Healthcare), according to the manufacturer's instructions. All monoclonal antibodies against protein S used in this study are described in the paper of Dahlbäck et al.[23] The protein S–containing fraction was applied to the equilibrated column (equilibration buffer: 50mM Tris-HCl [pH 7.5], 150mM NaCl, 2mM $CaCl_2$). After washing with 1M NaCl, the ionic strength was lowered by passing additional 3 column volumes of equilibration buffer over the column prior to elution of protein S with 50mM Tris-HCl (pH 7.5), 150mM NaCl, and 10mM EDTA. An excess of $CaCl_2$ was present in the tubes collecting the eluted protein S to neutralize the EDTA. The column was regenerated by stripping with 0.1M glycine-HCl (pH 2.7). Purified protein S was dialyzed in TBS and concentrated, as required.

Determination of protein S concentration by ELISA

Protein S concentrations in media were determined by an in-house ELISA. Polyclonal rabbit anti–protein S antibody (1 μg/mL; DAKO) was immobilized in a 96-well Nunc Maxisorp microplate in 50mM sodium carbonate buffer (pH 9.6) at 4°C overnight. Washing steps were performed in triplicate with 250 μL of phosphate-buffered saline, 0.1% Tween between each step. Wells were blocked with phosphate-buffered saline containing 3% bovine serum albumin (BSA; Sigma-Aldrich) for 2 hours. Bound protein S was detected by a mouse monoclonal antibody directed against the EGF3-4 domain of protein S, MK55, followed by a horseradish peroxidase (HRP)–conjugated goat anti–mouse antibody (DAKO). The plate was developed with 100 μL/well chromogenic substrate O-phenylene diamide dihydrochloride; OPD (Sigma-Aldrich), the enzymatic reaction was stopped with 50 μL/well 2M H_2SO_4, and the absorbance was read at 492 nm.

SDS-PAGE and Western blotting

SDS-PAGE and Western blotting were performed using standard techniques. Briefly, 4% to 12% or 10% precast NuPAGE Novex Bis-Tris gels (Invitrogen) were used. For silver staining and Western blotting, standard techniques were used. The SeeBlue-prestained marker from Invitrogen was

used. Protein S was detected either by a polyclonal rabbit anti–protein S antibody followed by an HRP-conjugated goat anti–rabbit antibody or by a monoclonal mouse antibody recognizing only γ-carboxylated Glu residues (American Diagnostica Inc), followed by a goat anti–mouse HRP-conjugated antibody (DAKO).

Phospholipid vesicle preparation

Phospholipids mixtures (Avanti Polar Lipids Inc) in chloroform were prepared and the chloroform was evaporated under a nitrogen stream. The phospholipids were resuspended in TBS or 25mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (pH 7.7), 150mM NaCl (HN) for the plasma and purified assays, respectively. Unilamellar phospholipids vesicles were obtained by sonicating the phospholipids in an ice container at amplitude 22% for 7 minutes. Extruded phospholipids were prepared as previously described[33] and used in the FVa inactivation assay. Synthetic phospholipids 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) were used in the plasma assay and the plate-binding assay. Natural phospholipids L-α-phosphatidylserine (PS; brain extract), L-α-phosphatidylethanolamine (PE; egg extract), and L-α-phosphatidylcholine (PC; egg extract) were used in the FVa inactivation and prothrombinase assays.

Binding of protein S to phospholipids

Phospholipids vesicles (DOPS/DOPC/DOPE, 20:60:20), 25 μg/mL, were coated as described for the ELISA. Washing steps were performed in triplicate with 250 μL/well TBS, 5mM $CaCl_2$, 0.3% BSA between each step and incubations were carried out in a plate shaker at 37°C. Wells were blocked with TBS, 5mM $CaCl_2$, 3% BSA for 2 hours. Protein S partially purified by barium citrate precipitation, 0 to 120nM, was incubated in the wells in duplicate for 2 hours and was detected with an HRP-conjugated anti–protein S antibody for 45 minutes (Affinity Biologicals). Data were analyzed with GraphPad Prism 4.03 and curves were fitted to a one-site binding hyperbola.

Binding of protein S to domain-specific monoclonal antibodies

Domain-specific monoclonal antibodies MK21 (Gla domain), MK54 (EGF1 domain), and MK61 (SHBG domain), 1 μg/mL, were coated as described for the ELISA. Washing steps were performed in triplicate with 250 μL of TBS, 3mM $CaCl_2$, 0.1% Tween between each step. Incubations were carried out in a plate shaker at 37°C for 1 hour unless stated otherwise. Wells were blocked with 200 μL of TBS, 3mM $CaCl_2$, 3% BSA for 2 hours. Protein S, 0 to 160nM was incubated in the plate for 1 hour and was detected by a rabbit polyclonal antibody against protein S followed by a goat anti–rabbit HRP-conjugated antibody.

