Novel determinants in coagulation pathophysiology:

reappraising the role of β2-glycoprotein I, factor seven activating protease and protein S

Ilze Dienava-Verdoold

Cover design: Ilze Dienava-Verdoold & Remco Verdoold.
An Art-Nouveau impression of a thrombin generation curve.

Typeset with Adobe Indesign.
Printed by: Wöhrmann Print Service, Zutphen, the Netherlands.

ISBN: 978-94-6203-079-4
e-mail: ilzedia@gmail.com

The research described in this thesis was performed at the Department of Plasma Proteins, Sanquin Researach, Amsterdam, the Netherlands.

Novel determinants in coagulation pathophysiology:

reappraising the role of β2-glycoprotein I, factor seven activating protease and protein S

Nieuwe determinanten in pathofysiologie van bloedstolling:
herwaardering van de rol van β2-glycoproteïne I, factor VII activerend protease en proteïne S
(met een samenvatting in het Nederlands)

Jauni noteicēji asinsreces patofizioloģijā: *izvērtējot β2-glikoproteīna I, VII faktora aktivējošās proteāzes un proteīna S lomu*
(ar kopsavilkumu latviešu valodā)

PROEFSCHRIFT

ter verkrijging van
de graad van doctor aan de Universiteit Utrecht,
op gezag van de rector magnificus, prof. dr. G. J. van der Zwaan,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen
op woensdag 29 augustus 2012 des middags te 12.45 uur

door

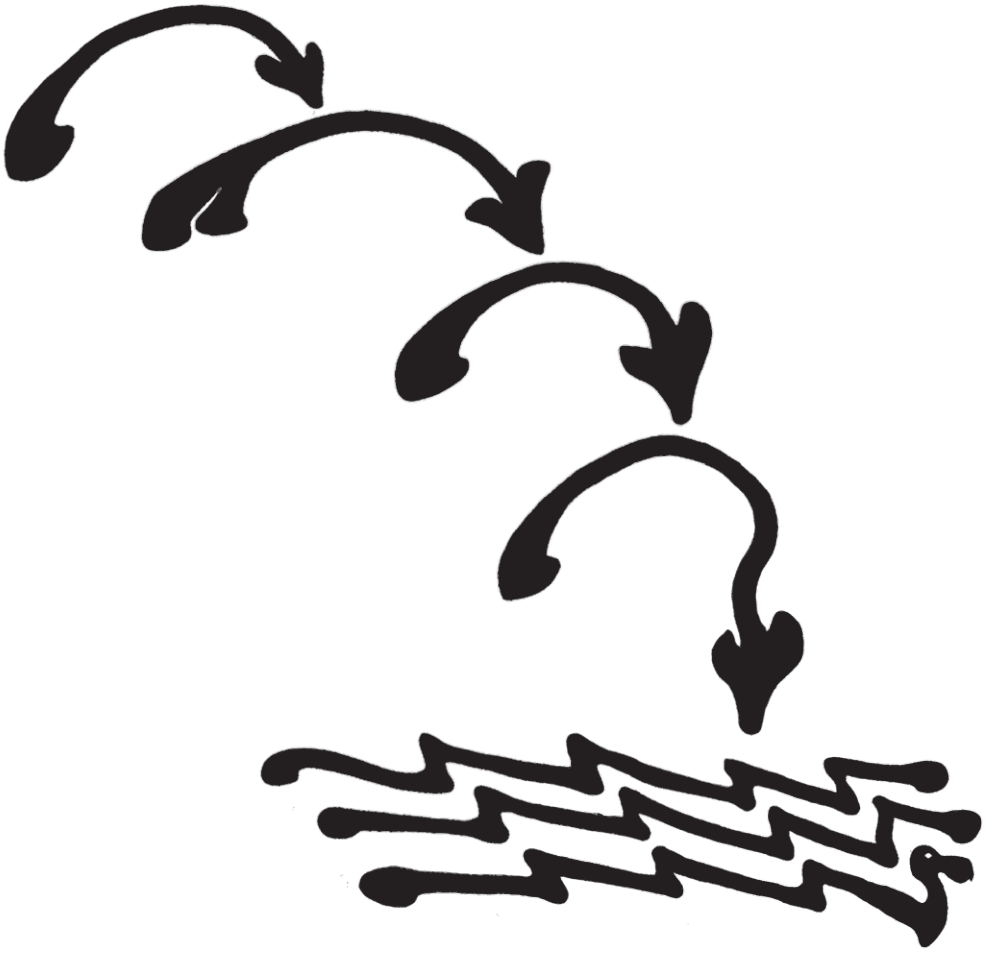
Ilze Dienava-Verdoold
geboren op 6 november 1982, te Riga, Letland

Promotor	Prof. dr. K. Mertens
Co-promotoren	dr. H. J. M. Brinkman dr. B. de Laat

Financial support by Sanquin Research for the publication of this thesis is gratefully acknowledged.

Manai ģimenei
Remco

Table of contents



Chapter 1
Introduction

The outline of this thesis

Blood coagulation is a highly important process that prevents blood loss and ensures restoration of the vascular integrity following a physical damage. It is tightly regulated; however, many pathological situations may occur that shift the haemostatic balance either towards thrombosis or bleeding. Inflammation conditions as in sepsis may lead to hypercoagulability while platelet consumption during tumour chemotherapy leads to bleeding. Autoimmune antibodies to various plasma proteins lead to either bleeding or thrombosis depending on their target antigen. In this thesis we study a selection of plasma proteins that have been implicated in (patho-) physiological situations leading to a disturbed haemostatic balance, i.e., β2-glycoprotein I, factor seven activating protease and protein S. The thrombin generation assay was instrumental in this research since it is capable of revealing activities of plasma proteins which are masked at high coagulation initiation stimuli used in clotting time based assays.

β2-glycoprotein I (β2GPI) is a protein with a yet enigmatic physiological role; however, its involvement in the antiphospholipid syndrome (APS) leading to thrombotic complications is beyond doubt. In **Chapter 2** we describe the generation and characterization of monoclonal antibodies against β2GPI which could be useful tools for future studies into the mechanisms and diagnostics of APS pathogenesis. The thrombin generation assay is used as tool to address the coagulation modulating potential of the constructed antibodies. Related to this topic, in **Chapter 3** we look deeper into the immunological mechanisms of β2GPI uptake and presentation of T-cell epitopes on dendritic cells which may trigger the autoimmune response.

Factor seven activating protease (FSAP) has been implicated in haemostasis by its ability to activate factor VII (FVII). In **Chapter 4** and **5** we use a recombinant FSAP protein with controlled activation to show that, surprisingly, FSAP is a rather poor activator of FVII in purified systems as well as in plasma. However, FSAP does have a boosting effect on thrombin generation in presence of low tissue factor concentrations. We employed the thrombin generation assay to discover that the procoagulant effect of FSAP is due to tight binding to and inhibition of one of the proteins implicated in coagulation inhibition: tissue factor pathway inhibitor (TFPI).

Even though the role of protein S in coagulation has been established as a cofactor to activated protein C and TFPI, there are several determinants that regulate protein S activity. We studied the effect of protein S cleavage on thrombin generation in **Chapter 6**. The relationship between platelet numbers in various disease states and protein S cleavage further suggests the implication of a yet unknown platelet protease in the modulation of protein S anticoagulant activity *in vivo*.

The specific questions explored in the individual chapters are summarized in "The questions addressed in this thesis" at the end of this chapter, after an overview of the processes involved in haemostasis, the description of the thrombin generation assay and of the main proteins discussed in this thesis.

General introduction on thrombin generation

Haemostatic system

The coagulation system is very ancient and has evolved before the divergence of vertebrates [1]. In mammals, including humans, the haemostatic system, an orchestrated collaboration of the vessel wall constituents, circulating blood cells and plasma proteins, is a complex system that safeguards from blood loss upon a blood vessel injury. Haemostasis ensures the integrity of the circulation by arrest of bleeding. It can be divided into two major events: primary- and secondary haemostasis. Primary haemostasis involves the adhesion of platelets to the damaged vessel wall and the subsequent formation of a platelet aggregate. Secondary haemostasis involves the formation of a fibrin clot among aggregating platelets on the damaged vessel wall. Both processes occur almost simultaneously and both are needed for optimal bleeding arrest. Upon healing of the damaged vessel wall, the blood clot is dissolved, i.e., removed in a process called fibrinolysis.

Primary haemostasis forms the first line of defence against bleeding. When activated by the nearby damage [2], endothelial cells locally release large quantities of ultra large (UL) von Willebrand factor (vWF) strings [3] from specialized vesicles called Weibel-Palade bodies [4]. These UL-vWF strings unfold under the stress of blood flow [5] and serve as an adhesive for circulating platelets, thus forming the initial platelet plug [6]. The interaction occurs between domain A1 of vWF and platelet membrane glycoprotein Ib-IX-V [7]. The captured platelets can subsequently bind the collagen exposed by the vessel wall damage [8]. This interaction gives the first activation signal to platelets which in turn promotes the release of additional activation messengers (ADP, thromboxane 2) for the neighbouring platelets (for a review see [9]). New platelets are recruited on the downstream side of the growing thrombus [10]. Cytoskeleton remodelling due to platelet activation leads also to contraction of platelets into a dense platelet plug, independent on fibrin formation and capable of withstanding elevated shear stress [11]. Activation of platelets also causes relocation of the negatively charged phospholipids, such as phosphatidylserine, from the inner to the outer platelet membrane, thus supporting the secondary haemostasis [12,13].

The secondary haemostasis is performed by coagulation factors present in blood plasma. In essence, the secondary haemostasis is a complex cascade of enzymatic events where one activated coagulation factor converts the following coagulation factor from zymogen to enzyme form. It is controlled by numerous positive and negative feedback mechanisms. The cascade form of the coagulation ensures amplification of the response at each step. Moreover, it also provides multiple targets for up- and down-regulation by enzymes and inhibitors, as well as redundancy to compensate for dysfunctional or missing components. The outcome of this cascade is the conversion of fibrinogen into a network of insoluble fibrin fibres securing the plug over the vessel wall damage.

The coagulation cascade is initiated by the exposure of tissue factor (TF) which is usually contained within the blood vessel wall (Fig. 1-2A). The exposed TF can bind and promote autoactivation of factor VII (FVII), thus forming the initiation complex of TF/FVIIa at the location of injury [14]. FVIIa with TF as its cofactor can then

activate factors IX and X (FIX and FX) [15]. Furthermore, FIXa in complex with activated factor VIII (FVIIIa) on negatively charged phospholipids can amplify the activation of FX [16]. Eventually, FXa in presence of activated factor V (FVa) on phospholipid membranes can activate factor II (FII, prothrombin) into thrombin [17]. The generated thrombin is able to cleave the terminal parts of fibrinogen; as a result the exposed ends of fibrin monomers bind to each other forming long and branched network of fibrin fibres. The formation of fibrin network is finalized by thrombin activated factor XIII (FXIIIa) which cross-links the fibrin strands and connects the loose ends [18].

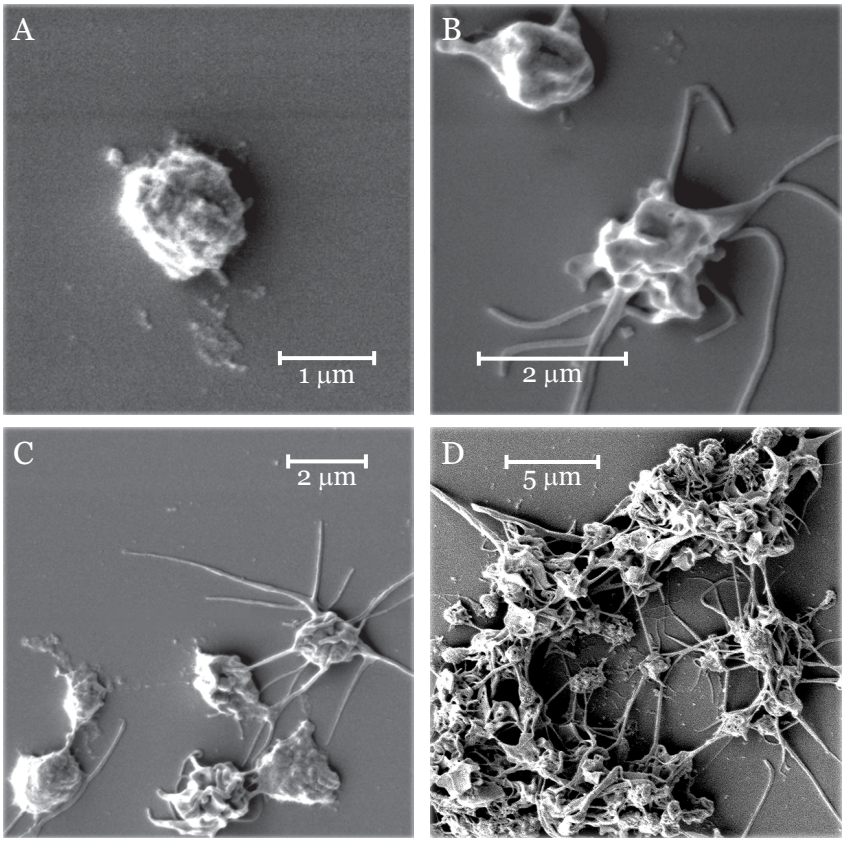


Figure 1-1 Platelet adherence and aggregation. *Isolated platelets from citrated blood were sedimented onto glass cover slips and permitted to aggregate during a 30 minute incubation step at room temperature in HEPES-buffered saline containing 10 mM K^+, 5 mM Ca^{2+} and 2 mM Mg^{2+}. The cover slips were then fixed with a mixture of paraformaldehyde and glutaraldehyde, washed with 100 mM phosphate buffer and then dehydrated by step-wise replacement of water with ethanol. Samples were then subjected to critical point drying, sputtered with gold and viewed under a Philips XL-30 ESEM (environmental scanning electron microscope). (A) A resting platelet; (B) an activated platelet adhered to the glass surface; (C) interaction between activated platelets leading to platelet aggregation (D) (I. Dienava-Verdoold, unpublished observations).*

The coagulation cascade is amplified by numerous forward and backward (feedback) activation processes (Fig. 1-2B). Thus, next to the forward activation of FVIII and FV by FXa (see review by Camire [19] and the references therein), the same coagulation cofactors can also be feedback-activated by thrombin [20]. Furthermore, thrombin can also activate Factor XI (FXI) which in turn can amplify the activation of FIX and the subsequent formation of FVIIIa/FIXa complex to generate more FXa [21]. In addition, FXa can perform feedback activation of FVII [21].

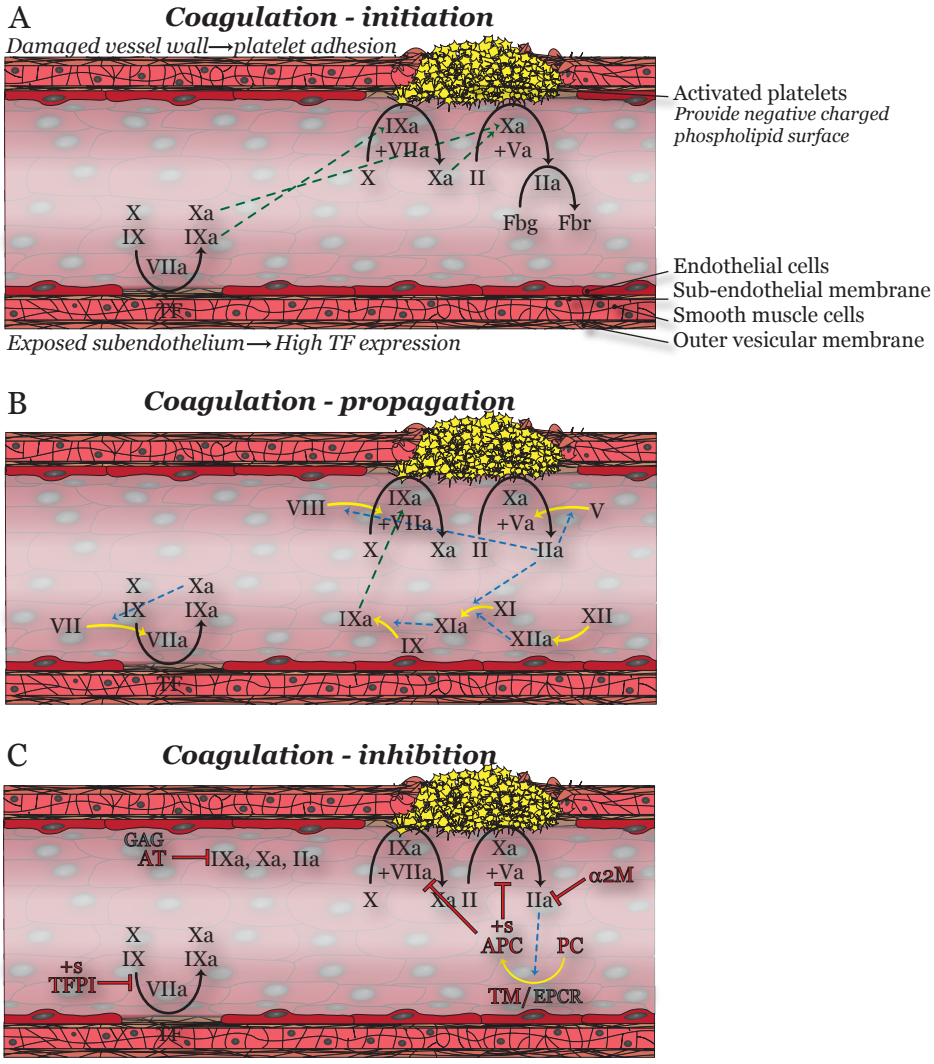


Figure 1-2 Schematic and simplified representation of reactions involved in coagulation. *(A) Coagulation initiation; (B) coagulation propagation and (C) coagulation inhibition.*

Historically the coagulation cascade was divided into two separate pathways which would then converge in a final common pathway. In the intrinsic (contact activation) pathway all the necessary components for coagulation cascade were present in blood itself, as observed by coagulation of non-anticoagulated blood in glass tubes in absence of any additives. The intrinsic pathway starts with activation of factor XII on negatively charged surfaces, and via the activation of FXI leads to activation of FIX with subsequent FXa generation. On the other hand, the extrinsic (tissue factor activated) pathway requires tissue factor as the external trigger and the TF/FVIIa complex which leads to activation of FX. The common pathway then continues with the FXa/FVa activating prothrombin into thrombin. However, now this division is considered rather artificial, although still useful in *in vitro* diagnostics to detect potential coagulation factor deficiency or dysfunction [23].

Naturally, there are also several inhibition mechanisms to provide control over the self-amplifying coagulation cascade (Fig. 1-2C). The first of them is tissue factor pathway inhibitor (TFPI), the gatekeeper of coagulation, ensuring that trace amounts of exposed TF do not trigger a full coagulation. The mechanism of inhibition by TFPI involves two steps [24]. First, TFPI binds to FXa via its second Kunitz domain. This interaction is slow in the sense that, although the initial binding is rapid, the enzyme-inhibitor complex undergoes a slow isomerisation step to a tight-binding complex [25]. Second, the FXa-TFPI complex binds to FVIIa-TF complex via the first Kunitz domain of TFPI to complete the inactive quaternary complex [24]. Protein S can serve as cofactor of TFPI [26], binding either its third Kunitz domain or carboxy-terminus and thus enhancing the affinity of TFPI for negatively charged phospholipids, e.g., on activated platelet surfaces [24]. However, once FXa is bound to FVa in the prothrombinase complex, it is protected from TFPI inhibition [27]. This underscores that TFPI only regulates the initial stages of coagulation [24].

In later stages of coagulation other inhibitory mechanisms are involved. Generated thrombin can bind to thrombomodulin (TM) exposed on the endothelial cell surface. This interaction prevents the procoagulant actions of thrombin, i.e., fibrin generation and feedback activation of FV and FVIII. TM-bound thrombin is able to activate endothelial cell protein C receptor (EPCR)-bound protein C into its activated form (APC). APC in presence of its cofactor protein S then proteolytically inactivates the coagulation cofactors FVIIIa and FVa [28]. Next to TM, also α2-macroglobulin (α2M) can absorb thrombin thus rendering it inactive [29], whereas antithrombin (AT) can bind and inactivate factors IXa, Xa and thrombin [30].

Once the fibrin plug has been formed and the damaged blood vessel wall healed, the thrombus must be lysed. Also in this case a finely tuned cascade of protease activation and inhibition ensures the generation of plasmin and its activity in proteolysis of fibrin (fibrinolysis). Plasmin normally circulates in plasma in the inactive form as plasminogen. Many proteases are capable of activating plasminogen, but the two best known are tissue type plasminogen activator (tPA) and urokinase plasminogen activator (uPA). The presence of fibrin ensures the activity of tPA, while uPA circulates in a single-chain form (scuPA) and needs to be cleaved to generate its fully active two-chain form (tcuPA) [31]. In either case the activation of plasminogen occurs directly on fibrin and ensures localized fibrinolysis. Fibrin-bound plasmin is also protected from its inhibitors, i.e., α2-antiplasmin, C1-inhibitor, α2M and AT. Eventually, the

activity of plasmin can be stopped by thrombin activatable fibrinolysis inhibitor (TAFI). Leukocytes ensure the final clearance of the thrombus [32].

The knowledge of the main coagulation pathways is essential as it provides a framework for addition of novel components that may be involved in these processes. Moreover, it leads to the necessity of an analysis tool that could help unravel the influence on coagulation by various other factors present in blood plasma.

The evolution of thrombography

One of the first thrombin generation tests used was developed by Biggs and Macfarlane [33]. It was rather labour-intensive since it required sub-sampling from clotting blood every 0.5 minutes and the measurement of the amount of generated thrombin in each sub-sample by clotting times in fibrinogen solution. The resulting curve appeared rather informative for various haemostatic abnormalities but obviously this procedure was not suitable for high throughput diagnostic application. Further attempts to improve the assay lead to the use of a chromogenic substrate of thrombin instead of fibrinogen and eventually measuring the thrombin generation continuously directly in the plasma [34]. However, the high affinity of the used substrate led to rapid substrate depletion and therefore a more slowly reacting substrate was required and eventually developed [35]. Furthermore, the measurement of optical density required for the chromogenic substrate cleavage was hampered by the fact that plasma becomes turbid upon fibrin clot formation. To eliminate this problem, Hemker and Béguin developed a method for continuous automated monitoring of fluorescent substrate Z-Gly-Gly-Arg-AMC cleavage by thrombin [36]. Even though the measurement was not influenced by the presence of platelets or clot formation, this system still suffered from some drawbacks. More efficient substrate cleavage lead to faster substrate depletion, and increasing amounts of free AMC caused some quenching of the fluorescent signal [37]. A previously observed phenomenon became a solution to the problem: it was shown that part of the thrombin forms a complex with α2M; the thrombin in this complex is biologically inactive but still capable of cleaving small substrates [29]. Thus, a calibrator was introduced in the thrombin generation assay, a fixed amount of thrombin-α2M complex. Each plasma sample could now be compared to its own calibrator measurement in time; this way it was possible to correct for the changes in the substrate cleavage rates caused by substrate depletion and the fluorescent signal quenching caused by the product (AMC) or plasma itself. The resulting assay is called Calibrated Automated Thrombography (CAT) [37].

Application of thrombography

Due to the fact that the CAT measurement enables high throughput automated reading and immediate derivation of the thrombin generation peaks, its advantage in research, diagnosis and treatment monitoring is obvious. It is now possible to directly assess and screen the efficiency of anticoagulant drugs, to monitor coagulation and platelet disorders, and to investigate the effect of various plasma proteins on thrombin generation [38].

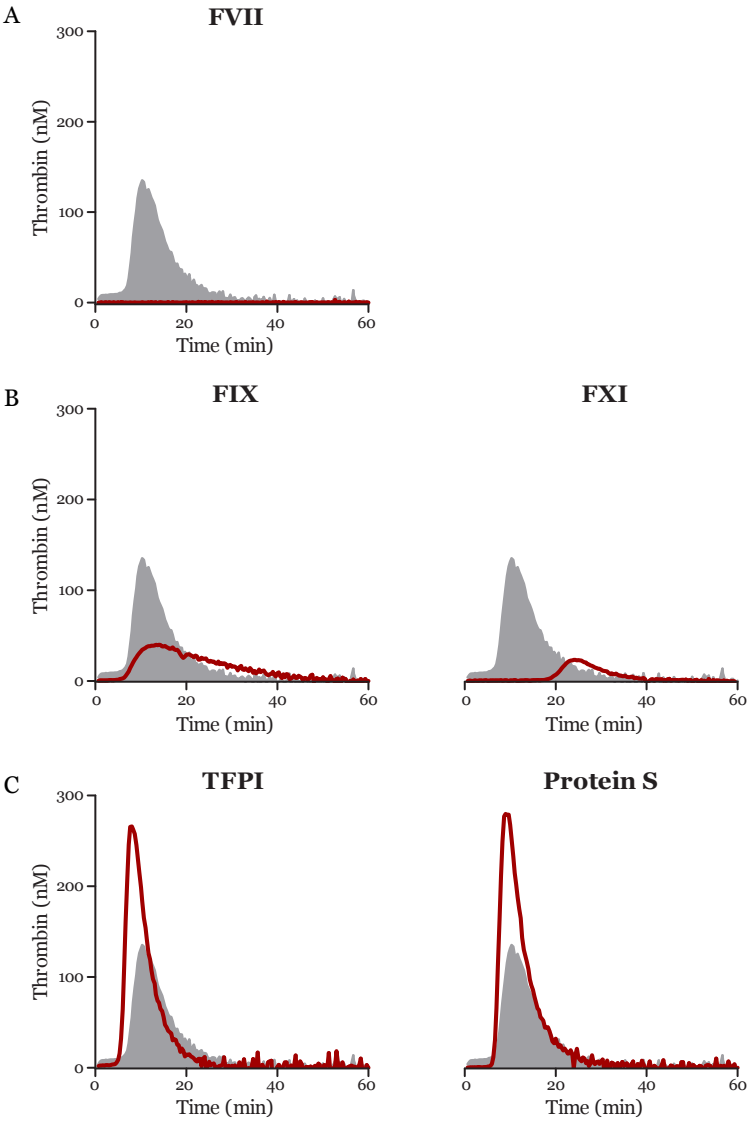


Figure 1-3 Thrombin generation profiles of various coagulation factor-deficient plasmas. *The plasmas were deficient in coagulation factors implicated in (A) the initiation phase, (B) the propagation phase and (C) in coagulation inhibition. Filled grey curve represents normal pooled plasma and the red line represents complete coagulation factor-deficient plasma. See* ***Chapter 5*** *for the experimental conditions. Thrombin generation was initiated by 1 pM TF, 4 µM PS/PC/PE and 15 mM* $CaCl_2$*. All plasmas used were microparticle-depleted. Coagulation factor-deficient plasmas were either prepared by immune depletion of normal pooled plasma (TFPI) or purchased from Hyphen Biomed (Protein S), Siemens (FIX), Haematologic Technologies (FVII and FXI).*

The currently most widely used clotting assays in coagulation disorder diagnostics provide us with information about only one dimension of haemostasis: the time until clotting occurs. In thrombin generation, however, that is only one of several

parameters that can be inferred from the thrombin generation curve. In addition to the lag time (clotting time), it is also possible to determine the thrombin peak height, time to peak, the slope of the peak and the endogenous thrombin potential (ETP). The peak height indicates the maximal intensity of thrombin generation. The time to peak compared to the lag time, sometimes also expressed as the slope of the peak, shows the maximal rate of thrombin generation. And finally the ETP, as the area under the curve, shows the total amount of generated thrombin [38]. The parameters of thrombin generation do not necessarily depend on each other. Procoagulant phenotype typically would be expressed as shortened lag time, increased peak and ETP [38]. However, in case of an antiphospholipid syndrome (APS), an autoimmune disease characterised by prothrombotic phenotype, a paradoxical increase in the clotting time is usually observed in the *in vitro* diagnostic assays. This is called lupus anticoagulant (LAC) activity. Analysis of thrombin generation curve in this syndrome shows that the lag time is indeed prolonged but peak height and ETP remain normal or increased [39]. Therefore, the more reliable indicators of hyper- or hypocoagulability are the peak height and ETP. In congenital deficiencies of coagulation factors (II, V, VII, VIII, IX, X and XI) the thrombin generation curve is either completely absent or characterized by markedly prolonged lag time and decreased peak height and ETP ([40] and Fig. 1-3 A and B). Moreover, the risk of bleeding is increased if ETP drops below 20% of the normal values [41]. Conversely, in plasmas deficient in coagulation inhibitors, such as TFPI and protein S, the lag time is very short and the peak height is greatly increased ([26] and Fig. 1-3C).

β2-glycoprotein I and antiphospholipid syndrome

Antiphospholipid syndrome (APS) is an autoimmune disease characterised by increased risk of thrombosis [42]. The main antigen for antiphospholipid (aPL) autoantibodies is β2-glycoprotein I (β2GPI), a highly abundant and conserved protein without a clear and well-defined function in haemostasis [43].

β2GPI is an anionic phospholipid binding protein synthesized in hepatocytes and circulates in plasma at high levels of 50–500 μg mL^{-1} [44]. It is a highly glycosylated protein and the molecular weight may vary due to the length of the sugar chains but generally it is around 50 kDa. β2GPI contains four N-glycosylation sites (Asn143, Asn164, Asn174 and Asn234) and one O-glycosylation site (Thr130); three of the carbohydrate chains are located on the inner curve and two – on the outer curve of the molecule [45]. The official gene name of β2GPI is Apolipoprotein H; however, this is a misnomer based on observations which could not be reproduced. β2GPI appeared not able to associate or to form complexes with various lipoproteins, an observation independent on health, presence of aPL antibodies or sepsis [46].

There have been several studies on the structure of β2GPI employing various methods. In 1999 two groups [45,47] independently resolved the crystal structure of β2GPI and both found five domains arranged in a fish-hook or J-shape (Fig. 1-4A). β2GPI belongs to a super-family of proteins containing repeating amino acid stretches termed complement control proteins, short consensus repeats or Sushi domains [45,47]. In the case of β2GPI, there are four regularly folded Sushi domains

and one aberrant Sushi domain connected with short interlinker sequences. The fifth domain contains an additional large positively charged patch for binding to negatively charged phospholipids and a hydrophobic loop for insertion into a phospholipid layer [45,47]. The study reported in 2002 on the solution structure of β2GPI by small-angle X-ray scattering [48] revealed an extra fold between domains II and III, thus giving the overall molecule an S-shape (Fig. 1-4B). In this model one of the carbohydrate chains also appeared to be shielding domain I; it gave the first idea about conformation changes necessary for antibody binding in the case of APS [49]. In 2010, electron microscopy on a dried β2GPI sample confirmed the existence of the fish-hook shape, albeit only after drastic treatment with high salt and extreme pH or after binding to aPL antibodies. Moreover, the predominant conformation of β2GPI in blood was a hitherto unknown conformation, a "closed" circular arrangement of the Sushi domains where domain V shields the aPL antibody epitope on domain I (Fig. 1-4C) [50]. However, the electron microscopy results with the dried β2GPI sample do not exclude the existence of the S-shape in solution. Taken together these results suggest that β2GPI is flexible and that the function and activity of β2GPI may be orchestrated by its conformation [43].

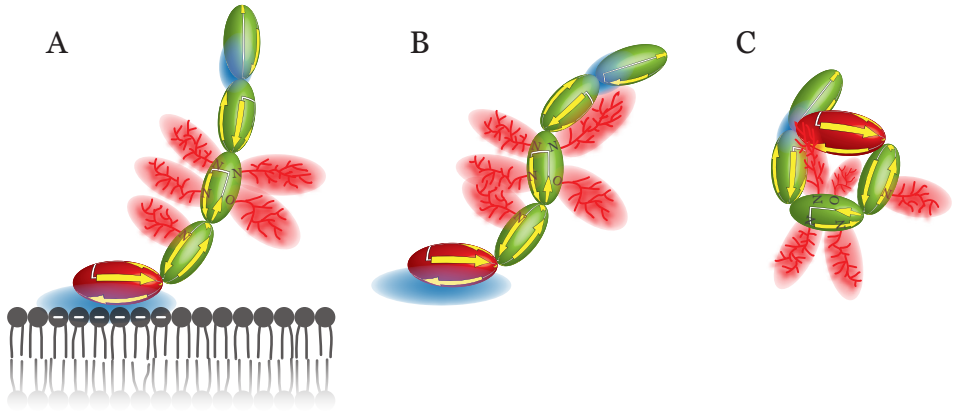


Figure 1-4 Comparison of β2GPI conformations. *The domains are numbered from top to bottom; domain V is depicted in red. The red branched structures represent carbohydrate chains and the positively charged regions in domain I and V are shown in blue. (A) The fish-hook conformation observed in crystallized protein [45,47]. Domain V interacts with the negatively charged phospholipid layer. (B) S-shape observed in solution structure by X-ray scattering [48]; one of the carbohydrate chains appears to shield the positively charged region in domain I. (C) The closed conformation of β2GPI [50]. Domain V interacts with and shields domain I in this conformation.*

It is interesting to note that β2GPI is highly conserved among species. A comparison of β2GPI amino acid sequences between 42 animal species, from a variety of mammals to nematodes, showed that β2GPI has about 80–99% homology among mammals, 16–60% homology among other vertebrates and a modest 14–17% homology with invertebrates [51]. However, certain epitopes were found to be even better conserved than the rest of the molecule. Those epitopes included the antibody binding site (Arg39–Arg43), the phospholipid binding loop (Cys281–Cys288) and a novel peptide sequence binding to lipopolysaccharides (LPS) (Leu313–Ala320) [51].

β2GPI: an abundant plasma protein with enigmatic function?

It is intriguing that a clearly defined function has not yet been attributed to β2GPI despite the fact that β2GPI is highly conserved and present in plasma in relatively high concentration. Moreover, clinical deficiency can occur without obvious health problems [43]. On the other hand, the reduced birth ratio of homozygous β2GPI-negative offspring and impaired thrombin generation in mice [52], clearly suggests a physiological role for β2GPI. However, *in vitro* studies on a role of β2GPI in haemostasis yielded contradictory results. Both procoagulant and anticoagulant effects as well as fibrinolytic properties were attributed to β2GPI (reviewed in [53]). Contradictory follow-up studies or lack thereof, however, cast doubt on the actual physiological relevance of those observations. On the other hand, more recent observations suggest an interaction between β2GPI and von Willebrand factor that inhibits platelet GPIb binding to the A1 domain of vWF and thus the platelet adhesion to UL-vWF strings [54]. Yet unpublished data suggest that this effect might have a protective function in, e.g., thrombotic thrombocytopenia purpura where hyperreactive von Willebrand factor is present (V. X. Du, G. van Os, J. A. Kremer Hovinga, I. Dienava-Verdoold, J. Wollersheim, Z. M. Ruggeri, R. Fijnheer, P. G. de Groot, and B. de Laat, unpublished data).

It has long been suspected that there is a connection between APS and infection. The 'two-hit' hypothesis of APS predicts that an infection may trigger the onset of thrombosis in the presence of aPL antibodies [55]. Alternatively, there may be a more direct interactions occurring, e.g., direct involvement of β2GPI in sepsis. Upon infection, the first defence against pathogens is conferred by the innate or non-specific immune response. Peptides derived from various proteins by neutrophil proteases display antibacterial activities [56]. Interestingly, peptides derived from domain V of neutrophil protease-processed β2GPI also show antibacterial properties. These domain V peptides contain a cluster of positively charged and hydrophobic amino acids, a feature that is common among the antibacterial peptides [57]. Further studies examined the interaction of β2GPI with lipopolysaccharides (LPS), a bacterial membrane constituent activating the host immune system [58]. These experiments showed a consumption and protective action of β2GPI during sepsis as well as in healthy volunteers after an LPS challenge [58]. On the molecular level, LPS caused opening of the globular and closed β2GPI molecule (Fig. 1-4 A and C) which in turn led to binding and internalization of LPS-β2GPI complexes by monocytes in LDL-receptor family dependent manner. As a consequence, in case of sepsis, β2GPI can act as a scavenger of LPS, thus neutralizing its effects [58].

In addition to LPS, other interactions also have been proposed to give physiological relevance to β2GPI. There are several studies indicating that β2GPI might bind to and facilitate clearance of apoptotic cells, either by binding directly to the negatively charged phosphatidylserine [59,60] or by binding to specific receptors on apoptotic cells [61]. Similarly, β2GPI can be involved in clearance of platelet-derived microparticles [62] or directly inhibit phospholipid membrane microvesiculation [62]. Both of these processes could be reverted by aPL antibodies [61,62]. A related function of β2GPI might be binding to and a subsequent promotion of clearance of oxidized LDL-particles (oxLDL) [64]; however, currently β2GPI–oxLDL complexes are only seen as markers and for APS-related atherothrombosis [64].

While the physiological role of β2GPI is still under investigation, there is no doubt about its role in the pathophysiology of APS. The wealth of data about the effects of β2GPI–antibody complexes have caused scientists to consider the aPL antibodies as gain-of-function antibodies, i.e., aPL antibodies induce new functions in an otherwise non-functional protein [43]. In patients with APS, β2GPI–antibody complexes can exert a wide range of pathophysiological responses. Binding to phospholipids can cause activation of endothelial cells, monocytes and platelets and, therefore, may lead to a procoagulant and proinflammatory phenotype [65]. In absence of cells, i.e., in an *in vitro* situation, the presence of aPL antibodies can induce a prolongation of clotting time, known as lupus anticoagulant (LAC) activity. This phenomenon has been explained by competition of aPL antibody-bound β2GPI with coagulation factors for binding to phospholipids [66]. The increase in the normally low affinity of β2GPI for phospholipids was attributed to the bivalency of β2GPI caused by antibody binding [67]. This hypothesis has been confirmed in experiments where a constructed dimeric β2GPI was able to mimic the effect of β2GPI–antibody complex in terms of affinity for phospholipids and LAC activity [68].

Due to the fact that aPL antibodies are present as a heterogeneous population in patients with APS, it remains notoriously hard to investigate their effects leading to the procoagulant phenotype. Therefore we tried to address this issue with producing a set of monoclonal anti- β2GPI antibodies derived from APS patients to provide tools for further APS pathogenesis research. Also little is known about the immunological mechanisms that cause the autoimmune response to β2GPI which we tried to solve by tracking the β2GPI in the antigen presenting cells.

Factor seven activating protease

Another protein with an ambiguous function in physiology is factor VII activating protease (FSAP), also known as hyaluronic acid binding protein 2 (HABP2) or plasma hyaluronan binding protein (PHBP). It was first reported in 1996 that a novel plasma serine protease could be isolated upon binding to hyaluronan [69]. These results showed that FSAP was a 560 amino acid long protease with a 23 amino acid signal peptide and that FSAP was predominantly synthesized in the liver [69]. The amino acid sequence of FSAP was found to be highly homologous to a hepatocyte growth factor activator (HGFA) [69] as well as to some other nonvitamin-K-dependent plasma proteins, i.e., FXII, tPA and uPA [70].

FSAP belongs to the S1 family of serine proteases. The amino acid sequence of FSAP suggests that it consists of three short epidermal growth factor (EGF) domains, a kringle domain and a serine protease domain (Fig. 1-5A) [69]. It is a common feature among serine proteases that the proteolytic domain is combined with additional N-terminal domains, such as apple, EGF-like, kringle, fibronectin, or sushi domains [71]. These domains ensure the zymogen activation mechanism by liberating a new N-terminus and usually remain attached by disulfide bonds [71]. Kringle and EGF-like domains participate in (pro-)enzyme–substrate or enzyme–cofactor binding; moreover, EGF-like domains may also bind Ca^{2+} or simply act as spacers between the protease domain and Gla domains if applicable [72,73].

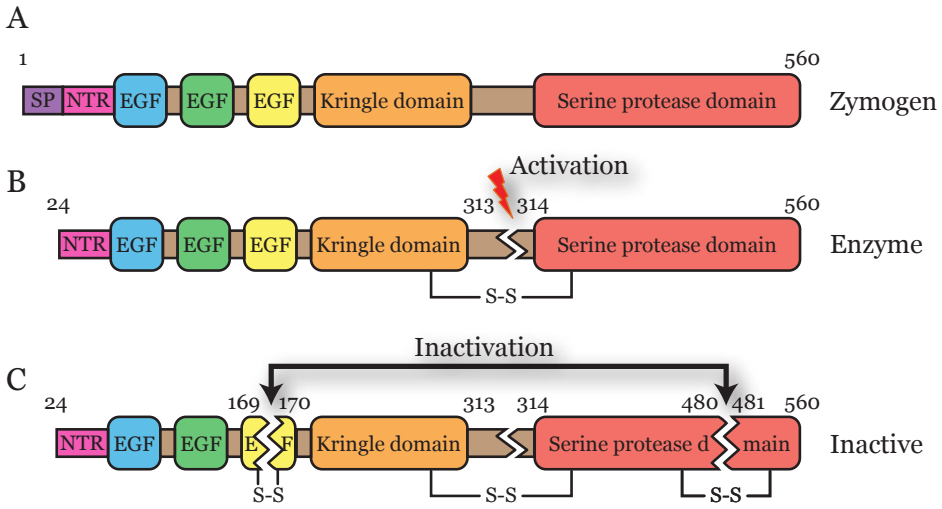


Figure 1-5 FSAP structure. *(A) Single-chain zymogen (pro-enzyme) form. (B) Cleavage after residue Arg313 creates the active two-chain (enzyme) form. (C) Further cleavage leads to inactivation.*

In FSAP, as in other serine proteases, the structure of the conserved catalytic domain can be compared to that of chymotrypsin. The catalytic serine protease domain is built of two six-stranded β-barrels and contains the conserved catalytic triad His362, Asp411 and Ser509 [71,74]. Although there are variations among the individual S1 serine proteases, they all contain eight surface loops that contribute to the substrate binding pocket [75].

Six separate loops are involved in the construction of the catalytic groove; loop Gln332–His345 [c30] (chymotrypsin numbering), loop Thr364–Arg369 [c60], loop Asp377–Glu387 [c70], loop Tyr398–Asn410 [c90], loop Val454–Leu465 [c140], and loop Tyr483–Ser490 [c170]. The remaining two loops indicated as c180 (Leu497–Asp503) and c220 (Gly534–Pro537) are located at the bottom of the protease, away from the catalytic core [71,74]. Based on the homology with other serine proteases these last two loops might be involved in stabilizing a Na^+ ion [71].

A Marburg I (MI) polymorphism, or rather a mutation, in the Na^+ binding loop (c220) G534E reduces the activity of the protein [76,77]. In many serine proteases Na^+ is important for stabilizing the Asp of the catalytic triad, therefore the Na^+ binding loop is one of the determinants for correct folding of the S1 specific binding pocket [78].

FSAP activation

Cleavage between the amino acids Arg313–Ile314 ensures transformation of single-chain FSAP into an active two-chain (tcFSAP) form of one 45 kDa fragment and one 25 kDa fragment linked by a disulfide bond [69]. The 25 kDa light chain contains the serine protease domain while the N-terminal region (NTR), three EGF domains and the kringle domain comprise the 45 kDa heavy chain (Fig. 1-5B) [69]. Further cleavage after Arg170, Arg171 and Arg480 inactivates FSAP (Fig. 1-5C) [79].

In the first studies only the two-chain form of FSAP could be isolated [69]. Further investigation revealed rapid autoactivation of single chain FSAP during purification [80]. Presence of Ca^{2+} ions or pH < 5.5 could stabilize the isolated pro-enzyme leading to slower conversion to tcFSAP and protection from degradation [80]. On the other hand, polyanions such as heparin, synthetic polyphosphate 65 or RNA [81,82] as well as positively charged polyamines like spermidine can support FSAP autoactivation complex assembly [83]. In summary, the view of FSAP autoactivation being supported by polyanions and polyamines resembles conditions observed during inflammation. Cell-derived RNA and polyamines can be released upon vascular cell damage and apoptosis [81,83]. However, RNA and polyamines fail to promote FSAP autoactivation in a plasma environment [84]. Instead, histones, released from dying cells or as part of neutrophil extracellular traps (NETs) support FSAP autoactivation *in vivo* [84]. Thus, pathophysiological situations where FSAP autoactivation could occur may include sepsis, myocardial infarction associated necrosis, acute respiratory distress syndrome as well as malignancies [81,83,85].