Thrombin generation assay by calibrated automated thrombography

Thrombin generation was assessed using a Fluoroscan Ascent FL Plate Reader (Thermo Lab System) in combination with Thrombinoscope software (SYNAPSE BV). Protein S–deficient plasma (Affinity Biologicals), 80 μL, was incubated with 65 μg of corn trypsin inhibitor (Haematologic Technologies Inc) per milliliter of plasma to inhibit contact activation, 50μM phospholipid vesicles (DOPS/DOPC/DOPE, 20:60:20), 1pM tissue factor (Dade Innovin; Dade Behring), 4 to 16nM APC (Enzyme Research Laboratories Ltd) with 0 to 120nM protein S, in a final volume of 100 μL (all concentrations are final). A polyclonal antibody against TFPI (Haematologic Technologies Inc), 100nM, was used to inhibit any protein S cofactor activity toward TFPI. Although protein S cofactor activity toward TFPI was not observed at low concentrations of APC, it was observed to a small extent when lower amounts of thrombin were generated (eg, at higher concentrations of APC in the presence of protein S). Polyclonal antibodies against protein S and protein C were used to show the specificity of the system and were from, respectively, DAKO and Sigma-Aldrich. Thrombin generation was initiated by automatic dispensation of 20 μL of 2.5mM Z-GlyArg-AMC-HCl (Bachem), 2.5% Me_2SO, 20mM Tris-HCl (pH 7.5), 60 mg/mL BSA, 100mM $CaCl_2$ into each well. The reaction was performed at 37°C and measurements were performed with an excitation and emission wavelength of 390 nm and 460 nm, respectively.

Protein S–dependent APC-mediated factor FVa inactivation assay

A previously prepared and reported[34] double mutant of FV, FV R506Q/R679Q, was activated with 0.5 U/mL human thrombin at 37°C for 10 minutes. The reaction was stopped by the addition of hirudin (5 U/mL, final concentration). The activated FVa variant was used as a substrate for APC to determine the ability of protein S to enhance cleavage of Arg306 of FVa by APC. The highly purified protein S preparations, 0 to 100nM, were incubated with 0.5nM APC, 25μM phospholipids vesicles (PS/PC/PE, 10:70:20), and 0.8nM FVa R506Q/R679Q in HN, 5mM $CaCl_2$, 5 mg/mL BSA ($HNBSACa^{2+}$) in a total volume of 50 μL (all concentrations are final). The solution was incubated at 37°C for 10 minutes, and the reaction was stopped by performing a 1:25-fold dilution in ice-cold $HNBSACa^{2+}$. The remaining FVa activity was measured in a prothrombinase assay.

Time course experiments were also performed to derive the rate constant of FVa cleavage. APC, 0.5 and 3nM, and 50nM protein S were then used and aliquots were quenched at intervals between 0 and 20 minutes. The remaining FVa activity was again measured in a prothrombinase assay. To calculate the pseudo–first-order rate constant for APC-mediated cleavage at Arg306, the previously reported equation[33] was used: $Va_t = Va_0 \times e^{-k306 \times t} + C \times Va_0 \times (1 - e^{-k306 \times t})$.

Va_t represents the cofactor activity determined at time *t*, Va_0 is the cofactor activity determined at time point 0, *C* is the remaining procoagulant cofactor activity of FVa after cleavage at position 306, and *k306* is the rate constant of cleavage at position 306.

Prothrombinase assay

An aliquot, 25 μL, of the FVa inactivation reaction was incubated with phospholipid vesicles (PS/PC 10:90) and FXa in the presence of $CaCl_2$. The reaction was initiated by the addition of prothrombin, in a final volume of 125 μL. The buffer contained HN and 0.5 mg/mL ovalbumin. Final concentrations were 50μM PS/PC, 5nM FXa, and 0.5μM prothrombin. The solution was incubated at 37°C for 2 minutes and the reaction was terminated by an 8-fold dilution in 50mM Tris-HCl (pH 7.9), 100mM NaCl, 20mM EDTA, 1% PEG 6000. The amount of thrombin generated was measured by cleavage of its chromogenic substrate S-2238 (Chromogenix) at 405 nm for 15 minutes at 30-second intervals.