Functions: from coagulation and fibrinolysis to a wide range of vascular activities

In 1999, soon after its discovery the group of Romisch reported two functions for FSAP: the ability to activate factor VII [86] and the ability to cleave and activate single-chain urokinase type plasminogen activator (scuPA) [87].

Further studies expanded our knowledge on the functional range of FSAP. As opposed to FSAP as a protease involved in coagulation and fibrinolytic cascades, effects on cellular level have been proposed. Consistent with the postulated activation of FSAP in inflammatory conditions, activated FSAP may promote vascular leakiness during acute lung injury [88]. Moreover, a two-way interaction was observed on apoptotic cells: FSAP activation by apoptotic cells and the subsequent FSAP-induced release of nucleosomes from late apoptotic cells [89,90]. Two distinct signalling pathways provoked by FSAP have been discovered that lead to cell activation: 1) activation of the bradykinin B2 receptor by FSAP-cleaved high molecular weight kininogen, and 2) activation by FSAP-cleaved basic fibroblast growth factor (bFGF) [91]. This translated to stimulation of proliferation and migration of fibroblasts but not endothelial cells (human umbilical cord endothelial cells, HUVEC) [85]. Furthermore, complex formation of FSAP with various growth factors and their subsequent proteolytic inactivation could be a mechanism by which properties of vascular cells are regulated [85]. Inhibition of platelet-derived growth factor BB (PDGF-BB) was found to inhibit neointima formation in atherosclerosis while the proteolytically inactive MI FSAP mutant was not able to do so [92]. Hampered PDGF-BB cleavage by MI FSAP could be the underlying mechanism explaining recognition of MI FSAP as a risk factor in cardiovascular disease [93]. Although FSAP is found in unstable coronary atherosclerotic plaques [94], its role either as protector or enabler of the plaque rupture remains to be established [95].

As can be noted from this review, since its discovery FSAP has gained in increasing wealth of functions but at the same time losing its specificity. Moreover, the physiological relevance of many of the aforementioned findings remains to be

established. Of particular interest in our research is the proposed procoagulant activity of FSAP. In the studies described in this thesis we investigated whether FSAP is capable of activating FVII and whether FSAP is relevant as a procoagulant factor.

Protein S

Unlike the previous two reviewed proteins, the role of protein S in physiology seems clear. It is a protein that plays an important part in anticoagulation processes. Protein S belongs to vitamin K-dependent coagulation factors. However, lacking the protease domain, it serves as non-enzymatic cofactor for coagulation inhibitors, such as activated protein C (APC) and tissue factor pathway inhibitor (TFPI). Protein S is a 635 amino acid long protein with the apparent mass of around 70 kDa [96].

Protein S (Fig. 1-6) contains a γ-carboxyglutamic acid rich (Gla) domain, a loop comprising the thrombin sensitive region (TSR), four EGF-like domains [96], but instead of a serine protease domain it contains a sex (or steroid) hormone binding globulin (SHBG)-like domain [97]. Each of the domains of protein S has a well defined role in protein S activity. The Gla domain ensures binding to phospholipid membranes and takes part in the APC-cofactor activity [98,99]. Both interactions occupy two different interfaces of the Gla domain [98]. As the name suggests, the loop comprising the thrombin sensitive region (TSR) is susceptible to cleavage by thrombin. Other proteases like FXa and elastase may cleave the TSR as well [100,101]. The TSR loop plays a role in binding phospholipids and APC cofactor activity by constraining the conformation of the Gla domain [102,103]. The TSR is followed by four EGF-like domains. As mentioned before, EGF-like domains usually serve as spacers and are important for Ca^{2+} binding [72]. It has been found that protein S contains several Ca^{2+} binding sites and three of them can be found in the EGF-like modules where they are implicated in proper protein S folding [104]. Among the four EGF-like domains, EGF1 and to a lesser extent also EGF2 are involved in binding to APC and hence in displaying APC-cofactor activity [105-107]. The carboxy-terminal SHBG-like domain also is important for the APC-cofactor activity as it may bind FV to promote the degradation of FVIIIa by APC [108]. SHBG domain also binds C4b-binding protein (C4b-BP) [109] leading to partial loss of protein S APC-cofactor function [110]. Isolated SHBG-like domain is able to fold properly and to bind C4b-BP, but is only able to support the APC cofactor function as part of the intact protein S molecule [109]. The SHBG-like domain contains conformation sensitive binding sites for bivalent metal ions [104,111]. Thus, it is possible that the SHBG-like domain also takes part in constraining the protein S molecule in the active conformation [109].

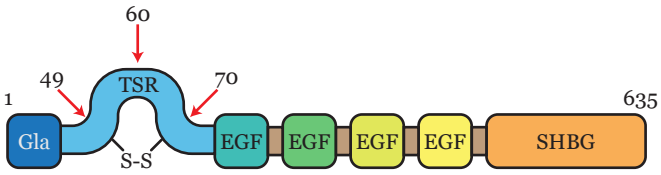


Figure 1-6 Protein S structure. *Arrows indicate cleavage sites for thrombin (Arg49, Arg70), FXa (Arg60) and elastase (Val73).*

How is protein S implicated in anticoagulation?

Protein S is a known determinant in anticoagulation processes. Individuals with protein S deficiency have a higher occurrence of thrombosis [112]. A similar prothrombotic phenotype was observed in heterozygous protein S deficient mice; however, homozygous deficiency was "incompatible with life" [113]. First it was found that protein S acts as a non-enzymatic cofactor to APC [114]. However, protein S displayed also APC-independent anticoagulant activity, recently attributed to enhancement of TFPI inhibition [26].

The anticoagulant protein C pathway belongs to the negative feedback control mechanisms of the coagulation cascade. When thrombin binds to thrombomodulin, the substrate specificity of thrombin is modulated towards protein C [115]. The activated protein C (APC) then quenches the coagulation cascade by cleaving and inactivating cofactors FVa and FVIIIa [28]. APC cleaves FVa at Arg306, Arg506 and Arg679 [116]) and FVIIIa at residues Arg336 and Arg562 [117]. Protein S is a cofactor to these proteolytic events. The Gla domain ensures anchoring of APC/protein S complex on phospholipid membranes [99]. Moreover, some studies suggest that protein S causes a conformation change in APC, thus bringing the active site of APC above phospholipid membranes [118,119]. In case of FVa inactivation, protein S potentiates APC on cleavage after Arg306 only [120]. At the same time, binding of FV to the SHBG domain of protein S is necessary to improve the APC-cofactor activity for inactivation of FVIII [121]. However, as mentioned before, C4b-BP is shown to interact with protein S and modulate its APC-cofactor activity. Earlier it was believed that binding of C4b-BP completely abolished the APC-cofactor activity [114] and only C4b-BP unbound (free) protein S was considered as the active form of protein S [122]. However, later it became apparent that binding to C4b-BP inhibited only part of protein S APC-cofactor activity in terms of FVa inactivation and FV-dependent FVIIIa inactivation, while retaining its cofactor activity to APC in factor VIIIa inactivation [110,123]. It is possible, that FV and C4b-BP compete for binding to the SHBG domain, thus explaining the partial loss of APC-cofactor activity observed with C4b-BP bound protein S [108-110].

Next to the APC-cofactor activity, protein S displays also APC-independent activity. There have been many attempts to characterize the APC-independent activity, linking it to the presence of protein S multimers [124], determining the effect of cleavage in the TSR loop [100] or assaying it at low procoagulant stimuli in thrombin generation [125]. However, the mechanism of APC-independent protein S activity was revealed only with the discovery that protein S could act as cofactor to TFPI inhibition [26]. In addition to the APC- and TFPI-cofactor activity, protein S also has direct anticoagulant activity by virtue of its inhibition of the assembly and functioning of FVIIIa/FIXa and FVa/FXa complexes [126–128]. Some investigators regard this direct anticoagulant activity of protein S as an *in vitro* artifact [129], a view disputed by others [130]. Further research into the TFPI cofactor activity has indicated that protein S selectively acts as cofactor only for FXa inhibition but not for the FVIIa/TF complex inhibition [131]. TFPI consists of three Kunitz type domains and a C-terminal tail. Kunitz-2 domain is required for interaction with FXa, Kunitz-1 – for FVIIa/TF [24] and Kunitz-3 domain is necessary to bind protein S [132]. TFPI is an inhibitor that acts at the onset of coagulation. TFPI inhibits FXa only in its

free form, before FXa is incorporated into the prothrombinase complex [24]. Once the coagulation cascade proceeds, the APC inhibitory pathway gains importance [133]. Similarly, in the thrombin generation assay the TFPI activity and the protein S cofactor activity for TFPI is manifested particularly at low TF concentrations, i.e., low initiation stimulus [133]. Eventually, the high affinity and Ca^{2+} dependent interaction suggested that C4b-BP bound protein S may have distinct functions which may also be unrelated to coagulation. With the Gla domain bound to phospholipids [134] and SHBG domain bound to C4b-BP [109], protein S might be able to localize C4b-BP to negatively charged phospholipids [135]. Phosphatidylserine exposed on apoptotic cells thus could recruit C4b via protein S-bound C4b-BP to ensure local regulation of complement activation on apoptotic cell surface [136].

In this thesis we will focus on the proteolytic modification of the anticoagulant activity of protein S. While limited proteolysis by targeted TSR cleavage has been recognised *in vitro* [100,137], the physiological significance of this event remains to be understood. Moreover, the actual source of the protease that might cleave protein S *in vivo* has remained unidentified [137].

The questions addressed in this thesis

In the current thesis we addressed the plasma proteins β2GPI, FSAP and protein S that may affect coagulation during inflammation, autoimmune disease or malignancy. Furthermore, we explored the value of thrombin generation assay in phenotyping the effects of these proteins that due to their multiple interactions with the coagulation system have proven hard to define by conventional coagulation assays. The general research questions have been summarized under "The outline of the thesis". The specific questions that were addressed in the individual chapters include:

Chapter 2: Is it possible to construct patient-derived monoclonal antibodies towards β2GPI that would represent part of the heterogeneous population of antibodies in patients with APS? What can we learn about the autoimmune antibodies in regard of their affinity and potency?

Chapter 3: Are dendritic cells able to present β2GPI peptides to T-cells on their outer surface, and if so, which β2GPI epitopes are presented?

Chapter 4: Is FSAP able to activate its proposed substrates: single-chain uPA and FVII?

Chapter 5: Does FSAP affect thrombin generation, and if so, what is the underlying mechanism by which FSAP modulates thrombin generation?

Chapter 6: Does *in vivo* protein S cleavage correlate with APC-dependent and APC-independent protein S anticoagulant activities as measured with the thrombin generation assay? Is it possible to correlate *in vivo* protein S cleavage with the putative protein S cleaving protease in platelets?

Chapter 7: The general discussion addresses the questions as to what extent do our results expand current knowledge on thrombin generation and the pathophysiology of coagulation disorders?

References

1. Davidson CJ, Tuddenham EG, McVey JH. 450 million years of hemostasis. *J Thromb Haemost*. 2003; **1**: 1487-94.
2. Wagner DD, Frenette PS. The vessel wall and its interactions. *Blood*. 2008; **111**: 5271-81.
3. Dong JF, Moake JL, Nolasco L, Bernardo A, Arceneaux W, Shrimpton CN, Schade AJ, McIntire LV, Fujikawa K, López JA. ADAMTS-13 rapidly cleaves newly secreted ultralarge von Willebrand factor multimers on the endothelial surface under flowing conditions. *Blood*. 2002; **100**: 4033-9.
4. Valentijn KM, Sadler JE, Valentijn JA, Voorberg J, Eikenboom J. Functional architecture of Weibel-Palade bodies. *Blood*. 2011; **117**: 5033-43.
5. Schneider SW, Nuschele S, Wixforth A, Gorzelanny C, Alexander-Katz A, Netz RR, Schneider MF. Shear-induced unfolding triggers adhesion of von Willebrand factor fibers. *Proc Natl Acad Sci U S A*. 2007; **104**: 7899-903.
6. Fredrickson BJ, Dong JF, McIntire LV, López JA. Shear-dependent rolling on von Willebrand factor of mammalian cells expressing the platelet glycoprotein Ib-IX-V complex. *Blood*. 1998; **92**: 3684-93.
7. De Luca M, Facey DA, Favaloro EJ, Hertzberg MS, Whisstock JC, McNally T, Andrews RK, Berndt MC. Structure and function of the von Willebrand factor A1 domain: analysis with monoclonal antibodies reveals distinct binding sites involved in recognition of the platelet membrane glycoprotein Ib-IX-V complex and ristocetin-dependent activation. *Blood*. 2000; **95**: 164-72.
8. Nuyttens BP, Thijs T, Deckmyn H, Broos K. Platelet adhesion to collagen. *Thromb Res*. 2011; **127**: S26-S9.
9. Rivera J, Lozano ML, Navarro-Núñez L, Vicente V. Platelet receptors and signaling in the dynamics of thrombus formation. *Haematologica*. 2009; **94**: 700-11.
10. Nesbitt WS, Westein E, Tovar-Lopez FJ, Tolouei E, Mitchell A, Fu J, Carberry J, Fouras A, Jackson SP. A shear gradient-dependent platelet aggregation mechanism drives thrombus formation. *Nat Med*. 2009; **15**: 665-73.
11. Ono A, Westein E, Hsiao S, Nesbitt WS, Hamilton JR, Schoenwaelder SM, Jackson SP. Identification of a fibrin-independent platelet contractile mechanism regulating primary hemostasis and thrombus growth. *Blood*. 2008; **112**: 90-9.
12. Williamson P, Bevers EM, Smeets EF, Comfurius P, Schlegel RA, Zwaal RFA. Continuous analysis of the mechanism of activated transbilayer lipid movement in platelets. *Biochemistry*. 1995; **34**: 10448-55.
13. Bevers EM, Comfurius P, van Rijn JLML, Hemker HC. Generation of prothrombin-converting activity and the exposure of phosphatidylserine at the outer surface of platelets. *Eur J Biochem*. 1982; **122**: 429-36.
14. Banner DW, D'Arcy A, Chene C, Winkler FK, Guha A, Konigsberg WH, Nemerson Y, Kirchhofer D. The crystal structure of the complex of blood coagulation factor VIIa with soluble tissue factor. *Nature*. 1996; **380**: 41-6.
15. Komiyama Y, Pedersen AH, Kisiel W. Proteolytic activation of human factors IX and X by recombinant human factor VIIa: effects of calcium, phospholipids, and tissue factor. *Biochemistry*. 1990; **29**: 9418-25.
16. Mertens K, van Wijngaarden A, Bertina RM. The role of factor VIII in the activation of human blood coagulation factor X by activated factor IX. *Thromb Haemost*. 1985; **54**: 654-60.
17. Lindhout T, Govers-Riemslag JWP, van de Waart P, Hemker HC, Rosing J. Factor Va-factor Xa interaction. Effects of phospholipid vesicles of varying composition. *Biochemistry*. 1982; **21**: 5494-502.
18. Mosesson MW. Fibrinogen and fibrin structure and functions. *J Thromb Haemost*. 2005; **3**: 1894-904.
19. Camire RM, Bos MHA. The molecular basis of factor V and VIII procofactor activation.

J Thromb Haemost. 2009; **7**: 1951-61.

20. Esmon CT, Lollar P. Involvement of thrombin anion-binding exosites 1 and 2 in the activation of factor V and factor VIII. *J Biol Chem*. 1996; **271**: 13882-7.
21. Kravtsov DV, Matafonov A, Tucker EI, Sun MF, Walsh PN, Gruber A, Gailani D. Factor XI contributes to thrombin generation in the absence of factor XII. *Blood*. 2009; **114**: 452-8.
22. Radcliffe R, Nemerson Y. Activation and control of factor VII by activated factor X and thrombin. Isolation and characterization of a single chain form of factor VII. *J Biol Chem*. 1975; **250**: 388-95.
23. Hoffman M. Remodeling the blood coagulation cascade. *J Thromb Thrombolys*. 2003; **16**: 17-20.
24. Crawley JTB, Lane DA. The haemostatic role of tissue factor pathway inhibitor. *Arterioscler Thromb Vasc Biol*. 2008; **28**: 233-42.
25. Huang ZF, Wun TC, Broze GJ. Kinetics of factor Xa inhibition by tissue factor pathway inhibitor. *J Biol Chem*. 1993; **268**: 26950-5.
26. Hackeng TM, Seré KM, Tans G, Rosing J. Protein S stimulates inhibition of the tissue factor pathway by tissue factor pathway inhibitor. *Proc Natl Acad Sci U S A*. 2006; **103**: 3106-11.
27. Mast AE, Broze GJJ. Physiological concentrations of tissue factor pathway inhibitor do not inhibit prothrombinase. *Blood*. 1996; **87**: 1845-50.
28. Dahlbäck B, Villoutreix BO. Regulation of blood coagulation by the protein C anticoagulant pathway. *Arterioscler Thromb Vasc Biol*. 2005; **25**: 1311-20.
29. Harpel PC. Studies on human plasma α2-macroglobulin-enzyme interactions. Evidence for proteolytic modification of the subunit chain structure. *J Exp Med*. 1973; **138**: 508-21.
30. Olson ST, Richard B, Izaguirre G, Schedin-Weiss S, Gettins PGW. Molecular mechanisms of antithrombin–heparin regulation of blood clotting proteinases. A paradigm for understanding proteinase regulation by serpin family protein proteinase inhibitors. *Biochimie*. 2010; **92**: 1587-96.
31. Weitz JI, Stewart RJ, Fredenburgh JC. Mechanism of action of plasminogen activators. *Thromb Haemost*. 1999; **82**: 974-82.
32. Cesarman-Maus G, Hajjar KA. Molecular mechanisms of fibrinolysis. *Br J Haematol*. 2005; **129**: 307-21.
33. Macfarlane RG, Biggs R. A thrombin generation test; the application in haemophilia and thrombocytopenia. *J Clin Pathol*. 1953; **6**: 3-8.
34. Hemker HC, Willems GM, Béguin S. A computer assisted method to obtain the prothrombin activation velocity in whole plasma independent of thrombin decay processes. *Thromb Haemost*. 1986; **56**: 9-17.
35. Rijkers DS, Wielders SJH, Tesser GI, Hemker HC. Design and synthesis of thrombin substrates with modified kinetic parameters. *Thromb Res*. 1995; **79**: 491-9.
36. Hemker HC, Giesen PL, Ramjee M, Wagenvoord R, Béguin S. The thrombogram: monitoring thrombin generation in platelet-rich plasma. *Thromb Haemost*. 2000; **83**: 589-91.
37. Hemker HC, Giesen P, Al Dieri R, Regnault V, de Smedt E, Wagenvoord R, Lecompte T, Béguin S. Calibrated automated thrombin generation measurement in clotting plasma. *Pathophysiol Haemost Thromb*. 2003; **33**: 4-15.
38. Hemker HC, Al Dieri R, De Smedt E, Béguin S. Thrombin generation, a function test of the haemostatic-thrombotic system. *Thromb Haemost*. 2006; **96**: 553-61.
39. Devreese K, Peerlinck K, Arnout J, Hoylaerts MF. Laboratory detection of the antiphospholipid syndrome via calibrated automated thrombography. *Thromb Haemost*. 2009; **101**: 185-96.
40. Duchemin J, Pan-Petesch B, Arnaud B, Blouch MT, Abgrall JF. Influence of coagulation factors and tissue factor concentration on the thrombin generation test in plasma.

Thromb Haemost. 2008; **99**: 767-73.

41. Hemker HC, Béguin S. Phenotyping the clotting system. *Thromb Haemost*. 2000; **84**: 747-51.
42. Miyakis S, Lockshin MD, Atsumi T, Branch DW, Brey RL, Cervera R, Derksen RHWM, De Groot PG, Koike T, Meroni PL, Reber G, Shoenfeld Y, Tincani A, Vlachoyiannopoulos PG, Krilis SA. International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). *J Thromb Haemost*. 2006; **4**: 295-306.
43. De Groot PG, Meijers JCM. β2-glycoprotein I: evolution, structure and function. *J Thromb Haemost*. 2011; **9**: 1275-84.
44. De Laat B, de Groot PG, Derksen RHWM, Urbanus RT, Mertens K, Rosendaal FR, Doggen CJM. Association between β2-glycoprotein I plasma levels and the risk of myocardial infarction in older men. *Blood*. 2009; **114**: 3656-61.
45. Bouma B, de Groot PG, van den Elsen JMH, Ravelli RBG, Schouten A, Simmelink MJA, Derksen RHWM, Kroon J, Gros P. Adhesion mechanism of human β2-glycoprotein I to phospholipids based on its crystal structure. *EMBO J*. 1999; **18**: 5166-74.
46. Ağar Ç, de Groot PG, Levels JHM, Marquart JA, Meijers JCM. β2-glycoprotein I is incorrectly named apolipoprotein H. *J Thromb Haemost*. 2009; **7**: 235-6.
47. Schwarzenbacher R, Zeth K, Diederichs K, Gries A, Kostner GM, Laggner P, Prassl R. Crystal structure of human β2-glycoprotein I: implications for phospholipid binding and the antiphospholipid syndrome. *EMBO J*. 1999; **18**: 6228-39.
48. Hammel M, Kriechbaum M, Gries A, Kostner GM, Laggner P, Prassl R. Solution structure of human and bovine β2-glycoprotein I revealed by small-angle X-ray scattering. *J Mol Biol*. 2002; **321**: 85-97.
49. De Laat B, Derksen RHWM, van Lummel M, Pennings MTT, de Groot PG. Pathogenic anti-β2-glycoprotein I antibodies recognize domain I of β2-glycoprotein I only after a conformational change. *Blood*. 2006; **107**: 1916-24.
50. Ağar Ç, van Os GMA, Mörgelin M, Sprenger RR, Marquart JA, Urbanus RT, Derksen RHWM, Meijers JCM, de Groot PG. β2-glycoprotein I can exist in two conformations: implications for our understanding of the antiphospholipid syndrome. *Blood*. 2010; **116**: 1336-43.
51. Ağar Ç, de Groot PG, Marquart JA, Meijers JCM. Evolutionary conservation of the lipopolysaccharide binding site of β2-glycoprotein I. *Thromb Haemost*. 2011; **106**: 1069-75.
52. Sheng Y, Reddel SW, Herzog H, Wang YX, Brighton T, France MP, Robertson SA, Krilis SA. Impaired thrombin generation in β2-glycoprotein I null mice. *J Biol Chem*. 2001; **276**: 13817-21.
53. Miyakis S, Giannakopoulos B, Krilis SA. β2-glycoprotein I - function in health and disease. *Thromb Res*. 2004; **114**: 335-46.
54. Hulstein JJJ, Lenting PJ, de Laat B, Derksen RHWM, Fijnheer R, de Groot PG. β2-glycoprotein I inhibits von Willebrand factor-dependent platelet adhesion and aggregation. *Blood*. 2007; **110**: 1483-91.
55. Shoenfeld Y, Blank M, Cervera R, Font J, Raschi E, Meroni PL. Infectious origin of the antiphospholipid syndrome. *Ann Rheum Dis*. 2006; **65**: 2-6.
56. Zasloff M. Antimicrobial peptides of multicellular organisms. *Nature*. 2002; **415**: 389-95.
57. Nilsson M, Wasylik S, Mörgelin M, Olin AI, Meijers JCM, Derksen RHWM, de Groot PG, Herwald H. The antibacterial activity of peptides derived from human β2-glycoprotein I is inhibited by protein H and M1 protein from *Streptococcus pyogenes*. *Mol Microbiol*. 2008; **67**: 482-92.
58. Ağar Ç, de Groot PG, Mörgelin M, Monk SDDC, van Os G, Levels JHM, de Laat B, Urbanus RT, Herwald H, van der Poll T, Meijers JCM. β2-glycoprotein I: a novel component of innate immunity. *Blood*. 2011; **117**: 6939-47.
59. Maiti SN, Balasubramanian K, Ramoth JA, Schroit AJ. β2-glycoprotein I-dependent

macrophage uptake of apoptotic cells: binding to lipoprotein receptor-related protein receptor family members. *J Biol Chem*. 2008; **283**: 3761-6.

60. Balasubramanian K, Schroit AJ. Characterization of phosphatidylserine-dependent β2-glycoprotein I - macrophage interactions. *J Biol Chem*. 1998; **273**: 29272-7.

61. Reed JH, Giannakopoulos B, Jackson MW, Krilis SA, Gordon TP. Ro 60 functions as a receptor for β2-glycoprotein I on apoptotic cells. *Arthritis Rheum*. 2009; **60**: 860-9.

62. Abdel-Monem H, Dasgupta SK, Le A, Prakasam A, Thiagarajan P. Phagocytosis of platelet microvesicles and β2-glycoprotein I. *Thromb Haemost*. 2010; **104**: 335-41.

63. Frank M, Sodin-Semrl S, Rozman B, Potocnik M, Kralj-Iglic V. Effects of low-molecular-weight heparin on adhesion and vesiculation of phospholipid membranes: a possible mechanism for the treatment of hypercoagulability in antiphospholipid syndrome. *Ann N Y Acad Sci*. 2009; **1173**: 874-86.

64. Matsuura E, Kobayashi K, Matsunami Y, Lopez LR. The immunology of atherothrombosis in the antiphospholipid syndrome: Antigen presentation and lipid intracellular accumulation. *Autoimmun Rev*. 2009; **8**: 500-5.

65. Pierangeli SS, Chen PP, Gonzalez EB. Antiphospholipid antibodies and the antiphospholipid syndrome: an update on treatment and pathogenic mechanisms. *Curr Opin Hematol*. 2006; **13**: 366-75.

66. Arnout J. Antiphospholipid syndrome: diagnostic aspects of lupus anticoagulants. *Thromb Haemost*. 2001; **86**: 83-91.

67. Willems GM, Janssen MP, Pelsers MMAL, Comfurius P, Galli M, Zwaal RFA, Bevers EM. Role of divalency in the high-affinity binding of anticardiolipin antibody–β2-glycoprotein I complexes to lipid membranes. *Biochemistry*. 1996; **35**: 13833-42.

68. Lutters BCH, Meijers JCM, Derksen RHWM, Arnout J, de Groot PG. Dimers of β2-glycoprotein I mimic the in vitro effects of β2-glycoprotein I–anti-β2-glycoprotein I antibody complexes. *J Biol Chem*. 2001; **276**: 3060-7.

69. Choi-Miura NH, Tobe T, Sumiya JI, Nakano Y, Sano Y, Mazda T, Tomita M. Purification and characterization of a novel hyaluronan-binding protein (PHBP) from human plasma: it has three EGF, a Kringle and a serine protease domain, similar to hepatocyte growth factor activator. *J Biochem*. 1996; **119**: 1157-65.

70. Sumiya J, Asakawa S, Tobe T, Hashimoto K, Saguchi K, Choi-Miura NH, Shimizu Y, Minoshima S, Shimizu N, Tomita M. Isolation and characterization of the plasma hyaluronan-binding protein (PHBP) gene (HABP2). *J Biochem*. 1997; **122**: 983-90.

71. Page M, Di Cera E. Serine peptidases: Classification, structure and function. *Cell Mol Life Sci*. 2008; **65**: 1220-36.

72. Stenflo J, Stenberg Y, Muranyi A. Calcium-binding EGF-like modules in coagulation proteinases: function of the calcium ion in module interactions. *Biochim Biophys Acta*. 2000; **1477**: 51-63.

73. Patthy L, Trexler M, Váli Z, Bányai L, Váradi A. Kringles: modules specialized for protein binding: Homology of the gelatin-binding region of fibronectin with the kringle structures of proteases. *FEBS Lett*. 1984; **171**: 131-6.

74. Stavenuiter F. Chapter 1 - General introduction. *Factor VII Activating Protease (FSAP): Functional implications of the "Marburg-1" polymorphism*. PhD thesis: University of Utrecht, 2010, 9-20.

75. Kolkman JA, Christophe OD, Lenting PJ, Mertens K. Surface loop 199–204 in blood coagulation factor IX Is a cofactor-dependent site involved in macromolecular substrate interaction. *J Biol Chem*. 1999; **274**: 29087-93.

76. Stavenuiter F, Meijer AB, Sellink E, Mertens K. Factor VII Activating Protease (FSAP): Functional Implications of the "Marburg-1" Polymorphism. *ASH Annual Meeting Abstracts*. 2009; **114**: 2126.

77. Römisch J, Feussner A, Nerlich C, Stöhr HA, Weimer T. The frequent Marburg I polymorphism impairs the pro-urokinase activating potency of the factor VII activating protease (FSAP). *Blood Coagul Fibrinolysis*. 2002; **13**: 433-41.

78. Underwood MC, Zhong D, Mathur A, Heyduk T, Bajaj SP. Thermodynamic linkage between the S1 site, the Na^+ site, and the Ca^{2+} site in the protease domain of human coagulation factor Xa. *J Biol Chem*. 2000; **275**: 36876-84.

79. Choi-Miura NH, Takahashi K, Yoda M, Saito K, Mazda T, Tomita M. Proteolytic activation and inactivation of the serine protease activity of plasma hyaluronan binding protein. *Biol Pharm Bull*. 2001; **24**: 448-52.

80. Kannemeier C, Feussner A, Stöhr H-A, Weisse J, Preissner KT, Römisch J. Factor VII and single-chain plasminogen activator-activating protease: activation and autoactivation of proenzyme. *Eur J Biochem*. 2001; **268**: 3789-96.

81. Nakazawa F, Kannemeier C, Shibamiya A, Song Y, Tzima E, Schubert U, Koyama T, Niepmann M, Trusheim H, Engelmann B, Preissner KT. Extracellular RNA is a natural cofactor for the (auto-)activation of Factor VII-activating protease (FSAP). *Biochem J*. 2005; **385**: 831-8.

82. Muhl L, Galuska SP, Oorni K, Hernandez-Ruiz L, Andrei-Selmer L-C, Geyer R, Preissner KT, Ruiz FA, Kovanen PT, Kanse SM. High negative charge-to-size ratio in polyphosphates and heparin regulates factor VII-activating protease. *FEBS J*. 2009; **276**: 4828-39.

83. Yamamichi S, Nishitani M, Nishimura N, Matsushita Y, Hasumi K. Polyamine-promoted autoactivation of plasma hyaluronan-binding protein. *J Thromb Haemost*. 2010; **8**: 559-66.

84. Yamamichi S, Fujiwara Y, Kikuchi T, Nishitani M, Matsushita Y, Hasumi K. Extracellular histone induces plasma hyaluronan-binding protein (factor VII activating protease) activation in vivo. *Biochem Biophys Res Commun*. 2011; **409**: 483-8.

85. Etscheid M, Kreß JA, Seitz R, Dodt J. The hyaluronic acid-binding protease: A novel vascular and inflammatory mediator? *Int Immunopharmacol*. 2008; **8**: 166-70.

86. Römisch J, Feussner A, Vermöhlen S, Stöhr HA. A protease isololated from human plasma activating Factor VII independent of tissue factor. *Blood Coagul Fibrinolysis*. 1999; **10**: 471-9.

87. Römisch J, Vermöhlen S, Feussner A, Stöhr HA. The FVII activating protease cleaves single-chain plasminogen activators. *Pathophysiol Haemost Thromb*. 1999; **29**: 292-9.

88. Mambetsariev N, Mirzapoiazova T, Mambetsariev B, Sammani S, Lennon FE, Garcia JGN, Singleton PA. Hyaluronic acid binding protein 2 is a novel regulator of vascular integrity. *Arterioscler Thromb Vasc Biol*. 2010; **30**: 483-90.

89. Zeerleder S, Zwart B, te Velthuis H, Stephan F, Manoe R, Rensink I, Aarden LA. Nucleosome-releasing factor: a new role for factor VII-activating protease (FSAP). *FASEB J*. 2008; **22**: 4077-84.

90. Stephan F, Hazelzet J, Bulder I, Boermeester M, van Till JW, van der Poll T, Wuillemin W, Aarden LA, Zeerleder S. Activation of factor VII-activating protease in human inflammation: a sensor for cell death. *Crit Care*. 2011; **15**: R110.

91. Kreß JA, Seitz R, Dodt J, Etscheid M. Induction of intracellular signalling in human endothelial cells by the hyaluronan-binding protease involves two distinct pathways. *Biol Chem*. 2006; **387**: 1275-83.

92. Sedding DG, Daniel J-M, Muhl L, Hersemeyer K, Brunsch H, Kemkes-Matthes B, Braun-Dullaeus RC, Tillmanns H, Weimer T, Preissner KT, Kanse SM. The G534E polymorphism of the gene encoding the factor VII–activating protease is associated with cardiovascular risk due to increased neointima formation. *J Exp Med*. 2006; **203**: 2801-7.

93. Ireland H, Miller GJ, Webb KE, Cooper JA, Humphries SE. The factor VII activating protease G511E (Marburg) variant and cardiovascular risk. *Thromb Haemost*. 2004; **92**: 986-92.

94. Parahuleva MS, Kanse SM, Parviz B, Barth A, Tillmanns H, Bohle RM, Sedding DG, Hölschermann H. Factor seven activating protease (FSAP) expression in human monocytes and accumulation in unstable coronary atherosclerotic plaques. *Atherosclerosis*. 2008; **196**: 164-71.

95. Kanse SM, Parahuleva MS, Muhl L, Kemkes-Matthes B, Sedding DG, Preissner KT. Factor VII-activating protease (FSAP): Vascular functions and role in atherosclerosis. *Thromb Haemost*. 2008; **99**: 286-9.

96. Lundwall A, Dackowski W, Cohen E, Shaffer M, Mahr A, Dahlbäck B, Stenflo J, Wydro R. Isolation and sequence of the cDNA for human protein S, a regulator of blood coagulation. *Proc Natl Acad Sci U S A*. 1986; **83**: 6716-20.

97. Gershagen S, Fernlund P, Lundwall Å. A cDNA coding for human sex hormone binding globulin. Homology to vitamin K-dependent protein S. *FEBS Lett*. 1987; **220**: 129-35.

98. Ahnström J, Andersson HM, Canis K, Norstrøm E, Yu Y, Dahlbäck B, Panico M, Morris HR, Crawley JTB, Lane DA. Activated protein C cofactor function of protein S: a novel role for a γ-carboxyglutamic acid residue. *Blood*. 2011; **117**: 6685-93.

99. Saller F, Villoutreix BO, Amelot A, Kaabache T, Le Bonniec BF, Aiach M, Gandrille S, Borgel D. The γ-carboxyglutamic acid domain of anticoagulant protein S is involved in activated protein C cofactor activity, independently of phospholipid binding. *Blood*. 2005; **105**: 122-30.

100. Heeb MJ, Griffin JH. Activated protein C-dependent and –independent anticoagulant activities of protein S have different structural requirements. *Blood Cells Mol Dis*. 2002; **29**: 190-9.

101. Morboeuf O, Borgel D, Gaussem P, Vincenot A, Pittet JL, Aiach M, Gandrille S. Characterization of cleaved plasma protein S with a monoclonal antibody-based assay. *Thromb Haemost*. 2000; **84**: 604-10.

102. Borgel D, Gaussem P, Garbay C, Bachelot-Loza C, Kaabache T, Liu WQ, Brohard-Bohn B, Le Bonniec B, Aiach M, Gandrille S. Implication of protein S thrombin-sensitive region with membrane binding via conformational changes in the γ-carboxyglutamic acid-rich domain. *Biochem J*. 2001; **360**: 499-506.

103. Saller F, Kaabache T, Aiach M, Gandrille S, Borgel D. The protein S thrombin-sensitive region modulates phospholipid binding and the γ-carboxyglutamic acid-rich (Gla) domain conformation in a non-specific manner. *J Thromb Haemost*. 2006; **4**: 704-6.

104. Persson KEM, Stenflo J, Linse S, Stenberg Y, Preston RJS, Lane DA, Rezende SM. Binding of Calcium to anticoagulant protein S: role of the fourth EGF module. *Biochemistry*. 2006; **45**: 10682-9.

105. He X, Shen L, Villoutreix BO, Dahlbäck B. Amino acid residues in thrombin-sensitive region and first epidermal growth factor domain of vitamin K-dependent protein S determining specificity of the activated protein C cofactor function. *J Biol Chem*. 1998; **273**: 27449-58.

106. Andersson HM, Arantes MJ, Crawley JTB, Luken BM, Tran S, Dahlbäck B, Lane DA, Rezende SM. Activated protein C cofactor function of protein S: a critical role for Asp95 in the EGF1-like domain. *Blood*. 2010; **115**: 4878-85.

107. Mille-Baker B, Rezende SM, Simmonds RE, Mason PJ, Lane DA, Laffan MA. Deletion or replacement of the second EGF-like domain of protein S results in loss of APC cofactor activity. *Blood*. 2003; **101**: 1416-8.

108. Nyberg P, Dahlbäck B, García de Frutos P. The SHBG-like region of protein S is crucial for factor V-dependent APC-cofactor function. *FEBS Lett*. 1998; **433**: 28-32.

109. Saposnik B, Borgel D, Aiach M, Gandrille S. Functional properties of the sex-hormone-binding globulin (SHBG)-like domain of the anticoagulant protein S. *Eur J Biochem*. 2003; **270**: 545-55.

110. Van de Poel RH, Meijers JC, Bouma BN. C4b-binding protein inhibits the factor V-dependent but not the factor V-independent cofactor activity of protein S in the activated protein C-mediated inactivation of factor VIIIa. *Thromb Haemost*. 2001; **85**: 761-5.

111. Heeb MJ, Prashun D, Griffin JH, Bouma BN. Plasma protein S contains zinc essential for efficient activated protein C-independent anticoagulant activity and binding to factor Xa, but not for efficient binding to tissue factor pathway inhibitor. *FASEB J*. 2009; **23**: 2244-53.

112. Duebgen S, Kauke T, Marschall C, Giebl A, Lison S, Hart C, Dick A, Spannagl M.

Genotype and laboratory and clinical phenotypes of protein S deficiency. *Am J Clin Pathol*. 2012; **137**: 178-84.

113. Saller F, Brisset AC, Tchaikovski SN, Azevedo M, Chrast R, Fernández JA, Schapira M, Hackeng TM, Griffin JH, Angelillo-Scherrer A. Generation and phenotypic analysis of protein S-deficient mice. *Blood*. 2009; **114**: 2307-14.

114. Dahlbäck B. Inhibition of protein Ca cofactor function of human and bovine protein S by C4b-binding protein. *J Biol Chem*. 1986; **261**: 12022-7.

115. Esmon CT. Thrombomodulin as a model of molecular mechanisms that modulate protease specificity and function at the vessel surface. *FASEB J*. 1995; **9**: 946-55.

116. Kalafatis M, Rand MD, Mann KG. The mechanism of inactivation of human factor V and human factor Va by activated protein C. *J Biol Chem*. 1994; **269**: 31869-80.

117. Fay PJ, Smudzin TM, Walker FJ. Activated protein C-catalyzed inactivation of human factor VIII and factor VIIIa. Identification of cleavage sites and correlation of proteolysis with cofactor activity. *J Biol Chem*. 1991; **266**: 20139-45.

118. Yegneswaran S, Smirnov MD, Safa O, Esmon NL, Esmon CT, Johnson AE. Relocating the active site of activated protein C eliminates the need for its protein S cofactor. *J Biol Chem*. 1999; **274**: 5462-8.

119. Hackeng TM, Yegneswaran S, Johnson AE, Griffin JH. Conformational changes in activated protein C caused by binding of the first epidermal growth factor-like module of protein S. *Biochem J*. 2000; **349**: 757-64.

120. Rosing J, Hoekema L, Nicolaes GAF, Thomassen MCLGD, Hemker HC, Varadi K, Schwarz HP, Tans G. Effects of protein S and factor Xa on peptide bond cleavages during inactivation of factor Va and factor VaR506Q by activated protein C. *J Biol Chem*. 1995; **270**: 27852-8.

121. Shen L, Dahlbäck B. Factor V and protein S as synergistic cofactors to activated protein C in degradation of factor VIIIa. *J Biol Chem*. 1994; **269**: 18735-8.

122. Griffin J, Gruber A, Fernandez J. Reevaluation of total, free, and bound protein S and C4b-binding protein levels in plasma anticoagulated with citrate or hirudin. *Blood*. 1992; **79**: 3203-11.

123. Maurissen LFA, Thomassen MC, Nicolaes GA, Dahlbäck B, Tans G, Rosing J, Hackeng TM. Re-evaluation of the role of the protein S-C4b binding protein complex in activated protein C-catalyzed factor Va-inactivation. *Blood*. 2008; **111**: 3034-41.

124. Seré KM, Janssen MP, Willems GM, Tans G, Rosing J, Hackeng TM. Purified protein S contains multimeric forms with increased APC-independent anticoagulant activity. *Biochemistry*. 2001; **40**: 8852-60.

125. Seré KM, Rosing J, Hackeng TM. Inhibition of thrombin generation by protein S at low procoagulant stimuli: implications for maintenance of the hemostatic balance. *Blood*. 2004; **104**: 3624-30.

126. Koppelman S, Hackeng T, Sixma J, Bouma B. Inhibition of the intrinsic factor X activating complex by protein S: evidence for a specific binding of protein S to factor VIII. *Blood*. 1995; **86**: 1062-71.

127. Van 't Veer C, Butenas S, Golden NJ, Mann KG. Regulation of prothrombinase activity by protein S. *Thromb Haemost*. 1999; **82**: 80-7.

128. Takeyama M, Nogami K, Saenko EL, Soeda T, Nishiya K, Ogiwara K, Yoshioka A, Shima M. Protein S down-regulates factor Xase activity independent of activated protein C: specific binding of factor VIII(a) to protein S inhibits interactions with factor IXa. *Br J Haematol*. 2008; **143**: 409-20.

129. Seré KM, Willems GM, Rosing J, Hackeng TM. Protein S multimers are generated in vitro and affect protein S structure-function analyses. *Semin Hematol*. 2006; **43**: S111-S20.

130. Heeb MJ, Radtke KP, Fernandez JA, Tonnu L. Plasma contains protein S monomers and multimers with similar direct anticoagulant activity. *J Thromb Haemost*. 2006; **4**: 2215-22.

131. Ndonwi M, Broze GJ. Protein S enhances the tissue factor pathway inhibitor inhibition

of factor Xa but not its inhibition of factor VIIa–tissue factor. *J Thromb Haemost*. 2008; **6**: 1044-6.

132. Ndonwi M, Tuley EA, Broze GJ. The Kunitz-3 domain of TFPI-alpha is required for protein S-dependent enhancement of factor Xa inhibition. *Blood*. 2010; **116**: 1344-51.

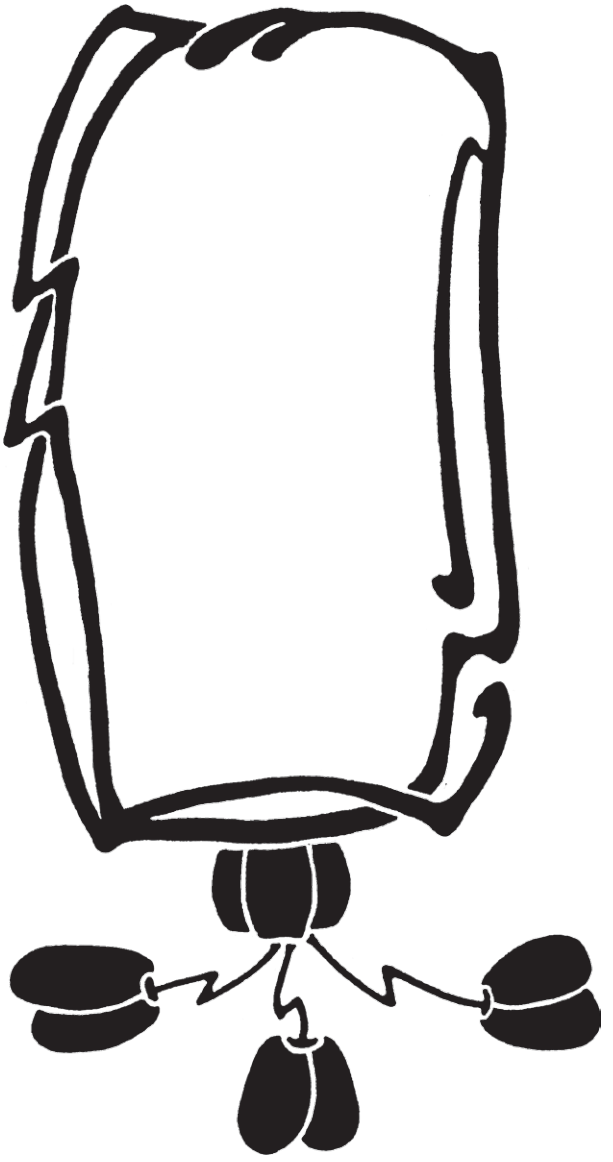
133. Hackeng TM, Maurissen LFA, Castoldi E, Rosing J. Regulation of TFPI function by protein S. *J Thromb Haemost*. 2009; **7**: 165-8.

134. Rezende SM, Lane DA, Mille-Baker B, Samama MM, Conard J, Simmonds RE. Protein S Gla-domain mutations causing impaired Ca^{2+}-induced phospholipid binding and severe functional protein S deficiency. *Blood*. 2002; **100**: 2812-9.

135. Schwalbe R, Dahlbäck B, Hillarp A, Nelsestuen G. Assembly of protein S and C4b-binding protein on membranes. *J Biol Chem*. 1990; **265**: 16074-81.

136. Webb JH, Blom AM, Dahlbäck B. Vitamin K-dependent protein S localizing complement regulator C4b-binding protein to the surface of apoptotic cells. *J Immunol*. 2002; **169**: 2580-6.

137. Brinkman HJM, Mertens K, van Mourik JA. Proteolytic cleavage of protein S during the hemostatic response. *J Thromb Haemost*. 2005; **3**: 2712-20.



Chapter 2

Patient-derived monoclonal antibodies directed towards β2-glycoprotein I display lupus anticoagulant activity

Ilze Dienava-Verdoold,[1] Mariette G. Boon-Spijker,[1] Philip G. de Groot,[2] Herm-Jan M. Brinkman,[1] Jan Voorberg,[1] Koen Mertens,[1,3] Ronald H. W. M. Derksen,[4] and Bas de Laat[1,2]

[1]Department of Plasma Proteins, Sanquin Research, Amsterdam, The Netherlands
[2]Clinical Chemistry and Hematology, University Medical Center Utrecht, Utrecht, The Netherlands
[3]Utrecht Institute of Pharmaceutical Sciences, Utrecht, The Netherlands
[4]Rheumatology and Clinical Immunology, University Medical Center Utrecht, Utrecht, The Netherlands.

J Thromb Haemost 2011; 9: 738–47.

Abstract

Background: Patients with antiphospholipid syndrome (APS) display a heterogeneous population of antibodies with β2-glycoprotein I (β2GPI) as the major antigen. *Objectives:* We isolated and characterized human mAbs directed against β2GPI from the immune repertoire of APS patients. *Methods:* Variable heavy chain repertoires from B cells from two APS patients with anti-β2GPI antibodies were cloned into the pHEN1-VLrep vector. Constructed full-length IgG antibodies were tested for lupus anticoagulant (LAC) activity and binding to β2GPI and its domains. *Results:* Two clones of each patient were selected on the basis of the reactivity of single-chain Fv (scFv) fragments displayed on phages towards full-length β2GPI and its isolated domain I. The affinity of selected antibodies for β2GPI was lost when transforming from phages to monovalent scFvs, and was regained when antibodies were constructed as complete IgG, indicating a role for bivalency in binding to β2GPI. Both selected clones from patient 2 recognized domain I of β2GPI, and for both clones selected from patient 1, binding required the presence of both domain I and domain II. All mAbs displayed LAC activity in both activated partial thromboplastin time-based and dilute Russell's viper venom test-based clotting assays and in thrombin generation. *Conclusions:* In this study, we show successful cloning of patient-derived mAbs that require domain I of β2GPI for binding, and that display LAC activity that is dependent on their affinity for β2GPI. These antibodies can help us to gain more insights into the pathogenesis of APS, and may facilitate standardization of APS diagnosis.

Introduction

The antiphospholipid syndrome (APS) is an acquired autoimmune disorder diagnosed by the simultaneous presence of thrombosis or pregnancy morbidity and antiphospholipid (aPL) antibodies [1]. aPL antibodies represent a heterogeneous group of antibodies that recognize various proteins with affinity for anionic phospholipids. The most common antigen for aPL antibodies is β2-glycoprotein I (β2GPI), but antibodies against prothrombin, protein C, protein S and annexin A5 have also been described as potential modulators of disease activity [2].

aPL antibodies are quite common in the general population, but only one-third of the individuals with persistent aPL antibody titres may develop APS-associated complications [3]. One explanation for this phenomenon is the so-called two-hit hypothesis of APS, stating that, besides aPL antibodies, the presence of an additional risk factor or trigger such as inherited thrombophilia or bacterial infection would be necessary to induce APS [3,4]. Alternatively, this phenomenon could be caused by the fact that pathogenic aPL antibodies may be present only in a proportion of the aPL antibody-positive population.

Many epitopes have been proposed for the pathogenic anti-β2GPI antibodies, but domain I, especially the epitope comprising residues Gly40–Arg43, is thought to be the most relevant in APS [5–7]. In addition to these residues, Asp8, Asp9, Arg39 and the domain I–II interlinker region could also be involved in pathogenic aPL antibody binding, resulting in a discontinuous epitope [8]. aPL antibodies cannot bind β2GPI

in solution, because the epitope for aPL antibodies on domain I is not surface-exposed, owing to the closed conformation [9,10]. β2GPI, on the other hand, can bind to anionic phospholipids via the positively charged patch on domain V, albeit with low affinity [11–13]. This binding induces a conformational change that causes exposure of the encrypted epitope in domain I. aPL antibodies can then bind and dimerize β2GPI to form a stable complex that has higher affinity than monomeric β2GPI for negatively charged phospholipids [7,13].

At present, there is no consensus on a pathogenic mechanism causing thrombosis in patients with anti-β2GPI antibodies. A wide range of pathogenic responses of β2GPI–antibody complexes has been observed [14]. In endothelial cells, these complexes can induce a procoagulant and inflammatory phenotype [15,16]. In monocytes, anti-β2GPI–β2GPI complexes can induce tissue factor expression [17]. Binding to the receptor ApoER2' or to the glycoprotein Iba subunit on platelets induces activation and increased adhesion to collagen [18]. Complement activation by aPL antibodies may contribute to a proinflammatory and procoagulant state of endothelial cells, and possibly to thrombosis in the placental vasculature and foetal loss [14]. In addition to cellular mechanisms, the aPL antibody–β2GPI complex can directly interfere with various pathways in the coagulation cascade. Inhibition of the anticoagulant factors may involve disruption of the interaction of activated protein C or annexin A5 with phospholipid membranes [19,20]. It is generally accepted that the clotting time prolongation that is used as a laboratory marker for the detection of APS occurs because of competition of anti-β2GPI–β2GPI complexes with coagulation factors for phospholipid binding [21].

So far, the characteristics of anti-β2GPI antibodies have only been determined by studying the properties of polyclonal antibodies present in plasma of APS patients. Several papers have reported the isolation of human mAbs that could be involved in APS pathogenesis [2,22,23]. Most of these studies have employed hybridoma technology to isolate anti-cardiolipin antibodies. Some antibodies did show moderate lupus anticoagulant (LAC) activity, but limited binding to β2GPI in the absence of cardiolipin [2,22,23]. Two studies reported the isolation of patient-derived anti-β2GPI single-chain Fv (scFv) and Fab fragments by phage display. These did bind to isolated β2GPI, but their LAC activity had not been investigated [24,25]. Recently, Prinz *et al.* reported finding a monoclonal anti-β2GPI antibody with LAC activity. However, the epitopes involved have not been identified [26]. In this study, we constructed full-length human mAbs from two patients with APS, employing phage display technology [27], and characterized these antibodies with regard to sequence, β2GPI binding, domain specificity and LAC activity. The results contribute molecular insights into aPL antibodies associated with APS.

Materials and methods

Preparation of full-length β2GPI and its domains

Human plasma-derived β2GPI was prepared as described previously [9], with some modifications. In short, fresh plasma was incubated batch-wise with 70 g of DEAE Sephadex A-50 per 900 mL of plasma. The unbound protein was applied to a HiTrap

SP FF Sepharose column. Salt gradient-eluted [9] fractions containing β2GPI were successively passed through a protein A Sepharose column and a protein G Sepharose column to remove IgG, and then through a heparin Sepharose FF column; this was followed by salt gradient elution. The final β2GPI eluate was dialyzed against Tris-buffered saline (TBS) (50 mM Tris, 150 mM NaCl, pH 7.4). Purity was more than 95% as assessed by SDS-PAGE.

Domain deletion mutants DI–II, DI–IV, DIII–V and DIV–V of β2GPI were a generous gift from M. Iverson of La Jolla Pharmaceutical Company (La Jolla, CA, USA); DI and DII–V were produced and purified according to the method described by Iverson *et al.* [5].

Patients

Two patients were selected on the basis of a history of recurrent thrombosis and the presence of anti-β2GPI and anti-domain I of β2GPI IgG antibodies in their plasma. Patient 1 had a history of arterial thrombosis, and patient 2 suffered from an episode of catastrophic APS. Both patients tested positive for anti-cardiolipin IgG antibodies, and displayed LAC activity in the dilute Russell's viper venom test (dRVVt) and the activated partial thromboplastin time (APTT) assay.

Preparation of patient-derived monoclonal anti-β2GPI antibodies

A phage display library was constructed from CD19-positive B cells isolated from peripheral blood of selected APS patients, essentially following the method described by Luken *et al.* [28]. The library was infected with KM13 helper phage (MRC Geneservice, Cambridge, UK) for production of phage particles [27]. The separate libraries for each VH family ranged in size from 0.35 to $3.5 \cdot 10^6$ and from 0.27 to $1.9 \cdot 10^6$ individual colonies for patient 1 and patient 2, respectively. Phages were selected for binding to plasma-derived β2GPI or its domain I coated on hydrophilic (Maxisorp; Nunc, Roskilde, Denmark) or hydrophobic (Costar #2595; Corning, NY, USA) multiwell plates, respectively. For isolated domain I, a hydrophobic plate is necessary to expose the epitope encompassing Gly40–Arg43 [7]. For full-length β2GPI, a hydrophilic plate was used because, on hydrophobic plates, binding of pathogenic APS antibodies to immobilized full-length β2GPI is hampered [9]. Bound phages were eluted with trypsin, and TG1 cells were infected with the eluted phages for the next selection round. Three rounds of selection were performed. Nucleotide sequencing of the VH and VL segments, isolation of scFv antibody fragments and conversion of selected VH and VL gene fragment combinations into full-length IgG1 molecules was carried out according to previously described methods [28,29]. Full-length antibodies were expressed in HEK293 cells, and purified from the collected cell culture supernatant with a protein A Sepharose affinity chromatography column, according to the manufacturer's instructions. Antibodies were dialyzed against TBS, and the purity was more than 95% as assessed by SDS-PAGE.

Binding characterization of monoclonal anti-β2GPI antibodies

An ELISA-based method [7] was used to characterize the patient-derived full-length

anti-β2GPI mAb binding to β2GPI and epitope mapping. In short, β2GPI domain-deleted mutants DI, DI–II, DI–IV, DII–V, DIII–V and DIV–V, as well as full-length β2GPI, were coated on hydrophilic and hydrophobic plates (for coating conditions, see figure legends). Monoclonal antibody CLB-Pro35 against the propeptide of von Willebrand factor [30] served as a negative control. Anti-β2GPI polyclonal IgG (Kordia, Leiden, The Netherlands) was used as a positive control. Bound antibodies were detected with anti-human, anti-mouse or anti-goat peroxidase-labelled conjugates for monoclonal anti-β2GPI antibodies, CLB-Pro35 or polyclonal antibodies against β2GPI, respectively. Detection of scFv bound to immobilized β2GPI or domain I was performed with peroxidase-labelled anti-myc tag antibody 9E10 (ATCC, Manassas, VA, USA).

The affinity of anti-β2GPI antibodies for soluble β2GPI was determined by antigen inhibition as described by Chukwuocha *et al.* [24]. The dissociation constant (K_d) was estimated according to the method of Friguet *et al.* [31].

Binding of our patient-derived full-length anti-β2GPI mAbs to immobilized β2GPI was also assessed in a plasma environment. Normal pooled plasma (NPP) from 40 healthy donors (blood bank division of our institute) was spiked with mAbs. Binding of antibodies to immobilized β2GPI was performed as described above. Additionally, binding to cardiolipin (Sigma-Aldrich, St Louis, MO, USA) immobilized on Polysorp multiwell plates (Nunc) was examined. In addition to these in-house assays, commercial anti-cardiolipin and anti-β2GPI ELISAs (Corgenix, Broomfield, CO, USA) were used according to the manufacturer's instructions.

Clotting assays

The LAC activity of patient-derived full-length anti-β2GPI mAbs was investigated in plasma. Antibodies were preincubated in NPP with or without purified β2GPI for 1 h at 37 °C. A simplified dRVVt was performed according to the protocol of the manufacturer (LA Screen; Life Diagnostics, Sydney, Australia). APTT was determined with a LAC-sensitive PTT-LA assay (Diagnostica Stago, Asnières, France). The mAb CLB-Pro35 served as a negative control. LA Control High plasma (Dade Behring, Marburg, Germany) was used as a LAC-positive control.

The impact of antibodies on thrombin generation was assessed by calibrated automated thrombography (CAT) [32], with commercially available mixtures of 5 or 1 pM tissue factor and 4 μM phospholipid vesicles (phosphatidylserine/phosphatidylcholine/phosphatidylethanolamine, 2 : 6 : 2 molar ratio) obtained from Thrombinoscope (Maastricht, The Netherlands). The amount of thrombin generated was calibrated against the thrombin calibrator from Thrombinoscope. Antibodies were preincubated with NPP for 1 h at 37 °C. The final plasma concentration was 50% (v/v).

Table 2-1 Nucleotide sequence characteristics of the isolated single-chain Fv (scFv) fragments.

ScFV	V-gene	Homology	D-gene	Homology	J-gene	Homology	CDR3	R	S
heavy chain									
P1-117	IGHV3-23*01	96.9 %	IGHD3-22*01	68.1 %	IGHJ4*02	82.6 %	16	8	1
P1-190	IGHV3-23*01	97.2 %	IGHD3-22*01	68.1 %	IGHJ4*02	82.6 %	16	7	1
P2-6	IGHV4-4*01	94.1 %	IGHD3-10*01	62.5 %	IGHJ4*02	89.1 %	12	13	4
P2-162	IGHV4-4*01	94.1 %	IGHD6-19*01	66.7 %	IGHJ4*02	86.9 %	12	10	7
light chain									
P1-117	IGKV1-9*01	96.1 %			IGKJ4*01	97.4 %	9	8	2
P1-190	IGKV1-5*01	92.8 %			IGKJ4*01	97.4 %	9	9	7
P2-6	IGLV2-14*01	96.5 %			IGLJ3*01	100 %	10	8	2
P2-162	IGLV2-8*01	93.1 %			IGLJ3*01	89.5 %	11	14	4

The germline gene segments (V-gene, D-gene and J-gene) used for assembly of patient heavy and donor light chains of anti-β2 glycoprotein I antibodies are shown. CDR3, amino acids in CDR3 region; R, amino acid replacements; S, silent mutations.

Heavy chain

	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
IGHV3-23*04	EVQLVESGGGLVQPGGSLRLSCAAS	**GFTFSSYA**	MSWVRQAPGKGLEWVSA	**ISGSGGST**	YYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYC	**AK**	
P1-117	Q----------	**------F-**	----------S	**------T-**	----------D--------I---	**--EHDSSGPPSMGFDY**	WGQGTLVTVS
P1-190	----------	**------F-**	----------S	**------T-**	----------D--------I---	**--EHDSSGPPSMGFDY**	WGQGTLVTVS
IGHV4-4*02	QVQLQESGPGLVKPSGTLSLTCAVS	**GGSISSSNW**	WSWVRQPPGKGLEWIGE	**IYHSGST**	NYNPSLKSRVTISVDKSKNQFSLKLSSVTAADTAVYYC	**AR**	
P2-6	----------T--	**----G--H-**	----------	**------I**	S----------M---N-E------FT----------	**--SMAGGFSFDY**	WGQGTLVTVS
P2-162	----------N----------	**-D----T--**	----------	**--E----**	---------LNM----------R----------	**--SVAGGFSFDY**	WGRGTLVTVS

Light chain

IGKV1-5*01	DIQMTQSPSTLSASVGDRVTITCRAS	**QSISSW**	LAWYQQKPGKAPKLLIY	**DAS**	SLESGVPSRFSGSGSGTEFTLTISSLQPDDFATYYC	**QQYNSYS**	
P1-190	--V----------	**----TL**	----------	**A--**	R-Q----------G---E-------	**-KLS--PLT**	FGGGTKLEIKRA
IGKV1-9*01	DIQLTQSPSFLSASVGDRVTITCRAS	**QGISSY**	LAWYQQKPGKAPKLLIY	**AAS**	TLQSGVPSRFSGSGSGTEFTLTISSLQPEDFATYYC	**QQLNSYP**	
P1-117	E-V----------F----I-------	**----N-**	----------	**---**	----------G-------S---	**-K-----LT**	FGGGTKLEIKRAAA
IGLV2-14*01	QSALTQPASVSGSPGQSITISCTGT	**SSDVGGYNY**	VSWYQQHPGKAPKLMIY	**EVS**	NRPSGVSNRFSGSKSGNTASLTISGLQAEDEADYYC	**SSYTSSSTL**	
P2-6	NFM----------	**--------F**	----------	**D--**	----------	**---------V**	FGGGTKLTVLGAA
IGLV2-8*01	QSALTQPPSASGSPGQSVTISCTGT	**SSDVGGYNY**	VSWYQQHPGKAPKLMIY	**EVS**	KRPSGVPDRFSGSKSGNTASLTVSGLQAEDEADYYC	**SSYAGSNNF**	
P2-162	HVI----A-V-------I-------	**-----A---**	------Y------V---	**--N**	----------D-------	**-----NS-SPV**	FGGGTKLTVLGAA

Figure 2-1 Amino acid sequences of the variable heavy and light chain segments of single-chain Fv (scFv) fragments. *Four selected scFv fragments from patients 1 and 2 are shown that recognized β2-glycoprotein I. FR1–4, framework regions; CDR1–3 (in bold), complementarity-determining regions; dashes indicate the amino acids identical to those in the germline sequence; differences from the germline sequence are shown. Nucleotide sequences from all clones are available from GenBank (GenBank accession numbers: HQ129860–HQ129867). CDR and FR regions were assigned to VH and VL sequences by V-QUEST (http://imgt.cines.fr).*

Statistical analysis

One-way ANOVA with Dunnett's post hoc test was performed with GraphPad Prism version 5.01 forWindows (GraphPad, La Jolla, CA, USA).

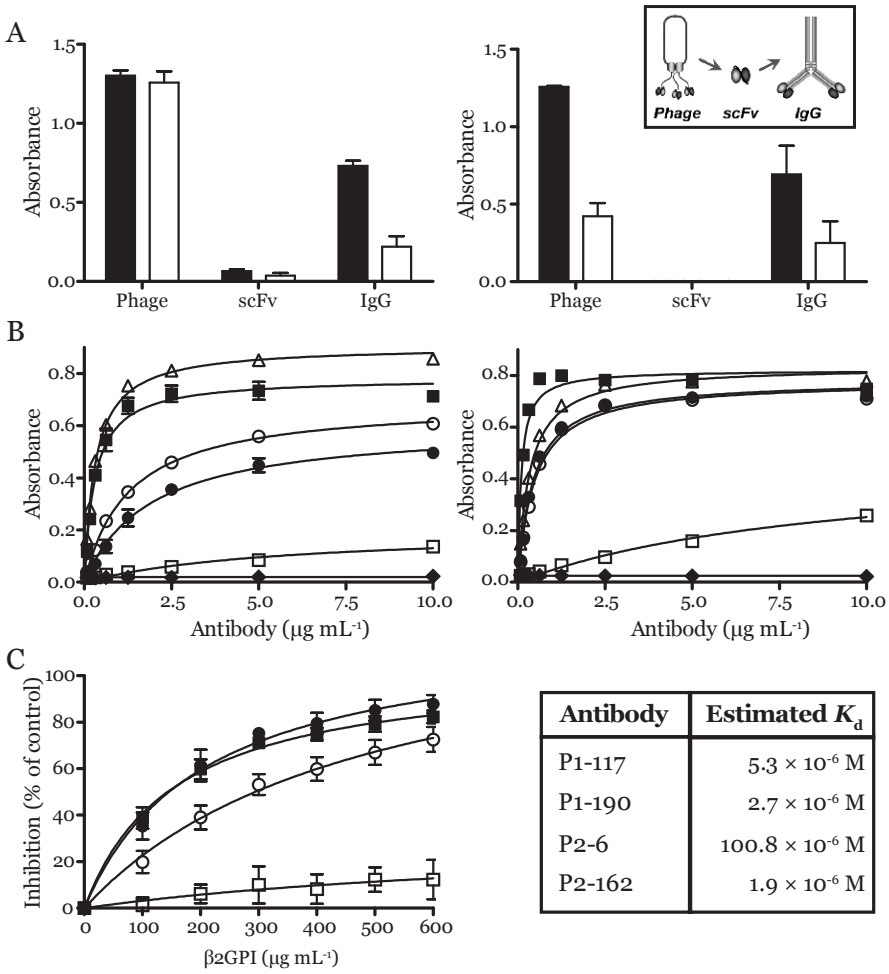
Results

Anti-β2GPI phage display library

A VH-gene phage library constructed from two APS patients was used to obtain human mAbs against β2GPI. As domain I of β2GPI is described as the epitope for antibodies involved in APS pathogenesis [7], phages were panned on full-length β2GPI or isolated domain I of β2GPI. Following three rounds of selection, 190 clones were picked from each patient and screened for binding to full-length β2GPI and isolated domain I of β2GPI. Nine clones from patient 1 consistently bound to full-length β2GPI, and seven clones also bound to isolated domain I of β2GPI. From patient 2, 81 clones were found that bound to full-length β2GPI and 43 clones that also recognized isolated domain I of β2GPI. All positive clones of patient 1 and 26 clones recognizing both full-length β2GPI and isolated domain I of β2GPI from patient 2 were subjected to nucleotide sequencing. Six distinct and complete scFv fragments were found for patient 1 and four for patient 2. From each patient, two clones showing high binding responses in the β2GPI ELISA were selected for further experiments. Their amino acid sequences and alignment with germline sequences are shown in Fig. 2-1 and Table 2-1. Both clones of patient 1 contained the VH domain of germline VH3-23, with an identical CDR3 region and a nearly identical hypermutation pattern, except for an extra mutation in the CDR1 region of P1-117. P1-190 used the light chain from the VK1-5 germline, whereas the VL domain of P1-117 was the most related in sequence to the VK1-9 germline. The VH domains of P2-6 and P2-162 were derived from germline VH4-4 with different hypermutation patterns. The similarity in the CDR3 region suggests that they were derived from the same parental B cell. P2-6 used the light chain derived from VL2-14 germline, whereas P2-162 was paired with a VL fragment homologous to the VL2-8 germline.

Binding characteristics of isolated scFv and full-length IgG constructs

When isolated scFvs were tested, hardly any binding to full-length β2GPI (Fig. 2-2A, left panel) or DI (Fig. 2-2A, right panel) was observed as compared with multiple scFv fragments displayed on phages. Surprisingly, binding was regained when full-length IgG was employed (Fig. 2-2A). IgG antibody binding to full-length β2GPI immobilized on a hydrophobic plate (Fig. 2-2B, left panel) showed that P2-6 had the highest binding for β2GPI, followed by both antibodies of patient 1. P2-162 showed the weakest binding to β2GPI. When β2GPI was immobilized on a hydrophilic plate, an increase in binding was observed for all patient-derived antibodies, although, again, P2-162 showed the lowest binding (Fig. 2-2B, right panel). Binding specificity was confirmed by the ability of soluble β2GPI to inhibit antibody interactions with immobilized β2GPI (Fig. 2-2C). The strongest inhibition was achieved by P1-117 and P2-6, and the weakest inhibition was achieved by P2-162. These data were



Antibody	Estimated K_d
P1-117	5.3×10^{-6} M
P1-190	2.7×10^{-6} M
P2-6	100.8×10^{-6} M
P2-162	1.9×10^{-6} M

Figure 2-2 Binding of human mAbs to β2-glycoprotein I (β2GPI). *(A) Comparison of binding to full-length β2GPI (left) and domain I (right) between single-chain Fv (scFv) fragments presented on phage particles, isolated scFv fragments and full-length IgGs. Undiluted phage suspension, 35 μg mL⁻¹ scFv and 10 μg mL⁻¹ IgG of P2-6 (black bars) and P2-162 (white bars) were used. Data represent mean ± range of two separate experiments. For patient 1, we omitted the testing of scFv fragments and proceeded directly to the construction of full-length antibodies. Inset: schematic representation of scFv displayed on phage, isolated scFv and full-length IgG. (B) Concentration-dependent binding of antibodies to β2GPI. Plasma-derived β2GPI was coated on a hydrophobic (left) or hydrophilic (right) plate at 5 μg mL⁻¹ in phosphate-buffered saline. Plates were blocked with 4% (w/v) bovine serum albumin and then incubated with mAbs P1-117 (closed circle), P1-190 (open circle), P2-6 (closed square) and P2-162 (open square) at a concentration range of 0.08–10 μg mL⁻¹. Polyclonal anti-β2GPI (triangle) antibody and CLB-Pro35 (diamond) were used as positive and negative control, respectively. Data represent mean ± range of two separate experiments. (C) Affinity of antibodies for soluble β2GPI. Left: inhibition of antibody binding to immobilized β2GPI by soluble β2GPI. Antibodies were used at concentrations of half-maximal binding (P1-117, 0.5 μg mL⁻¹ [closed circle]; P1-190, 0.5 μg mL⁻¹ [open circle]; P2-6, 0.1 μg mL⁻¹ [closed square]; P2-162, 10 μg mL⁻¹ [open square]) and preincubated with increasing concentrations of β2GPI. Data represent mean ± range of two separate experiments. Right: the estimated* K_d *for mAbs.*

used to calculate the dissociation constants. The estimated K_d values were in the low micromolar range, except for P2-162, which displayed 20–50-fold higher K_d values than the other antibodies (Fig. 2-2C, right panel). Apparently, our patient-derived mAbs bind to β2GPI by a mechanism that involves multivalent interactions. Moreover, the micromolar affinities of our antibodies are comparable to the low affinities of the actual autoantibodies in APS patients, and compatible with the continued presence of β2GPI at micromolar concentrations in plasma [24,33].

Epitope mapping was performed with a panel of β2GPI domain-deleted mutants. P1-117 and P1-190 reacted with DI–II, DI–IV and full-length β2GPI, but not with DI, DII–V, DIII–V and DIV–V (Fig. 2-3A). These results suggest that the epitope for patient 1 antibodies requires the presence of both domains I and II. In contrast, P2-6 and P2-162 reacted with DI, DI–II, DI–IV and full-length β2GPI, but not with DII–V, DIII–V and DIV–V (Fig. 2-3B). Therefore, we conclude that the antibodies derived from patient 2 differ from those derived from patient 1 in that they do specifically recognize domain I of β2GPI.

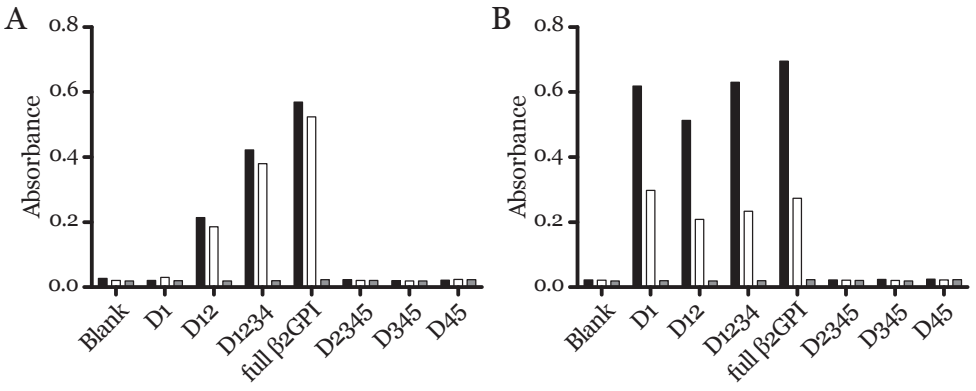


Figure 2-3 Epitope mapping of full-length IgG antibodies. *Epitope mapping on hydrophobic plates of patient 1 antibodies P1-117 (black bars) and P1-190 (white bars) (A) or patient 2 antibodies P2-6 (black bars) and P2-162 (white bars) (B) was performed with a panel of β2-glycoprotein I (β2GPI) domain-deleted mutants. Plasma-derived β2GPI or its domain deletion mutants were coated on a hydrophobic or a hydrophilic plate at 5 μg mL^{-1} in phosphate-buffered saline. Plates were blocked with 4% (w/v) bovine serum albumin, and then incubated with mAbs at 10 μg mL^{-1}. Similar results were obtained on hydrophilic plates, except for binding to domain I. CLB-Pro35 (gray bars) was used as a negative control. All β2GPI domain-deleted mutants were recognized by polyclonal anti-β2GPI antibody.*

Solid-phase binding assays were performed to assess binding of full-length IgG constructs to β2GPI as well as cardiolipin in a plasma environment. In addition to our in-house assays, commercial ELISAs from Corgenix were used. All antibodies showed binding to immobilized cardiolipin to a similar extent in both assays (Fig. 2-4A,B). With regard to binding to immobilized β2GPI, both patient 1 antibodies and P2-6 showed binding to β2GPI in both the commercial and in-house assays (Fig. 2-4C,D). It was striking, however, that P2-162 showed low binding in the in-house assay (Fig. 2-4D), whereas its response in the commercial assay was only slightly lower than that of the other three antibodies (Fig. 2-4C). Taking these findings

together, in plasma our antibodies showed binding to both immobilized β2GPI and cardiolipin.

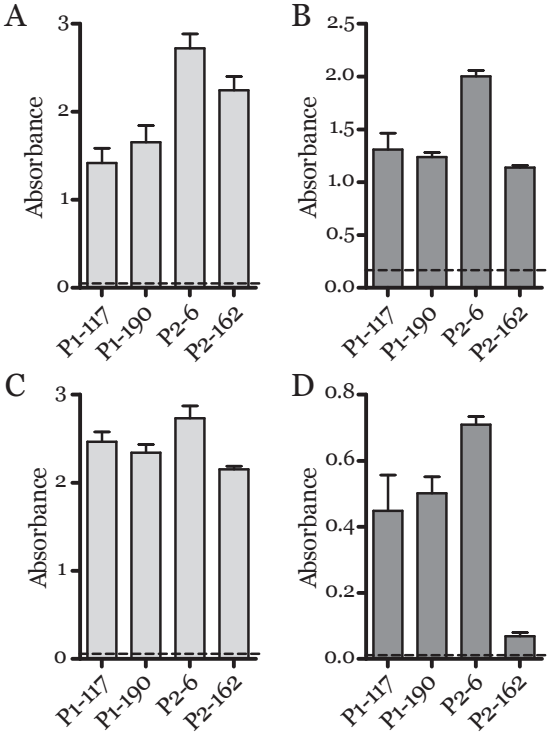


Figure 2-4 *Binding comparison between in-house and commercial solid-phase assays. Commercial (A) and in-house (B) anti-cardiolipin ELISAs and commercial (C) and in-house (D) anti-β2-glycoprotein I (β2GPI) ELISAs. Antibodies were used at a concentration of 2 μg mL^{-1} in normal pooled plasma. Dashed lines indicate the absorbance of negative serum controls. Data are expressed in absorbance units, and represent mean ± range of two separate experiments.*

LAC activity of full-length IgG constructs

Several assays were used to assess the LAC activity of the patient-derived anti-β2GPI mAbs. The results are summarized in Table 2-2. We used two clotting assays that are recommended for the diagnostics of APS and that employ low levels of phospholipids: the dRVVt, representing the common pathway of coagulation, and the APTT-based assay, which evaluates the contact activation pathway. In addition, we assessed thrombin generation (CAT method) initiated by tissue factor. P1-117, P1-190 and P2-6 showed substantial LAC activity, although lower than that of the positive control reference plasma, as became apparent from the increase in clotting time (dRVVt and APTT). Of the three CAT parameters, the lag time was also prolonged (Table 2-2). P2-162 displayed low LAC activity in the APTT assay, but its effect on the dRVVt and CAT lag time lacked statistical significance.

In addition to the lag time, the CAT parameters endogenous thrombin potential (ETP) and thrombin peak height were evaluated. The effect of the antibodies on these parameters proved to be limited and inconsistent. P2-6 showed some apparent increase in ETP and peak value, but this was also observed for the negative control antibody CLB-Pro35. Therefore, the stimulatory effect of P2-6 cannot be unambiguously attributed to its interaction with β2GPI. P1-117, P1-190 and P2-162 displayed reduction of the thrombin peak level, but their effect on the ETP was only statistically significant if calculated against the buffer control (Table 2-2). We conclude that, of the CAT parameters assessed, the lag time, but not ETP or thrombin peak, respond to our anti-β2GPI antibodies. Moreover, the prolongation of the lag time paralleled the clotting time prolongation observed in the dRVVt and the APTT assay.

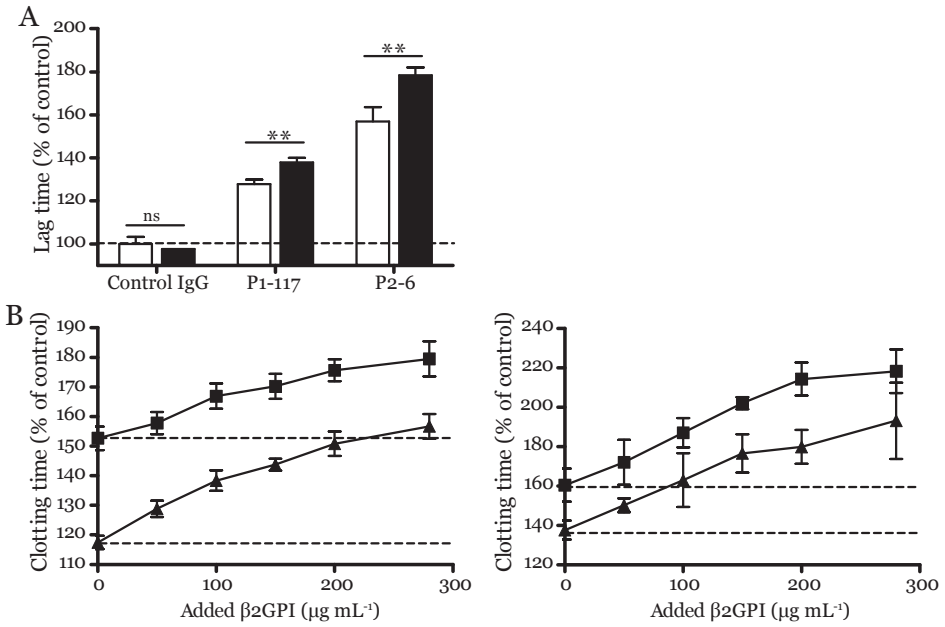


Figure 2-5 The effect of added β2-glycoprotein I (β2GPI) on the lupus anticoagulant (LAC) activity. *(A) Influence of added β2GPI on thrombin generation (calibrated automated thrombography). Antibodies were used at a concentration of 200 μg mL^{-1} plasma. β2GPI was added at a concentration of 75 μg mL^{-1} plasma (black bars). One picomolar tissue factor was used for thrombin generation initiation. (B) Plasma-derived β2GPI at increasing concentrations was added to normal plasma spiked with plasma containing 240 μg mL^{-1} antibody P1-117 (triangle) or P2-6 (square). After 1 h of incubation at 37 °C, LAC activity was assayed with the dilute Russell's viper venom test (left) or an activated partial thromboplastin time assay (right). Dashed lines show the LAC activity of antibodies without added β2GPI. Measurements were performed in triplicate; data are represented as mean ± standard deviation. Statistical significance: NS, not significant; **P < 0.01.*

Table 2-2 Antibody performance in clotting assays and thrombography.

Antibody	**dRVVt**		**APTT**		**CAT**					
added	***clotting time, s***		***clotting time, s***		***lag time, min***		***ETP, nM·min***		***peak, nM***	
None	48.4 ± 2.0		38.7 ± 0.9		2.55 ± 0.01		1867 ± 15		281 ± 4	
Control	47.9 ± 0.8^{NS}	[0.98]	39.5 ± 0.2^{NS}	[1.01]	2.55 ± 0.16^{NS}	[1.00]	1995 ± 49*	[1.07]	301 ± 4***	[1.07]
P1-117	61.7 ± 3.1***	[1.27]	58.0 ± 2.4***	[1.49]	3.33 ± 0.16***	[1.31]	1763 ± 38$^{NS\ (***)}$	[0.94]	244 ± 2***	[0.87]
P1-190	59.0 ± 1.9***	[1.22]	52.8 ± 0.4***	[1.37]	3.66 ± 0.16***	[1.44]	1810 ± 44$^{NS\ (**)}$	[0.97]	230 ± 1***	[0.82]
P2-6	74.3 ± 2.4***	[1.53]	60.9 ± 0.6***	[1.63]	3.77 ± 0.16***	[1.48]	2083 ± 67$^{***\ (NS)}$	[1.12]	304 ± 6$^{***\ (NS)}$	[1.08]
P2-162	49.4 ± 1.0^{NS}	[1.02]	42.8 ± 0.2**	[1.07]	2.77 ± 0.16^{NS}	[1.09]	1851 ± 56$^{NS\ (*)}$	[0.99]	262 ± 6***	[0.93]
LA High	87.4 ± 0.8***	[1.79]	90.1 ± 5.5***	[2.26]	no data		no data		no data	

*APTT, activated partial thromboplastin time; CAT, calibrated automated thrombography; dRVVt, dilute Russell's viper venom test; ETP, endogenous thrombin potential; NS, not significant. Antibodies were used at a concentration of 320 μg mL^{-1} in plasma. CLB-Pro35 was used as a negative control IgG. LA (Control) High, a lupus anticoagulant-positive reference plasma, was used as a positive control. For CAT analysis, 5 pM tissue factor was used for thrombin generation initiation. Measurements were performed in triplicate. Data are represented as mean ± standard deviation. The ratio against parameters obtained in the absence of added antibody is shown in parentheses. P-values were calculated against parameters obtained from incubation without added antibody. P-values were also calculated against control antibody, and are indicated in parentheses when different: NS, not significant; *P < 0.05; **P < 0.01; ***P < 0.001.*

As aPL antibodies exert their action via β2GPI, we were interested in determining what would be the effect of additional β2GPI in the assays. A prolongation of the lag time by additional β2GPI was observed by thrombography (Fig. 2-5A). Similarly, in both the dRVVt and the APTT assay, an increase in β2GPI concentration caused a gradual increase in clotting time prolongation (Fig. 2-5B). The addition of β2GPI without any of the antibodies did not affect either the APTT, dRVVt result or the lag time in thrombin generation (not shown). The results show that additional β2GPI has a potentiating effect on the LAC activity of patient-derived antibodies.

Antibody titration in the presence of added β2GPI revealed that the LAC activity caused by patient-derived antibodies was concentration-dependent (Fig. 2-6). P2-6 displayed the strongest LAC activity in both clotting assays, whereas P2-162 had very low LAC activity. P1-117 and P1-190 showed practically identical LAC activity in the dRVVt that was lower than that of P2-6. However, in the APTT assay, P1-190 retained its LAC activity, whereas P1-117 displayed LAC activity that was comparable to that of P2-6. Taking these findings together, our antibodies showed β2GPI-dependent LAC activity in various clotting assays that relates to their binding to β2GPI. The patient-derived antibodies that displayed the highest LAC activity (P1-117 and P2-6) also displayed the highest affinity for β2GPI. On the other hand, P2-162 revealed very low affinity for β2GPI and showed the lowest LAC activity. This suggests that our patient-derived antibodies display LAC activity that relates to their binding to β2GPI.

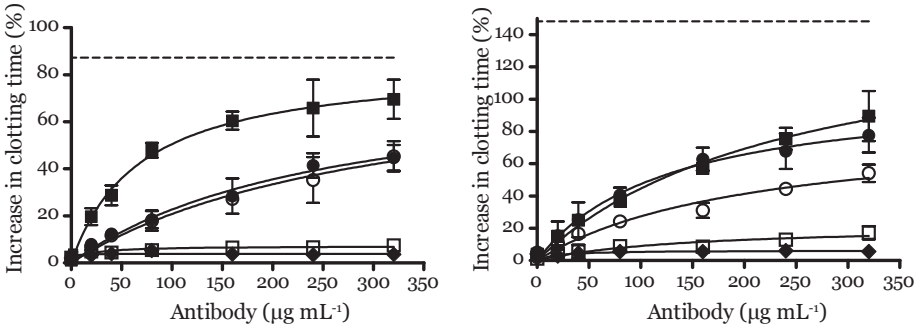


Figure 2-6 Lupus anticoagulant (LAC) activity of antibodies in the presence of added β2-glycoprotein I (β2GPI). *Normal plasma was incubated for 1 h at 37 °C with various concentrations of antibodies P1-117 (closed circle), P1-190 (open circle), P2-6 (closed square), P2-162 (open square) and CLB-Pro35 (diamond) as control IgG in the presence of plasma containing 150 μg mL⁻¹ β2GPI. LAC activity was assayed with the dilute Russell's viper venom test assay (left) or an activated partial thromboplastin time assay (right). Measurements were performed in triplicate; error bars represent mean ± standard deviation. The dashed line represents the LAC activity of positive control plasma (LA Control High) without added β2GPI.*

Discussion

aPL antibodies are a heterogeneous population of antibodies recognizing different antigens. Even within one antigen, such as β2GPI, different antibodies

have been reported to bind to different parts of the molecule [7]. Because of this heterogeneity, studies with patient antibodies are usually performed with a pool of purified antibodies, without knowledge of exactly which population of antibodies is responsible for an observed effect. Therefore, we designed a study for the construction of patient-derived mAbs directed against β2GPI. In this study, we show the successful construction of human monoclonal anti-β2GPI antibodies that display LAC activity related to their affinity for β2GPI. These antibodies were cloned from the immunoglobulin variable heavy chain repertoire of two patients with APS, with the use of phage display technology.

Domain I of β2GPI is thought to be the most relevant in APS pathogenesis among all of the epitopes proposed for pathogenic anti-β2GPI antibodies [5–8]. A discontinuous epitope on domain I has been suggested, comprising residues Asp8–Asp9, Arg39–Arg43 and the domain I–II interlinker region [8]. Epitope mapping (Fig. 2-3) revealed that both antibodies of patient 2 bound specifically to domain I. This was not surprising, because they were derived from phages that were deliberately selected as being positive against isolated domain I. In contrast, both antibodies of patient 1 were derived from phages that were positive for binding to intact β2GPI, but not to isolated domain 1. Nevertheless, the epitope of these antibodies did involve domain I, albeit in combination with domain II (Fig. 2-3). It seems likely that the epitope of the patient 1 antibodies comprises the domain I and domain II linking region, and, as such, it could be similar to the epitope identified by Ioannou *et al.* [8]. However, a domain II-dependent conformational epitope in domain I would be another possibility. Further epitope fine-mapping studies are necessary to identify the precise region(s) on β2GPI. The notion that patient 1 antibodies require both domain I and domain II for binding indicates that it may not be sufficient to screen for pathogenic antibodies in an assay based on recognition of isolated domain I of β2GPI only. Future studies are needed to investigate whether an assay based on the recognition of both domains is, indeed, superior.