Results

Screening of protein S mutants for APC cofactor activity in plasma

Concentrated and dialyzed conditioned media samples containing WT protein S and the 27 protein S variants were normalized with respect to protein S concentration, using the protein S–specific ELISA. Western blotting revealed single-band protein S in all samples (nonreducing conditions).

To assess the APC cofactor activity of protein S, thrombin generation was assessed by calibrated automated thrombography (CAT) using protein S–deficient plasma supplemented with recombinant WT protein S and its variants in conditioned media. Under the conditions of the assay, APC (0-10nM) did not affect peak, endogenous thrombin potential (ETP), lag time, or time to peak, when coagulation was initiated in protein S–deficient plasma (Figure 1A). Concentrated conditioned media from cells not expressing protein S had no influence on thrombin generation (data not shown). When 120nM WT protein S was progressively introduced into the assay, dose-dependent effects were observed,

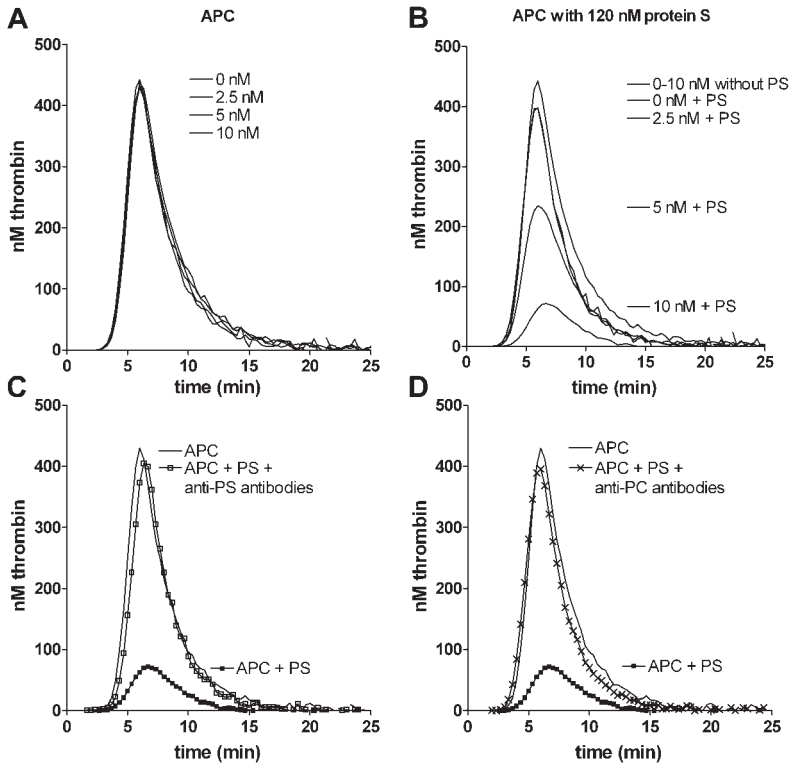

Figure 1. Effect of APC and protein S on thrombin generation. Thrombin generation was performed in protein S–deficient plasma with 100nM inhibitory antibodies against TFPI. Up to 10nM APC had no effect on thrombin generation in the absence of protein S. All concentrations generate lines that are superimposable (A). After addition of 120nM protein S (at 0-10nM APC), an APC dose-dependent effect was observed (B). The top single line represents 0 to 10nM APC in the absence of protein S. Protein S in the presence of no or 2.5nM APC generated lines that were superimposable. Conditions used are noted adjacent to the peaks to which they refer. The anticoagulant effect of 10nM APC and 120nM protein S was inhibited by polyclonal antibodies against protein S (C) or against protein C (D). PS indicates protein S; PC, protein C. Representative experiments are shown (n = 3).

primarily on reduction of the peak and of ETP values (Figure 1B). The extent of inhibition of peak and ETP was dependent both on the concentration of APC and protein S. The higher the APC concentration, the less protein S was necessary to completely inhibit thrombin generation. In these experimental conditions, 10nM APC and 120nM protein S reduced both the peak and ETP by approximately 84%. To verify that those effects were a direct consequence of APC cofactor activity of protein S, polyclonal antibodies against either protein S (Figure 1C) or protein C (Figure 1D) were used. These inhibited the anticoagulant APC–protein S effect.

The screening of the protein S variants for their APC cofactor activity was performed at high concentration of APC (16nM) to achieve almost complete inhibition of thrombin generation with 100nM WT protein S (Figure 2). These screening experiments identified a single point mutant in EGF1 of protein S, D95A, which had a severely impaired APC cofactor activity. Under all conditions used, the reduction in cofactor activity was greater than that observed for the previously reported variant with 7 amino acids exchanged (prothrombin residue swap), GLA2/Face2.