One limitation of our study was that our patient-derived antibodies may not fully represent the original patient antibodies, as we used the variable light chain repertoire from a naïve donor. Moreover, phage display generates random immunoglobulin variable heavy and light chain combinations, and also the original class and subclass characteristics of antibodies are lost. However, selection of antibodies *in vivo* is mimicked by multiple stringent selection rounds that help to identify specific binding scFvs from a large pool of possible combinations [34]. Furthermore, the pathogenicity of aPL antibodies has been attributed to the IgG heavy chain [35]. The VH domains of both clones of patient 2 were derived from germline VH4-4, whereas both clones of patient 1 contained the VH domain of germline VH3-23. It is interesting to note that the VH3-23 is the most commonly used VH germline in IgM producing B cells, regardless of antigen [36]. Furthermore, this germline has also been identified in other human mAbs with LAC activity or reactivity towards anionic phospholipids or β2GPI, generated by hybridomas or phage display technology [2,25,36]. The IgG light chain may have some impact on the pathogenicity of aPL antibodies as well, because, in our experiments, patient 1 antibodies with identical CDR3 regions but slightly differing light chains showed minor differences in affinity for β2GPI and in LAC activity. However, changed somatic hypermutation patterns

in heavy chains and slightly different light chains of patient 2 antibodies may explain the pronounced differences in antibody binding and activity between P2-6 and P2-162.

Soluble β2GPI may have a closed circular conformation that prevents epitope G40–R43 of domain I from interacting with anti-β2GPI antibodies [10]. Pathogenic anti-β2GPI antibodies in plasma from APS patients, therefore, may recognize β2GPI only after a conformational change, as induced, for example, by interaction of β2GPI with a hydrophilic surface [9]. Accordingly, our patient-derived mAbs showed increased binding to β2GPI immobilized on a hydrophilic surface as compared with β2GPI immobilized on a hydrophobic surface (Fig. 2-2B). However, the reduction in response on hydrophobic plates was not as drastic as previously observed with type A (pathogenic) antibodies [9], suggesting that the conformational change is not crucial for epitope recognition. Another aspect of the pathogenesis of APS is β2GPI dimerization. The intrinsically low-affinity aPL antibodies need a high density of immobilized β2GPI to ensure dimerization of β2GPI and subsequent bivalent binding [13,33]. Dimers of β2GPI can also mimic the effects of antibody–β2GPI complexes *in vitro* [37]. Interestingly, all scFv clones recognized β2GPI and its domain I predominantly as phages that display multiple copies of scFv, or as full-length IgG molecules displaying two copies of scFv. In contrast, a monovalent scFv displayed weak interaction with β2GPI (Fig. 2-2A). This finding supported the notion that bivalency or multivalency, in the case of phages, is important in antibody binding to β2GPI [13,33].

Assessment of the LAC activity of antibody-spiked plasma samples in various clotting assays showed that the human mAbs prolonged the clotting time in clotting assays and lag time in thrombography (Table 2-2) in a dose-dependent and β2GPI-dependent manner (Figs 2-5 and 2-6). Moreover, the ratios of prolongation were comparable among the various assays, and were in accordance with the antibody affinity for β2GPI (Fig. 2-2C), with higher-affinity antibodies showing stronger LAC activity. However, the LAC activity of our cloned mAbs added to normal plasma at a concentration of 320 μg mL^{-1} was still below that observed for polyclonal antibodies present in APS plasma (Fig. 2-6). The rationale behind this phenomenon is not clear, as the exact concentration of pathogenic antiβ2GPI antibodies in plasma of APS patients is not known. It is possible that a mixture of different antibodies present in APS plasma is more potent in displaying LAC activity than isolated mAbs. In addition, the LAC activity of our cloned mAbs was investigated in NPP, whose composition might differ from that of APS plasma. Pertinent to this view is the notion of differences in the glycosylation of β2GPI between APS patients and healthy controls [38]. This suggests that the properties of β2GPI itself might also contribute to increased LAC activity of antibodies present in APS plasma.

One striking finding is the limited number of scFVs that were identified in our study. We have previously used the same methods to analyze the immune repertoire of patients with antibodies against ADAMTS13 [28], and with antibodies against factor VIII [39,40]. The various scFVs isolated in those studies displayed much higher affinity for their target antigen (typical K_d values between 10^{-7} and 10^{-9} M) than the anti-β2GPI antibodies in our study (Fig. 2-2C). It seems possible that the phage display procedure, with its multiple panning steps, more easily detects high-affinity scFV-expressing phages.

Consequently, the P1 and P2 antibodies that we identified may reflect only a fraction of the anti-β2GPI repertoire of these patients. Nevertheless, these mAbs seem to be typical APS-related antibodies, as they show evidence for the need for dimerization of β2GPI. Moreover, in addition to β2GPI binding, they display LAC activity in a variety of assays used in the laboratory diagnosis of APS (Fig. 2-4; Table 2-2). As such, these mAbs may provide useful tools for the standardization of assays for the detection of aPL antibodies.

Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

References

1. Miyakis S, Lockshin MD, Atsumi T, Branch DW, Brey RL, Cervera R, Derksen RHWM, de Groot PG, Koike T, Meroni PL, Reber G, Shoenfeld Y, Tincani A, Vlachoyiannopoulos PG, Krilis SA. International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). *J Thromb Haemost*. 2006; **4**: 295-306.
2. Chukwuocha RU, Zhu M, Cho CS, Visvanathan S, Hwang KK, Rahman A, Chen PP. Molecular and genetic characterizations of five pathogenic and two non-pathogenic monoclonal antiphospholipid antibodies. *Mol Immunol*. 2002; **39**: 299-311.
3. Farmer-Boatwright MK, Roubey RA. Venous thrombosis in the antiphospholipid syndrome. *Arterioscler Thromb Vasc Biol*. 2009; **29**: 321-5.
4. Shoenfeld Y, Blank M, Cervera R, Font J, Raschi E, Meroni PL. Infectious origin of the antiphospholipid syndrome. *Ann Rheum Dis*. 2006; **65**: 2-6.
5. Iverson GM, Victoria EJ, Marquis DM. Anti-β2 glycoprotein I (β2GPI) autoantibodies recognize an epitope on the first domain of β2GPI. *Proc Natl Acad Sci U S A*. 1998; **95**: 15542-6.
6. Iverson GM, Reddel S, Victoria EJ, Cockerill KA, Wang YX, Marti-Renom MA, Sali A, Marquis DM, Krilis SA, Linnik MD. Use of single point mutations in domain I of β2-glycoprotein I to determine fine antigenic specificity of antiphospholipid autoantibodies. *J Immunol*. 2002; **169**: 7097-103.
7. De Laat B, Derksen RHWM, Urbanus RT, de Groot PG. IgG antibodies that recognize epitope Gly40-Arg43 in domain I of β2-glycoprotein I cause LAC, and their presence correlates strongly with thrombosis. *Blood*. 2005; **105**: 1540-5.
8. Ioannou Y, Rahman A. Domain I of β2-glycoprotein I: its role as an epitope and the potential to be developed as a specific target for the treatment of the antiphospholipid syndrome. *Lupus*. 2010; **19**: 400-5.
9. De Laat B, Derksen RHWM, van Lummel M, Pennings MT, de Groot PG. Pathogenic anti- β2-glycoprotein I antibodies recognize domain I of β2-glycoprotein I only after a conformational change. *Blood*. 2006; **107**: 1916-24.
10. Ağar Ç, van Os GMA, Mörgelin M, Sprenger RR, Marquart JA, Urbanus RT, Derksen RHWM, Meijers JCM, de Groot PG. β2-glycoprotein I can exist in two conformations: implications for our understanding of the antiphospholipid syndrome. *Blood*. 2010; **116**: 1336-43.
11. Bouma B, de Groot PG, van den Elsen JM, Ravelli RB, Schouten A, Simmelink MJ, Derksen RHWM, Kroon J, Gros P. Adhesion mechanism of human β2-glycoprotein I to phospholipids based on its crystal structure. *EMBO J*. 1999; **18**: 5166-74.

12. Schwarzenbacher R, Zeth K, Diederichs K, Gries A, Kostner GM, Laggner P, Prassl R. Crystal structure of human β2-glycoprotein I: implications for phospholipid binding and the antiphospholipid syndrome. *EMBO J*. 1999; **18**: 6228-39.

13. Willems GM, Janssen MP, Pelsers MM, Comfurius P, Galli M, Zwaal RF, Bevers EM. Role of divalency in the high-affinity binding of anticardiolipin antibody-β2-glycoprotein I complexes to lipid membranes. *Biochemistry*. 1996; **35**: 13833-42.

14. Pierangeli SS, Chen PP, Gonzalez EB. Antiphospholipid antibodies and the antiphospholipid syndrome: an update on treatment and pathogenic mechanisms. *Curr Opin Hematol*. 2006; **13**: 366-75.

15. Del Papa N, Sheng YH, Raschi E, Kandiah DA, Tincani A, Khamashta MA, Atsumi T, Hughes GRV, Ichikawa K, Koike T, Balestrieri G, Krilis SA, Meroni PL. Human β2-glycoprotein I binds to endothelial cells through a cluster of lysine residues that are critical for anionic phospholipid binding and offers epitopes for anti-β2-glycoprotein I antibodies. *J Immunol*. 1998; **160**: 5572-8.

16. Dignat-George F, Camoin-Jau L, Sabatier F, Arnoux D, Anfosso F, Bardin N, Veit V, Combes V, Gentile S, Moal V, Sanmarco M, Sampol J. Endothelial microparticles: a potential contribution to the thrombotic complications of the antiphospholipid syndrome. *Thromb Haemost*. 2004; **91**: 667-73.

17. Wolberg AS, Roubey RA. Mechanisms of autoantibody-induced monocyte tissue factor expression. *Thromb Res*. 2004; **114**: 391-6.

18. Pennings MTT, Derksen RHWM, van Lummel M, Adelmeijer J, VanHoorelbeke K, Urbanus RT, Lisman T, de Groot, PG. Platelet adhesion to dimeric β2-glycoprotein I under conditions of flow is mediated by at least two receptors: glycoprotein Ibalpha and apolipoprotein E receptor 2'. *J Thromb Haemost*. 2007; **5**: 369-77.

19. Safa O, Esmon CT, Esmon NL. Inhibition of APC anticoagulant activity on oxidized phospholipid by anti-β2-glycoprotein I monoclonal antibodies. *Blood*. 2005; **106**: 1629-35.

20. Rand J, Wu X. Antibody-mediated disruption of the annexin-V antithrombotic shield: a new mechanism for thrombosis in the antiphospholipid syndrome. *Thromb Haemost*. 1999; **82**: 649-55.

21. Arnout J. Antiphospholipid syndrome: diagnostic aspects of lupus anticoagulants. *Thromb Haemost*. 2001; **86**: 83-91.

22. Lai CJ, Rauch J, Cho CS, Zhao Y, Chukwuocha RU, Chen PP. Immunological and molecular analysis of three monoclonal lupus anticoagulant antibodies from a patient with systemic lupus erythematosus. *J Autoimmun*. 1998; **11**: 39-51.

23. Pierangeli SS, Liu X, Espinola R, Olee T, Zhu M, Harris NE, Chen PP. Functional analyses of patient-derived IgG monoclonal anticardiolipin antibodies using *in vivo* thrombosis and *in vivo* microcirculation models. *Thromb Haemost*. 2000; **84**: 388-95.

24. Chukwuocha RU, Hsiao ET, Shaw P, Witztum JL, Chen PP. Isolation, characterization and sequence analysis of five IgG monoclonal anti-β2-glycoprotein I and anti-prothrombin antigen-binding fragments generated by phage display. *J Immunol*. 1999; **163**: 4604-11.

25. Languren M, Becerril B, Cabral AR, Fernandez-Altuna LE, Pascual V, Hernandez-Ramirez DF, Cabiedes, J. Characterization of monoclonal anti-β2-glycoprotein I and anti-prothrombin antibody fragments generated by phage display from a patient with primary antiphospholipid syndrome. *J Autoimmun*. 2006; **26**: 57-65.

26. Prinz N, Hauser F, Lorenz M, Lackner KJ, von Landenberg P. Structural and functional characterization of a human IgG monoclonal antiphospholipid antibody. *Immunobiology*. 2010; **216**: 145–51.

27. Azzazy HME, Highsmith WE. Phage display technology: clinical applications and recent innovations. *Clin Biochem*. 2002; **35**: 425-45.

28. Luken BM, Kaijen PHP, Turenhout EAM, Kremer Hovinga JA, van Mourik JA, Fijnheer R, Voorberg J. Multiple B-cell clones producing antibodies directed to the spacer and disintegrin/thrombospondin type-1 repeat 1 (TSP1) of ADAMTS13 in a patient with acquired thrombotic thrombocytopenic purpura. *J Thromb Haemost*. 2006; **4**: 2355-64.

29. Pos W, Luken BM, Kremer Hovinga JA, Turenhout EAM, Scheiflinger F, Dong JF, Fijnheer R, Voorberg, J. VH1-69 germline encoded antibodies directed towards ADAMTS13 in patients with acquired thrombotic thrombocytopenic purpura. *J Thromb Haemost*. 2009; **7**: 421-8.

30. Borchiellini A, Fijnvandraat K, ten Cate JW, Pajkrt D, van Deventer SJH, Pasterkamp G, Meijer-Huizinga F, Zwart-Huinink L, Voorberg J, van Mourik JA. Quantitative analysis of von Willebrand factor propeptide release *in vivo*: effect of experimental endotoxemia and administration of 1-deamino-8-D-arginine vasopressin in humans. *Blood*. 1996; **88**: 2951-8.

31. Friguet B, Chaffotte AF, Djavadi-Ohaniance L, Goldberg ME. Measurements of the true affinity constant in solution of antigen-antibody complexes by enzyme-linked immunosorbent assay. *J Immunol Methods*. 1985; **77**: 305-19.

32. Van Veen JJ, Gatt A, Bowyer AE, Cooper PC, Kitchen S, Makris M. Calibrated automated thrombin generation and modified thromboelastometry in haemophilia A. *Thromb Res*. 2009; **123**: 895-901.

33. Sheng Y, Kandiah DA, Krilis SA. Anti-β2-glycoprotein I autoantibodies from patients with the "antiphospholipid" syndrome bind to β2-glycoprotein I with low affinity: dimerization of β2-glycoprotein I induces a significant increase in anti-β2-glycoprotein I antibody affinity. *J Immunol*. 1998; **161**: 2038-43.

34. Cabiedes J, Cabral AR. Anti- β2-glycoprotein-I antibodies in scFv format. *Autoimmun Rev*. 2005; **4**: 289-95.

35. Blank M, Waisman A, Mozes E, Koike T, Shoenfeld Y. Characteristics and pathogenic role of anti-β2-glycoprotein I single-chain Fv domains: induction of experimental antiphospholipid syndrome. *Int Immunol*. 1999; **11**: 1917-26.

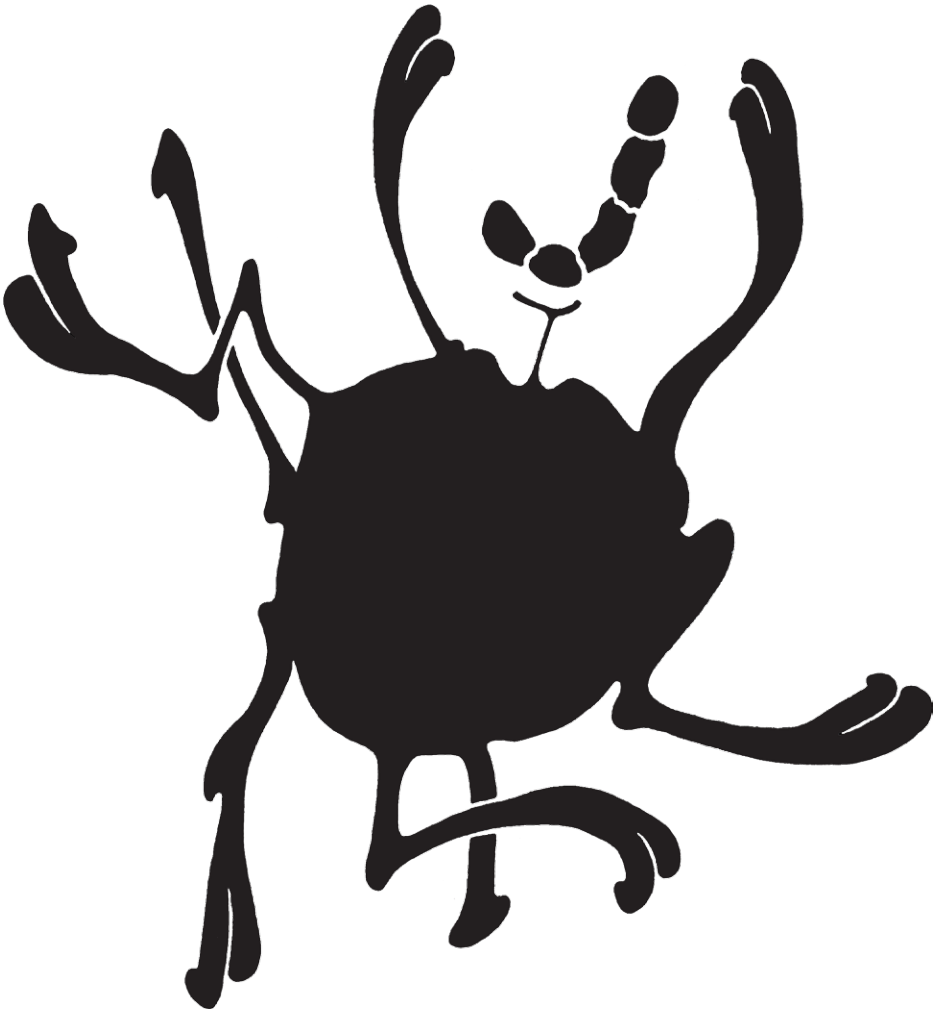
36. Giles IP, Haley JD, Nagl S, Isenberg DA, Latchman DS, Rahman A. A systematic analysis of sequences of human antiphospholipid and anti-β2-glycoprotein I antibodies: The importance of somatic mutations and certain sequence motifs. *Semin Arthritis Rheum*. 2003; **32**: 246-65.

37. Lutters BCH, Meijers JCM, Derksen RHWM, Arnout J, de Groot PG. Dimers of β2-glycoprotein I mimic the *in vitro* effects of β2-glycoprotein I–anti-β2-glycoprotein I antibody complexes. *J Biol Chem*. 2001; **276**: 3060-7.

38. Kondo A, Miyamoto T, Yonekawa O, Giessing AM, Osterlund EC, Jensen ON. Glycopeptide profiling of β2-glycoprotein I by mass spectrometry reveals attenuated sialylation in patients with antiphospholipid syndrome. *J Proteomics*. 2009; **73**: 123-33.

39. Van den Brink EN, Turenhout EAM, Bovenschen N, Heijnen BGAD, Mertens K, Peters M, Voorberg J. Multiple VH genes are used to assemble human antibodies directed toward the A3-C1 domains of factor VIII. *Blood*. 2001; **97**: 966-72.

40. Van den Brink EN, Bril WS, Turenhout EAM, Zuurveld M, Bovenschen N, Peters M, Yee, TT, Mertens K, Lewis, DA, Ortel, TL, Lollar, P, Scandella D, Voorberg J. Two classes of germline genes both derived from the VH1 family direct the formation of human antibodies that recognize distinct antigenic sites in the C2 domain of factor VIII. *Blood*. 2002; **99**: 2828-34.



Chapter 3

Endocytosis and presentation of β2-glycoprotein I by dendritic cells

Ilze Dienava-Verdoold,[1] Simon D. van Haren,[2] Herm-Jan M. Brinkman,[1] Jan Voorberg,[2] Alexander B. Meijer,[1,3] Koen Mertens,[1,3] and Eszter Herczenik[2]

[1]Department of Plasma Proteins, Sanquin Research, Amsterdam, The Netherlands
[2]Sanquin-AMC Landsteiner Laboratory and Van Creveld Laboratory, Amsterdam, The Netherlands
[3]Utrecht Institute for Pharmaceutical Sciences, Utrecht University, Utrecht, The Netherlands

Manuscript in preparation

Abstract

Autoantibodies targeting β2-glycoprotein I (β2GPI) are strongly correlated with thrombotic complications in patients with antiphospholipid syndrome. Generation of β2GPI-specific B cell responses requires endocytosis and subsequent presentation of β2GPI-derived peptides on MHC class II molecules by antigen presenting cells to CD4+ T cells. Here, we investigated the uptake of β2GPI by human monocyte derived immature dendritic cells. Our findings show that β2GPI is rapidly internalized by iDCs in a Ca^{2+}-dependent manner. Blocking experiments using sucrose suggest that β2GPI is internalized via receptor-mediated endocytosis. The majority of endocytosed β2GPI is present in early endosomes where it co-localizes with constitutively recycling macrophage mannose receptor. We also explored whether β2GPI-derived peptides are presented on MHC class II molecules employing dendritic cells derived of HLA-typed normal individuals. Unexpectedly, only a limited number of peptides derived of domain V of β2GPI was presented on MHC class II molecules. One of the peptides identified overlapped with a previously identified binding site for acidic phospholipids and lipopolysaccharide (LPS). These findings show that despite its rapid endocytosis only a limited number of β2GPI-derived peptides is presented on MHC class II molecules.

Introduction

Antiphospholipid syndrome (APS) is an autoimmune disorder associated with venous and arterial thrombosis as well as recurrent foetal loss [1]. It has now well-recognized that β2-glycoprotein I (β2GPI) provides the principal target of antiphospholipid antibodies that develop in patient with APS [2]. β2-glycoprotein I (also known as apolipoprotein H) is an anionic phospholipid binding protein that is comprised of 5 complement control protein (CCP) domains [3]. Domain V of β2GPI contains a positively charged exposed surface and a flexible hydrophobic loop that both have been implicated in its binding to anionic phospholipids [3,4]. The three-dimensional structure of β2GPI revealed a "J-shaped" or "fish-hook" like structure with domain V oriented away from the other 4 CCP domains [3,5,6]. Several studies have shown that β2GPI can exist in two different conformations; the circulating form is a "closed" structure that is preserved through the interaction between domain I and V of β2GPI [7]. Upon binding to anionic surfaces or cardiolipin, or upon exposure to high pH or high salt concentrations β2GPI is converted to an "open" configuration [7–9].

Several lines of evidence suggest that conformational changes in β2GPI are crucially involved in the pathogenesis of APS. It has been well established that pathogenic antibodies directed towards residues Arg39 to Arg43 in domain I of β2GPI comprise a major fraction of anti-β2GPI antibodies [10,11]. Interestingly, exposure of this antigenic determinant requires a conformational change of β2GPI that is induced by its immobilization on hydrophilic surfaces [2,12]. Similarly, exposure of a cryptic CD4+ T cell epitope in domain V is dependent on binding of β2GPI to anionic phospholipids [13]. Peptide mapping studies showed that this dominant CD4+ T cell is contained within residues 276–290 (KVSFFCKNKEKKCSY) which comprises part of the phospholipid binding site of β2GPI [14].

CD4+ T cell responses promote affinity maturation and Ig class-switching. Analysis of anti-β2GPI antibodies at the clonal level using phage display have shown that the variable region of these antibodies have been modified by somatic hypermutation strongly suggesting functional involvement of β2GPI-specific CD4+ T cells in the production of high affinity antibodies by B cells [15,16]. Although different classes and subclasses of anti-β2GPI antibodies have been detected the presence of high affinity anti-β2GPI IgGs has been suggested to correlate with thrombosis [11,17].

The induction of immune responses requires endocytosis and processing of β2GPI protein by antigen presenting cells and the subsequent antigen-derived peptide presentation on MHC class II molecules to CD4+ T cells. β2GPI has been implicated to bind to multiple cell surface structures such as heparan sulfates, annexin II and low density lipoprotein (LDL) receptor family members [1]. Here, we explored the uptake and processing of β2GPI by antigen presenting cells using immature human monocyte derived dendritic cells (iDCs) as a model system. Our findings show that β2GPI is rapidly internalized in a dose-dependent manner by iDCs. Despite its rapid and efficient endocytosis, only a limited number of β2GPI-derived peptides were presented on MHC class II molecules. Apparently, β2GPI-derived peptides do not efficiently compete with peptides derived from other endogenous and exogenous antigens for binding to MHC class II molecules on antigen presenting cells.

Materials and Methods

Reagents

The following reagents were used in this study: penicillin and streptomycin (Invitrogen, Paisley, Scotland), anti-human CD206 (MR) and CD91 (LRP1) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-early endosomal antigen-1 (EEA-1, BD Biosciences, San Jose, CA, USA), human serum albumin (HSA, Cealb, Sanquin Blood Supply, Amsterdam, The Netherlands), anti-human dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN, AbD Serotec, Germany), EDTA, sucrose, saponin from Quillaja Bark, mannan, and LPS from E. coli (Sigma-Aldrich, St. Louis, MO, USA), CD14 microbeads and human Fc receptor blocking reagent (MACS, Miltenyi Biotech, Auburn, CA, USA) blocking antibody LEAF anti-MR clone 15.2 and isotype control (Biolegend, San Diego, Ca, USA). Hybridoma L243 (anti-HLA-DR) was obtained from ATCC (Wesel, Germany). Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden); Cellgro cell culture medium, human recombinant GM-CSF and IL-4 (Cellgenix Technology Transfer, Freiburg, Germany) were used for generation of immature human monocyte-derived DCs (iDCs).

Generation of iDCs

Human iDCs were generated as described previously [18]. Blood was drawn from healthy volunteers in accordance with the Dutch regulations and following approval from Sanquin Ethical Advisory Board and was in agreement with the Declaration of Helsinki. The blood was collected in sterile and pyrogen-free 10 mL tubes containing EDTA as anticoagulant. Ficoll-Paque Plus density gradient separation was

performed to collect the Peripheral Blood Mononuclear Cells (PBMCs), according to manufacturer's instructions. The monocytes were purified from the PBMC fraction by magnetic separation with CD14 magnetic beads. The isolated monocytes were cultured in Cellgro cell culture medium supplemented with GM-CSF (1000 U mL^{-1}) and IL-4 (800 U mL^{-1}) at a concentration of 2.5×10^6 cells per 3 mL per well in a 6-well culture plate (BD Falcon, NJ, USA). After four to six days of culturing, iDCs were collected for experiments.

Purification of β2GPI

β2GPI was purified from freshly drawn, citrated human plasma essentially as described [16]. The final β2GPI eluate was dialyzed against phosphate-buffered saline (PBS). Purity was more than 95% as assessed by SDS-PAGE. Carbohydrate moieties present on β2GPI were identified using lectins *Galanthus nivalis* agglutinin (GNA) specific for terminal (1-3), (1-6) or (1-2) linked mannose, *Sambucus nigra* agglutinin (SNA) specific for (2-6) galactose linked sialic acid, *Maacka amurensis* agglutinin (MAA) specific for (2-3) galactose linked sialic acid, Peanut agglutinin (PNA) specific for core disaccharide ggalactose (1-3) N-acetylgalactosamine, and *Datura stramonium* agglutinin (DSA) specific for galactose (1-4) N-acetyl glucosamine all contained in the DIG glycan differentiation kit from Roche Applied Science (Penzberg, Germany).

Fluorescent labelling of β2GPI

β2GPI was fluorescently labelled either with fluorescein isothiocyanate (FITC) for flow cytometry experiments, or with Alexa Fluor 488 for confocal microscopy analysis following the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). The free dye was separated from the conjugate by spin filter loaded with gel resin provided in the kit. Finally, the degree of labelling of the purified conjugate was determined at 280 nm and 494 nm.

β2GPI uptake and cell surface binding by iDCs

To investigate if β2GPI is endocytosed by DCs, the cells were incubated with different concentrations of β2GPI-FITC (typically 1, 5, 10, 50, 100 nM) in Cellgro medium for 30 min at 37 °C. Medium was added as a negative control. After washing DCs with PBS containing 0.5% HSA (PBS-HSA 0.5%), the fluorescent intensity was measured by flow cytometry using LSRII (BD Biosciences, Uppsala, Sweden) and data were analyzed by GraphPad Prism 5.01 software (San Diego, CA, USA) and FlowJo 7.6.5 (Tree Start Inc, Ashland, OR, USA). To compare uptake with cell surface binding, DCs were incubated with β2GPI-FITC dilutions (typically 5 and/or 50 nM) for 30 min at 4 °C and 37 °C.

Confocal microscopy analysis

The uptake of β2GPI by DCs was visualized using confocal microscopy. Wells of Lab-Tek™ Chamber Slides (Nunc, Langenselbold, Germany) were coated with fibronectin

overnight at 37 °C. Then, the chambers were washed once with Cellgro medium. DCs were collected from the culture plates and 500 µL cell suspension (1×10^6 per mL in Cellgro medium supplemented with 1000 U mL^{-1} GM-CSF and 800 U mL^{-1} IL-4) was added to each chamber and incubated for at least 4 hours at 37 °C to ensure adhesion. After two careful washing steps with medium, the chambers were incubated with 100 nM Alexa Fluor 488 conjugated β2GPI or medium (control) for various time points (0, 2, 5, 10, 20 and 30 minutes) at 37 °C. The chambers were carefully washed with PBS and the cells were fixed with fresh 3.7 % paraformaldehyde in PBS for 20 min at room temperature (RT). Subsequently, fixed DCs were incubated for 1 h at RT with antibodies against early endosome antigen-1 (EEA-1), dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), LDL receptor related protein (LRP1) or mannose receptor (MR) diluted in PBS-HSA 0.5% containing 0.05% saponin and Fc receptor blocking reagent at a dilution of 1:500. After two washing steps with PBS-HSA 0.5%, Alexa Fluor 568 conjugated goat-anti mouse IgG was added as secondary antibody for 30 min at RT. Images were acquired by confocal microscopy (Zeiss LSM 510 META microscope) and the data were analyzed with Zen 2008 or LSM Image Browser softwares (Carl Zeiss MicroImaging GmbH, Jena, Germany).

Inhibition of β2GPI uptake by DCs

For blockage experiments, DCs (0.25×10^6 cells per sample) were preincubated with various antagonists before β2GPI-FITC was added for 30 min at 37 °C (uptake) or 4 °C (binding). To examine whether the uptake of β2GPI is receptor-mediated or Ca^{2+}-dependent, DCs were preincubated for 15 min at 37 °C with 0.75 M sucrose or 25 mM EDTA, respectively.

Purification of β2GPI peptides presented on HLA-DR of DCs

HLA-DR molecules were purified from DCs that were first pulsed with 100 nM β2GPI for 5 hours, and afterwards maturated overnight using 1 µg mL^{-1} LPS. Then, the cells were lysed and HLA-DR molecules were affinity-purified using antibody L243 as described previously [18]. The eluted peptide repertoire was analyzed by mass spectrometry using a LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific Inc., Bremen, Germany) and identified by Sequest search algorithm with Proteome Discoverer release version 1.1 software (Thermo Fisher Scientific Inc., Bremen, Germany) [18]. During the Sequest search we allowed a mass deviation of 20 ppm. In general mass deviations were below 3 ppm. Fragment mass tolerance was 0.8 Da. All peptides with a charge state of 2 have a minimal Xcorr score of 1.4; for peptides with a charge state 3 minimal Xcorr score is 1.6, and for charge state 4 minimal Xcorr score 1.8. All peptides not complying with these criteria were excluded.

Results

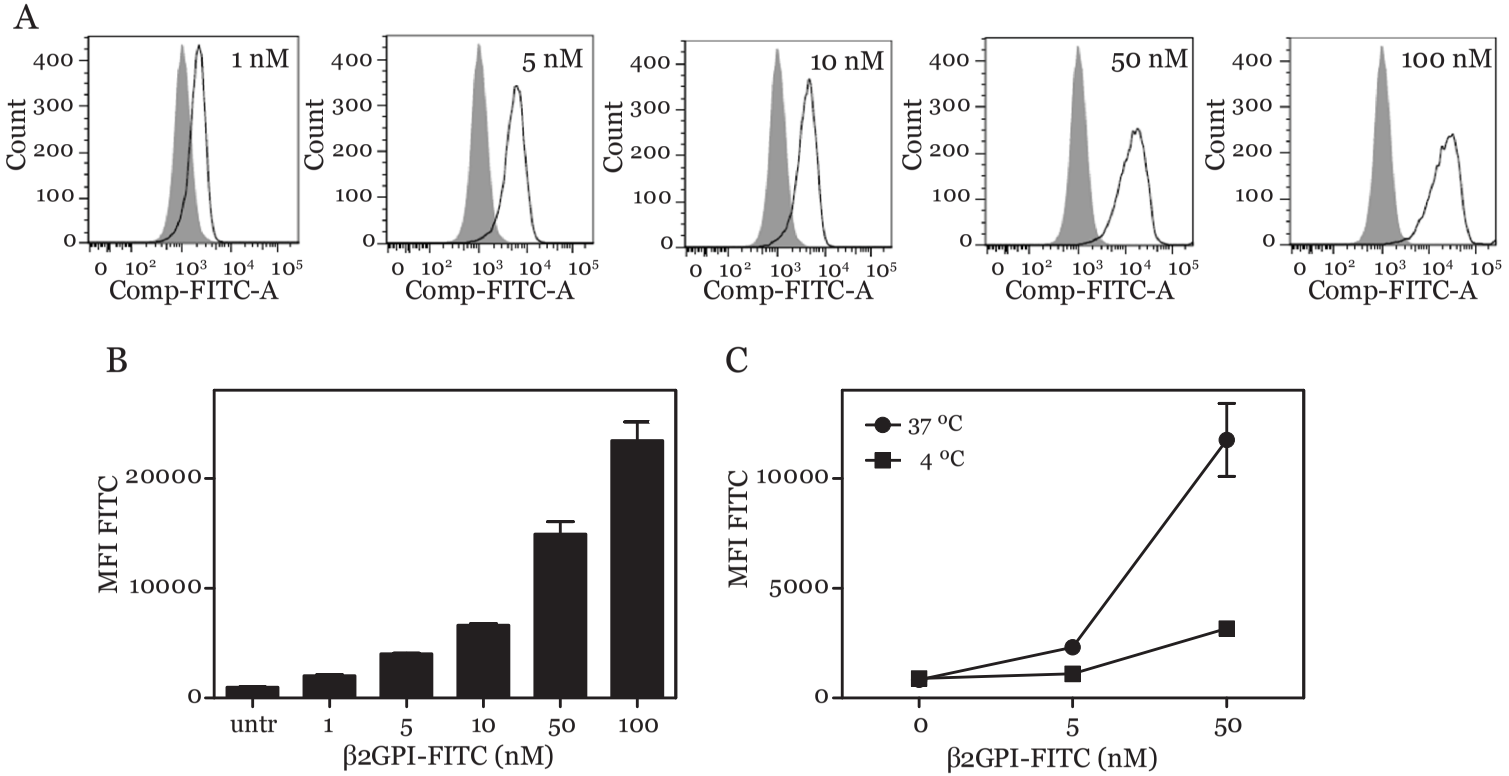


Figure 3-1 β2GPI is rapidly internalized by immature dendritic cells (DCs). *(A) β2GPI-FITC was added to DCs for 30 minutes at 37 °C at increasing concentrations (0, 1, 5, 10, 50, 100 nM) and the cells were analyzed by FACS. (B) Quantification of β2GPI–FITC uptake employing FACS. (C) Temperature-dependent uptake of β2GPI by iDCs. Uptake and cell surface binding of β2GPI was determined by incubating 0, 5 or 50 nM β2GPI-FITC at 37 °C (uptake and binding) or 4 °C (binding only). Data are represented as mean of at least three independent experiments ± standard deviation.*

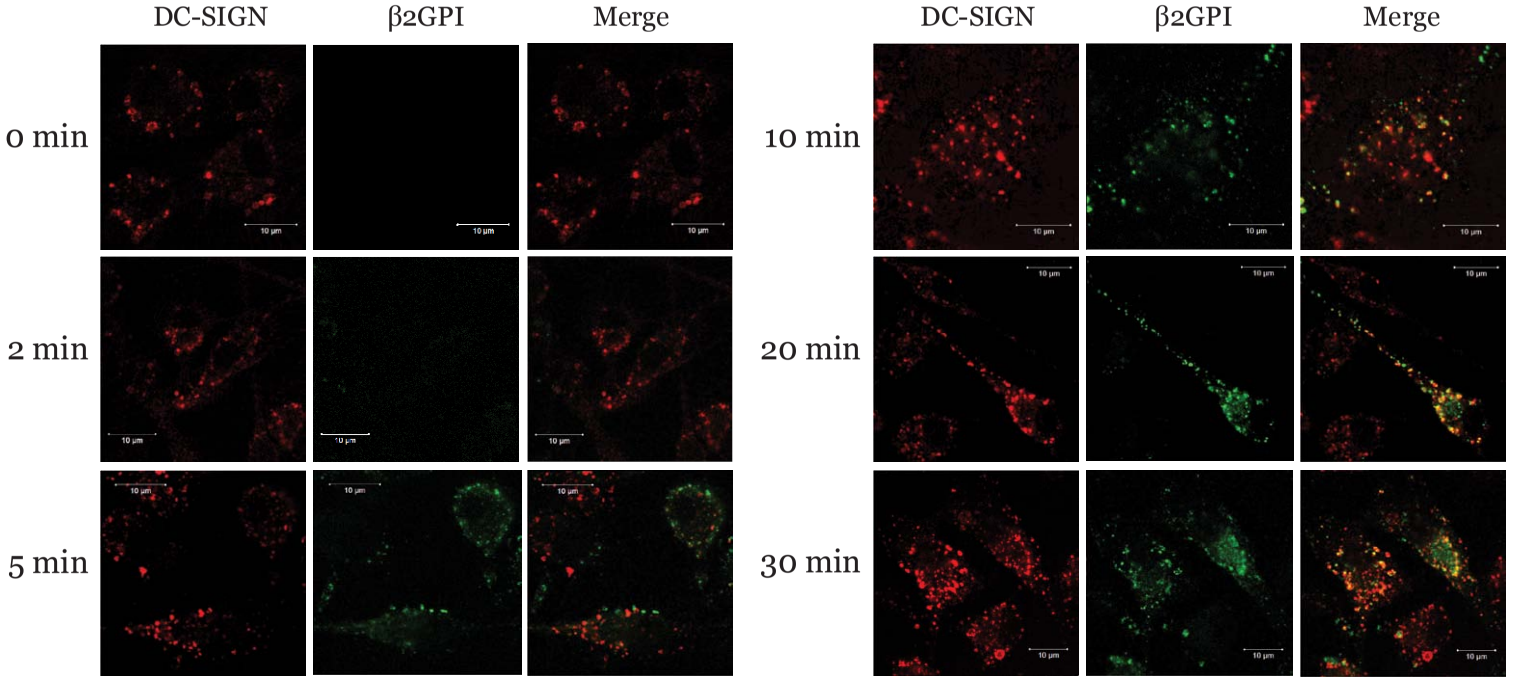


Figure 3-2 β2GPI is transferred to early endosomes following its uptake by DCs. *100 nM β2GPI-488 was added to DCs on fibronectin-coated glass slides for 0, 2, 5, 10, 20 and 30 minutes at 37 °C. The cells were subsequently fixed, stained for early endosome marker EEA-1 (in red) and analyzed by confocal microscopy.*

Endocytosis of β2GPI by immature DCs

To study whether β2GPI is endocytosed by immature DCs, we incubated these cells with increasing concentrations of FITC-labelled β2GPI. A concentration-dependent increase in the uptake of β2GPI was observed (Fig. 3-1A,B). To determine the ratio of cell surface binding and endocytosis, β2GPI-FITC was incubated with DCs either at 4 °C (binding) or at 37 °C (binding and uptake). Incubation at 4 °C resulted in a reduced signal when compared to that observed for incubation at 37 °C (Fig. 3-1C,D). The mean fluorescence intensity (MFI) of DCs after incubation with 50 nM β2GPI-FITC at 4°C was only 32% of that observed at 37 °C (Fig. 3-1D). These results suggest that following its binding to surface components on iDCs the majority of β2GPI is rapidly internalized.

To confirm the uptake of β2GPI, we incubated DCs that were previously seeded on fibronectin-coated glass chamber slides with 100 nM Alexa Fluor 488-conjugated β2GPI (β2GPI-488) for various time points (0, 2, 5, 10, 20 and 30 minutes) and analyzed the uptake of β2GPI-488 by confocal microscopy. At time points 0 and 2 minutes no internalized β2GPI was detected in iDCs. Peripheral patches of fluorescent β2GPI were observed at 5 minutes (Fig. 3-2). No co-localization of β2GPI-488 and the early endosome marker EEA-1 was observed at this time-point. This suggests that fluorescent signals observed at 5 minutes correspond to cell surface-bound β2GPI and/or to early endocytic events. At 10, 20 and 30 minutes we found that the majority of β2GPI-488 was present within vesicles that also stained for EEA-1. These data suggest that β2GPI is rapidly internalized by iDCs and that after 10-20 minutes the majority of endocytosed β2GPI is present in early endosomes.

We subsequently addressed whether endocytosed β2GPI co-localized with LRP1 and MR which are both expressed on iDCs. Partial co-localization of LRP1 and endocytosed β2GPI was observed (Fig. 3-3A). Co-staining of β2GPI and MR also revealed partial co-localization indicating that β2GPI is targeted to a population of endocytic vesicles that also incorporates LRP1 and MR (Fig. 3-3B).

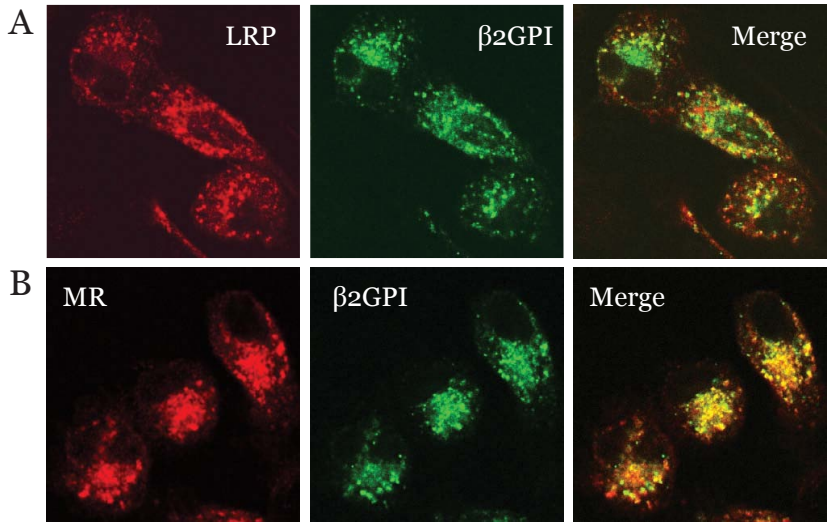


Figure 3-3 Co-localization of internalized β2GPI with MR and LRP1. *(A) Intracellular localization of internalized β2GPI and LRP1. (B) Intracellular localization of internalized β2GPI and MR.*

β2GPI is taken up by iDCs through Ca^{2+}-dependent receptor-mediated endocytosis

DCs internalize antigens through various mechanisms, including receptor mediated endocytosis or receptor-independent fluid phase macropinocytosis [19]. DCs were preincubated with high concentrations of sucrose which interferes with clathrin-mediated endocytosis through disruption of clathrin microcages on the inner part of the plasma membrane [20]. Preincubation of iDCs with 0.75 M sucrose resulted in a significant reduction in the amount of internalized β2GPI-FITC (Fig. 3-4A). Analysis by confocal microscopy confirmed that uptake of β2GPI was blocked upon preincubation of iDCs with 0.75 M sucrose (Fig. 3-4C). To study the involvement of Ca^{2+}-dependent processes in β2GPI uptake, we preincubated iDCs with 25 mM EDTA prior to the addition of β2GPI-FITC. Pretreatment with 25 mM EDTA resulted in 70% reduction in uptake of β2GPI by iDCs. Analysis using confocal microscopy confirmed that the endocytosis of β2GPI is abolished by pre-treatment with 25 mM EDTA (Fig. 3-4B).