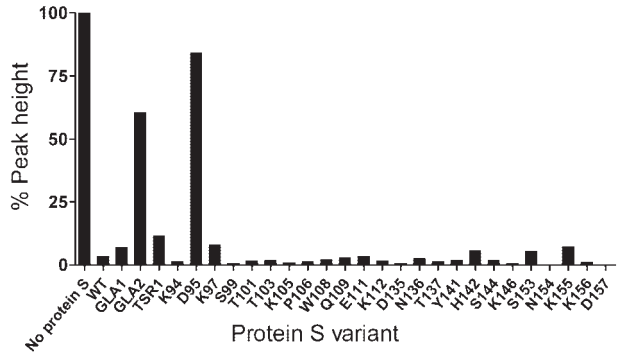

Figure 2. Screening of protein S variants for APC cofactor activity. The APC cofactor activity of protein S was evaluated at 16nM APC and 100nM protein S by CAT. The peak height in the absence of protein S was set to 100%. A high concentration of APC, leading to almost complete inhibition of thrombin generation with 100nM WT protein S, was chosen specifically for screening purposes as this allows widening of the assay window at which mutants with reduced APC cofactor activity are visualized. Results were confirmed by evaluating protein S (at 60 and 90nM) cofactor activity toward 4 or 9nM APC.

Evaluation of the importance of Asp95 for APC cofactor activity of protein S in plasma

To further evaluate the APC cofactor activity of protein S D95A, a titration (0-120nM) of the mutant (Figure 3B) was performed alongside WT protein S (Figure 3A), using 9nM APC. The protein S D95A had a severely impaired APC cofactor activity, a finding replicated when different concentrations of APC were used (results not shown). In these experimental conditions, whereas 120nM WT protein S reduced peak and ETP by approximately 80%, 120nM protein S D95A reduced peak and ETP by only approximately 10%. To confirm the importance of Asp95 in protein S APC cofactor function, Asp95 was also substituted with Asn, rather than Ala. Asn is structurally more similar to Asp than Ala and it can also be β-hydroxylated. When the protein S D95N mutant was titrated, it was observed that it also had severely reduced APC cofactor activity (Figure 3C) with 120nM D95N inhibiting peak and ETP by approximately 20%. All results in Figure 3A through C were conducted with protein S and its variants contained in concentrated conditioned media. To confirm that they were not influenced by the media, WT protein S and the protein S D95A variant were purified to homogeneity using anion exchange and affinity chromatography (see Figure 3D inset for SDS-PAGE gel visualized with silver staining). Addition of 90nM purified WT protein S to protein S–deficient plasma in the presence of 9nM APC appreciably attenuated thrombin generation (Figure 3D), replicating the findings with WT protein S in conditioned media. Moreover, addition