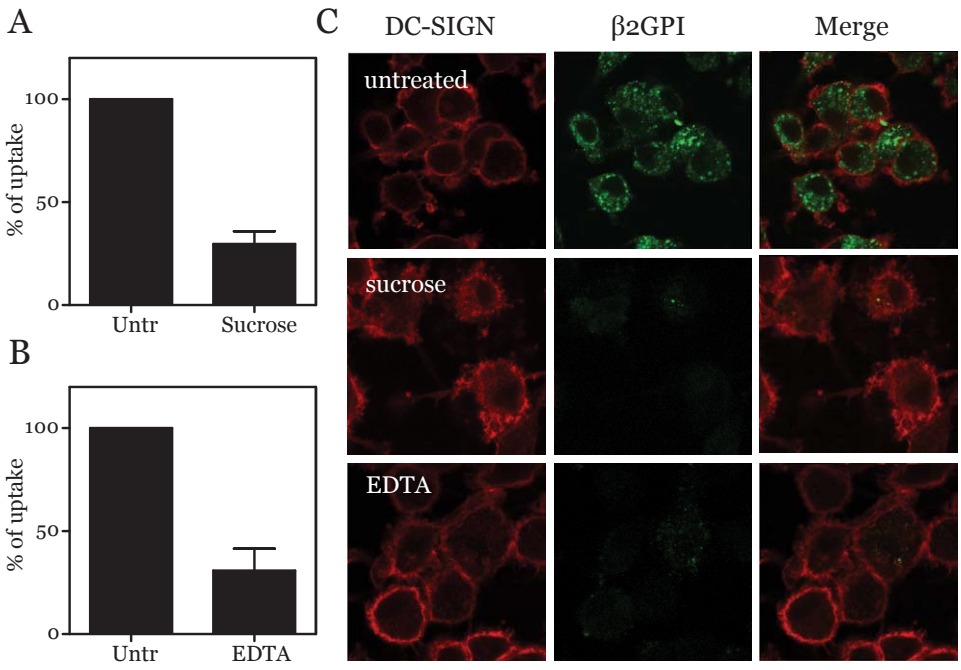


Figure 3-4 β2GPI is internalized by receptor mediated endocytosis. *DCs were first preincubated with 0.75 M sucrose (**A**) or 25 mM EDTA (**B**), then 50 nM β2GPI-FITC was added to the cells for 30 minutes at 37 °C or 4 °C. The samples were analyzed by FACS. The results are expressed as percentages of uptake by untreated cells (100%). Data are represented as mean of at least three independent experiments ± standard deviation. (**C**) 100 nM β2GPI-488 was added to DCs that were previously seeded on fibronectin-coated glass slides and preincubated with sucrose or EDTA. The cells were stained for cell surface receptor DC-SIGN (in red) and analyzed by confocal microscopy.*

β2GPI-derived peptides presented on MHC class II molecules

Antigen presenting cells continuously sample their environment and present internalized antigens on MHC class I and II molecules for presentation to T lymphocytes [19]. Also endogenous antigens derived from the endo-lysosomal pathway and other subcellular compartments are continuously presented on MHC class II [21]. We have previously established a protocol for analysis of MHC class II-presented peptides on antigen pulsed dendritic cells [18]. To explore the repertoire of β2GPI-presented peptides on MHC class II, iDCs were pulsed for 5 hours with 100 nM β2GPI and then maturated overnight employing 1 μg mL^{-1} of LPS. HLA-DR molecules were subsequently purified and bound peptides were eluted and analyzed by mass spectrometry. Dendritic cells generated from three HLA-typed healthy donors were used for these studies. As expected, the majority of the presented peptides did not derive from β2GPI (Table 3-1). The number of MHC class II peptides derived from these three donors varied between 4800 and 7200 (Table 3-1). Unexpectedly, only a limited number of β2GPI-derived peptides were identified for the three donors analyzed. For donor 1 (DRB1*0301/0701) two peptides (VPVKKATVVYQGERV; 266–280 and VPKCFKEHSSLAFWK; 322–336) were identified both originating of domain V. A single peptide (ATVVYQGERVKIQEKFK; 271–286) partially overlapping with one of the domain V-derived peptides of donor 1 was recovered from the second donor (DRB1*0101/1101). To address the reproducibility of our approach we analyzed iDCs derived from the third donor with the same DRB1-alleles as donor 1. The same VPKCFKEHSSLAFWK peptide was identified using iDCs from this donor (see Table 3-1). The other peptide (VPVKKATVVYQGERV) identified in donor 1 was not identified on MHC class II molecules of β2GPI-pulsed iDCs of donor 3. Taken together, our results demonstrate that upon endocytosis of β2GPI, a small number of domain V-derived peptides is presented on MHC class II molecules on the surface of DCs.

Table I: β2GPI peptides presented on MHC class II.

Haplotype	Peptide	Domain	Charge	Xcorr	Total number of peptides
DRB1* 0301/0701	VPVKKATVVYQGERV	266-280 (domain V)	2	1.48	7272
	VPKCFKEHSSLAFWK	322-336 (domain V)	4	2.55	
DRB1* 0101/1101	ATVVYQGERVKIQEKF	271-286 (domain V)	4	1.85	4863
DRB1* 0301/0701	VPKCFKEHSSLAFWK	322-336 (domain V)	4	2.03	5462

Table shows β2GPI peptide repertoires of three different donors. The columns demonstrate haplotype of the donors, amino acid sequences of the β2GPI peptides, domain(s) where the peptides are located within β2GPI and the obtained charge and Xcorr values of the SEQUEST analysis. The total number of MHC class II presented peptides is also displayed.

Discussion

In this study, we demonstrate that β2GPI is endocytosed and that β2GPI-derived peptides are presented on MHC class II molecules on dendritic cells. Autoreactive CD4+ T cells against β2GPI initiate the generation of pathogenic anti-β2GPI antibodies in patients with APS [22]. As yet, the underlying mechanisms in the initiation of immune responses against β2GPI remain unidentified [2]. Here, we show that β2GPI is rapidly endocytosed by dendritic cells in a Ca^{2+}-dependent manner; modulation of the uptake of β2GPI by cellular receptor on iDCs is suggested by the partial inhibition of its uptake by sucrose. DCs express numerous pattern recognition receptors such as C-type lectins that bind glycan structures in a Ca^{2+}-dependent manner. We performed a glycan analysis with various lectins including GNA (*Galanthus nivalis* agglutinin), SNA (*Sambucus nigra* agglutinin), MAA (*Maacka amurensis* agglutinin), PNA (Peanut agglutinin), DSA (*Datura stramonium* agglutinin) (Fig. 3-5). This analysis revealed that GNA, SNA and PNA interact with β2GPI, which indicates the presence of terminal mannose (GNA), sialic acid (SNA) and terminal β-galactose (PNA) within β2GPI. These results raise the possibility that β2GPI is internalized by virtue of its ability to interact with lectins expressed on the surface of iDCs. Terminal mannose-residues provide a potential binding site for the macrophage mannose receptor and DC-SIGN which are highly expressed on iDCs [23]. Preincubation of DCs with mannan (at a concentration of 1 mg mL^{-1}) or a blocking antibody directed towards the macrophage mannose receptor (clone 15.2 at 100 μg mL^{-1}) resulted in a 40% inhibition of the uptake of β2GPI (data not shown). A recent study provided evidence for attenuated sialylation of β2GPI derived from patients with APS [24]. Whether sialic acid binding immunoglobulin-like lectins (Siglecs) are involved in endocytosis of β2GPI by antigen presenting cells requires further study [25].

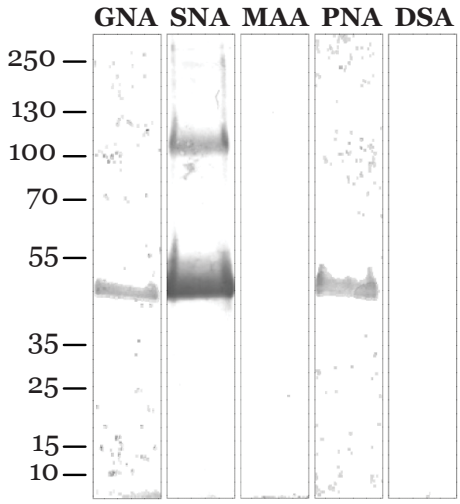


Figure 3-5 Glycan analysis of purified β2GPI. *Glycosylation pattern of β2GPI was determined using the DIG glycan differentiation kit. Lectins analyzed included GNA (galanthus nivalis agglutinin), SNA (Sambucus nigra agglutinin), MAA (Maacka amurensis agglutinin), PNA (Peanut agglutinin) and DSA (Datura stramonium agglutinin).*

Previous work has shown that dimeric but not monomeric β2GPI can interact with multiple LDL receptor family members [26,27]. It should be noted that we used monomeric β2GPI in this study. LDL receptor family members may not be able to promote the uptake of monomeric β2GPI on DCs as a result of a closed β2GPI conformation in which key interactive residues are buried and therefore are not capable of interacting with the complement type repeats on these receptors [3,7]. In "closed" β2GPI domains I and V are non-covalently linked thereby shielding the antibody binding site in domain I and the anionic phospholipids and LPS binding site in domain V [3,28]. In the "open" conformation these sites in domain I and V become exposed and this may result in an alternative mechanism of uptake as compared to "closed" β2GPI.

Analysis of β2GPI-pulsed DCs revealed that following uptake of β2GPI, these cells are capable of intracellular processing and subsequent presentation of β2GPI-derived peptides on MHC class II. As outlined previously, functional presentation of β2GPI –derived peptides is essential for the activation of β2GPI-reactive CD4+ T cells. In this study we identified three different domain V-derived peptides using β2GPI-pulsed APC from three unrelated donors (Table 3-1). In a pilot study performed in an untyped donor, also two domain I derived peptides were recovered (data not shown). Interestingly, two HLA-DRB1 identical donors presented the same Val322–Lys336 peptide. One of these donors presented another peptide (Val266–Val280) that partially overlapped with a Ala271–Phe286 peptide presented by a HLA-DRB1 non-identical donor. One of the peptides (Val322–Lys336) contains the conserved AFWKTDA sequence in β2GPI, which overlaps with a LPS binding domain and a binding site for anionic phospholipids in domain V of β2GPI [28]. Arai and co-workers have previously isolated autoreactive CD4+ T cell clones directed towards amino acid sequence KVSFFCKNKEKKCSY (295–309) in domain V of β2GPI [14]. Proliferation of CD4+ T cell clones reactive with this peptide were restricted by DRB4*0103 [14]. In none of the donors analyzed in our study this particular peptide was presented. Kuwana *et al.* showed that presentation of this "cryptic" peptide was dramatically enhanced in the presence of anionic phospholipids [13]. It will be interesting to determine whether addition of anionic phospholipids to β2GPI will increase the presentation of this particular peptide on MHC class II. Under our experimental conditions the number of peptides presented on β2GPI-pulsed DCs is low (despite efficient endocytosis of β2GPI). We anticipate that conformational changes in β2GPI result in exposure of "cryptic" determinants (such as 276–290) that will evoke CD4+ T cell responses following their presentation on MHC class II molecules eventually resulting in the onset of APS.

Acknowledgements

We would like to thank Maaike Hanegraaf for her technical help with purification of β2GPI from human plasma.

References

1. Meroni PL, Borghi MO, Raschi E, Tedesco F. Pathogenesis of antiphospholipid syndrome: understanding the antibodies. *Nat Rev Rheumatol*. 2011; **7**: 330-9.
2. De Groot PG, Urbanus RT. The significance of auto-antibodies against β2-glycoprotein I. *Blood*. 2012.
3. De Groot PG, Meijers JCM. β2-glycoprotein I: evolution, structure and function. *J Thromb Haemost*. 2011; **9**: 1275-84.
4. Hunt J, Krilis S. The fifth domain of β2-glycoprotein I contains a phospholipid binding site (Cys281-Cys288) and a region recognized by anticardiolipin antibodies. *J Immunol*. 1994; **152**: 653-9.
5. Bouma B, de Groot PG, van den Elsen JMH, Ravelli RBG, Schouten A, Simmelink MJA, Derksen RHWM, Kroon J, Gros P. Adhesion mechanism of human β2-glycoprotein I to phospholipids based on its crystal structure. *EMBO J*. 1999; **18**: 5166-74.
6. Schwarzenbacher R, Zeth K, Diederichs K, Gries A, Kostner GM, Laggner P, Prassl R. Crystal structure of human β2-glycoprotein I: implications for phospholipid binding and the antiphospholipid syndrome. *EMBO J*. 1999; **18**: 6228-39.
7. Ağar Ç, van Os GMA, Mörgelin M, Sprenger RR, Marquart JA, Urbanus RT, Derksen RHWM, Meijers JCM, de Groot PG. β2-glycoprotein I can exist in two conformations: implications for our understanding of the antiphospholipid syndrome. *Blood*. 2010; **116**: 1336-43.
8. Hammel M, Schwarzenbacher R, Gries A, Kostner GM, Laggner P, Prassl R. Mechanism of the interaction of β2-glycoprotein I with negatively charged phospholipid membranes. *Biochemistry*. 2001; **40**: 14173-81.
9. De Laat B, van Berkel M, Urbanus RT, Siregar B, de Groot PG, Gebbink MF, Maas C. Immune responses against domain I of β2-glycoprotein I are driven by conformational changes: Domain I of β2-glycoprotein I harbors a cryptic immunogenic epitope. *Arthritis Rheum*. 2011; **63**: 3960-8.
10. Iverson GM, Reddel S, Victoria EJ, Cockerill KA, Wang YX, Marti-Renom MA, Sali A, Marquis DM, Krilis SA, Linnik MD. Use of single point mutations in domain I of β2-glycoprotein I to determine fine antigenic specificity of antiphospholipid autoantibodies. *J Immunol*. 2002; **169**: 7097-103.
11. De Laat B, Derksen RHWM, Urbanus RT, de Groot PG. IgG antibodies that recognize epitope Gly40–Arg43 in domain I of β2-glycoprotein I cause LAC, and their presence correlates strongly with thrombosis. *Blood*. 2005; **105**: 1540-5.
12. De Laat B, Derksen RHWM, van Lummel M, Pennings MTT, de Groot PG. Pathogenic anti-β2-glycoprotein I antibodies recognize domain I of β2-glycoprotein I only after a conformational change. *Blood*. 2006; **107**: 1916-24.
13. Kuwana M, Matsuura E, Kobayashi K, Okazaki Y, Kaburaki J, Ikeda Y, Kawakami Y. Binding of β2-glycoprotein I to anionic phospholipids facilitates processing and presentation of a cryptic epitope that activates pathogenic autoreactive T cells. *Blood*. 2005; **105**: 1552-7.
14. Arai T, Yoshida K, Kaburaki J, Inoko H, Ikeda Y, Kawakami Y, Kuwana M. Autoreactive CD4+ T-cell clones to β2-glycoprotein I in patients with antiphospholipid syndrome: preferential recognition of the major phospholipid-binding site. *Blood*. 2001; **98**: 1889-96.
15. Chukwuocha RU, Hsiao ET, Shaw P, Witztum JL, Chen PP. Isolation, characterization and sequence analysis of five IgG monoclonal anti-β2-glycoprotein-I and anti-prothrombin antigen-binding fragments generated by phage display. *J Immunol*. 1999; **163**: 4604-11.

16. Dienava-Verdoold I, Boon-Spijker MG, de Groot PG, Brinkman HJM, Voorberg J, Mertens K, Derksen RHWM, de Laat B. Patient-derived monoclonal antibodies directed towards β2-glycoprotein I display lupus anticoagulant activity. *J Thromb Haemost*. 2011; **9**: 738-47.

17. Čučnik S, Kveder T, Ulcova G, Swadzba J, Musial J, Valesini G, Avčin T, Rozman B, Božič B. The avidity of anti-β2-glycoprotein I antibodies in patients with or without antiphospholipid syndrome: a collaborative study in the frame of the European forum on antiphospholipid antibodies. *Lupus*. 2011; **20**: 1166-71.

18. Van Haren SD, Herczenik E, ten Brinke A, Mertens K, Voorberg J, Meijer AB. HLA-DR-presented peptide repertoires derived from human monocyte-derived dendritic cells pulsed with blood coagulation factor VIII. *Mol Cell Proteomics*. 2011; **10**: M110.002246.

19. Trombetta ES, Mellman I. Cell biology of antigen processing in vitro and in vivo. *Annu Rev Immunol*. 2005; **23**: 975-1028.

20. Heuser JE, Anderson RG. Hypertonic media inhibit receptor-mediated endocytosis by blocking clathrin-coated pit formation. *J Cell Biol*. 1989; **108**: 389-400.

21. Nedjic J, Aichinger M, Mizushima N, Klein L. Macroautophagy, endogenous MHC II loading and T cell selection: the benefits of breaking the rules. *Curr Opin Immunol*. 2009; **21**: 92-7.

22. Kuwana M. β2-glycoprotein I: antiphospholipid syndrome and T-cell reactivity. *Thromb Res*. 2004; **114**: 347-55.

23. Herczenik E, van Haren SD, Wroblewska A, Kaijen P, van den Biggelaar M, Meijer AB, Martinez-Pomares L, ten Brinke A, Voorberg J. Uptake of blood coagulation factor VIII by dendritic cells is mediated via its C1 domain. *J Allergy Clin Immunol*. 2012; **129**: 501-9.e5.

24. Kondo A, Miyamoto T, Yonekawa O, Giessing AM, Osterlund EC, Jensen ON. Glycopeptide profiling of β2-glycoprotein I by mass spectrometry reveals attenuated sialylation in patients with antiphospholipid syndrome. *J Proteomics*. 2009; **73**: 123-33.

25. Crocker PR, Paulson JC, Varki A. Siglecs and their roles in the immune system. *Nat Rev Immunol*. 2007; **7**: 255-66.

26. Pennings MTT, van Lummel M, Derksen RHWM, Urbanus RT, Romijn RA, Lenting PJ, de Groot PG. Interaction of β2-glycoprotein I with members of the low density lipoprotein receptor family. *J Thromb Haemost*. 2006; **4**: 1680-90.

27. Van Lummel M, Pennings MTT, Derksen RHWM, Urbanus RT, Lutters BCH, Kaldenhoven N, de Groot PG. The binding site in β2-glycoprotein I for ApoER2' on platelets is located in domain V. *J Biol Chem*. 2005; **280**: 36729-36.

28. Ağar Ç, de Groot PG, Marquart JA, Meijers JCM. Evolutionary conservation of the lipopolysaccharide binding site of β2-glycoprotein I. *Thromb Haemost*. 2011; **106**: 1069-75.

Chapter 4

Factor seven activating protease (FSAP): does it activate factor VII?

Fabian Stavenuiter,[1,2] Ilze Dienava-Verdoold,[1] Mariette G. Boon-Spijker,[1] Herm-Jan M. Brinkman,[1] Alexander B. Meijer,[1,3] and Koen Mertens,[1,2,3]

[1]Department of Plasma Proteins, Sanquin Research, Amsterdam, The Netherlands
[2]Van Creveld Laboratory of UMC-Utrecht and Sanquin, Amsterdam, The Netherlands
[3]Department of Pharmaceutics, Utrecht Institute of Pharmaceutical Sciences (UIPS), Utrecht, The Netherlands
[4]Rheumatology and Clinical Immunology, University Medical Center Utrecht, Utrecht, The Netherlands.

J Thromb Haemost 2012; 10: 859–66.

Abstract

Background: Factor seven activating protease (FSAP) was initially reported as an activator of single-chain urokinase-type plasminogen activator (scuPA) and factor VII (FVII). Subsequently, numerous additional substrates have been identified, and multiple other biological effects have been reported. Due to the apparent lack of specificity, the physiological role of FSAP has become increasingly unclear. Rigorous studies have been limited by the difficulty of obtaining intact FSAP from blood or recombinant sources. *Objectives*: Our aim was to produce intact recombinant human FSAP, and to assess its role as a trigger of coagulation and fibrinolysis. *Results*: Expression of wild-type FSAP in various mammalian cells invariably resulted in the accumulation of degraded FSAP due to autoactivation and degradation. To overcome this problem, we constructed a variant in which Arg313 at the natural activation site was replaced by Gln, creating a cleavage site for the bacterial protease thermolysin. HEK293 cells produced $FSAP^{R313Q}$ in its intact form. Thermolysin-activated FSAP displayed the same reactivity toward the substrate S-2288 as plasma-derived FSAP, and retained its ability to activate scuPA. Polyphosphate and heparin increased V_{max} by 2–3-fold, without affecting K_m (62 nM) of scuPA activation. Surprisingly, FVII activation by activated FSAP proved negligible, even in the presence of calcium ions, phospholipid vesicles and recombinant soluble tissue factor. On membranes of 100% cardiolipin FVII cleavage did occur, but this resulted in transient activation and rapid degradation. *Conclusions*: While FSAP indeed activates scuPA, FVII appears remarkably resistant to activation. Therefore, reappraisal of the putative role of FSAP in haemostasis seems appropriate.

Introduction

Factor seven activating protease (FSAP) is the precursor of a serine protease that has several putative interactions with the haemostatic system. FSAP was first described in 1996 as plasma hyaluronan binding protein (PHBP), a novel serine protease with the ability to activate single-chain pro-urokinase (scuPA) [1,2]. Subsequently, others have purified a so far unknown factor VII (FVII) activating protease, designated FSAP, which later proved identical to PHBP [3]. These findings suggested that FSAP, being a trigger of both coagulation and fibrinolysis, might play a key role in haemostasis and thrombosis. Apart from FVII and scuPA, FSAP has been reported to cleave multiple other substrates, including fibrinogen and high-molecular-weight kininogen [4,5]. More recently, FSAP has been attributed a role in vascular biology and atherosclerosis [6,7] and in autoimmune disease [8]. More than a decade since its discovery, FSAP now seems a broad-spectrum protease with a multitude of substrates in a variety of cross-linked biological pathways. With its decreasing specificity, the 'true' physiological role of FSAP has become increasingly unclear.

FSAP circulates in plasma at a concentration of about 12 μg mL^{-1} [1,2]. FSAP is notoriously difficult to isolate in its single-chain form, because it is highly sensitive to proteolysis and autocatalytic degradation [1,3,9]. FSAP displays a discrete domain structure, and comprises three epidermal growth factor domains (EGF1–3), one kringle domain and a serine protease domain, representing a total molecular mass of

approximately 70 kDa [1]. Like other serine proteases, FSAP is activated by limited proteolysis. This involves cleavage between Arg313 and Ile314 at the N-terminal region of the catalytic domain, resulting in a ~45 kDa heavy chain and ~25 kDa light chain held together by a disulfide bond [1,9]. The activation into two-chain FSAP has been described to result from autocatalytic cleavage, a process that is stimulated by polyanions such as heparin and polyamine [10,11]. Due to its sensitivity to autodegradation, most purified FSAP preparations are heterogeneous, comprising multiple peptides of varying size. This raises the possibility that contaminants that are co-purified from plasma contribute to the disparate roles of FSAP as reported.

The aim of the present study was to produce recombinant FSAP suitable for functional studies, and to re-assess its role as a trigger of coagulation and fibrinolysis. To overcome the problem of autoactivation and subsequent degradation, we constructed an FSAP variant in which Arg313 at the natural activation site was replaced by Gln, creating a cleavage site for the bacterial protease thermolysin. This approach allowed us to isolate recombinant FSAP in its intact, single-chain form, which then could be activated under controlled conditions by thermolysin. Studies using purified proteins confirmed that activated FSAP indeed activates scuPA. In contrast, we found little evidence in favour of a role in FVII activation.

Materials and methods

Materials

CNBr-Sepharose 4B, DEAE-Sephadex A-50, Q-Sepharose FF and Heparin-Fractogel were from Amersham Pharmacia Biotech Nederland (Roosendaal, the Netherlands). Optimem-I medium, penicillin/streptomycin and DNA restriction enzymes were obtained from Life Technologies (Breda, the Netherlands). Microtitre plates and cell culture flasks were purchased from Nunc (Roskilde, Denmark). Oligonucleotide primers, 293 Freestyle® cell culture system, Insulin-Transferrin-Selenium-X (ITS) supplement, restriction enzymes, pcDNA3.1(–) vector, DMRIE-C, Geneticin (G418) and phosphoramidon disodium salt were supplied by Invitrogen (Breda, the Netherlands). Foetal calf serum (FCS) was from Thermo Fisher Scientific (Breda, the Netherlands). The synthetic substrates H-(D)-Ile-Pro-Arg-pNA (S-2288) and pyroGlu-Gly-Arg-pNA (S-2444) were from Chromogenix (Milan, Italy). Brain phosphatidylserine (PS), brain phosphatidylethanolamine (PE) and phosphatidylcholine (PC) were obtained from Avanti Polar Lipids Inc. (Alabaster, AL, USA). Thermolysin from *Bacillus thermoproteolyticus*, cardiolipin, polyphosphate type 65 (Poly-P65), soybean trypsin inhibitor and benzamidine were from Sigma-Aldrich (Zwijndrecht, the Netherlands). Aprotinin (Trasylol) was from Roche Diagnostics (Almere, the Netherlands) and heparin from LEO Pharmaceuticals (Weesp, the Netherlands). Purified human scuPA was obtained from Kordia Life Sciences (Leiden, the Netherlands). Activated human factor X (FXa) was from Enzyme Research Laboratories (South Bend, IN, USA). Recombinant soluble tissue factor (sTF) fragment 33–251 was from ProSpec-Tany Technogene Ltd (Ness-Ziona, Israel). Ovalbumin was from Serva (Heidelberg, Germany).

Plasma-derived human FSAP

Human FSAP was purified from cryoprecipitate-depleted plasma (Sanquin Plasma Products, Amsterdam, the Netherlands). Plasma was supplied with benzamidine to a final concentration of 10 mM, and then incubated batch-wise with pre-swollen DEAE-Sephadex A-50 (30 mL per L plasma) for 90 min at room temperature. The matrix was then collected in a column, and extensively washed with 125 mM NaCl, 10 mM benzamidine, 10 mM trisodium citrate, pH 7.0. The column was extensively washed using the same buffer containing 0.5 M NaCl, and bound protein was eluted by washing buffer containing 2 M NaCl. FSAP-containing fractions were pooled, dialyzed against 50 mM NaCl, 10 mM benzamidine, 10% (v/v) glycerol, 20 mM Hepes (pH 6.0) and loaded onto a Heparin-Fractogel column (29 mL) equilibrated in the same buffer. The column was eluted using a gradient from 0.05 to 2 M NaCl in 10 mM benzamidine, 20 mM Hepes, pH 6.0, and FSAP-containing fractions were pooled and concentrated on a QSepharose FF column (2 mL) by elution with 2 M NaCl, 10 mM benzamidine, 50 mM Tris-HCl (pH 8.0). Concentrated FSAP was dialyzed against 250 mM NaCl, 50% (v/v) glycerol, 50 mM Tris-HCl (pH 8.0), and stored at -20 °C.

Recombinant FSAP variants

The complete open reading frame of human wild-type FSAP was produced by PCR from human liver quick-clone cDNA (BD Biosciences, Breda, the Netherlands) using the primers (a) 5'-TTAGGATCCGCAAAGATGTTTGCCAGGATGTCTGATCTC-3' (sense) and (b) 5'-ATTGGTACCGAAGACAGTACCTTAGAAGCCACT-3' (antisense), including *Bam*HI and *Kpn*I restriction sites (underlined), respectively. The resulting PCR fragment was digested with *Bam*HI and *Kpn*I and then ligated into the pcDNA3.1(–) vector. The wild-type FSAP plasmid then was used as the template for site-directed mutagenesis with the QuickChange® kit (Stratagene, La Jolla, CA,USA). Plasmid encoding $FSAP^{S509A}$ was constructed using the primers 5'-GACACCTGCCAGGGTGACGCTGGAGGCCCCCTGACCTGT-3' (sense) and 5'-ACAGGTCAGGGGGCCTCCAGCGTCACCCTGGCAGGTGTC-3' (antisense), and plasmid encoding the $FSAP^{R313Q}$ variant using the primers 5'-GCAGAGAGGAAGATCAAGCAAATCTATGGAGGCTTTAAG-3' (sense) and 5'-CTTAAAGCCTCCATAGATTTGCTTGATCTTCCTCTCTGC-3' (antisense). All constructs were verified by sequence analysis. Plasmids encoding wild-type FSAP were transfected into LoVo-, HEK293- and CHO-cells using DRMIE-C, and positive clones were expanded in DMEM-F12 with 10% (v/v) FCS and 500 μg mL^{-1} G418. Stable cell lines were maintained on DMEM-F12 containing 10% (v/v) FCS or 1% (v/v) ITS. $FSAP^{S509A}$ and $FSAP^{R313Q}$ were transiently expressed in 293-F cells using the Freestyle® system according to the manufacturer's instructions. FSAP amino acid numbering is as given in UniProtKB accession code Q14520, and includes the signal peptide.

Recombinant FSAP was purified by immuno-affinity chromatography using anti-FSAP4 [8] coupled to CNBr-Sepharose 4B (5 mg per mL gel). After loading, the column was washed with 1 M NaCl, 50 mM Tris-HCl, pH 8.0, followed by a wash with 100 mM NaCl, 15% (v/v) ethylene glycol, 50 mM Tris-HCl, pH 8.0. Bound

FSAP was eluted with 150 mM NaCl, 55% ethylene glycol, 10% (v/v) glycerol and 25 mM Lysine, pH 11, and immediately neutralized using 1.5 M Imidazole (pH 7.0). Remaining contaminants were removed on a Q-Sepharose FF column, employing a gradient from 0.15 to 1 M NaCl in 5% (v/v) glycerol, 50 mM Tris-HCl, pH 8.0. FSAP fractions were pooled and concentrated on a second Q-Sepharose FF column (1 mL) by elution with 1 M NaCl, 5% (v/v) glycerol, 50 mM Tris-HCl, pH 8.0. Concentrated $FSAP^{S509A}$ was dialyzed against 150 mM NaCl, 50 mM Tris-HCl, pH 8.0. $FSAP^{R313Q}$ was dialyzed against 150 mM NaCl, 5% (v/v) glycerol, 10 mM MES, pH 5.0. Purified recombinant FSAP was stored in aliquots at -20 °C.

For all experiments $FSAP^{R313Q}$ (700 nM) was freshly activated by incubation with thermolysin (3 nM) for 2 h at 30 °C in 150 mM NaCl, 5% (v/v) glycerol, 0.01% (v/v) Tween-80, 100 μM $CaCl_2$, 50 nM $ZnCl_2$, 10 mM MES, pH 5.0. The reaction was stopped by the addition of phosphoramidon (10 μM), and activation was checked by amidolytic activity and SDS/PAGE. Controls demonstrated that the components from the activation mixture did not interfere with FSAP amidolytic activity, scuPA activation and FVII cleavage in the presence of cardiolipin. Activated $FSAP^{R313Q}$ was stored on ice and used within 30 min.

Other methods

Human FVII was isolated from plasma as previously described for FVIIa [12], with the exception that after cryoprecipitation, the DEAE-Sephadex and Heparin-Sepharose chromatography steps were replaced by a single immunoaffinity chromatography step using antibody 2G3 [13]. FVII was stored in 150 mM NaCl, 5% (v/v) glycerol, 10 mM MES (pH 5.0), at -20 °C. FSAP antigen was determined by standard ELISA techniques, using monoclonal anti-FSAP4 [8] as primary antibody (1 μg per well) and peroxidase-labelled polyclonal anti-FSAP-IgG (0.2 μg mL^{-1}) for detection. Polyclonal anti-FSAP was produced in rabbits by Biogenes GmbH (Berlin, Germany), using the purified plasma-derived FSAP described above. Concentrations of purified proteins were measured by the Bradford method [14] using human albumin as a reference. Molar concentrations were calculated from protein values using a molecular size of 70 kDa for FSAP, and 55 kDa for FVII. Concentrations of uPA were expressed in International Units based on the International Standard for HMW uPA, encoded 87/594 (NIBSC, South Mimms, UK). Lipid vesicles were prepared by sonification as described [15]. SDS/PAGE was performed using 10% or precast 4–12% gradient gels (Invitrogen). Protein was reduced using 20 mM 1,4 dithiotreitol for 5 min at 100 °C. Protein bands of reduced FSAP were excised and subjected to N-terminal sequencing (Eurosequence, Groningen, the Netherlands). For detecting FVII proteolysis, immunoblotting was performed using rabbit polyclonal antibody against human FVIIa [12] that is predominantly recognizing the FVIIa heavy chain. Bands were visualized by electro-chemiluminescence (ECL; Roche Diagnostics, Mannheim, Germany). Amidolytic activity of FVIIa toward S-2288 was determined in the presence of 50 nM sTF. Substrate hydrolysis was converted into FVIIa concentration using fully FXa-activated FVII as a reference. Kinetic parameters of S-2288 cleavage and scuPA activation were calculated using GraphPad Prism® software (GraphPad, La Jolla, CA, USA).

Results

Plasma-derived human FSAP

Given its sensitivity to autoactivation and autodegradation [1,3,9], it was not surprising that FSAP was not obtained as a single-chain polypeptide. The reduced protein (Fig. 4-1A) comprised even more constituents than originally reported [1]. The identity of the individual fragments was verified by N-terminal sequencing, and the sequences are given in Fig. 4-1(A). Ser28 was found as the N-terminus of the fragments of approximately 45, 25 and 18 kDa, and represents one of the two reported N-termini after cleavage of the signal peptide [9]. The 25 kDa band further comprised a second fragment, starting with Arg171, apparently due to cleavage in the N-terminal section of FSAP (the heavy chain). The same N-termini were found in the 18 kDa band, together with Ile314 and Ser320, which represent the N-terminus of the protease domain and a truncated form thereof missing six residues. Finally, the 11 kDa band was identified as a C-terminal fragment from the protease domain, starting at Gln481. The non-reduced protein has an apparent molecular size of 60 kDa instead of 70 kDa as expected (Fig. 4-1A), suggesting that one or more fragments had been further truncated from the C-terminal end. Apparently, our purification procedure yielded pure but cleaved FSAP. While this material did display reactivity towards S-2288 (data not shown), it was considered suitable for immunization purposes only (see Materials and methods).

Recombinant human FSAP

In an attempt to overcome the heterogeneity of plasma-derived FSAP, we expressed recombinant FSAP in mammalian cell lines. These were varying from low to high expression of furin-like endoprotease activity [16], and included human colon carcinoma line (LoVo cells), Chinese Hamster Ovary (CHO) cells, Baby Hamster Kidney (BHK) cells, and Human Embryonic Kidney cells (HEK-293). By ELISA very low antigen levels were detected in medium from BHK and LoVo cells, while CHO and HEK293 cells showed relatively high expression (μg mL^{-1} range). Recombinant human FSAP could be easily purified using immunoaffinity chromatography (see Materials and methods). Wild-type human FSAP was obtained as a 70 kDa species that was even more degraded than FSAP from human plasma (Fig. 4-1B). Degradation could not be prevented by supplementing the medium with a variety of serine protease inhibitors, including benzamidine, PPACK, Pefabloc, Soy Bean Trypsin Inhibitor, Fut-175 (Futhan) and C1-inhibitor (data not shown). In contrast, no degradation was observed upon expression of FSAP with a replacement of Ser509 to Ala in its active site (Fig. 4-1C). This identifies autocatalysis as the cause of FSAP degradation during expression in mammalian cells. At the same time, this implies that production of wild-type human recombinant FSAP in its intact form would remain difficult, if possible at all. The conversion of the single chain FSAP zymogen into the active two-chain enzyme involves cleavage of theArg313–Ile314 bond [1]. We therefore introduced an Arg313 to Gln substitution, which renders this site resistant to cleavage by serine proteases. Indeed, $FSAP^{R313Q}$ could now be purified in its intact, single-chain form (Fig. 4-1D). The observation that FSAP degradation

requires both Ser509 in the active site and Arg313 at the scissile bond involved in zymogen activation confirms that the heterogeneity observed (Fig. 4-1A,B) results from autoactivation.

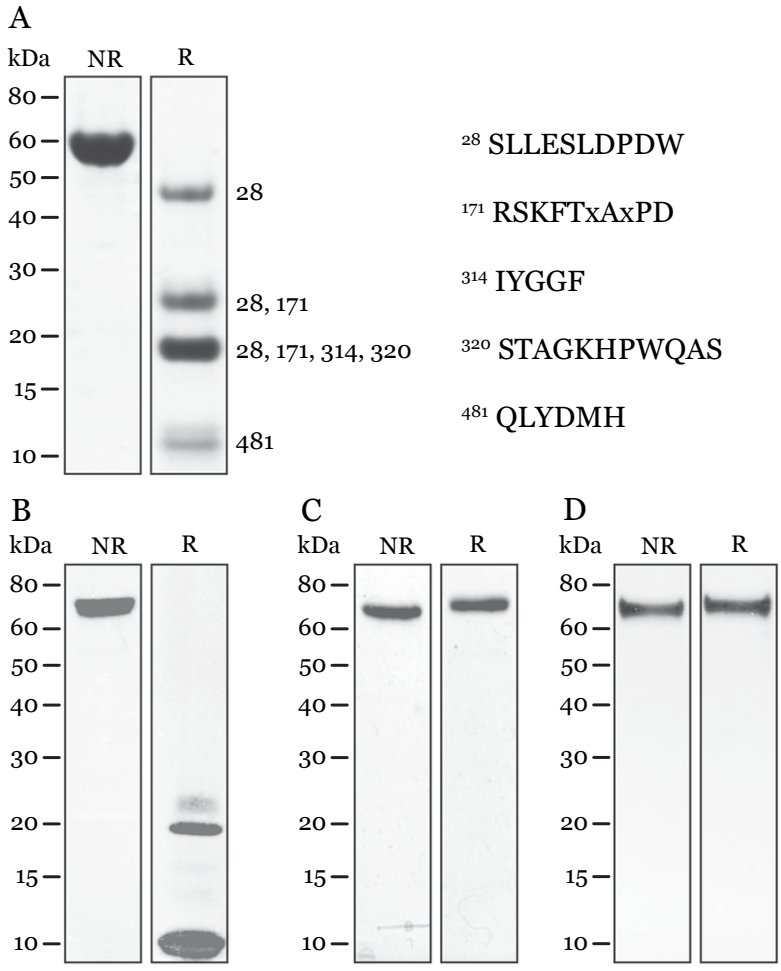


Figure 4-1 Characterization of purified factor seven activating protease (FSAP). *Plasma-derived FSAP (5 µg per lane, panel A) and recombinant FSAP variants (1 µg per lane, panels B–D) were analyzed by 10% SDS/PAGE under non-reducing (NR) and reducing (R) conditions, and protein was visualized by Coomassie Blue (A) or silver staining (B–D). Recombinant wild-type FSAP (B) was from CHO cells, and FSAP^{S509A} (C) and FSAP^{R313Q} (D) from 293-F cells. Individual bands of reduced plasma-derived FSAP were cut out for N-terminal sequencing, and the numbers of the identified N-termini are indicated. Numbering includes the 23 amino acid signal peptide [1].*

Activation of FSAP^{R313Q} by thermolysin

The Arg to Gln substitution created a suitable target site (Gln313–Ile314) for the bacterial metalloprotease thermolysin. In order to suppress the intrinsic instability

of FSAP^{R313Q} once activated, activation was performed at pH 5, and the activated protein was used within 30 min. Each series of experiments required FSAP^{R313Q} to be freshly activated. As shown in Fig. 4-2 (inset), activation by thermolysin resulted in a two-chain species with a heavy chain of approximately 45 kDa and a light chain of the expected molecular size of 25 kDa. The recombinant enzyme efficiently cleaved the chromogenic substrate S-2288, with a K_m of 0.36 ± 0.06 mM (mean ± SD) and a V_{max} of 5.7 ± 0.1 µM min^{-1}. Plasma-derived FSAP displayed a similar K_m value, but much lower activity (data not shown), presumably because it was largely degraded. These data demonstrate that thermolysin converts FSAP^{R313Q} into the two-chain enzymatic form. Reactivity against S-2288, combined with SDS/PAGE, was used to verify the enzyme concentration as estimated from protein content in all individual experiments using activated FSAP^{R313Q}.

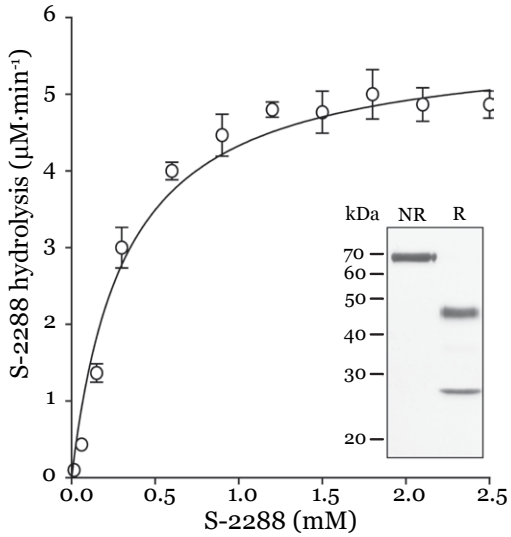


Figure 4-2 Amidolytic activity of activated factor seven activating protease (FSAP)^{R313Q}. *Activated FSAP^{R313Q} (10 nM) was incubated with S-2288 (0.5–2.5 mM) at 37 °C in a buffer containing 0.2% (w/v) BSA, 150 mM NaCl, 5 mM $CaCl_2$, 50 mM Tris-HCl, pH 8.0. Initial rates of S-2288 cleavage are plotted as mean ± SD of three to four experiments, and fitted to the Michaelis–Menten equation. SDS/PAGE (1 µg per lane, silver staining) confirmed that activated FSAP^{R313Q} consists of two chains of approximately 45 and 25 kDa (see inset).*

Activation of scuPA by activated FSAP^{R313Q}

As various studies reported that plasma-derived FSAP activates scuPA [2,9,17], we first assessed the reactivity of activated FSAP^{R313Q} towards this substrate. As shown in Fig. 4-3, two-chain FSAP^{R313Q} readily activated scuPA. The K_m for scuPA activation was estimated to be 62 ± 7 nM (mean ± SD), and V_{max} was 24 ± 2 IU mL^{-1} min^{-1}. Activation was stimulated 2–3-fold by polyanions such as polyphosphate and heparin. These co-factors did not affect K_m, but increased the calculated V_{max} to 48 and 64 IU mL^{-1} min^{-1}, respectively. The presence of 100 µM phospholipid vesicles consisting of PS/

PE/PC (20/20/60 mol %) or 100% cardiolipin did not accelerate scuPA activation to any appreciable extent (data not shown). These data demonstrate that recombinant thermolysin-activated, two-chain FSAP^{R313Q} does display reactivity towards scuPA as previously reported for plasma-derived FSAP.