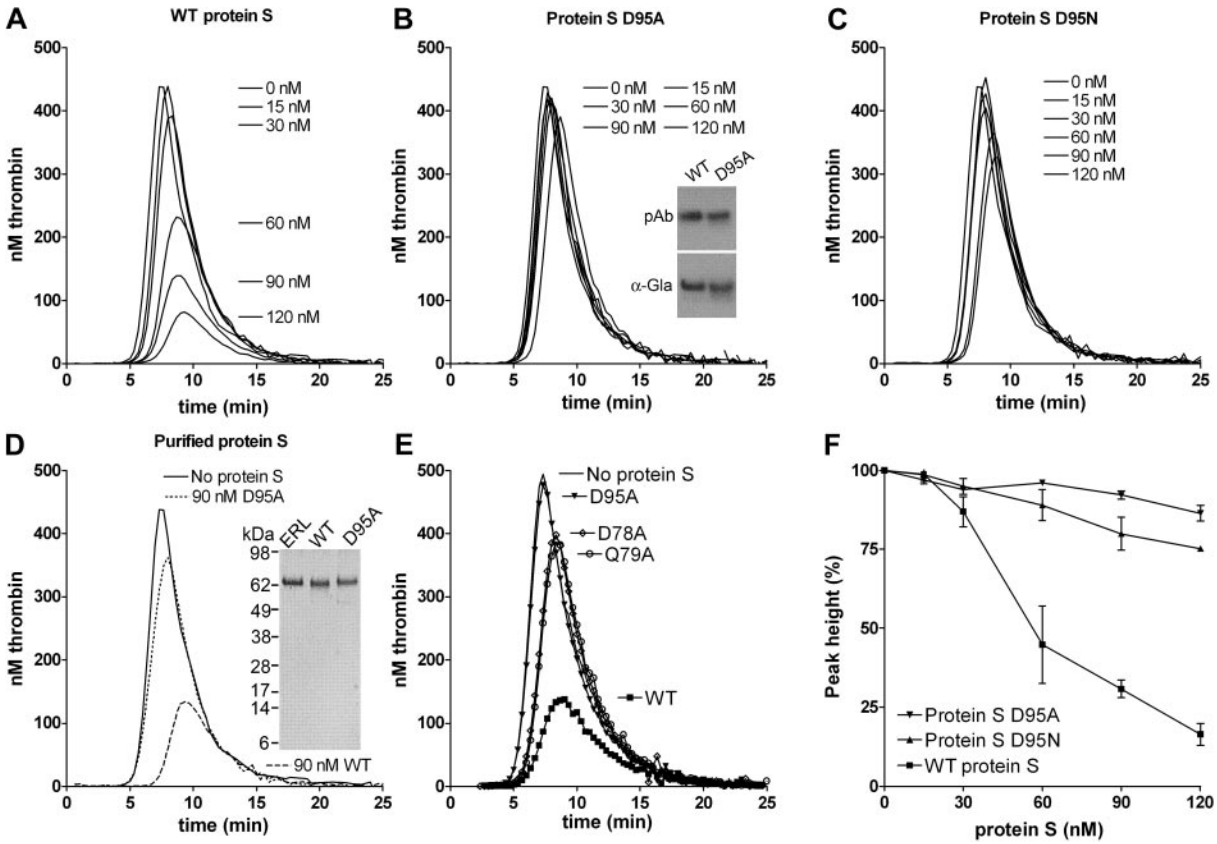

Figure 3. Effect of WT protein S, D95A, D95N, D78A, and Q79A variants on thrombin generation. Thrombin generation was measured in protein S–deficient plasma supplemented with 9nM APC, 100nM antibodies against TFPI, and 0 to 120nM WT protein S (A), protein S D95A (B), protein S D95N (C), or 90nM purified WT (dashed line) or purified protein S D95A (dotted line; D). Protein S concentrations are positioned adjacent to the peaks to which they refer. The cofactor activity of 60nM WT protein S and protein S variants D95A, D78A, and Q79A was compared at 9nM APC (E). Typical experiments are shown (n = 3). Whereas the cofactor activity of WT protein S is highly dependent on the APC concentration used (Figure 1B), that of protein S D95A is not, explaining the difference in fold activity between WT protein S and protein S D95A in Figures 2 and 3. Dose-response data from titrations with WT protein S, protein S D95A, and protein S D95N in the presence of 9nM APC are shown in panel F (data are expressed as mean ± SD of 2 independent experiments performed in duplicate). Inset in panel B shows recognition of WT protein S and protein S D95A in media by polyclonal antibodies and a monoclonal antibody recognizing only γ-carboxylated Gla domains. Inset in panel D shows the SeeBlue-prestained marker, plasma-purified protein S from Enzyme Research Laboratories Ltd (lane 1), purified recombinant WT protein S (lane 2), and purified protein S D95A (lane 3) visualized with silver staining.

of 90nM purified protein S D95A to APC produced only a minimal effect on thrombin generation (Figure 3D).

When analyzing a model[35] of Gla-TSR-EGF1 domains of protein S, 2 residues with a solvent-exposed R residue in close proximity to that of Asp95 were identified, Asp78 and Gln79. These were also mutated to alanine and expressed. When protein S D78A and Q79A were analyzed in the thrombin generation assay alongside WT protein S, a severely reduced APC cofactor activity was also observed (Figure 3E). This further confirms the importance of this region of EGF1 in APC cofactor activity of protein S. Dose-response experiments of WT protein S, protein S D95A, and protein S D95N, shown in Figure 3F, confirm reduced APC cofactor function of these variants.

Phospholipid binding of WT protein S and protein S D95A

As phospholipid binding is a prerequisite for protein S function, the binding of WT protein S and of protein S D95A to phospholipids was evaluated. Partially purified (using barium citrate precipitation) protein S, 0 to 120nM, was incubated for 2 hours on a plate coated with 25 μg/mL phospholipids and was detected by an HRP-conjugated antibody against protein S. As shown in Figure 4, both WT protein S and protein S D95A were able to bind phospholipids, with apparent dissociation constant (Kd_{app}) of 5.69 plus or minus 1.24 and 9.54 plus or minus 2.26nM, respectively (Table 2). Kd_{app} values were analyzed by the Mann-Whitney test and were found not to be statistically different ($P > .05$). Binding was observed in the presence of Ca^{2+}, but not in the presence of EDTA or in the absence of phospholipids, as expected. These

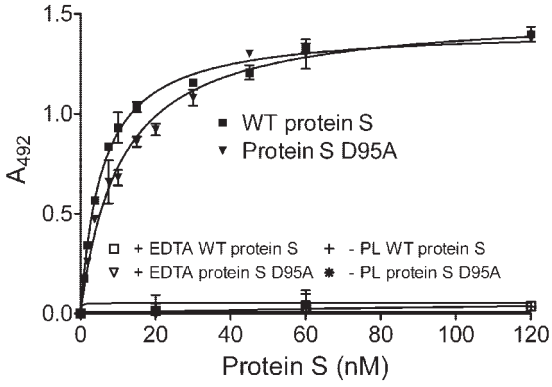

Figure 4. Binding of protein S to phospholipid surfaces. Protein S (0-120nM) was incubated in a plate coated with 25 μg/mL phospholipids. Bound protein S was detected with an HRP-conjugated polyclonal antibody against protein S. A representative experiment is shown. The apparent Kd values, 5.69 ± 1.24 and 9.54 ± 2.26nM for WT protein S and protein S D95A, respectively, were obtained by calculating the mean ± SD of 3 independent experiments performed in duplicate. PL indicates phospholipids.

Table 2. Binding of protein S to phospholipids and domain-specific monoclonal antibodies

	Phospholipid vesicles	MK21 (Gla)	MK54 (EGF1)	MK61 (SHBG)
WT protein S	5.69 ± 1.24	2.18 ± 0.97	0.81 ± 0.11	5.31 ± 1.11
Asp95 protein S mutant	9.54 ± 2.26	2.53 ± 1.06	0.83 ± 0.03	5.39 ± 0.54

Kd_{app} values (nM) of WT protein S and the Asp95 protein S mutant for phospholipid vesicles and conformational domain-specific monoclonal antibodies are expressed as mean plus or minus SD of 3 independent experiments performed in duplicate. Binding of WT protein S and the Asp95 mutant to phospholipids and domain-specific monoclonal antibodies was analyzed by Mann-Whitney test; results were not statistically significant ($P > .05$).

findings are broadly consistent with previously reported Kd values for protein S binding to phospholipid surfaces.[17-19,36,37] Our results suggest the loss of APC cofactor activity observed for protein S D95A is not due to loss of binding to phospholipid surfaces.

Binding to domain-specific monoclonal antibodies

Binding of protein S to conformational domain-specific antibodies was evaluated to assess the integrity of the domain structure and folding. Protein S in concentrated conditioned media was incubated in a plate coated with monoclonal antibodies recognizing either the Gla domain (MK21), the EGF1 domain (MK54), or the C-terminal SHBG domain (MK61). Bound protein S was detected with polyclonal antibodies. Binding curves were fitted with a one-site binding equation, and the Kd_{app} values were obtained. Kd_{app} values were analyzed by the Mann-Whitney test and were found not to be statistically different ($P > .05$). Table 2 represents the mean plus or minus SD Kd_{app} values of 3 independent experiments performed in duplicate. The results suggest that mutation of Asp95 does not result in a significant change in the domain structure of protein S.

Protein S enhancement of APC-mediated cleavage of FVa at Arg306

The FVa variant, R506Q/R679Q, was used to evaluate protein S–enhanced APC-mediated cleavage at Arg306 in FVa. FVa inactivation was performed in the presence or absence of 0.5nM APC and a titration of purified protein S (0-100nM) was performed. After 10 minutes, the remaining FVa activity was measured in the prothrombinase assay. WT protein S efficiently enhanced APC-mediated cleavage at Arg306 of FVa, in contrast to the D95A mutant (Figure 5A).

To quantify the rate of cleavage at FVa Arg306 in the presence and absence of protein S, time course experiments were performed (Figure 5B). In the absence of protein S, or in the presence of protein S D95A, 3nM APC was used and aliquots were quenched at different time points (0-20 minutes). In the presence of WT protein S, the APC concentration was lowered to 0.5nM, as APC is efficiently enhanced by protein S. The remaining FVa activity at all time points was measured in the prothrombinase assay. Using the FVa inactivation curves obtained, the apparent pseudo–first-order rate constants were calculated and corrected for the APC concentrations used. Under these experimental conditions, protein S had no effect on FVa activity in the absence of APC (data not shown). APC-mediated cleavage at FVa Arg306 was enhanced by WT protein S by 13.9-fold plus or minus 3.6-fold, whereas protein S D95A was only able to enhance APC by 1.8-fold plus or minus 0.4-fold.