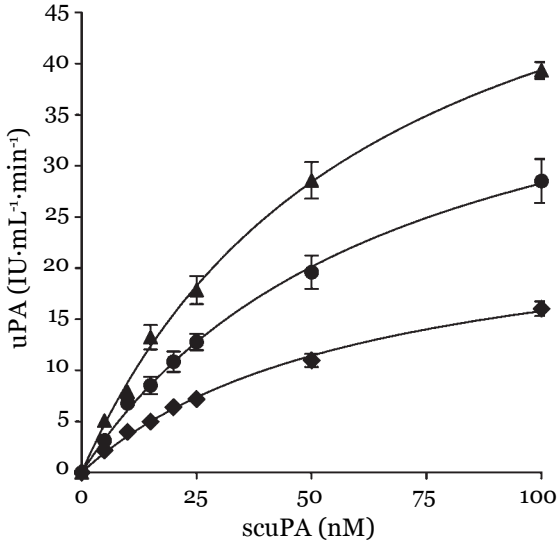


Figure 4-3 Activation of single-chain urokinase-type plasminogen activator (scuPA) by activated factor seven activating protease (FSAP)^{R313Q}. *Varying amounts of scuPA (5–100 nM) were incubated with activated FSAP^{R313Q} (0.25 nM) in a buffer containing 100 mM NaCl, 0.1% BSA, 0.01% (v/v) Tween-80, 5 mM $CaCl_2$, 20 mM Tris-HCl (pH 7.5) at 37 °C. Incubations were performed in the absence of co-factors (diamonds), and in the presence of 10 IU mL^{-1} of heparin (triangles) or 5 μM PolyP65 (circles) in a final volume of 75 μL. After 10 min, reactions were stopped by adding 25 μL of aprotinin (1 mM final concentration) in the same buffer, and uPA formation was quantified as described in Materials and methods. Data are given as mean ± SD of at least four independent experiments.*

Activated FSAP^{R313Q} is a poor FVII activator

Assessment of FVII activation is complicated by the fact that FVII is subject to autoactivation [18,19] and activation by FXa and thrombin, which precludes the use of regular clotting assays or thrombin generation systems. We therefore assessed FVII activation by FSAP using purified FVII, and monitored FVIIa directly by its amidolytic activity toward S-2288. In the presence of FXa and phosholipids, FVII was nearly completely converted into FVIIa in 30 min (Fig. 4-4). In contrast, no FVII activation could be detected in the presence of thermolysin-activated, two-chain FSAP^{R313Q}. We considered the possibility that FSAP may be more reactive against FVII in the presence of sTF. With increasing sTF concentrations, some FVIIa-like activity became detectable at a very low level (Fig. 4-4, inset). Control experiments indicated that amidolytic activity under these conditions was partially FSAP-independent, and the data in Fig. 4-4 (closed squares) were corrected for this background activity. However, because rates were close to the detection limit, the significance of these

low FVIIa levels remains unclear. If this indeed represents FSAP-dependent FVII activation, it remains negligible in comparison with the effect of FXa. Taking into account the concentration differences between FSAP (40 nM) and FXa (2 nM) in these experiments (see Fig. 4-4), FXa-dependent FVIIa formation proved to be at least 1000-fold faster.

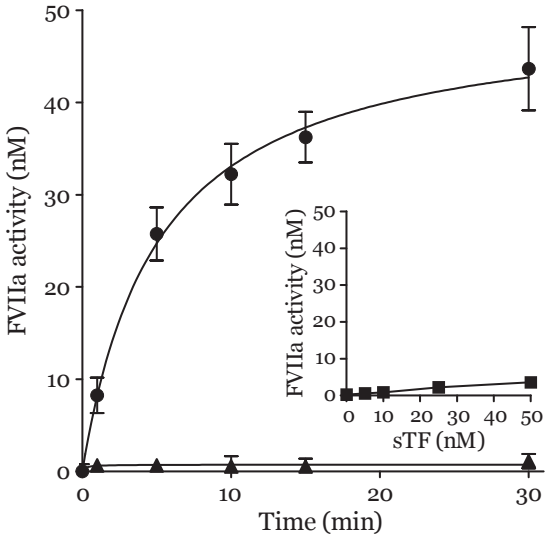


Figure 4-4 Activation of human factor (F) VII by FXa and activated factor seven activating protease (FSAP)^{R313Q} in the absence and presence of sTF. *Purified FVII (50 nM) was incubated at 37 °C in a buffer containing 5 mM $CaCl_2$, 150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 0.2% (w/v) ovalbumin, 100 μM phospholipids (PS/PC/PE) and 2 nM FXa (circles) or 40 nM activated FSAP^{R313Q} (triangles). Samples were drawn and immediately diluted 5-fold in the same buffer containing either 2 μM aprotinin or 5 μM SBTI to block FSAP or FXa activity, respectively. FVIIa amidolytic activity was determined as described in Materials and methods. The inset shows the effect of varying concentrations of sTF on the activity of 40 nM activated FSAP^{R313Q} under the same conditions. Reactions were stopped after 30 min. Data were corrected for residual S-2288 hydrolysis in the absence of FSAP, and represent the mean of two to three independent experiments.*

Proteolytic activation of FVII activation was further assessed by SDS/PAGE followed by immunoblotting employing polyclonal antibodies against the FVIIa heavy chain. In agreement with the lack of FVIIa activity in Fig. 4-4, FVII proved resistant against cleavage by two-chain FSAP^{R313Q} (Fig. 4-5A,B). In the absence of phospholipids no FVII cleavage was observed, even when two-chain FSAP^{R313Q} was used at concentrations at a large excess over FVII (up to 400 nM, see Fig. 4-5A). In the presence of phospholipids (Fig. 4-5B), FVII activation did occur for the positive control FXa. In contrast, no FVII proteolysis was detected using activated FSAP^{R313Q}. The lipid membranes used in these experiments consisted of PS/PE/PC (20/20/60 mol %) vesicles, which should provide an appropriate surface for most lipid-dependent steps in the coagulation cascade. The absence of FVII proteolysis under these conditions, in combination with the data of Fig. 4-4, supports the conclusion that two-chain FSAP^{R313Q} is a poor FVII activator.

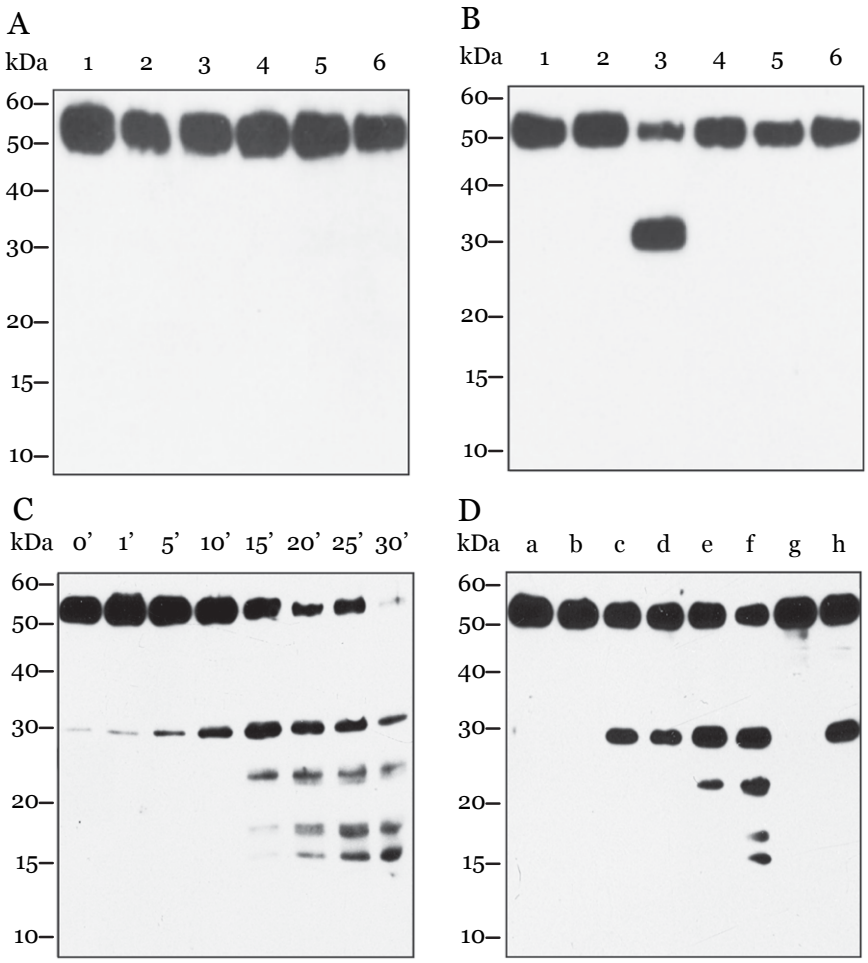


Figure 4-5 Sensitivity of human factor (F) VII to cleavage by activated factor seven activating protease (FSAP)^{R313Q}. *Purified FVII (50 nM) was incubated in a buffer containing 5 mM $CaCl_2$, 150 mM NaCl, 50 mM Tris-HCl (pH 7.4) at 37 °C in the absence of lipids (A), in the presence of 100 μM PS/PE/PC (B), or in the presence of 100 μM cardiolipin (C, D). In panels A and B, the additions were: FSAP activation buffer (lane 1), 400 nM non-activated FSAP^{R313Q} (lane 2), 2 nM FXa (lane 3), and activated FSAP^{R313Q} in concentrations of 40, 200 and 400 nM (lanes 4, 5 and 6, respectively), and the reactions were stopped after 30 min by the addition of SDS/PAGE sample buffer. In panel C, FVII (50 nM) was incubated with activated FSAP^{R313Q} (40 nM) in the presence of cardiolipin, and at varying time-points (1–30 min) SDS/PAGE sample buffer was added. In panel D, FVII (50 nM) was incubated with activated FSAP^{R313Q} in concentrations of 1, 2, 4, 8, 20 and 40 nM (lanes a, b, c, d, e and f, respectively) for 30 min. Controls were FSAP activation buffer (lane g) and 2 nM of FXa with 100 μM of PS/PE/PC instead of cardiolipin (lane h). Samples were analyzed under reducing conditions and FVII was visualized using anti-heavy chain antibody as described in Materials and methods.*

Cardiolipin promotes FVII cleavage by activated FSAPR313Q

Because polyanions support the activity of two-chain FSAP towards scuPA, we examined whether these would support FVII activation as well. Addition of heparin or polyphosphate at concentrations that accelerated scuPA activation (Fig. 4-3) did not yield any FVII cleavage, neither alone, nor in combination with PS/PE/PC vesicles (data not shown). Replacing PS by cardiolipin (up to 40 mol %) did not support FVII cleavage either. However, FVII cleavage did occur on a fully anionic membrane surface consisting of cardiolipin only. Cardiolipin preparations proved variable with regard to the extent of FVII cleavage occurring, presumably due to the inherent instability of these membranes in the presence of calcium ions. A typical pattern is shown in Fig. 4-5(C). The initial product was a 30 kDa derivative, which was further degraded into smaller fragments of 23, 16 and 15 kDa, until almost no intact FVII was left after 30 min. The extent of FVII cleavage on cardiolipin membranes suggested that activated FSAP^{R313Q} should also cleave FVII at much lower concentration than the 40 nM used in Fig. 4-5(C). Indeed, upon varying the concentration of two-chain FSAP^{R313Q}, substantial formation of the 30 kDa derivative was observed at enzyme concentrations as low as 4–8 nM (lanes c and d in Fig. 4-5D). The 30 kDa fragment has the same mobility as the FXa-cleaved FVII heavy chain (lane h), and most likely represents FVIIa. It seems evident however, that FSAP also cleaves additional sites in the FVII heavy chain, resulting in FVII degradation. These data suggest that on cardiolipin membranes FVIIa may occur as an intermediate during FSAP-dependent FVII degradation.

Discussion

Since its discovery in 1996 [1], the physiological role of FSAP has predominantly been explored using heterogeneous preparations comprising multiple peptides. In the present study, human plasma-derived FSAP proved similarly degraded. We identified five different N-termini, at positions 28, 171, 314, 320 and 481 (Fig. 4-1A), which were also reported previously [1,3,9]. While intact FSAP can be isolated from plasma employing low pH or high concentrations of urea, this is rapidly activated and further degraded once brought to more physiological conditions [8,11,17]. This is consistent with autoactivation and autocatalytic degradation, as has been described for a variety of other serine protease precursors, including FVII [18,19]. The implication therefore is that the expression and purification of the recombinant protein suffers from the same problem [20]. Indeed, we obtained wild-type human FSAP from various mammalian cells as a 70 kDa species that upon reduction proved consistently degraded (Fig. 4-1B). The finding that the active site substitution variant FSAP^{S509A} was obtained as a single-chain protein (Fig. 4-1C) implies that cleavage is indeed due to autocatalysis, and not to an endogenous endopeptidase that cleaves the basic sequence RKIKR^{313}-I^{314} at the activation site.

The problem of autoactivation and degradation was overcome by the Arg313 to Gln substitution (Fig. 4-1D), which makes the Arg313–Ile314 scissile bond resistant to serine proteases, and at the same time creates a cleavage site for the bacterial metalloprotease thermolysin. This strategy has previously been used for the expression of complement factor C1r, another serine protease that displays autoactivation [21].

The advantage of this approach is that it allows for the expression and isolation of single-chain FSAP^{R313Q}, which subsequently can be activated using thermolysin. The disadvantage is that FSAP^{R313Q}, once activated, is inherently instable due to its autocatalytic activity. Thus, two-chain FSAP^{R313Q} needs to be freshly activated for each series of experiments. Although we monitored individual preparations by amidolytic activity and SDS/PAGE, this nevertheless introduces some variability between experiments. Activated FSAP^{R313Q} comprises the wild-type serine protease domain, and a heavy chain ending with Gln instead of Arg as the C-terminus. Employing activated FSAP^{R313Q} we could confirm that FSAP is an effective activator of scuPA [2,4], and that this cleavage is stimulated by polyanionic components such as heparin and polyphosphate [10,11]. The kinetic parameters of scuPA activation (Fig. 4-3) suggest that FSAP would be equally as effective as established scuPA activators such as tissue-type plasminogen activator [22]. This seems compatible with a role of FSAP in fibrinolysis and/or vascular biology as earlier proposed [2,4,6,17].

It seems obvious to expect that FSAP, as its name suggests, would activate FVII. Our data demonstrate, however, that activated FSAP^{R313Q} does not activate purified FVII (Figs 4-4 and 4-5A,B), even at very high enzyme concentrations and in the presence of PS-containing membranes that support typical lipid-dependent steps in the coagulation cascade. This is surprising, because the FSAP literature generally refers to its FVII activating properties and its potential role as tissue factor-independent initiator of the FVII-dependent coagulation pathway (for review, see [6] and references therein). In retrospect, this notion appears to be based on limited experimental evidence. One study reported activation of purified FVII by FSAP in FX-deficient plasma, as monitored by a commercial clotting assay using recombinant soluble tissue factor [3]. While subsequent papers have been referring to this finding, we are not aware of any experimental confirmation by others. In our studies using two-chain FSAP^{R313Q}, we monitored FVII proteolysis and FVIIa amidolytic activity, because more complex, FX-containing assay systems, although more sensitive, could promote FVII autoactivation or reciprocal FVII activation by FXa and other activated coagulation factors. From our experiments using purified proteins we can only conclude that FSAP is an extremely poor FVII activator in comparison with, for instance, FXa (Figs 4-4 and 4-5B). However, we cannot exclude the possibility that FVII activation by FSAP requires a plasma cofactor that is lacking in our purified system. We addressed the possibility that effective activation of FVII by FSAP would require tissue factor, but sTF proved unable to achieve this (Fig. 4-4, inset). Two-chain FSAP^{R313Q} differs from wild-type activated FSAP in carrying Gln instead of Arg as the C-terminus of the non-catalytic heavy chain. However, it seems unlikely that this could have any impact on substrate recognition by the serine protease domain.

An intriguing observation is that two-chain FSAP^{R313Q} does cleave FVII on cardiolipin membranes (Fig. 4-5C,D). The relevance of this finding remains unclear. Cardiolipin is an anionic phospholipid that is present in the inner mitochondrial membrane, although it may also occur in small amounts in plasma in association with lipoproteins [23]. In our experiments, membranes containing 40 mol % or less cardiolipin were unable to support FVII cleavage to any appreciable extent. This makes a role of cardiolipin-supported FVII cleavage in plasma unlikely. It seems conceivable, however, that suitable cardiolipin-rich membranes may be exposed

upon cell damage or apoptosis. While this would be compatible with a role of FSAP in association with apoptotic cells [8], the link with FVII activation, if any, remains unclear. Further studies will be needed to reassess the role of FSAP in the coagulation cascade. Usage of thermolysin-activated FSAP^{R313Q} may prove instrumental for this purpose.

Acknowledgements

We thank E. Sellink and J. Klein-Gebbinck for expert technical assistance, and P. P.F.M. Clijsters for initiating this study. This work was supported by the Trombosestichting Nederland, grant number TSN 2005-4.

Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

References

1. Choi-Miura NH, Tobe T, Sumiya J, Nakano Y, Sano Y, Mazda T, Tomita M. Purification and characterization of a novel hyaluronan-binding protein (PHBP) from human plasma: it has three EGF, a kringle and a serine protease domain, similar to hepatocyte growth factor activator. *J Biochem* 1996; **119**: 1157-65.
2. Römisch J, Vermöhlen S, Feussner A, Stöhr HA. The FVII activating protease cleaves single-chain plasminogen activators. *Haemostasis* 1999; **29**: 292-9.
3. Römisch J, Feussner A, Vermöhlen S, Stöhr HA. A protease isolated from human plasma activating factor VII independent of tissue factor. *Blood Coagul Fibrinolysis* 1999; **10**: 471-9.
4. Choi-Miura NH, Yoda M, Saito K, Takahashi K, Tomita M. Identification of the substrates for plasma hyaluronan binding protein. *Biol Pharm Bull* 2001; **24**: 140-3.
5. Etscheid M, Beer N, Fink E, Seitz R, Dodt J. The hyaluronan-binding serine protease from human plasma cleaves HMW and LMW kininogen and releases bradykinin. *Biol Chem* 2002; **383**: 1633-43.
6. Kanse SM, Parahuleva M, Muhl L, Kemkes-Matthes B, Sedding D, Preissner KT Factor VII-activating protease (FSAP): vascular functions and role in atherosclerosis. *Thromb Haemost* 2008; **99**: 286-9.
7. Etscheid M, Kress J, Seitz R, Dodt J. The hyaluronic acid-binding protease: a novel vascular and inflammatory mediator? *Int Immunopharmacol* 2008; **8**: 166-70.
8. Zeerleder S, Zwart B, te Velthuis H, Stephan F, Manoe R, Rensink I, Aarden LA. Nucleosome-releasing factor: a new role for factor VII-activating protease (FSAP). *FASEB J* 2008; **22**: 4077-84.
9. Choi-Miura NH, Takahashi K, Yoda M, Saito K, Mazda T, Tomita M. Proteolytic activation and inactivation of the serine protease activity of plasma hyaluronan binding protein. *Biol Pharm Bull* 2001; **24**: 448-52.
10. Muhl L, Galuska SP, Oörni K, Hernández-Ruiz L, Andrei-Selmer LC, Geyer R, Preissner KT, Ruiz FA, Kovanen PT, Kanse SM. High negative charge-to-size ratio in polyphosphates and heparin regulates factor VII-activating protease. *FEBS J* 2009; **276**: 4828-39.

11. Yamamichi S, Nishitani M, Nishimura N, Matsushita Y, Hasumi K. Polyamine-promoted autoactivation of plasma hyaluronan-binding protein. *J Thromb Haemost* 2010; **8**: 559-66.

12. Mertens K, Briët E, Giles AR. The role of factor VII in haemostasis: infusion studies of factor VIIa in a canine model of factor VIII deficiency. *Thromb Haemost* 1990; **64**: 138-44.

13. Van 't Veer C, Hackeng TM, Delahaye C, Sixma JJ, Bouma BN. Activated factor X and thrombin formation triggered by tissue factor on endothelial cell matrix in a flow model: effect of the tissue factor pathway inhibitor. *Blood* 1994; **84**: 1132-42.

14. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976; **72**: 248-54.

15. Mertens K, Cupers R, van Wijngaarden A, Bertina RM. Binding of human blood-coagulation Factors IXa and X to phospholipid membranes. *Biochem J* 1984; **223**: 599-605.

16. Bennett BD, Denis P, Haniu M, Teplow HD, Kahn S, Louis J-C, Citron M, Vassar R. A furin-like convertase mediates propeptide cleavage of BACE, the Alzheimer's β-secretase. *J Biol Chem* 2000; **275**: 37712-17.

17. Kannemeier C, Feussner A, Stöhr HA, Weisse J, Preissner KT, Römisch J. Factor VII and single-chain plasminogen activator-activating protease: activation and autoactivation of the proenzyme. *Eur J Biochem* 2001; **268**: 3789-96.

18. Pedersen AH, Lund-Hansen T, Bisgaard-Frantzen H, Olsen F, Petersen LC. Autoactivation of human recombinant coagulation factor VII. *Biochemistry* 1989; **28**: 9331-6.

19. Nakagaki T, Foster DC, Berkner KL, Kisiel W. Initiation of the extrinsic pathway of blood coagulation: evidence for the tissue factor dependent autoactivation of human coagulation factor VII. *Biochemistry* 1991; **30**: 10819-24.

20. Muhl L, Hersemeyer K, Preissner KT, Weimer T, Kanse SM. Structure-function analysis of factor VII activating protease (FSAP): sequence determinants for heparin binding and cellular functions. *FEBS Lett* 2009; **583**: 1994-8.

21. Lacroix M, Ebel C, Kardos J, Dobó J, Gál P, Závodszky P, Arlaud GJ, Thielens NM. Assembly and enzymatic properties of the catalytic domain of human complement protease C1r. *J Biol Chem* 2001; **276**: 36233-40.

22. Hoylaerts M, Rijken DC, Lijnen HR, Collen D. Kinetics of the activation of plasminogen by human tissue plasminogen activator: role of fibrin. *J Biol Chem* 1982; **257**: 2912-9.

23. Deguchi H, Fernández JA, Hackeng TM, Banka CL, Griffin JH. Cardiolipin is a normal component of human plasma lipoproteins. *Proc Natl Acad Sci USA* 2000; **97**: 1743-8.

Chapter 5

Factor seven activating protease, but not its Marburg I variant, promotes thrombin generation at low tissue factor levels by interacting with tissue factor pathway inhibitor

*Ilze Dienava-Verdoold,[1] *Fabian Stavenuiter,[1,2] Femke Stephan,[3] Alexander B. Meijer,[1,4] Herm-Jan M. Brinkman,[1] and Koen Mertens[1,2,4]
* These authors contributed equally to this study

[1]Department of Plasma Proteins, Sanquin Research, Amsterdam, The Netherlands
[2]Van Creveld Laboratory of UMC-Utrecht and Sanquin, Amsterdam, The Netherlands
[3]Department of Immunopathology, Sanquin Research, Amsterdam, The Netherlands
[4]Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences (UIPS), Utrecht University, Utrecht, The Netherlands

Manuscript in preparation

Abstract

The Marburg I polymorphism in the *HAPB2* gene encodes a G534E substitution in the serine protease domain of hyaluronan-binding protein-2 (HABP2), or factor seven activating protease (FSAP). This mutation has been associated with an increased risk of cardiovascular disease and venous thromboembolism. Whereas earlier studies attributed this to its factor VII (FVII) activating potential, we recently reported that activated FSAP fails to effectively activate FVII in a purified system. In the present study we used thrombography to assess the role of FSAP and its G534E variant in plasma. Activated FSAP proved capable of stimulating thrombin generation, but its effect was restricted to tissue factor concentrations in the sub pM range. This required the presence of tissue factor pathway inhibitor (TFPI), suggesting that activated FSAP potentiates thrombin generation by down-regulating TFPI. Indeed, using isolated recombinant proteins we found that TFPI is a fast-acting, competitive inhibitor of FSAP. The activated G534E variant, which displayed more than 10-fold reduced enzymatic activity, lacked effective inhibition by TFPI. In accordance with reduced TFPI interaction, thrombin generation was only marginally affected by $FSAP^{G534E}$. While the significance of FSAP as regulator of TFPI remains questionable, it seems evident that the Marburg I polymorphism is not associated with any procoagulant potential.

Introduction

Factor seven activating protease (FSAP) is the zymogen of a serine protease that has been discovered in 1996 as plasma hyaluronan-binding protein, or hyaluronan binding protein-2 (HABP2) [1]. This protein was found to be an activator of single-chain urokinase-type plasminogen activator (scuPA), and factor VII (FVII), suggesting a key role in haemostasis and thrombosis [2,3]. Subsequently, numerous additional functions for FSAP have been proposed, including inhibition of vascular smooth muscle cell proliferation and migration, suggesting a role in vascular biology as well [4,5]. The pleiotropic role of FSAP has created confusion with regard to its biological function. Within the *HABP2* gene two polymorphisms have been identified, called Marburg I and Marburg II [6]. The former encodes a Gly to Glu substitution at position 534 in the FSAP protease domain, and has been described to be a risk factor for venous thromboembolism [7–9], although others have challenged this view [10–12]. The prothrombotic effect of the G534E substitution has been explained by reduced scuPA activation, in combination with normal FVII activation [6]. Using isolated proteins, we recently reported that FSAP does not effectively activate FVII at all [13]. This leaves the possibility that FSAP and its G534E variant contribute to thrombin generation in a FVII-independent manner. In the present study, we addressed this issue employing various *in vitro* methods, including thrombography in plasma.

Methods

Proteins

Normal single chain FSAP (scFSAP) was constructed and expressed as the $FSAP^{R313Q}$ variant, which can be activated under control of the bacterial metalloprotease thermolysin [13]. Activation into the two-chain form (tcFSAP) was performed as described [13]. The Marburg I (G534E) substitution was introduced in the wild-type sequence using the QuickChange® mutagenesis kit and oligonucleotide primers 5'AGCTGGGGCCTGGAGTGTGAGAAGAGGCCAGGGGTCTAC3' (sense) and 5'GTAGACCCCTGGCCTCTTCTCACACTCCAGGCCCCAGCT3' (antisense). The $FSAP^{G534E}$ variant was expressed in CHO-cells, and purified and characterized as described in detail elsewhere [13]. Purified $FSAP^{G534E}$ proved spontaneously activated and was fully processed into the two-chain form. N-terminal sequencing (Eurosequence, Groningen, The Netherlands) identified Ile314 as the N-terminus of the 25 kDa light chain, demonstrating that $tcFSAP^{G534E}$ had been cleaved at the authentic activation site. Recombinant full length TFPI was expressed in mammalian cells and purified as described [14]. Recombinant activated FVII (FVIIa) was the 2nd International Standard for FVIIa concentrate 07/228 (NIBSC, South Mimms, UK).

Functional characterization

Thrombin generation was assessed by the calibrated automated thrombogram assay as described in detail elsewhere [15], with the exception that normal microparticle-free plasma was depleted of FSAP using Sepharose-coupled antibody anti-FSAP4 [16], and reconstituted with scFSAP, tcFSAP, or $tcFSAP^{G543E}$ (12 μg mL^{-1}) prior to thrombography. For some experiments, plasma was further depleted of TFPI using an immobilized antibody against the Kunitz-2 domain (anti-TFPI MW1847, Sanquin Reagents, Amsterdam, The Netherlands). FVII-deficient plasma was obtained from Haematologic Technologies Inc., Essex Junction, VT, USA, and depleted of microparticles and FSAP as described above. All other materials and analytical methods have been described previously [13].

Results and discussion

Effect of FSAP on thrombin generation involves TFPI

Thrombography was performed using normal, but FSAP-depleted plasma that was reconstituted with either non-activated scFSAP, or activated tcFSAP. Upon systematically lowering the TF concentration a contribution of tcFSAP became apparent at 1 pM TF, and this became more prominent at lower TF concentrations (Fig. 5-1A). At the lowest TF concentration (0.03 pM) thrombin generation only occurred in the presence of tcFSAP, but not of scFSAP or in the buffer control. Thus, the effect of tcFSAP is reflected by decreased lag time and increased peak height of thrombin generation. The FSAP concentration used in Fig. 5-1A (12 μg mL^{-1}, or 0.17 μM) equals the normal plasma concentration [6] and as such reflects the situation where all plasma FSAP would be converted into the activated form. The effects on thrombin generation parameters was dose-dependent (Fig. 5-1B), and

less pronounced at lower tcFSAP concentrations. To what extent FSAP activation occurs under physiological conditions remains unclear, but it seems likely that our observations relate to a stressed system, with a tcFSAP concentration that is non-physiologically high. In the absence of TF, tcFSAP did not display any thrombin generation (not shown). This indicates that FSAP, once activated, is able to modulate the TF-induced thrombin formation, but is not able to initiate coagulation by itself.

The procoagulant effect of FSAP was remarkable in view of our recent finding that FSAP is a poor FVII activator [13]. We considered the possibility that FVII activation by tcFSAP might require a cofactor that is present in plasma, but missing in our previous study with isolated proteins. However, the same effect on peak height and lag time occurred in FVII-deficient plasma supplemented with recombinant FVIIa (Fig. 5-1C). This implies that the effect of tcFSAP on thrombin generation in plasma does not involve FVII activation, and that the target of FSAP should be sought downstream of FVIIa in the coagulation cascade.

It has been well established that TF-induced thrombin generation is under tight control of the Kunitz-type inhibitor TFPI [17,18]. In concert with the anticoagulant protein S, it increases lag time and decreases peak height in thrombin generation [19], which is opposite to the effect of FSAP that we observed. Thus, tcFSAP might counteract TFPI in thrombin generation. This would seem compatible with recent reports that TFPI inhibits FSAP activity [20], and that FSAP cleaves and inactivates TFPI [21]. Indeed, tcFSAP did not display its effect in TFPI-deficient plasma, while this was restored by full-length TFPI in a dose-dependent manner (Fig. 5-1D). Apparently, the procoagulant effect of FSAP finds its basis in down-regulating TFPI instead of activating FVII.

Functional characterisation of the Marburg I variant FSAP

The "new" role of FSAP implies that the current view that the G534E variant displays a prothrombotic phenotype because of its FVII activating properties [6,8,9] needs to be reconsidered. The G534E variant was expressed in CHO cells, and proved spontaneously activated into its two-chain form (Fig. 5-2A, inset). Its reduced enzymatic activity was apparent from the lack of full autodegradation as displayed by normal FSAP [13], by its strongly reduced amidolytic activity towards S-2288 (Fig. 5-2A) and its reduced scuPA activation (Fig. 5-2B). While S-2288 cleavage by normal tcFSAP was readily inhibited by TFPI, only minor inhibition of tcFSAP^{G534E} was observed (Fig. 5-2C). As visualized in a classical Dixon plot [22], TFPI displayed competitive inhibition with a K_i of 8 nM (Fig. 5-2D). Moreover, inhibition was nearly instantaneous, and due to binding, but not cleavage of TFPI (Fig. 5-2E). These data demonstrate that the G534E substitution not only disrupts FSAP enzymatic activity, but also its interaction with TFPI. Therefore, the G534E variant should lack the TFPI-dependent effect on thrombin generation as well. Indeed, thrombin peak height and lag time were similar to that of scFSAP, while tcFSAP displayed the typical response (Fig. 5-2F).

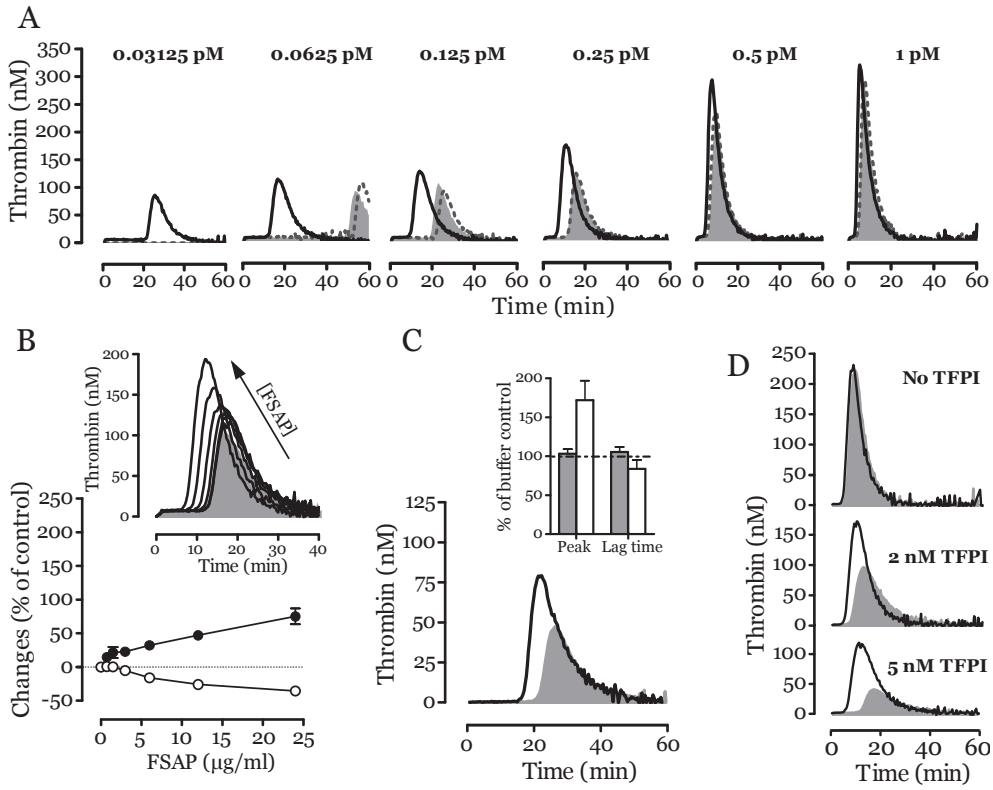


Figure 5-1 Effect of FSAP on thrombin generation. *(A) Thrombography was performed in FSAP-depleted plasma that had been reconstituted with 12 µg mL^{-1} scFSAP (broken lines) or tcFSAP (solid lines). Plasma was 1:1 diluted with buffer (50 mM Tris, 150 mM NaCl, pH 7.4) containing a fixed amount of phospholipids (final concentration 4 µM), varying amounts of recombinant tissue factor, and a mixture of $CaCl_2$ and fluorogenic substrate as described [15]. Buffer controls are indicated by filled grey profiles. (B) Effect of varying concentrations of tcFSAP on lag time (open circles) and thrombin peak height (closed circles) in the presence of 0.2 pM of TF. Data represent mean ± SD from three experiments. The inset shows a single set of individual thrombin generation profiles (grey area represents buffer control). (C) Thrombin generation in FSAP- and FVII-depleted plasma that was reconstituted with FSAP (12 µg mL^{-1}) and FVIIa (10 nM) at 0.2 pM TF. The inset shows the effect of tcFSAP (open bars) and scFSAP (grey bars) on peak height and lag time. Data represent mean ± SD from three experiments. (D) Thrombin generation in TFPI- and FSAP-depleted plasma reconstituted with tcFSAP (12 µg mL^{-1}) and 0 (top), 2 (centre) or 5 (bottom) nM of TFPI at a TF concentration of 0.2 pM. Thrombin generation in the absence and presence of tcFSAP is depicted as solid lines and filled grey profiles, respectively.*

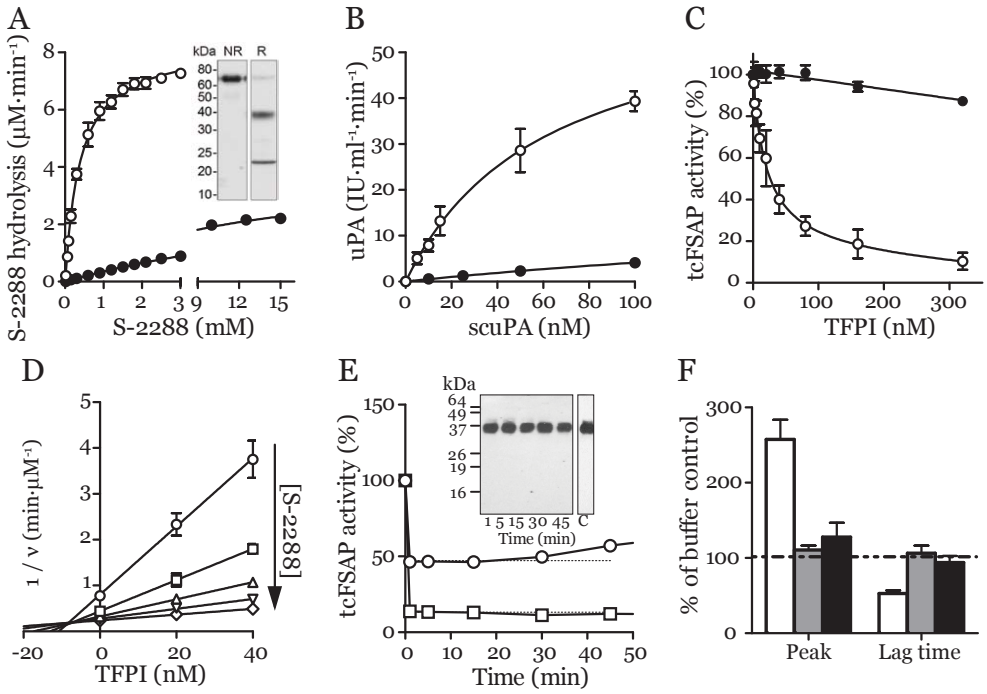


Figure 5-2 Comparison of normal FSAP with the Marburg I variant. *(A) Amidolytic activity of normal tcFSAP (10 nM, open circles) and tcFSAP^{G534E} (25 nM, closed circles) towards substrate S-2288 at 37 ºC in a buffer containing 0.2% (w/v) bovine serum albumin, 150 mM NaCl, 5 mM $CaCl_2$, 50 mM Tris-HCl (pH 8.0). The inset shows 10% SDS/PAGE of purified tcFSAP^{G534E} (1 µg/lane, silver staining) under non-reducing (NR) and reduced (R) conditions. (B) Activation of scuPA by normal tcFSAP (open circles) and tcFSAP^{G534E} (closed circles) at a concentration of 0.25 nM in the presence of 10 IU ml^{-1} of heparin. Experimental details were as described [13]. (C) 3 nM of normal tcFSAP (open circles) or 30 nM of tcFSAP^{G534E} (closed circles) were incubated with S-2288 (0.4 mM) and varying amounts of TFPI at 37 ºC in a buffer containing 0.1% (w/v) Tween-80, 150 mM NaCl, 5 mM $CaCl_2$, 50 mM Tris-HCl (pH 8.0). The initial rate of substrate was recorded and residual activity was plotted against the TFPI concentration. (D) Dixon plot [22] of inhibition experiments of normal tcFSAP (3 nM) at S-2288 concentrations increasing from 0.04 mM (circles) to 0.1 mM (squares), 0.2 mM (triangles), 0.4 mM (inverted triangles) and 0.8 mM (diamonds), showing that K_i was 8 nM. (E) Time course of tcFSAP (3 nM) inhibition 30 (circles) or 300 nM (squares) of TFPI under the same conditions as in panels C and D. The inset shows immunoblot analysis of subsamples of inhibition experiments at 30 nM TFPI. TPFI was visualized using antibody against Kunitz-3 domain (MW1845, Sanquin Reagents, Amsterdam, The Netherlands). Lane C represents a control of TFPI in the absence of tcFSAP. (F) Thrombin generation in FSAP- depleted plasma reconstituted with FSAP (12 µg mL^{-1}) at 0.2 pM TF. Peak height and lag time are shown for tcFSAP (open bars), scFSAP (grey bars), and tcFSAP^{G534E} (closed bars). In all panels, data represent mean ± SD from at least three experiments.*

Concluding remarks

The present study shows that FSAP, by virtue of its fast and high affinity association with TFPI, is capable of disrupting the regulatory role of TFPI in the TF pathway of coagulation. The relevance hereof seems questionable, however, because it is restricted to very low tissue factor concentrations and high levels of FSAP activation. Whether or not this mechanism may impact on cell-associated TFPI as well remains an open question. At this point it seems appropriate to abandon the term FSAP and rename the protein to its gene HABP2 [13,23]. With regard to the Marburg I polymorphism in the *HABP2* gene, our data imply that the current rationale in favour of a procoagulant phenotype is no longer justified.

Acknowledgements

We thank Dr. Ida Hilden (Novo Nordisk, Måløv, Denmark) for providing TFPI, and prof. L.A. Aarden and dr. S. Zeerleder for their interest in this work. Part of the study was supported by Trombosestichting Nederland, grant number TSN 2005-4.

Author contributions

I.D.-V., F. Stavenuiter, H.J.M.B., A.B.M. and KM designed the study, I.D.-V., F. Stephan and H.J.B.M. performed experiments, and I.D.-V., H.J.M.B. and K.M. wrote the paper.

References

1. Choi-Miura NH, Tobe T, Sumiya JI, Nakano Y, Sano Y, Mazda T, Tomita M. Purification and characterization of a novel hyaluronan-binding protein (PHBP) from human plasma: it has three EGF, a Kringle and a serine protease domain, similar to hepatocyte growth factor activator. *J Biochem*. 1996; **119**: 1157-65.
2. Choi-Miura NH, Yoda M, Saito K, Takahashi K, Tomita M. Identification of the substrates for plasma hyaluronan binding protein. *Biol Pharm Bull*. 2001; **24**: 140-3.
3. Römisch J, Feussner A, Vermöhlen S, Stöhr HA. A protease isololated from human plasma activating Factor VII independent of tissue factor. *Blood Coagul Fibrinolysis*. 1999; **10**: 471-9.
4. Etscheid M, Kreß JA, Seitz R, Dodt J. The hyaluronic acid-binding protease: A novel vascular and inflammatory mediator? *Int Immunopharmacol*. 2008; **8**: 166-70.
5. Mambetsariev N, Mirzapoiazova T, Mambetsariev B, Sammani S, Lennon FE, Garcia JGN, Singleton PA. Hyaluronic acid binding protein 2 is a novel regulator of vascular integrity. *Arterioscler Thromb Vasc Biol*. 2010; **30**: 483-90.
6. Römisch J, Feussner A, Nerlich C, Stöhr HA, Weimer T. The frequent Marburg I polymorphism impairs the pro-urokinase activating potency of the factor VII activating protease (FSAP). *Blood Coagul Fibrinolysis*. 2002; **13**: 433-41.
7. Willeit J, Kiechl S, Weimer T, Mair A, Santer P, Wiedermann CJ, Römisch J. Marburg I polymorphism of factor VII-activating protease. *Circulation*. 2003; **107**: 667-70.
8. Sedding DG, Daniel J-M, Muhl L, Hersemeyer K, Brunsch H, Kemkes-Matthes B, Braun-Dullaeus RC, Tillmanns H, Weimer T, Preissner KT, Kanse SM. The G534E

polymorphism of the gene encoding the factor VII–activating protease is associated with cardiovascular risk due to increased neointima formation. *J Exp Med*. 2006; **203**: 2801-7.

9. Hoppe B, Tolou F, Radtke H, Kiesewetter H, Dörner T, Salama A. Marburg I polymorphism of factor VII-activating protease is associated with idiopathic venous thromboembolism. *Blood*. 2005; **105**: 1549-51.

10. van Minkelen R, de Visser MCH, Vos HL, Bertina RM, Rosendaal FR. The Marburg I polymorphism of factor VII-activating protease is not associated with venous thrombosis. *Blood*. 2005; **105**: 4898-9.

11. Gulesserian T, Hron G, Endler G, Eichinger S, Wagner O, Kyrle PA. Marburg I polymorphism of factor VII-activating protease and risk of recurrent venous thromboembolism. *Thromb Haemost*. 2006; **95**: 65-7.

12. Franchi F, Martinelli I, Biguzzi E, Bucciarelli P, Mannucci PM. Marburg I polymorphism of factor VII-activating protease and risk of venous thromboembolism. *Blood*. 2006; **107**: 1731.

13. Stavenuiter F, Dienava-Verdoold I, Boon-Spijker MG, Brinkman HJM, Meijer AB, Mertens K. Factor seven activating protease (FSAP): does it activate factor VII? *J Thromb Haemost*. 2012; **10**: 859-66.