Discussion

We initially constructed and expressed 27 protein S variants with mutations in the Gla, TSR, EGF1, and EGF2 domains and evaluated their ability to enhance APC-mediated anticoagulant activity in plasma. The anticoagulant function of these 27 variants, and the subsequently expressed variants D78A, D79A, and D95N, were assessed by a thrombin generation assay, the specificity of which was demonstrated using polyclonal antibodies against either protein S or protein C that completely inhibited the anticoagulant response observed when adding protein S in the presence of APC. The advantage of the thrombin generation assay conducted in plasma is that it shows very clearly how APC is heavily dependent on protein S.[38] In the absence of protein S, 10nM APC has no effect on thrombin generation. In contrast, in the presence of protein S, maximal anticoagulant effects with near ablation of thrombin generation are obtainable with APC concentrations less than 10nM.

Our initial screening results identified 2 protein S variants with appreciable reduction in APC cofactor function. These were the already reported GLA2/Face2 variant (a 7-residue composite mutant in the Gla domain) and the novel D95A point variant in the EGF1 domain. The former variant, reported by Saller et al, retains binding to phospholipids and yet has appreciably reduced APC cofactor activity.[19] It is suggested that the 7 residues substituted in this variant collectively present a face of the Gla domain to APC and form a potential contact region. It was selected for investigation here because it is a well-characterized protein S variant with

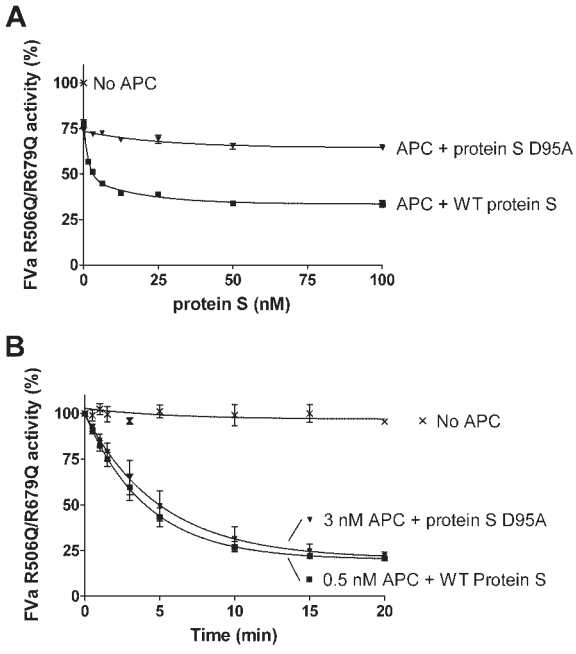

Figure 5. Protein S enhancement of APC-mediated cleavage of FVa in Arg306. Protein S (0-120nM) in the presence of 0.5nM APC was incubated with 0.8nM FVa R506Q/R679Q in the presence of phospholipids for 10 minutes. The remaining FVa actvity was measured with a prothrombinase assay. Results are plotted as mean ± SD from 3 independent experiments performed in duplicate (A). A time course experiment was performed to calculate the apparent pseudo–first-order rate constants of WT protein S and protein S D95A. It is observed that approximately 6-fold more APC is needed in the presence of protein S D95A to obtain a similar amount of APC-mediated FVa R506Q/R679Q inactivation as with WT protein S (B).

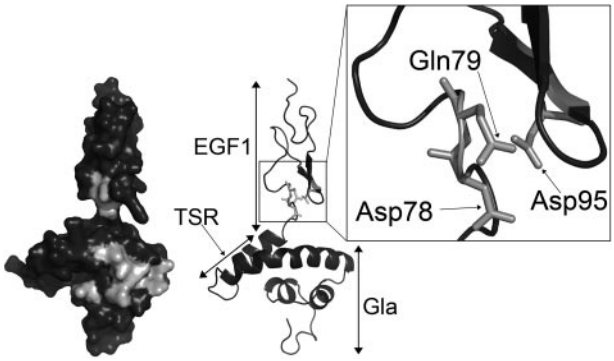

Figure 6. Location of Asp78, Gln79, and Asp95 within the protein S Gla-TSR-EGF1 model. Domains are labeled on the right side of the model. Residues mutated in the GLA2 variant, Asp78, Gln79, and Asp95, are in light gray on the left side surface model. Residues Asp78, Gln79, and Asp95 are highlighted by the box to show their proximal spatial location. The model is taken from Villoutreix et al.[35]

substantially reduced APC cofactor activity and could therefore act as a representative control for a dysfunctional protein S. Saller et al,[19] however, did not assess GLA2/Face2 APC cofactor activity in the CAT assay. We can confirm a reduction in APC cofactor activity in plasma, assessed by CAT. The important novel results here, however, concerned the protein S D95A variant, which was almost devoid of APC cofactor activity in plasma. The natural protein S variants T103N[39] and K155E[40,41] have been reported to have reduced APC cofactor activity. We could not observe any significant reduction in APC cofactor activity of protein S T103A in the CAT assay. It is, however, important to point out that defective APC cofactor activity of protein S T103N was not observed in the APC-mediated inactivation of WT-FVa.[39] In addition, at higher concentrations of APC (8nM), no difference was observed between WT protein S and protein S T103N in the FVIIIa degradation assay.[39] We did, however, observe a decrease in K155A cofactor activity toward APC, although this was quite moderate under conditions used in the screening assay.

Analysis of protein S function is complicated by its propensity to multimerize upon purification.[42,43] Accordingly, we have performed APC cofactor assays with unpurified protein S in concentrated conditioned media as well as with fully purified preparations. In the event, our results were all consistent. Both forms of the protein S D95A variant, purified or in concentrated conditioned media, had an appreciable reduction in APC cofactor activity compared with the respective WT protein S preparation. Although it has not been formally assessed, in all comparison experiments carried out, the protein S D95A variant had less APC cofactor than the GLA2/Face2 variant. This suggests that Asp95 in protein S may occupy a pivotal position with respect to its interaction with APC. The importance of Asp95 in APC cofactor activity was further confirmed by the severely reduced activity observed when mutating to alanine the 2 residues in close proximity to Asp95, Asp78, and Gln79. Asp78, Gln79, and Asp95 are conserved across species with the exception of Asp78, which is replaced by the structurally similar Asn78 in birds. Using a working model of the Gla-TSR-EGF1 domains of protein S, we demonstrate the likely proximal spatial location of these 3 residues (Figure 6) and their relationship to GLA2/Face2 residues.

We have investigated the mechanism of reduction of activity of protein S Asp95 variants. Whereas EGF1 of protein S has not been shown to contain a calcium-binding site (in contrast to EGF2, EGF3, and EGF4),[44] Asp95 could be part of a partially conserved calcium-binding motif. We therefore mutated Asp95 to Asn rather than Ala. Asn is structurally similar to Asp, can be β-hydroxylated, and is able to coordinate a calcium ion (as has been observed in EGF2, EGF3, and EGF4). As the protein S D95N variant also had reduced activity, however, it is unlikely that a calcium-binding site is disrupted. Furthermore, whereas Asp95 is known to be β-hydroxylated, it has previously been shown that β-hydroxylation itself is not a requirement for anticoagulant activity of protein S.[45] Our evidence favors a direct effect of Asp95 in APC cofactor activity. Alternatively, the dramatic effect on protein S activity of the Asp95 residue substitution could be through a conformational repositioning of other functional domains. To assess this, we performed binding to phospholipids, as this property underpins all protein S function. Plate binding assays, however, indicated no major functional defect on phospholipid binding. Furthermore, domain-specific monoclonal antibody binding to WT and mutant protein S (against Gla domain, EGF1 domain, and the C-terminal SHBG domain) was also normal, suggesting that substitution of Asp95 does not reduce APC cofactor function by disrupting adjacent domain structure.

A current view of protein S–enhanced APC cofactor activity suggests a functional repositioning of the APC cleavage site away from FVa Arg506 toward FVa Arg306.[9] We therefore performed a FVa inactivation assay using a FVa variant, FVa R506Q/R679Q, that cannot be cleaved at position 506 and 679. This allowed us to analyze directly cleavage at Arg306 by APC, which is at the site enhanced mainly by protein S. Using both concentration-dependent and time course assays, we were able to confirm reduced cleavage of this variant by APC in the presence of protein S D95A.

The available results therefore suggest that Asp95 of protein S may play an important and a direct role in APC recognition, resulting in enhanced APC function. Our results are compatible with the study performed by Hackeng et al,[27] who used the isolated EGF1 domain of protein S to functionally disrupt the protein S and APC interaction. They suggested EGF1 as a potentially important APC contact site on protein S. Our results suggest that Asp95 constitutes a critical residue within EGF1, mediating the APC cofactor function. Together with Asp78 and Gln79, Asp95 could form the principal functional interaction site for APC.

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Authorship

Contribution: H.M.A., D.A.L., S.M.R., B.D., and J.T.B.C. designed the research, analyzed the results, and wrote the paper; and H.M.A., M.J.A., S.M.R., B.M.L., and S.T. performed the experiments.

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