14. Pedersen AH, Nordfang O, Norris F, Wiberg FC, Christensen PM, Moeller KB, Meidahl-Pedersen J, Beck TC, Norris K, Hedner U. Recombinant human extrinsic pathway inhibitor. Production, isolation, and characterization of its inhibitory activity on tissue factor-initiated coagulation reactions. *J Biol Chem*. 1990; **265**: 16786-93.

15. Dienava-Verdoold I, Boon-Spijker MG, de Groot PG, Brinkman HJM, Voorberg J, Mertens K, Derksen RHWM, de Laat B. Patient-derived monoclonal antibodies directed towards β2-glycoprotein I display lupus anticoagulant activity. *J Thromb Haemost*. 2011; **9**: 738-47.

16. Zeerleder S, Zwart B, te Velthuis H, Stephan F, Manoe R, Rensink I, Aarden LA. Nucleosome-releasing factor: a new role for factor VII-activating protease (FSAP). *FASEB J*. 2008; **22**: 4077-84.

17. Crawley JTB, Lane DA. The haemostatic role of tissue factor pathway inhibitor. *Arterioscler Thromb Vasc Biol*. 2008; **28**: 233-42.

18. Huang ZF, Wun TC, Broze GJ. Kinetics of factor Xa inhibition by tissue factor pathway inhibitor. *J Biol Chem*. 1993; **268**: 26950-5.

19. Hackeng TM, Rosing J. Protein S as cofactor for TFPI. *Arterioscler Thromb Vasc Biol*. 2009; **29**: 2015-20.

20. Stephan F, Dienava-Verdoold I, Bulder I, Wouters D, Mast AE, Te Velthuis H, Aarden LA, Zeerleder S. Tissue factor pathway inhibitor is an inhibitor of factor VII-activating protease. *J Thromb Haemost*. 2012; **10**: 1165-71.

21. Kanse SM, Declerck PJ, Ruf W, Broze G, Etscheid M. Factor VII–activating protease promotes the proteolysis and inhibition of tissue factor pathway inhibitor. *Arterioscler Thromb Vasc Biol*. 2012; **32**: 427-33.

22. Dixon M. The determination of enzyme inhibitor constants. *Biochem J*. 1953; **55**: 170-1.

23. McVey JH. FSAP: Does it do what it says on the tin? *J Thromb Haemost*. 2012; **10**: 857-8.

Chapter 6

Platelet-mediated proteolytic down-regulation of the anticoagulant activity of protein S in individuals with haematological malignancies

Ilze Dienava-Verdoold[1], Marina R. Marchetti[2], Liane C.J. te Boome[3,4], Laura Russo[2], Anna Falanga[2], Harry R. Koene[3,5], Koen Mertens[1,6], Herm-Jan M. Brinkman[1]

[1]Department of Plasma Proteins, Sanquin Research, Amsterdam, The Netherlands
[2]Immunohematology and Transfusion Medicine Department, Ospedali Riuniti di Bergamo, Bergamo, Italy
[3]Department of Hematology, Academical Medical Center, University of Amsterdam, Amsterdam, The Netherlands
[4]Present address: Department of Hematology, Academic Medical Center, University of Utrecht, Utrecht, The Netherlands
[5]Pressent address: Department of Internal Medicine, St. Antonius Hospital, Nieuwegein, The Netherlands
[6]Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences, Utrecht University, Utrecht, The Netherlands

Thromb Haemost. 2012; 107: 468–76.

Summary

The natural anticoagulant protein S contains a so-called thrombin-sensitive region (TSR), which is susceptible to proteolytic cleavage. We have previously shown that a platelet-associated protease is able to cleave protein S under physiological plasma conditions *in vitro*. The aim of the present study was to investigate the relation between platelet-associated protein S cleaving activity and *in vivo* protein S cleavage, and to evaluate the impact of *in vivo* protein S cleavage on its anticoagulant activity. Protein S cleavage in healthy subjects and in thrombocytopenic and thrombocythaemic patients was evaluated by immunological techniques. Concentration of cleaved and intact protein S was correlated to levels of activated protein C (APC)-dependent and APC-independent protein S anticoagulant activity. In plasma from healthy volunteers 25% of protein S is cleaved in the TSR. While in plasma there was a clear positive correlation between levels of intact protein S and both APC-dependent and APC-independent protein S anticoagulant activities, these correlations were absent for cleaved protein S. Protein S cleavage was significantly increased in patients with essential thrombocythaemia (ET) and significantly reduced in patients with chemotherapy-induced thrombocytopenia. In ET patients on cytoreductive therapy, both platelet count and protein S cleavage returned to normal values. Accordingly, platelet transfusion restored cleavage of protein S to normal values in patients with chemotherapy-induced thrombocytopenia. In conclusion, proteases from platelets seem to contribute to the presence of cleaved protein S in the circulation and may enhance the coagulation response *in vivo* by down-regulating the anticoagulant activity of protein S.

Introduction

Protein S, a vitamin K-dependent plasma protein, is one of the molecules involved in down-regulation of the coagulation cascade. The importance of protein S as natural coagulation inhibitor is underscored by the occurrence of fatal consumptive coagulopathy in the embryonic stage of homozygous protein S-deficient mice, while heterozygous deficient mice exhibit a thrombotic phenotype [1,2]. In humans, heterozygous protein S deficiency is associated with the development of both venous and arterial thrombosis [3,4]. The anticoagulant activities ascribed to protein S are numerous. Well established is the activated protein C (APC)-cofactor activity of protein S that refers to the cofactor function of protein S in the proteolytic inactivation of both factor (F) Va and FVIIIa by APC [5]. More recently, protein S has been recognized as cofactor for tissue factor pathway inhibitor (TFPI) in the inhibition of the tissue factor (TF)/FVIIa/FXa complex [6]. Protein S may also display a direct anticoagulant activity, that refers to the ability of protein S to inhibit the assembly and activity of FVIIIa/FIXa and FVa/FXa complexes [7,8].

Protein S contains a so-called thrombin-sensitive region (TSR) comprising residues Cys47–Ala75, that is susceptible to proteolytic cleavage. *In vitro* studies employing purified proteins in plasma free systems have revealed that FXa, thrombin and elastase are able to cleave protein S at the TSR. FXa cleaves at Arg60 only [9–12], while thrombin cleaves at Arg49 and Arg70 [10–12]. Elastase is reported to cleave protein S adjacent the protease sensitive loop at Val73 [13]. It has been firmly established

that upon TSR cleavage at any of these sites the APC-cofactor activity of protein S is abolished [9–11,14]. TSR cleavage may also down-regulate the direct anticoagulant activity of protein S [9], with respect to cleavage at Arg60, an observation challenged by others [11]. Whether the tissue factor pathway inhibitor (TFPI)-cofactor activity of protein S is also affected upon TSR cleavage is presently unknown. The full impact of *in vivo* TSR cleavage on the role of protein S as an anticoagulant in the generation of thrombin in plasma, therefore, remains to be elucidated.

In plasma, a single-chain molecule as well as a two chain form of protein S can be detected [13,15]. This two-chain variant is the result of *in vivo* cleavage at position Arg60 [13]. To date, the enzyme responsible for the presence of TSR-cleaved protein S in the circulation is not known. Indeed, the so far described proteases targeting the TSR of protein S *in vitro*, thrombin, FXa and elastase do not cleave protein S in plasma under normal physiological conditions [12,13,16]. Moreover, the documented *in vivo* cleavage at Arg60 excludes any contribution of thrombin and elastase to the presence of cleaved protein S in the circulation. Mitchell and Salem [17] have reported protein S cleaving protease activity in platelets. Our group confirmed and extended this observation by showing that a protease from platelets is able to cleave protein S in plasma [12]. Concurrent with the presence of Arg60 cleaved protein S in plasma, cleavage of protein S by the platelet protease occurred at Arg60 [12]. We hypothesized that platelets might contribute to the presence of cleaved protein S in the circulation. Recently we described the occurrence of acquired APC resistance in patients with essential thrombocythaemia (ET) [18], a phenomenon to which platelet-mediated proteolysis of protein S might be accountable for.

The aim of this study was to extend prior observations on the modulation of the anticoagulant activity of protein S by limited proteolysis *in vivo*. Particularly, we investigated whether a correlation exists between platelet count and levels of cleaved protein S in the circulation of individuals with pathological conditions characterized by low platelet counts, as occurred in patients under chemotherapy, and high platelet count, i.e., in patients with ET. The present study provides new insights into the contribution of platelets in the regulation of thrombin generation under normal and pathological conditions.

Materials and Methods

Study subjects

The study group consisted of 106 randomly selected healthy Dutch blood bank donors (51 males / 55 females), 34 patients with chemotherapy-induced thrombocytopenia (17 males / 17 females), nine patients (4 males / 5 females) with idiopathic thrombocytopenia and 24 patients (11 males / 13 females) with ET. The chemotherapy-induced thrombocytopenia group consisted of patients with haematological malignancies treated with myelo-suppressive chemotherapy i.e., multiple myeloma (11 individuals), acute myeloid leukaemia (11 individuals), myelodysplastic syndrome (6 individuals), acute lymphoblastic lymphoma (2 individuals) and non-Hodgkin lymphoma (4 individuals). Patients were treated in the Amsterdam Academical Medical Center with high dose chemotherapy

according to standard protocols. Blood samples were taken prior to and during thrombocytopenia. We also included samples from patients with chemotherapy-induced thrombocytopenia collected within 48 hours (h) after platelet support (leukocyte-depleted platelet concentrate containing 350 × 10^9 platelets per unit, Sanquin Blood Supply Foundation, Amsterdam, the Netherlands). Blood samples from ET patients and the corresponding group of 24 healthy controls (16 males / 8 females), were collected at the Bergamo Thrombosis and Hemostasis Center. At the time of blood collection, five ET patients were on aspirin treatment and 17 on cytoreductive treatment with hydroxyurea (HU). The present study was conducted with the approval of the ethical committees of the involved medical institutions and all blood samples were obtained after informed consent. The procedures followed were in accordance with the Helsinki declaration of 1975 as revised in 2000.

Blood collection and plasma preparation

Venous blood samples were collected into siliconized tubes containing trisodium citrate (0.129 M, 1/9 v/v). Differential blood cell counts were determined by a Sysmex-XE 2100 hematology analyzer (Sysmex, Kobe, Japan) or a NE800 Analyzer (Dasit, Milan, Italy). Plasma was separated by centrifugation of whole blood for 15 min at 4,000 g at room temperature, aliquoted, snap-frozen and stored at -80 °C until testing.

Determination of cleaved protein S in plasma by western blotting

Protein S was immune-precipitated from plasma samples using a home-made rabbit anti-human protein S IgG [12] coupled to CNBr-sepharose. Intact and cleaved protein S was then separated by electrophoresis on 7.5 % polyacrylamide gels under reducing conditions. Protein bands were visualized by immunoblotting with HRP-labelled sheep anti-human protein S IgG. The bands on immunoblot were stained by ECL (Roche Diagnostics GmbH, Mannheim, Germany) and quantified using the Image-J analysing software (National Institute of Health, Bethesda, MD, USA). Percentage of cleavage was calculated from the digitally quantified intact and cleaved protein S bands. Some experiments were performed with plasma in which protein S was cleaved with 100 nM thrombin [12]. Coloured protein molecular weight markers (Rainbow™ RPN756) were from Amersham Life Science (Buckinghamshire, UK).

Determination of cleaved protein S in plasma by ELISA

Total and intact protein S antigen levels were measured in citrated plasma samples by in-house ELISAs, as described [12]. For both determinations, a home-made polyclonal antibody against human protein S was used as catching antibody. A commercially available HRP-labelled sheep-anti human protein S IgG (Affinity Biologicals Inc, Ancaster, Ontario, Canada) or HRP-labelled monoclonal antibody (MoAb) CLB-PS18 [12,19] was used to detect total protein S. HRP-labelled MoAb CLB-PS52 was employed as detection antibody for intact protein S. This MoAb is directed against the protein S peptide sequence Phe40–Ala59 and recognizes TSR-intact protein S only [12,19]. Our reference plasma (pooled citrated plasma from

40 large quantity donations, Blood bank division of our Institute) contained 346 nM total protein S (estimated concentration) [20] and 225 nM intact protein S (calculated from the relative value of 35 % cleaved protein S in our plasma pool as determined by Western blotting) and was used for the calibration curve in both ELISAs. The levels of cleaved protein S in plasma samples were calculated from the determined levels of total and intact protein S.

Determination of protein S anticoagulant activity

Protein S activity was measured both as APC-dependent and APC-independent activity by the calibrated automated thrombogram (CAT) assay [21] as described by Serè *et al.* [22] with some modifications. Thrombin generation was performed in polystyrene round-bottom 96 well-microtiter plates (Greiner Bio-one, ref. no. 650161, Kremsmuenster, Austria) in a final volume of 120 ml. Typically, prewarmed reaction mixtures contained 60 ml plasma, 20 ml additives (APC, MoAb CLB-PS13) diluted in Tris buffered saline (50 mM Tris, 150 mM NaCl, pH 7.4) supplemented with 1 % w/v albumin and 20 ml of a TF/phospholipid mixture (Thrombinoscope, Maastricht, The Netherlands). Final concentration of TF and phospholipid vesicles (phosphatidylserine / phosphatidylcholine / phosphatidylethanolamine, 2/6/2 molar ratio) was 1 pM and 4 mM, respectively. Reaction was started with 20 µl of a mixture of the fluorogenic substrate (Z-Gly-Gly-Arg-AMC; Bachem, Bubendorf, Switzerland) and $CaCl_2$ (final concentration 0.5 mM and 15 mM respectively). Substrate hydrolysis, monitored by recording the fluorescence signal at 460 nm with excitation at 390 nm in a plate fluorimeter (Fluoroscan Acsent, Thermo Labsystems, Helsinki, Finland), was correlated with thrombin concentrations using the thrombin calibrator from Thrombinoscope as internal standard. The APC cofactor activity of protein S was measured as the % decrease in peak height of the thrombin generation curve in the presence of 0.7 nM APC (Enzyme Research Laboratories, Uplands Swansea, UK), a concentration of APC that reduced peak thrombin by 50% in normal pooled plasma. The APC-independent anticoagulant activity of protein S was measured as the % increase in peak height of the thrombin generation curve in the presence of our inhibiting anti-protein S MoAb CLB-PS13 [22-24]. Titration experiments in normal pooled plasma revealed maximal enhanced peak thrombin values at IgG concentrations > 15 mg mL^{-1}. Data reported in the present study were obtained with 100 mg mL^{-1} MoAb CLB-PS13. Specificity of the assays was confirmed in plasmas deficient in protein S (Hyphen Biomed, Neuville-sur-Oise, France) or protein C (Siemens Healthcare Diagnostics, Deerfield, IL USA) and in normal plasma supplemented with 50 mg mL^{-1} of an inhibitory anti-protein C polyclonal antibody. Anti-protein C IgG was obtained by immunizing rabbits with human protein C according to standard laboratory protocols. Titration experiments revealed that 50 mg mL^{-1} anti protein C IgG was able to inhibit up to 50 nM APC as measured by CAT

Some experiments were performed with intact and cleaved protein S supplemented in protein S deficient plasma. Intact protein S was purified from human plasma as described [12]. TSR-cleaved protein S was obtained by incubating purified intact protein S with a-thrombin (prepared as described) [12] in a 100 / 1 molar ratio in Tris buffered saline for 2.5 h at 37 °C. Subsequently, thrombin was irreversibly inhibited with D-Phe-Pro-Arg-chloromethyl ketone (PPACK, Bachem) and excess

of inhibitor was removed by dialysis against Tris buffered saline. The preparation contained cleaved protein S only, as judged by SDS-PAGE.

Protein S activity in plasma samples was also evaluated by a commercial available functional assay of protein S (Instrumentation Laboratory, Milan, Italy) by measuring the degree of prolongation of a prothrombin time in the presence of TF, phospholipids, calcium ions and APC according to the manufacturer's instruction.

Statistical analysis

Results are expressed as mean ± SD. Student's t-test was employed to compare the difference between the mean values of different groups. Differences were considered significant at a p-value < 0.05. Correlation and linear regression statistical analysis was performed using the Graphpad Prism 5 package (La Jolla, CA, USA).

Results

Quantification of circulating cleaved protein S in healthy individuals

To investigate the occurrence of cleaved protein S in plasma, immunoprecipitates from plasma of 12 healthy subjects were subjected to SDS-PAGE under reducing conditions. The immunoblot (Fig. 6-1A) revealed the presence of two forms of protein S in plasma with an estimated apparent molecular weight of 86 and 77 kDa, respectively. Fig. 6-1B shows that upon incubation of plasma with thrombin the upper band disappeared while the intensity of the lower band increased, indicating the upper band as intact protein S and the lower band as heavy chain of TSR cleaved protein S [15]. To exclude protein S cleavage post-venipuncture, blood was collected both in the absence and presence of 55 mM di-isopropylfluorophosphate (DFP) [12]. The analysis of protein S in these two types of plasma samples gave a similar portion of cleaved protein S (i.e., 31% and 32%, respectively; data not shown), indicating that the portion of cleaved protein S measured in plasma truly reflects the *in vivo* cleavage of protein S. We then measured the percentage of cleaved protein S by ELISA in a population of 106 healthy subjects. The results as depicted in Fig. 6-1C show that the percentage of cleaved protein S ranged between 5 and 51 %, with an average of 25 ± 8% (mean ± SD).

Effect of in vivo protein S cleavage on its anticoagulant activities

Fig. 6-2 shows the result of measurements on the impact of TSR cleavage on the anticoagulant activities of protein S by the CAT assay performed with purified intact and thrombin-cleaved protein S added to protein S-deficient plasma. Plasma containing intact protein S showed sensitivity in the CAT assay for both APC and the anti-protein S MoAb CLB-PS13 (Fig. 6-2A, left panel and Fig. 6-2B). On the contrary, protein S-deficient plasma supplemented with TSR-cleaved protein S was insensitive to the addition of APC or MoAb CLB-PS13 (Fig. 6-2A, right panel and Fig. 6-2B), similar as occurred in protein S deficient plasma alone (Fig. 6-2B).

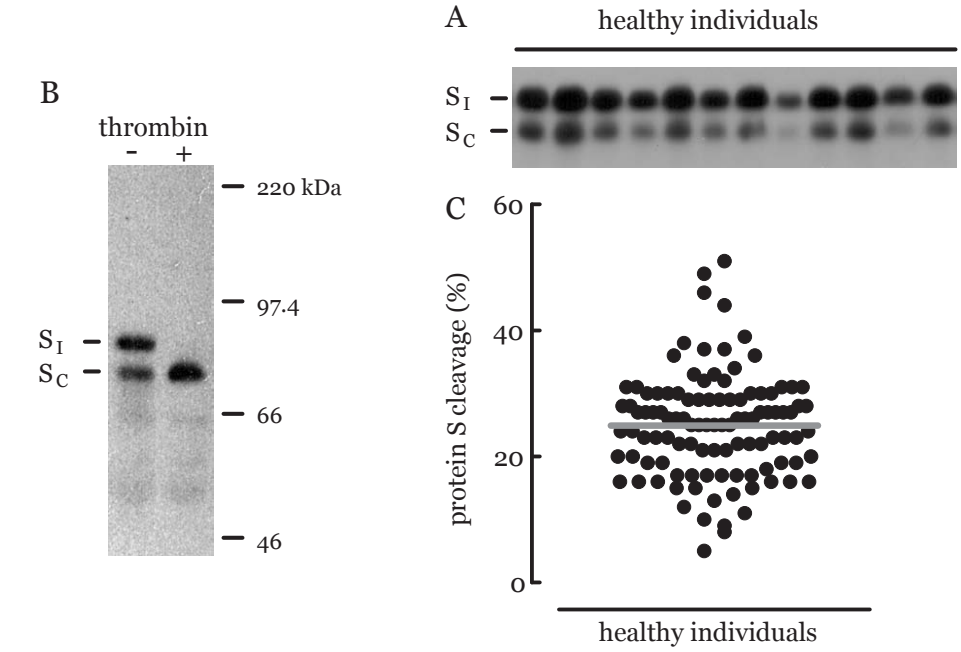


Figure 6-1 Presence of cleaved protein S in plasma from healthy individuals. *(A) Immunoblot of protein S immune-precipitated from plasma from 12 healthy volunteers. Si, intact protein S. Sc, cleaved protein S. (B) Immunoblot of protein S immune-precipitated from normal pooled plasma incubated for 1 hour in the absence and presence of thrombin (100 nM). (C) Protein S cleavage examined by ELISA in plasma from 106 healthy individuals.*

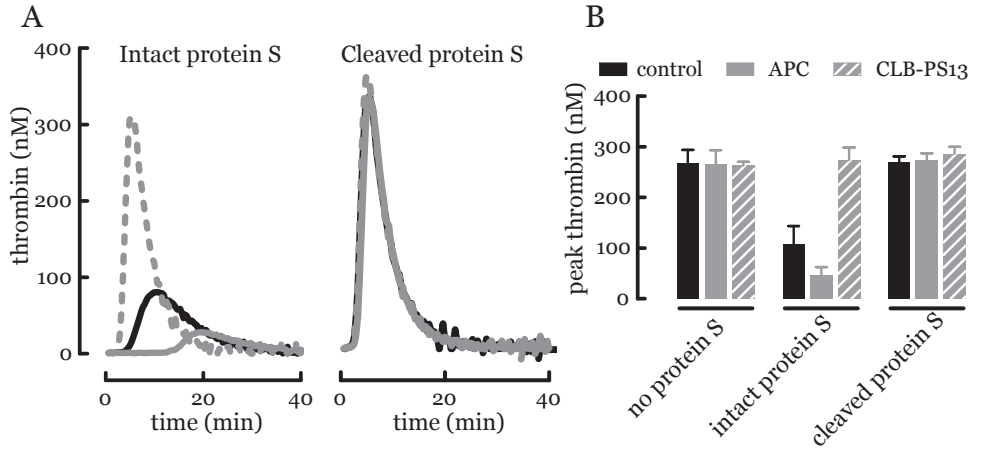


Figure 6-2 Influence of intact and TSR-cleaved protein S on thrombography. *(A) Representative thrombin generation profiles (CAT assay) obtained with protein S-deficient plasma supplemented with 140 nM intact protein S or thrombin-cleaved protein S in the absence (solid black curve) and presence (striped gray curve) of MoAb CLB-PS13 (100 µg mL^{-1}) or APC (0.7 nM) (solid gray curve). (B) Peak thrombin value obtained with the CAT assay in protein S deficient plasma, in absence or in presence of intact or TSR-cleaved protein S. The experiments were performed in the absence (black bar) and presence of (solid gray bar) APC (0.7 nM) or (striped gray bar) MoAb CLB-PS13 (100 µg mL^{-1}). Data are expressed as mean ± SD of three different experiments.*

We subsequently analyzed plasma from a subset of 47 healthy individuals (Fig. 6-3). Correlating the change in peak thrombin in the CAT assay upon CLB-PS13 or APC addition with levels of intact protein S in these plasma samples yielded a positive correlation for both CLB-PS13 (Fig. 6-3, left upper panel) and APC (Fig. 6-3, middle upper panel). For cleaved protein S this relationship was absent (Fig. 6-3, lower left and middle panels). A similar correlation was observed in a different subset of normal plasma samples when tested in a prothrombin time based assay for functional protein S (Fig. 6-3, right upper and lower panels). These results indicate that the anticoagulant activities displayed by protein S in plasma are attributable to intact protein S only.

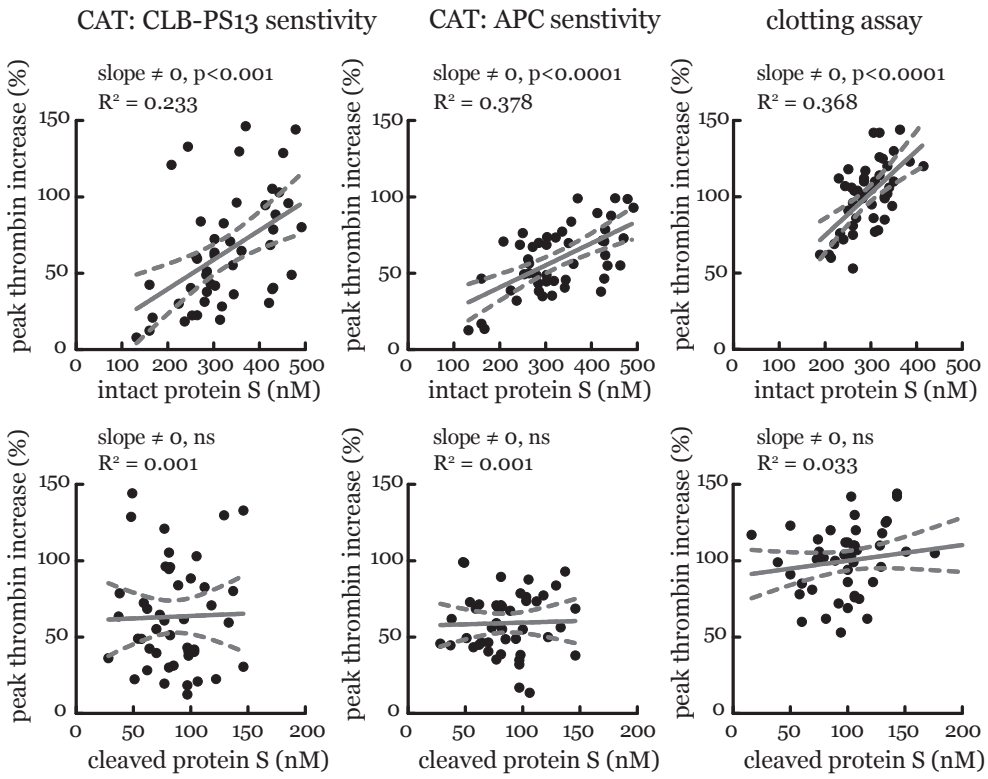


Figure 6-3 Correlation of protein S anticoagulant activities with levels of intact protein S (upper panels) and cleaved protein S (lower panels) in plasma samples from healthy individuals. *Sensitivity to CLB-PS13 (APC-independent anticoagulant activity, left upper and lower panel) and sensitivity to APC (APC-dependent anticoagulant activity, middle upper and lower panel), as determined by CAT, is expressed as % change in peak thrombin relative to peak thrombin values in the absence of CLB-PS13 and APC. Protein S activity as determined by a prothrombin time based assay (right upper and lower panel) is expressed as % of normal. Deming linear regression was used to investigate whether the correlation is significantly non-zero. Dashed lines represent the 95 % confidence interval.*

Contribution of circulating platelets to protein S cleavage *in vivo*

Experiments were performed to correlate *in vivo* protein S cleavage with circulating platelets. In 45 healthy individuals showing a wide range of protein S cleavage (6–48 %), no correlation was observed between platelet count (133–361 × 10^9 L^{-1}) and protein S cleavage (Fig. 6-4A). Such a correlation was also absent for white blood cells (WBC count 4–9 × 10^9 L^{-1}; R^2 0.03) (data not shown). On the other hand, a clear correlation between platelet count and protein S cleavage was observed in blood samples from 44 patients with haematological malignancies randomly taken during HU treatment or high-dose chemotherapy (Fig. 6-4B). The effect of platelet number on protein S cleavage was most pronounced at platelet counts below 50 × 10^9 L^{-1} and protein S cleavage tend to a maximum value at platelet counts above normal.

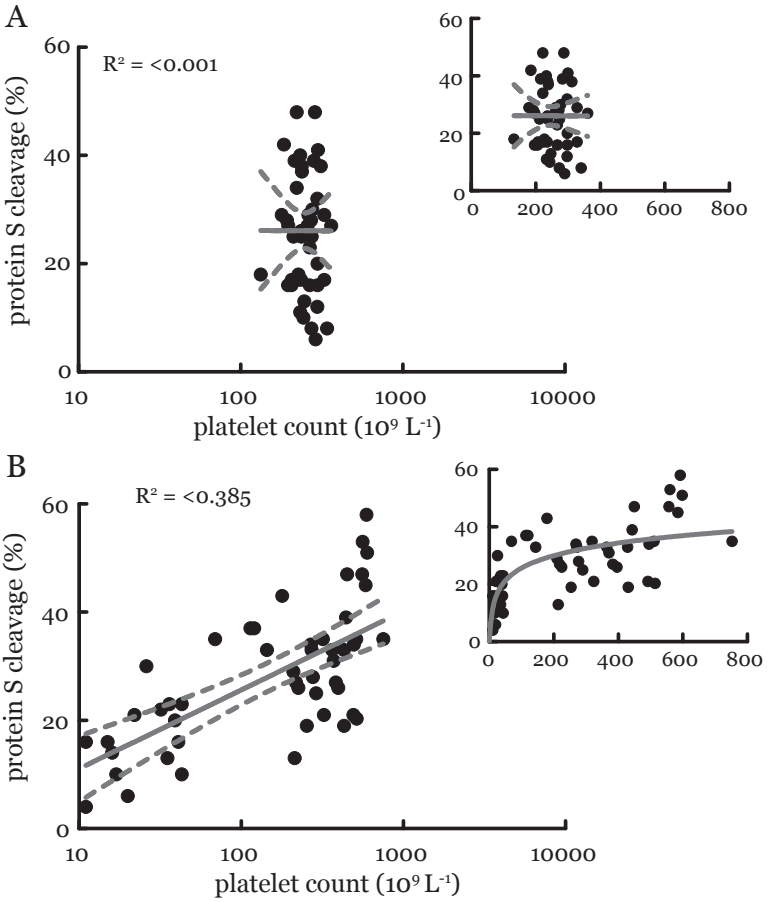


Figure 6-4 Semi logarithmic plots and linear plots (insets) showing the correlation between platelet count and protein S cleavage in 45 healthy individuals (A) and 44 chemotherapy treated patients not receiving platelet support (B). *In total 49 samples from 17 HU-treatment patients and 27 patients on high dose chemotherapy were included. Blood samples were randomly taken during treatment. Nonlinear regression analysis was performed employing the following equation: Y=Yintercept + Slope*log(X). Semi logarithmic plots and linear plots all show the 95% confidence interval (dashed line).*

Table 6-1 Cleavage of protein S in thrombocytopenia and thrombocytosis

Subjects		Protein S cleavage (%)	Total protein S (nM)
Study 1: Essential Thrombocythaemia			
Control	(n = 24)	29 ± 11	388 ± 73
Thrombocytosis	(n = 7)	40 ± 15*	305 ± 65*
Platelet count normalization (HU)	(n = 9)	28 ± 6†	343 ± 76
Study 2: Chemotherapy-induced thrombocytopenia			
Control	(n = 16)	32 ± 9	386 ± 79
Thrombocytopenia	(n = 15)	16 ± 7*	411 ± 64
Thrombocytopenia + platelet support	(n = 11)	35 ± 14†	436 ± 77
Study 3: Idiopathic Thrombocytopenia			
Control	(n = 6)	26 ± 8	275 ± 65
Thrombocytopenia	(n = 9)	29 ± 4	340 ± 96

*Study 1: Protein S cleavage and levels of total protein S in plasma samples from patients with Essential Thrombocythaemia and not taking any platelet reducing agent at a platelet count > 800 × 10^9 L^{-1} (WBC count 6–18 × 10^9 L^{-1}) and at normalizing platelet count upon cytoreductive treatment with hydroxyurea (HU) (platelet count 290–443 × 10^9 L^{-1}; WBC count 5–10 × 10^9 L^{-1}). Control samples were taken from healthy volunteers (platelet count 133–328 × 10^9 L^{-1}; WBC count 5–9 × 10^9 L^{-1}). Study 2: Protein S cleavage in plasma samples from patients with a haematological malignancy and with chemotherapy-induced thrombocytopenia (platelet count < 50 × 10^9 L^{-1}; WBC count < 2 × 10^9 L^{-1}) before and after platelet transfusion. Control samples were taken from patients at a platelet count > 100 × 10^9 L^{-1} (WBC count 3–52 × 10^9 L^{-1}). Study 3: Protein S cleavage in patients with idiopathic thrombocytopenia (platelet count < 50 × 10^9 L^{-1}). Control samples were taken from individuals with a history of idiopathic thrombocytopenia (platelet count > 100 × 10^9 L^{-1}). Mean ± SD of n individuals examined * P<0.05 as compared to controls, † P<0.05 as compared to untreated thrombocythaemic patients or patients at thrombocytopenia without platelet support.*

Table 6-1 shows protein S cleavage at conditions characterized by extreme high and low platelet counts. In seven ET patients not taking any platelet reducing agent (platelet count > 800 × 10^9 L^{-1}) protein S cleavage was significantly increased compared to healthy controls (40%, p<0.05). Differently, in nine ET patients at normalizing platelet count upon cytoreductive treatment with HU (platelet count 290–443 × 10^9 L^{-1}), protein S cleavage was significantly reduced compared to no-HU treated patients (28%, p<0.05) and similar to that in respective healthy control subjects (Table 6-1). Accordingly, high-dose chemotherapy-induced thrombocytopenia (platelet count < 50 × 10^9 L^{-1}) (n=15) was associated with a significantly reduced protein S cleavage (16%, p<0.05) as compared to the control subjects (Table 6-1). In our HU-treated patient population, WBC count (5–10 × 10^9 L^{-1}) remained in the normal range and a correlation of WBC number with protein S cleavage was absent (data not shown). Thrombocytopenia induced by high dose chemotherapy, however, was inevitably associated with a drop in WBC count (0–2

$\times 10^9$ L^{-1}). In order to exclude a possible contribution of WBC to protein S cleavage, we therefore performed the measurement of protein S in a subgroup of 11 patients with chemotherapy-induced thrombocytopenia during platelet transfusion. In these subjects the cleavage of protein S rose within the normal range (Table 6-1). Level of total protein S was not affected upon platelet transfusion (Table 6-1). In order to discriminate between reduced thrombopoiesis and peripheral platelet destruction, we included nine patients with idiopathic thrombocytopenia. In these patients with autoantibody-mediated peripheral platelet destruction, protein S cleavage was in the normal range (Table 6-1). Collectively, our data suggests a correlation between thrombopoiesis and *in vivo* cleavage of protein S.

Discussion

This study corroborates and extends prior observations on the origin and anticoagulant activity of cleaved protein S in the circulation. Cleaved protein S accounted for 25% of total protein S in plasma (Fig. 6-1). The inability of appropriate protease inhibitors in the blood collection tube to prevent protein S cleavage (this study and ref [13,15]) suggests that protein S cleavage indeed occurs prior to blood sampling. Since platelets contain a protease able to cleave protein S [12,17], platelets may contribute to the presence of cleaved protein S in the circulation. This issue was addressed using plasma from patients with chemotherapy-induced thrombocytopenia. A significantly reduced cleavage of protein S at a platelet count $< 50 \times 10^9$ L^{-1} suggests that indeed platelets contribute to protein S cleavage *in vivo* (Table 6-1). Strong evidence for platelets as *in vivo* source of protein S cleaving protease was provided by the observation that in patients with suppressed platelet production, protein S cleavage was restored to normal values upon platelet transfusion (Table 6-1). Additional evidence was provided by the observation of increased protein S cleavage in patients with ET at platelets counts $> 800 \times 10^9$ L^{-1} and normalization of both parameters upon cytoreductive therapy (Table 6-1). Cleaved protein S present in plasma is completely inactive as an anticoagulant (Figs. 6-2,6-3). Therefore, cleavage of protein S provoked by platelet infusion may contribute to the beneficial effect of platelet transfusion in the treatment of bleeding episodes. On the other hand, cleavage of protein S provoked by high platelet count, e.g., in ET, may promote thrombosis.

Our previously published *in vitro* study [12] clearly points towards platelets as source of the protein S cleaving proteolytic activity in plasma. Merit of the present study resides in the *in vivo* approach in correlating platelet count with protein S cleavage. A positive correlation between protein S cleavage and platelet count, although undisclosed in healthy individuals, was evident in patients with haematological malignancies (Fig. 6-4). Our results show that the effect of platelet number on protein S cleavage is most prominent at platelet count extremes. In healthy individuals, the platelet count range is too narrow to argue either in favour or against a correlation of protein S cleavage with platelet count. In this group, variability in extent of protein S cleavage is apparently due to inter-individual variability in protein S cleaving activity per se. However, it cannot be fully ruled out that a correlation between platelet count and protein S cleavage is restricted to pathological conditions associated with haematological malignancies. Also, contribution of proteases from blood cells other

than platelets had to be taken into account. Neutrophil elastase, e.g., is known to degrade protein S, a process, however, that is strongly suppressed in plasma [13,16]. High-dose chemotherapy-induced thrombocytopenia is not only associated with a reduction in protein S cleavage but also with inevitable low white blood cell counts. Nevertheless, normalization of protein S cleavage upon transfusion of leukocyte-depleted platelet concentrate to chemotherapy-treated patients (Table 6-1) excludes contribution of an *in vivo* cellular source of protein S cleaving protease activity other than platelets. Contribution of donor protein S, nonetheless, should be taken into account. It can be calculated that cleaved donor protein S from plasma present in the transfusion bag as preservative may account, on average, for a 2% increase in the portion of cleaved protein S measured in the patient samples upon platelet transfusion. This amount still gives a 17% increase in the portion of cleaved protein S on the account of transfused platelets. It has been reported that platelets contain 1.6 ng protein S per 10^9 platelets [25]. Assuming that all protein S in transfused platelets is released in the circulation, one bolus of 350×10^9 transfused platelets may increase the total concentration of plasma protein S by 2.54 nM, an amount too low to significantly contribute to levels of total protein S in transfused patients. Whether platelet-derived protein S is intact or cleaved remain to be elucidated. However, even if donor platelet-derived protein S is fully cleaved and completely secreted from the transfused platelets, the portion of circulating cleaved protein S is increased by less than 1%. The increase in the portion of circulating TSR-cleaved protein S upon platelet transfusion, therefore, is predominantly attributable to proteolytic activity associated with transfused platelets.

The average portion of cleaved protein S that we observed (Fig. 6-1) in our Dutch population of 106 healthy control subjects (25 ± 8%), is more than twice as high as the 10% reported by Borgel *et al.* [26]. The cause of this apparent discrepancy is unclear, but it might be a methodological issue. In the Borgel study, a MoAb specific for Arg60-cleaved protein S was used in ELISA [13]. In our study, cleavage is based on the protein profile on Western blot and loss of response in ELISA with our MoAb CLB-PS52 that specifically recognizes TSR-intact protein S [12,19]. Most intriguing is the observation that the effect of platelet number on protein S cleavage was most pronounced at platelet counts below 50×10^9 L^{-1} and that protein S cleavage tend to a maximum value at platelet counts above normal (Fig. 6-4B). This observation might be explained by a reduction in biomass (including the putative protein S cleaving protease) per platelet at increased thrombopoiesis [27]. Alternatively, since two thirds of the total amount of protein S is bound to C4b-binding protein (C4BP) [20], C4BP-bound protein S might be protected from cleavage by the platelet-associated protein S cleaving activity.

The rationale for the presence of cleaved protein S in the circulation is presently unknown. Possibly, controlled protein S cleavage is required to keep the activity of circulating protein S within acceptable limits. Although several studies show loss of protein S anticoagulant activity upon TSR cleavage *in vitro*, the impact of TSR cleavage on the APC-independent anticoagulant activity of protein S has never been investigated in plasma. Also, *in vivo* cleavage of protein S has never been correlated to thrombin generation in the CAT assay. This assay has been successfully used by others for the separate measurement of APC-dependent and APC-independent

protein S anticoagulant activity [22,28]. The mechanistic background behind the measurement of the APC cofactor activity of protein S lies in the observation that in plasma APC is completely dependent on protein S [29,30]. For the measurement of the APC-independent cofactor activity of protein S by CAT we employed our inhibiting MoAb CLB-PS13. This MoAb is directed against the Gla-domain of protein S [23] and recognizes both intact and TSR cleaved protein S [19]. In a study by Serè *et al.* [22], CLB-PS13 was found to increase thrombin generation in the CAT assay in the absence of APC; a phenomenon that later turned out to correspond with the TFPI cofactor activity of protein S [6]. The results of our experiments show that TSR-cleaved protein S has no detectable APC-dependent and APC-independent anticoagulant activity in plasma (Fig. 6-3). Indeed, uncleaved protein S is able to block TF-initiated thrombin generation while plasma containing only TSR-cleaved protein S is devoid of protein S anticoagulant activity with a concomitant hypercoagulable state (Fig. 6-2) as observed in plasma from type I and type III protein S-deficient individuals [28]. Because of the impact of protein S cleavage on its anticoagulant activity, an association of protein S cleavage with haematological disorders seems conceivable. However, cleaved protein S did not emerge as risk factor for thrombosis in a case-control study including 87 patients with VTE [26]. On the other hand, a pathologically increased protein S cleavage has been observed in disseminated intravascular coagulation [13]. In addition, increased cleavage of protein S in ET may contribute to the occurrence of an acquired APC-resistance phenotype in these patients [18].

Research from the past decades have notified that although protein S deficiency is associated with the development of thrombotic events, the relevance of protein S as marker in coagulation or as a therapeutic target may be limited. On the other hand, in vivo protein S cleavage has largely been ignored and studies on the mechanism of in vivo cleavage of protein S may reveal new strategies in the prevention and treatment of coagulation disorders. Employing plasma samples from healthy individuals and patients with either thrombocytopenia or thrombocytosis, we are the first to describe a relationship between in vivo protein S cleavage, protein S anticoagulant activity and platelet-associated protein S cleaving activity. In addition to protein S, the concept of cellular proteases as modulators of the activity of clotting factors have also been suggested for FV, FVIII, FX and TF [31–35]. To date, the role of the protein S cleaving protease in haemostasis is only speculative. Vessel trauma needs a rapid response to injury reaction with locally triggered thrombin generation. Down-regulation of the anticoagulant activity of protein S by platelet-associated protein S cleaving activity may guarantee full activity of TF-FVIIa, FIXa-FVIIIa and FXa-FVa complexes required for the thrombin generation burst.

What is known about this topic?

- In plasma, protein S circulates in two forms: a single-chain molecule and a two-chain variant as the result of cleavage in the protease sensitive loop (TSR).
- *In vitro* studies with purified proteins have shown reduced protein S anticoagulant activity upon TSR cleavage, an observation that needs to be verified *in vivo*.
- Well established TSR cleaving proteases (i.e., thrombin, FXa) do not cleave protein S under physiological plasma conditions.
- A platelet protease was found to cleave the TSR in plasma during tissue factor-induced clotting *in vitro*. This protease might be responsible for the presence of cleaved protein S *in vivo*.

What does this paper add?

- Levels of circulating intact but not TSR-cleaved protein S show a positive correlation with both APC-dependent and APC-independent protein S anticoagulant activity.
- A pathological increased or decreased thrombopoiesis correlate with the extent of *in vivo* TSR cleavage.
- Cleavage of protein S induced by platelet infusion may contribute to the beneficial effect of platelet transfusion in the treatment of bleeding episodes.

Acknowledgement

The authors thank C.M.E. Eckmann and T. Brummel (Dept. of Blood Coagulation, Sanquin Diagnostics, Amsterdam) for providing the data of Fig. 6-3, right upper and lower panel.

References

1. Saller F, Brisset AC, Tchaikovski SN, Azevedo M, Chrast R, Fernández JA, Schapira M, Hackeng TM, Griffin JH, Angelillo-Scherrer A. Generation and phenotypic analysis of protein S-deficient mice. *Blood*. 2009; **114**: 2307-14.
2. Burstyn-Cohen T, Heeb MJ, Lemke G. Lack of protein S in mice causes embryonic lethal coagulopathy and vascular dysgenesis. *J Clin Invest*. 2009; **119**: 2942-53.
3. Bombeli T, Basic A, Fehr J. Prevalence of hereditary thrombophilia in patients with thrombosis in different venous systems. *Am J Hematol*. 2002; **70**: 126-32.
4. Mahmoodi BK, Brouwer JL, Veeger NJ, van der Meer J. Hereditary deficiency of protein C or protein S confers increased risk of arterial thromboembolic events at a young age: results from a large family cohort study. *Circulation*. 2008; **118**: 1659-67.
5. Dahlbäck B, Villoutreix BO. Regulation of blood coagulation by the protein C anticoagulant pathway: novel insights into structure-function relationships and molecular recognition. *Arterioscler Thromb Vasc Biol*. 2005; **25**: 1311-20.
6. Hackeng TM, Rosing J. Protein S as cofactor for TFPI. *Arterioscler Thromb Vasc Biol*.

2009; **29**: 2015-20.

7. Heeb MJ, Prashun D, Griffin JH, Bouma BN. Plasma protein S contains zinc essential for efficient activated protein C-independent anticoagulant activity and binding to factor Xa, but not for efficient binding to tissue factor pathway inhibitor. *FASEB J*. 2009; **23**: 2244-53.

8. Takeyama M, Nogami K, Saenko EL, Soeda T, Nishiya K, Ogiwara K, Yoshioka A, Shima M. Protein S down-regulates factor Xase activity independent of activated protein C: specific binding of factor VIII(a) to protein S inhibits interactions with factor IXa. *Br J Haematol*. 2008; **143**: 409-20.

9. Long GL, Lu D, Xie RL, Kalafatis M. Human protein S cleavage and inactivation by coagulation factor Xa. *J Biol Chem*. 1998; **273**: 11521-6.

10. Borgel D, Gaussem P, Garbay C, Bachelot-Loza C, Kaabache T, Liu WQ, Brohard-Bohn B, Le Bonniec B, Aiach M, Gandrille S. Implication of protein S thrombin-sensitive region with membrane binding via conformational changes in the gamma-carboxyglutamic acid-rich domain. *Biochem J*. 2001; **360**: 499-506.

11. Heeb MJ, Griffin JH. Activated protein C-dependent and -independent anticoagulant activities of protein S have different structural requirements. *Blood Cells Mol Dis*. 2002; **29**: 190-9.

12. Brinkman HJ, Mertens K, van Mourik JA. Proteolytic cleavage of protein S during the hemostatic response. *J Thromb Haemost*. 2005; **3**: 2712-20.

13. Morboeuf O, Borgel D, Gaussem P, Vincenot A, Pittet JL, Aiach M, Gandrille S. Characterization of cleaved plasma protein S with a monoclonal antibody-based assay. *Thromb Haemost*. 2000; **84**: 604-10.

14. Oates AM, Salem HH. The binding and regulation of protein S by neutrophils. *Blood Coagul Fibrinolysis*. 1991; **2**: 601-7.

15. Dahlbäck B. Purification of human vitamin K-dependent protein S and its limited proteolysis by thrombin. *Biochem J*. 1983; **209**: 837-46.

16. Eckle I, Seitz R, Egbring R, Kolb G, Havemann K. Protein S degradation in vitro by neutrophil elastase. *Scand J Clin Lab Invest*. 1993; **53**: 281-8.

17. J Mitchell CA, Salem HH. Cleavage of protein S by a platelet membrane protease. *J Clin Invest*. 1987; **79**: 374-9.

18. Marchetti M, Castoldi E, Spronk HM, van Oerle R, Balducci D, Barbui T, Rosing J, Ten Cate H, Falanga A. Thrombin generation and activated protein C resistance in patients with essential thrombocythaemia and polycythemia vera. *Blood*. 2008; **112**: 4061-8.

19. Meijer-Huizinga F, Mertens K, van Mourik JA. Isolation and characterization of single-chain protein S. *Thromb Haemost*. 1994; **72**: 408-14.

20. Griffin JH, Gruber A, Fernández JA. Reevaluation of total, free, and bound protein S and C4b-binding protein levels in plasma anticoagulated with citrate or hirudin. *Blood*. 1992; **79**: 3203-11.

21. Hemker HC, Giesen P, Al Dieri R, Regnault V, de Smedt E, Wagenvoord R, Lecompte T, Béguin S. Calibrated automated thrombin generation measurement in clotting plasma. *Pathophysiol Haemost Thromb*. 2003; **33**: 4-15.

22. Seré KM, Rosing J, Hackeng TM. Inhibition of thrombin generation by protein S at low procoagulant stimuli: implications for maintenance of the hemostatic balance. *Blood*. 2004; **104**: 3624-30.

23. Hackeng TM, Hessing M, van 't Veer C, Meijer-Huizinga F, Meijers JC, de Groot PG, van Mourik JA, Bouma BN. Protein S binding to human endothelial cells is required for expression of cofactor activity for activated protein C. *J Biol Chem*. 1993; **268**: 3993-4000.

24. Hackeng TM, van 't Veer C, Meijers JC, Bouma BN. Human protein S inhibits prothrombinase complex activity on endothelial cells and platelets via direct interactions with factors Va and Xa. *J Biol Chem*. 1994; **269**: 21051-8.

25. Schwarz HP, Heeb MJ, Wencel-Drake JD, Griffin JH. Identification and quantitation of protein S in human platelets. *Blood*. 1985; **66**:1452-5.

26. Borgel D, Reny JL, Fischelis D, Gandrille S, Emmerich J, Fiessinger JN, Aiach M. Cleaved protein S (PS), total PS, free PS, and activated protein C cofactor activity as risk factors for venous thromboembolism. *Clin Chem*. 2003; **49**: 575-80.

27. Damon AL, Scudder LE, Gnatenko DV, Sitaraman V, Hearing P, Jesty J, Bahou WF. Altered bioavailability of platelet-derived factor VIII during thrombocytosis reverses phenotypic efficacy in haemophilic mice. *Thromb Haemost*. 2008; **100**: 1111-22.

28. Castoldi E, Maurissen LF, Tormene D, Spiezia L, Gavasso S, Radu C, Hackeng TM, Rosing J, Simioni P. Similar hypercoagulable state and thrombosis risk in type I and type III protein S-deficient individuals from families with mixed type I/III protein S deficiency. *Haematologica*. 2010; **95**: 1563-71.

29. Bertina RM, van Wijngaarden A, Reinalda-Poot J, Poort SR, Bom VJ. Determination of plasma protein S, the protein cofactor of activated protein C. *Thromb Haemost*. 1985; **53**: 268-72.

30. Malm K, Dahlbäck B, Arnljots B. Prevention of thrombosis following deep arterial injury in rats by bovine activated protein C requiring co-administration of bovine protein S. *Thromb Haemost*. 2003; **90**: 227-34.

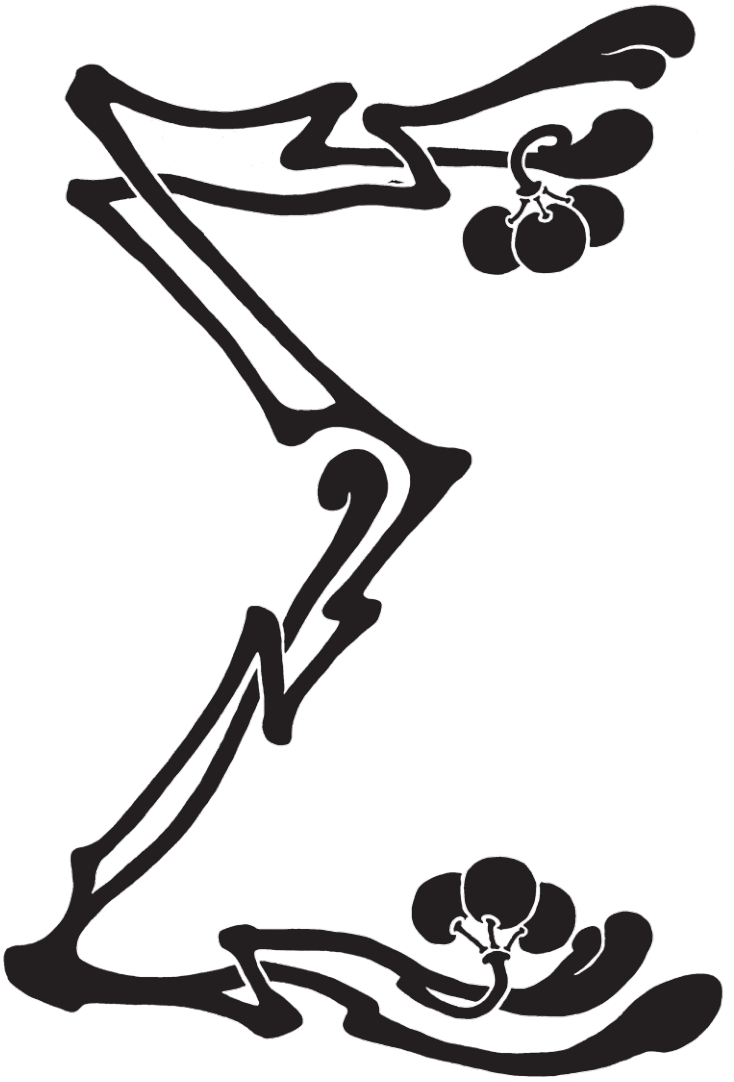
31. Camire RM, Kalafatis M, Tracy PB. Proteolysis of factor V by cathepsin G and elastase indicates that cleavage at Arg1545 optimizes cofactor function by facilitating factor Xa binding. *Biochemistry*. 1998; **37**: 11896-906.

32. Gale AJ, Rozenshteyn D. Cathepsin G, a leukocyte protease, activates coagulation factor VIII. *Thromb Haemost*. 2008; **99**: 44-51.

33. Plescia J, Altieri DC. Activation of Mac-1 (CD11b/CD18)-bound factor X by released cathepsin G defines an alternative pathway of leucocyte initiation of coagulation. *Biochem J*. 1996; **319**: 873-9.

34. Reinhardt C, von Brühl ML, Manukyan D, Grahl L, Lorenz M, Altmann B, Dlugai S, Hess S, Konrad I, Orschiedt L, Mackman N, Ruddock L, Massberg S, Engelmann B. Protein disulfide isomerase acts as an injury response signal that enhances fibrin generation via tissue factor activation. *J Clin Invest*. 2008; **118**: 1110-22.

35. Nogami K, Ogiwara K, Matsumoto T, Nishiya K, Takeyama M, Shima M. Mechanisms of human neutrophil elastase-catalysed inactivation of factor VIII(a). *Thromb Haemost*. 2011; **105**: 968-80.



Chapter 7
General discussion

Naturally occurring genetic variations together with targeted gene knock-down in mice and supported by extensive *in vitro* analysis have disclosed the major proteins implicated in the formation of thrombin (**Chapter 1**, Fig. 1-2, [1]). In addition to the major known coagulation factors, there are many additional factors that can modulate the haemostatic balance towards bleeding or thrombosis. Some of them take part in maintaining the haemostatic balance in physiological conditions, for example, heparins, polyphosphates and various endothelial cell membrane components [2,3]. Others spring into action at various pathophysiological conditions and further promote the development of clinically significant thrombosis or bleeding problems. Inflammation, sepsis, major trauma or surgery, malignancies, autoimmune diseases are examples of conditions that go hand in hand with a disrupted haemostatic balance. A clear understanding of the pathophysiology of bleeding disorders requires the evaluation of potential new determinants in the haemostasis system.

In this thesis we have explored the contributing mechanisms to haemostatic abnormalities in various pathological states associated with β2-glycoprotein I (β2GPI), factor VII activating protease (FSAP), and with cleavage of protein S. While there is not yet consensus about involvement of β2GPI in coagulation, its associated antiphospholipid syndrome (APS), an autoimmune disease, is characterized by a prothrombotic phenotype. The activation of FSAP might occur predominantly in cases of severe inflammation, such as sepsis, where subsequently the active FSAP could contribute to the prothrombotic phenotype. And finally, the cleavage of protein S could serve to maintain the fine line between bleeding and haemostasis, whereas a disrupted balance, e.g., in case of malignancies may contribute to complications in either way. However, these proteins are notoriously hard to phenotype and therefore require highly sensitive assays to determine their activities and the mechanisms involved therein.

Solving the haemostatic riddles with thrombography

The recent revival of the global thrombin generation assay (TGA) has provided a worthy tool to supplement the *in vivo* data of coagulation research ([4], **Chapter 1**, Fig. 1-3). For instance, thrombography has supported the *in vivo* findings that FXIIa-mediated FXI activation does not play a role in normal haemostasis [5]. Direct feedback activation of FXI by thrombin is now believed to play a role in the amplification phase of thrombin generation [6]. Similarly, the observation of reduced thrombin generation in FVIII or FIX deficient plasma at low TF concentration only [4] explains why individuals with haemophilia A or B are prone to haemorrhages in joints and skeletal muscle which are both sites associated with low levels of TF [7]. Thrombography also has been used to reveal the hypercoagulable plasma state associated with deficiency of natural anticoagulants and allowed to phenotype the anticoagulant activities of protein C, protein S and tissue factor pathway inhibitor (TFPI). For example, *in vitro* experiments with protein S in thrombin generation in plasma revealed that protein S is able to exert anticoagulant activity that is independent of APC [8], an observation recently established in a baboon thrombosis model [9].

The thrombography approach as employed in the studies described in this thesis appeared successful for FSAP and allowed to expose its real target in coagulation cascade, TFPI (**Chapter 5**). Also for protein S, TGA showed the clear difference between intact and cleaved protein S in terms of their anticoagulant activity (**Chapter 6**). However, TGA failed to provide novel insights into APS pathogenesis (**Chapter 2**), presumably because the pathways leading to thrombosis in APS heavily lean on the cellular factors lacking in our experimental setup. The various lessons learned from our studies are summarized in Fig. 7-1 and will be further discussed separately.

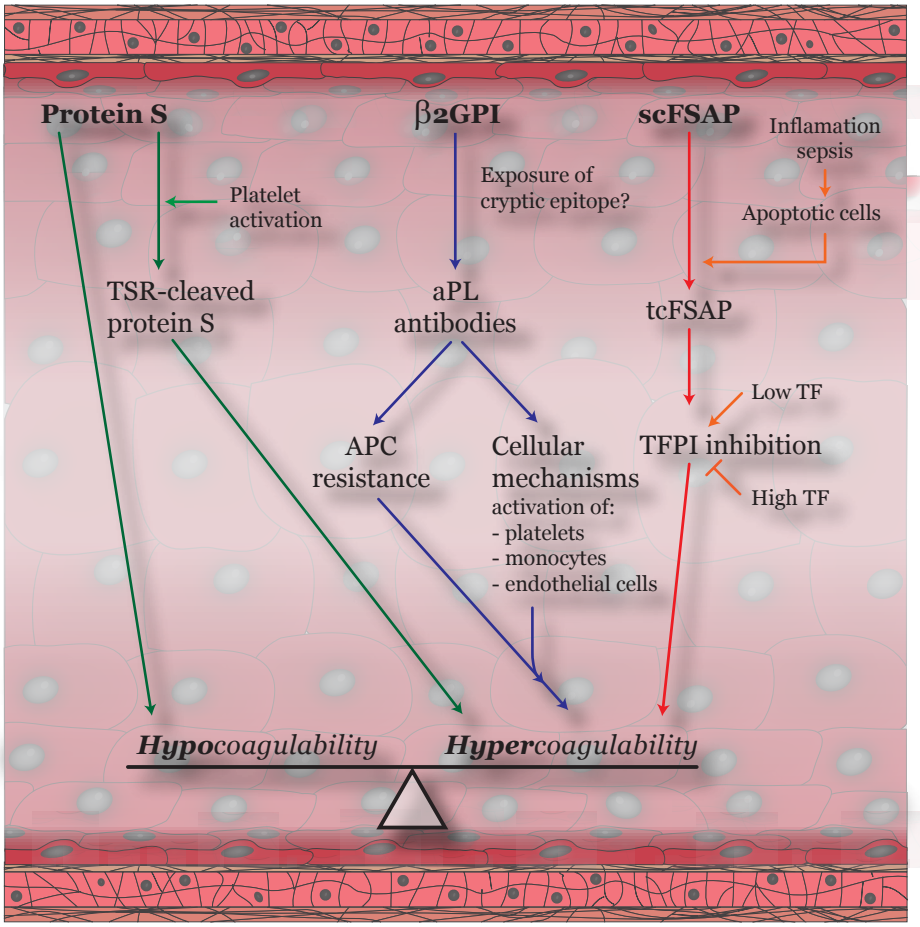


Figure 7-1 A scheme summary showing the proposed mechanisms of how FSAP, antibodies against β2GPI and cleavage of protein S may affect the haemostatic balance.

β2-glycoprotein I and the antiphospholipid syndrome

β2-glycoprotein I (β2GPI) is the predominant autoantigen for antiphospholipid (aPL) antibodies implicated in the antiphospholipid syndrome [10]. The heterogeneous nature of these antibodies complicates the research on APS pathophysiology as reflected by the variety of proposed mechanisms that may cause APS associated thrombosis [11]. Therefore, we constructed monoclonal aPL antibodies against β2GPI derived by phage display from two patients with APS (**Chapter 2**). To characterize these antibodies, we performed coagulation assays, including thrombin generation assay, in normal plasma spiked with the constructed monoclonal antibodies. However, the effect in TGA was limited and did not yield more information than what can be inferred from the characteristic Lupus anticoagulant (LAC) phenotype as observed in diagnostic tests (**Chapter 2**). Apart from a prolonged lag time, the other parameters that could be inferred from the thrombin generation curve were comparable to normal plasma alone (**Chapter 2**).

Unlike other pathogenic autoantibodies, APS antibodies do not have an inhibitory role but instead form complexes with β2GPI and exhibit various pathophysiological activities [10]. The paradoxical and characteristic property of aPL antibodies is their ability to prolong clotting time in the *in vitro* setting [12], as was also observed in **Chapter 2**. This observation has a rather simple rationalization, namely, competition for the limited available negatively charged phospholipids in the assay [12]. This is further explained by the increase in affinity for phospholipids upon antibody binding [13]. However, this *in vitro* phenomenon does not meet APS associated thrombosis. It has been reported that for TGA peak height/lag time ratio was a better predictive of thromboembolic risk in APS than lag time alone [14]. A possible mechanism for thrombosis in APS patients may include resistance to acquired activated protein C [15]. The thrombin generation assay has been applied to analyze APC resistance in APS patients [16]; however, our experiments in this direction did not yield reproducible results (data not shown).

While one might argue that the in **Chapter 2** observed effects in TGA were not very strong, it should be noted that a large part of thrombosis pathogenesis in APS may rely on cellular mechanisms. The aPL antibody–β2GPI complexes, for example, are capable of activating platelets [17,18] and monocytes leading to tissue factor expression [19]. Endothelial cell perturbation, either by direct binding of aPL antibodies to these cells [20] or via aPL antibody-triggered complement activation [21], may further promote the thrombophilic state [22]. Also, increased microvesiculation from monocytes, platelets [23] and endothelial cells can contribute towards thrombophilia in APS [24]. Therefore, further research on the thrombogenic properties of the constructed aPL monoclonal antibodies described in **Chapter 2** should include cellular elements as well.

A remarkable feature of aPL antibodies that revealed from experiments described in **Chapter 2** was the relationship between their LAC activity and affinity toward β2GPI. Antibodies in APS patients usually display low affinity for β2GPI, which is consistent with the high β2GPI levels in plasma and the fact that no circulating

antibody–β2GPI complexes are found in APS patients [25]. We observed that from our constructed monoclonal antibodies those with relative lower affinity towards β2GPI displayed insignificant LAC activity while for antibodies with relative high affinity for β2GPI their LAC activity was considerable. It can be speculated that the antibody affinity for β2GPI may further indicate their potency to generate thrombotic complications in APS patients.

In the study described in **Chapter 2** it is striking that although many single chain Fv fragments were initially isolated from the B-cell repertoire of APS patients by phage display, extensive and repeated testing resulted in selection of only four clones that were consistently binding β2GPI. Moreover, also the binding epitopes of these antibodies were limited to domain I with or without domain II (**Chapter 2**). We therefore further focussed on etiological aspects of APS and examined β2GPI uptake by dendritic cells (DCs) and presentation on MHC II class molecules (**Chapter 3**). Dendritic cells play a central role in the immunological processes. DCs are proficient in uptake, degradation and subsequent peptide presentation of various endogenous and exogenous antigens [26]. $CD4^+$ T cells will recognize the peptides presented on MHC class II molecules and depending on whether the presented antigen is either endogenous or foreign, the T cells will undergo apoptosis or will proliferate to promote antigen-specific antibody production [27]. Our results clearly revealed β2GPI uptake and subsequent residue presentation by DCs. Employing a novel mass spectrometry (MS) approach [28], we were able to identify β2GPI peptides presented on HLA-DR molecules. We found a short list of predominantly domain V peptides which resonates with earlier studies where also several albeit different domain V peptides were distinguished among a panel of β2GPI peptides used to stimulate T-cells [29]. These peptides only serve to stimulate CD4+ T-cell activity and therefore are not necessarily the epitopes recognised by antibodies [30]. This means that presentation of domain V peptides does not exclude generation of domain I recognizing anti-β2GPI auto-antibodies.

Despite the rapid uptake of β2GPI, only a limited number HLA-DR exposed β2GPI peptides were detected. In order to be able to visualize uptake, fluorochrome-labelled β2GPI was used in the experiments described in **Chapter 3**. In peptide presentation experiments, however, unmodified β2GPI was used. Contribution of used fluorescent tags to the observed rapid internalization kinetics of β2GPI can not be excluded. On the other hand, the limited amount of detected β2GPI peptides may indicate that either T cells require only particular exposed β2GPI peptides to trigger further immune response. It is also possible that we have been looking at native β2GPI instead of a variety of aberrant β2GPI forms suggested to be implicated in the autoimmune response of APS [29,31,32]. There is a long probed paradox in APS: if β2GPI is so abundantly present in plasma (50–500 μg mL^{-1}), how and why the autoimmune response is triggered only in a fraction of individuals? Many believe that it is an issue of exposure of cryptic epitopes that are usually shielded from immunity [27,32,33]. Probing the APS patient derived T cell response towards β2GPI has repeatedly revealed that the immune response towards native β2GPI is low [29,33,34]. On the other hand, the immunogenic potential of β2GPI becomes higher if T cells are stimulated with DCs presenting β2GPI epitopes that were hitherto shielded by phospholipids [29,33] or by the native conformation. To test the latter

hypothesis both *in vivo* and *in vitro*, β2GPI has been treated with cardiolipin [32] or carbohydrates leading to β2GPI variants with higher DC stimulating and antibody response eliciting potential [31]. It is therefore interesting to note that patients with APS display a differently glycosylated β2GPI, that may include reduced sialylation [35]. Whether or not this leads to increased uptake and presentation by DCs remains an open question.

In summary, the research presented in **Chapters 2** and **3** has mainly lead to inspiring results provoking further investigation on APS pathogenesis. Now that we have established the uptake and presentation of β2GPI, it would be interesting to further explore whether a different set of peptides is presented in case of modifications of β2GPI. Furthermore, in order to link the presented peptides with the autoimmune response provoked in APS, their capability of stimulating CD4+ T cells should be investigated. Our constructed APS patient-derived monoclonal antibodies may serve in diagnostics. A large fraction of APS patients have an intermediate LAC activity and therefore pose a challenge in consistent APS diagnostics [36]. This means that rather than using high affinity anti-β2GPI antibodies as diagnostic standards, our monoclonal antibodies with the modest LAC activity could help to standardize detection of intermediate LAC activity in APS patients. Moreover, they could serve in further investigation of thrombosis pathogenesis in APS. Since our antibodies may represent an aPL antibody subset with mild LAC activity, it is nevertheless important to know how such antibodies contribute to the thrombotic complications in APS.

FSAP and inflammation-associated thrombosis

Factor VII activating protease (FSAP) is a serine protease with a wide range of proteolytic activities and suggested to be implicated in a variety of biological processes including coagulation, fibrinolysis, apoptosis and vascular regulation through several distinct cell activation pathways. As the name suggests, FSAP was initially suggested to have an involvement in coagulation as a physiological activator of FVII [37], an observation that has been widely quoted but never reproduced. However, in **Chapter 4** of this thesis we have succeeded to give a final answer to the growing doubt about the FVII-cleaving activity of active (two-chain) FSAP.

Studies on FSAP functions are hampered by the inherent instability of the protein and heterogeneous preparations leading to misinterpretation of the acquired data. The use of the recombinant FSAP variant R313Q that allows controlled activation by thermolysin proved a valuable tool for dissecting the activity of FSAP (**Chapter 4**) and its significance in thrombin generation (**Chapter 5**). The findings reported in **Chapter 4** show that FSAP can not be regarded as a physiological activator of FVII. No FVII activating properties were found for FSAP in either cleavage or FVIIa activity assays. In the study presented in **Chapter 5** TGA served as a useful tool, providing the sensitivity necessary to research the involvement of FSAP in coagulation. The TGA experimental setup, for instance, enables lowering the TF concentration to a minimum. The conditions restricted to very low TF concentration appeared essential in detecting that two-chain (tc-) FSAP does have a procoagulant effect in plasma that is totally independent of FVII activation. Activated FSAP in itself is not able to trigger coagulation as no thrombin generation was observed in the

presence of tcFSAP and complete absence of TF. This finding suggests that tcFSAP may amplify thrombin generation by activating coagulation factors other than FVII and prothrombin. Alternatively, activated FSAP may bind and neutralize inhibitors of blood coagulation. In **Chapter 5** we observed that the procoagulant effect of tcFSAP is actually caused by the interaction between FSAP and TFPI, resulting in TFPI inhibition. Therefore, the binding and consumption of TFPI is required for the procoagulant activity of FSAP. Indeed, studies by Stephan *et al.* [38] have supported this notion by showing that TFPI is a potential inhibitor of the by activated FSAP induced nucleosome release from apoptotic cells. Remarkably, Kanse *et al.* reported proteolytic degradation of TFPI by FSAP and suggested that this TFPI degradation may explain the observed procoagulant effect of activated FSAP [39]. On the other hand, even though proteolysis of TFPI by FSAP may occur eventually, the thrombin generation data together with inhibition kinetics data indicate that TFPI inhibition by FSAP is very rapid. TFPI must act at the very onset of thrombin generation when the levels of FXa and TF/FVIIa are still very low. Therefore it is rather unrealistic that the time frame needed for cleavage, at least 30 minutes as shown by Kanse *et al.* [39], is of impact in affecting the onset of thrombin generation. Thus, *in vivo* activation of FSAP may lead to rapid inhibition of TFPI thereby eliminating the inhibitory gatekeeper of thrombin generation at its onset.

In **Chapter 5** we also analyzed the Marburg I mutation of FSAP (MI FSAP), a G534E substitution which yields a proteolytically impaired version of the protein. Even though the proteolytic activity of MI FSAP is diminished, nevertheless it is not fully abolished. Furthermore, the affinity of MI FSAP for TFPI is strongly reduced, thus hindering the procoagulant effect of FSAP in thrombin generation. In any case, the statement about MI FSAP as retaining its ability to activate FVII [40] is not true. Therefore we can speculate that MI FSAP is a risk factor for cardiovascular disease not because of its suggested procoagulant activity but because of its cellular effects.

A curious unpublished side-observation from the thrombin generation experiments is connected with the proposed involvement of FSAP in fibrinolysis. One of the substrates for tcFSAP is the single chain urokinase type plasminogen activaton (scuPA) [41]. Cleavage of scuPA creates the proteolytically active form of uPA which can further activate plasminogen that lead to degradation of the fibrin network [42]. It is easy to macroscopically observe fibrin clot formation in plasma by increase in opacity. Supplementing coagulating plasma with tissue type plasminogen activator (tPA) caused rapid lysis of the fibrin clot. However, after thrombin generation analysis in the presence of tcFSAP, the resulting fibrin clot did not dissolve (I. Dienava-Verdoold, unpublished observation). Therefore, the actual involvement of FSAP in fibrinolysis is a matter that asks for a more thorough investigation.

The physiological conditions required for and the extent of FSAP activation in healthy subjects is unknown; however, the pathophysiological activation of FSAP in sepsis has been demonstrated [43]. This leads to the region of interplay between sepsis-induced inflammation and coagulation. Local coagulation and fibrin deposition at the infection site may be beneficial by entrapping the infection and limiting its propagation, while at the same time it can cause grave damage by causing disseminated intravascular coagulation [44]. Inflammation and coagulation pathways cross in many ways, e.g., tissue factor expressed by activated

monocytes and proinflammatory cytokines triggering the coagulation cascade [44]. Anticoagulants, such as TFPI, may reduce inflammation-associated thrombosis by limiting thrombin generation and intracellular thrombin signalling. Therefore TFPI may be administered as a drug to patients exhibiting severe sepsis [45]. On the other hand, TFPI is sensitive to proteolytic inactivation, either by endogenous (mast cell or neutrophil) or pathogen proteases [45–47]. Therefore, it is conceivable that FSAP activated during sepsis may also contribute to TFPI down regulation, with TFPI inhibition by tight binding to tcFSAP preceding the eventual proteolytic inactivation.

TSR-targeted protein S proteolysis and the haemostatic balance

Protein S is gaining increasing recognition as an anticoagulant factor, with activated protein C (APC) cofactor activity [48] as well as APC-independent activity which at least partly can be attributed to TFPI cofactor activity [49]. It was already known that in mice homozygous protein S deficiency is lethal and heterozygous deficiency leads to a prothrombotic phenotype [50]. Also in humans heterozygous protein S deficiency is associated with thrombophilia [51,52]. However, only recently high protein S levels (but not protein C levels) were correlated with a higher incidence of bleeding episodes [53]. It would have been interesting to evaluate TFPI levels as well, since those have been found to correlate with protein S levels in patients [54]. It is possible that initially the role of protein S in the anticoagulant pathways was downplayed due to the lack of awareness of key modulators of its activity. Indeed, research by Seré *et al.* [8] revealed the APC independent anticoagulant activity of protein S only under experimental conditions allowing thrombin generation at low procoagulant stimuli. Recent research by Heeb *et al.* suggests that binding of Zn^{2+} to protein S is an important regulator of its conformation and activity, both *in vitro* [55] and *in vivo* in a baboon thrombosis model [9]. Similarly, the recognition of implications of *in vivo* protein S cleavage is only slowly starting to emerge. In **Chapter 6** of this thesis we have examined protein S cleavage and found that the anticoagulant activity of intact and cleaved protein S in plasma in a thrombin generation assay has the same difference as reported for that between Zn^{2+}-containing and Zn^{2+}-devoid protein S and that between normal plasma and protein S deficient plasma. Thus, intact as well as Zn^{2+}-containing protein S preparations display potent, concentration dependent anticoagulant activity while cleaved or Zn^{2+}-deficient protein S is devoid of anticoagulant properties (**Chapter 6** and [9]). Clearly, these are two distinct mechanisms by which the anticoagulant activity protein S is regulated; however, it indicates how sensitive protein S is to such modulators and how poorly characterized preparations of protein S may lead to underestimation of its anticoagulant ability.

In 2005 our group has found that the proteases like thrombin and FXa, known to cleave protein S in the thrombin sensitive region (TSR) *in vitro*, are not capable of cleaving protein S under plasma conditions [56]. Instead, a yet unidentified protease exposed upon platelet activation can cleave the TSR of protein S in plasma [56]. In **Chapter 6** we linked the *in vitro* data with the *in vivo* situation by correlating platelet number with the extent of *in vitro* cleavage of protein S. This correlation with

platelet number were shown in clinical situations like malignancy or its treatment, namely, myeloproliferative neoplasms leading to increase in platelet numbers and chemotherapy-induced thrombocytopenia.

Patients with various types of malignancies have a higher chance of developing thrombotic complications, such as deep vein thrombosis, venous thromboembolism and pulmonary embolism. In fact, thrombotic complications without other known causes or risk factors (idiopathic venous thromboembolism) can serve as predictor of occult malignancy [57,58]. Although the exact causes of coagulation activation are not known, inflammatory responses and tumour procoagulant molecules are likely involved [57]. Furthermore, analysis of thrombin generation in plasma from patients with various tumour types revealed a great variety of procoagulant profiles [59]. However, the causal relationship with thrombosis becomes stronger as myeloproliferative malignancies are considered. Polycythaemia vera (PV) and essential thrombocythaemia (ET) are two related malignancies characterized by overpopulation of haematopoietic progenitor cells that may lead to more severe tumour types, such as acute myeloid lymphoma. In patients with PV the red cell mass is increased while in ET platelet number is increased; however, both malignancies are characterized by qualitative as well as quantitative platelet abnormalities [60]. In PV and ET, platelet, leukocyte and endothelium activation and interactions may contribute to the prothrombotic phenotype [60]. Thrombin generation assay has been employed with great success to reveal that, compared to healthy donor platelets, neoplastic platelets in these disorders indeed have a higher thrombin generation potential, at least in part explained by higher TF expression on the platelet surface [61]. In **Chapter 6** we show that increased protein S cleavage in presence of abnormally high platelet counts may also contribute to this higher TGA potential.

On the other side of the haemostatic balance, chemotherapy-induced thrombocytopenia is mostly induced by aggressive chemotherapy against myeloid malignancies causing bone marrow failure with subsequent thrombocytopenia lasting 2–3 weeks [62], while also chemotherapy against non-myeloid, solid tumours can have the same effect, albeit with lower incidence [63]. Nearly a half of patients with chemotherapy-induced thrombocytopenia may develop bleeding complications and half of these cases are clinically significant bleeding episodes [64]. Platelet transfusion is a common prophylactic treatment in these cases, although the full impact and side effects of it are still not fully understood [62]. In **Chapter 6**, a reduced protein S cleavage was demonstrated in patients with thrombocytopenia while prophylactic platelet transfusions also restored normal levels of protein S cleavage. Thus, the modulation of protein S cleavage by platelets may have a potentiating effect on the bleeding phenotype associated with chemotherapy-induced thrombocytopenia or thrombotic phenotype in malignant platelet proliferation.

It is a striking observation that no correlation between platelet count and proteolysis of protein S in the TSR was observed in the healthy population. One reason might be that the normal platelet count range is too narrow and the normal cleaved protein S range is too wide to observe any significant correlation. Therefore significant changes in protein S cleavage might be visible only at the pathological extremes of platelet counts, i.e., below 50×10^9 cells mL^{-1} and above 800 × cells mL^{-1}. In addition, our group has previously found that platelets need to be activated to support protein

S cleavage [56]. Therefore it is possible that the more activated malignant platelets in ET and PV [61] have also a better protein S cleavage potential.

Although the in **Chapter 6** employed ELISA assay using an antibody recognising only intact protein S and the used western blot gives more precise information about the extent of protein S cleavage, the thrombin generation assay provided valuable information about the impact of protein S cleavage in terms of its anticoagulant activity. On the other hand, although it is easy to modify TGA conditions to allow determination of APC cofactor activity [8] or TFPI cofactor activity [49], ELISA still serves as a simple and higher throughput assay for intact and thus active anticoagulant protein S.

In conclusion

The research presented in this thesis has allowed us to evaluate novel determinants in coagulation pathophysiology. The haemostatic balance is finely tuned; however, many pathways may skew the balance towards bleeding or thrombosis. In this thesis we have identified diverse mechanisms that may lead to a procoagulant phenotype, as illustrated in Fig. 7-1. Exposure of cryptic epitopes of β2GPI with subsequent uptake and presentation on dendritic cells may lead to formation of autoantibodies which in turn induce a thrombotic phenotype via various cellular and non-cellular mechanisms. FSAP, activated during inflammation, may promote thrombosis by inhibiting TFPI at the onset of thrombin generation. Proteolytic cleavage of TSR by a platelet protease abolishes the anticoagulant activity of protein S. This may represent another mechanism of how platelet activation and increased platelet number contribute to hypercoagulability.

References

1. Mackman N. Tissue-specific hemostasis in mice. *Arterioscler Thromb Vasc Biol*. 2005; **25**: 2273-81.
2. Pendurthi UR. Size matters for polyP to clot. *Blood*. 2010; **116**: 4042-3.
3. Sundaram M, Qi Y, Shriver Z, Liu D, Zhao G, Venkataraman G, Langer R, Sasisekharan R. Rational design of low-molecular weight heparins with improved *in vivo* activity. *Proc Natl Acad Sci U S A*. 2003; **100**: 651-6.
4. Duchemin J, Pan-Petesch B, Arnaud B, Blouch MT, Abgrall JF. Influence of coagulation factors and tissue factor concentration on the thrombin generation test in plasma. *Thromb Haemost*. 2008; **99**: 767-73.
5. Kravtsov DV, Matafonov A, Tucker EI, Sun MF, Walsh PN, Gruber A, Gailani D. Factor XI contributes to thrombin generation in the absence of factor XII. *Blood*. 2009; **114**: 452-8.
6. Matafonov A, Sarilla S, Sun MF, Sheehan JP, Serebrov V, Verhamme IM, Gailani D. Activation of factor XI by products of prothrombin activation. *Blood*. 2011; **118**: 437-45.
7. Mackman N. The role of tissue factor and factor VIIa in hemostasis. *Anesth Analg*. 2009; **108**: 1447-52.
8. Seré KM, Rosing J, Hackeng TM. Inhibition of thrombin generation by protein S at low procoagulant stimuli: implications for maintenance of the hemostatic balance. *Blood*.

2004; **104**: 3624-30.

9. Heeb MJ, Marzec U, Gruber A, Hanson SR. Antithrombotic activity of protein S infused without activated protein C in a baboon thrombosis model. *Thromb Haemost*. 2012; **107**: 690-8.

10. De Groot PG, Meijers JCM. β2-glycoprotein I: evolution, structure and function. *J Thromb Haemost*. 2011; **9**: 1275-84.

11. Pierangeli SS, Chen PP, Gonzalez EB. Antiphospholipid antibodies and the antiphospholipid syndrome: an update on treatment and pathogenic mechanisms. *Curr Opin Hematol*. 2006; **13**: 366-75.

12. Arnout J. Antiphospholipid syndrome: diagnostic aspects of lupus anticoagulants. *Thromb Haemost*. 2001; **86**: 83-91.

13. Willems GM, Janssen MP, Pelsers MMAL, Comfurius P, Galli M, Zwaal RFA, Bevers EM. Role of divalency in the high-affinity binding of anticardiolipin antibody–β2-glycoprotein I complexes to lipid membranes. *Biochemistry*. 1996; **35**: 13833-42.

14. Devreese K, Peerlinck K, Hoylaerts MF. Thrombotic risk assessment in the antiphospholipid syndrome requires more than the quantification of lupus anticoagulants. *Blood*. 2010; **115**: 870-8.

15. Safa O, Esmon CT, Esmon NL. Inhibition of APC anticoagulant activity on oxidized phospholipid by anti-β2-glycoprotein I monoclonal antibodies. *Blood*. 2005; **106**: 1629-35.

16. Liestøl S, Sandset PM, Mowinckel MC, Wisløff F. Activated protein C resistance determined with a thrombin generation-based test is associated with thrombotic events in patients with lupus anticoagulants. *J Thromb Haemost*. 2007; **5**: 2204-11.

17. Pennings MTT, Derksen RH, van Lummel M, Adelmeijer J, VanHoorelbeke K, Urbanus RT, Lisman T, de Groot PG. Platelet adhesion to dimeric β2-glycoprotein I under conditions of flow is mediated by at least two receptors: glycoprotein Ibalpha and apolipoprotein E receptor 2'. *J Thromb Haemost*. 2007; **5**: 369-77.

18. Shi T, Giannakopoulos B, Yan X, Yu P, Berndt MC, Andrews RK, Rivera J, Iverson GM, Cockerill KA, Linnik MD, Krilis SA. Anti-β2-glycoprotein I antibodies in complex with β2-glycoprotein I can activate platelets in a dysregulated manner via glycoprotein Ib-IX-V. *Arthritis Rheum*. 2010; **54**: 2558-67.

19. Wolberg AS, Roubey RAS. Mechanisms of autoantibody-induced monocyte tissue factor expression. *Thromb Res*. 2004; **114**: 391-6.

20. Romay-Penabad Z, Aguilar-Valenzuela R, Urbanus RT, Derksen RHWM, Pennings MTT, Papalardo E, Shilagard T, Vargas G, Hwang Y, de Groot PG, Pierangeli SS. Apolipoprotein E receptor 2 is involved in the thrombotic complications in a murine model of the antiphospholipid syndrome. *Blood*. 2011; **117**: 1408-14.

21. Pierangeli SS, Girardi G, Vega-Ostertag M, Liu X, Espinola RG, Salmon J. Requirement of activation of complement C3 and C5 for antiphospholipid antibody-mediated thrombophilia. *Arthritis Rheum*. 2005; **52**: 2120-4.

22. Del Papa N, Sheng YH, Raschi E, Kandiah DA, Tincani A, Khamashta MA, Atsumi T, Hughes GRV, Ichikawa K, Koike T, Balestrieri G, Krilis SA, Meroni PL. Human β2-glycoprotein I binds to endothelial cells through a cluster of lysine residues that are critical for anionic phospholipid binding and offers epitopes for anti-β2-glycoprotein I antibodies. *J Immunol*. 1998; **160**: 5572-8.

23. Nagahama M, Nomura S, Kanazawa S, Ozaki Y, Kagawa H, Fukuhara S. Significance of anti-oxidized LDL antibody and monocyte-derived microparticles in anti-phospholipid antibody syndrome. *Autoimmunity*. 2003; **36**: 125-31.

24. Dignat-George F, Camoin-Jau L, Sabatier F, Arnoux D, Anfosso F, Bardin N, Veit V, Combes V, Gentile S, Moal V, Sanmarco M, Sampol J. Endothelial microparticles:

a potential contribution to the thrombotic complications of the antiphospholipid syndrome. *Thromb Haemost*. 2004; **91**: 667-73.

25. De Laat B, Derksen RHWM, van Lummel M, Pennings MTT, de Groot PG. Pathogenic anti-β2-glycoprotein I antibodies recognize domain I of β2-glycoprotein I only after a conformational change. *Blood*. 2006; **107**: 1916-24.

26. Savina A, Amigorena S. Phagocytosis and antigen presentation in dendritic cells. *Immunol Rev*. 2007; **219**: 143-56.

27. Lanzavecchia A. How can cryptic epitopes trigger autoimmunity? *J Exp Med*. 1995; **181**: 1945-8.

28. Van Haren SD, Herczenik E, ten Brinke A, Mertens K, Voorberg J, Meijer AB. HLA-DR-presented peptide repertoires derived from human monocyte-derived dendritic cells pulsed with blood coagulation factor VIII. *Mol Cell Proteomics*. 2011; **10**: M110.002246.

29. Ito H, Matsushita S, Tokano Y, Nishimura H, Tanaka Y, Fujisao S, Mitsuya H, Hashimoto H, Nishimura Y. Analysis of T cell responses to the β2-glycoprotein I-derived peptide library in patients with anti-β2-glycoprotein I antibody-associated autoimmunity. *Hum Immunol*. 2000; **61**: 366-77.

30. Lanzavecchia A. Antigen-specific interaction between T and B cells. *Nature*. 1985; **314**: 537-9.

31. Buttari B, Profumo E, Capozzi A, Facchiano F, Saso L, Sorice M, Riganò R. Advanced glycation end products of human β2-glycoprotein I modulate the maturation and function of DCs. *Blood*. 2011; **117**: 6152-61.

32. De Laat B, van Berkel M, Urbanus RT, Siregar B, de Groot PG, Gebbink MF, Maas C. Immune responses against domain I of β2-glycoprotein I are driven by conformational changes: Domain I of β2-glycoprotein I harbors a cryptic immunogenic epitope. *Arthritis Rheum*. 2011; **63**: 3960-8.

33. Kuwana M, Matsuura E, Kobayashi K, Okazaki Y, Kaburaki J, Ikeda Y, Kawakami Y. Binding of β2-glycoprotein I to anionic phospholipids facilitates processing and presentation of a cryptic epitope that activates pathogenic autoreactive T cells. *Blood*. 2005; **105**: 1552-7.

34. Arai T, Yoshida K, Kaburaki J, Inoko H, Ikeda Y, Kawakami Y, Kuwana M. Autoreactive CD4+ T-cell clones to β2-glycoprotein I in patients with antiphospholipid syndrome: preferential recognition of the major phospholipid-binding site. *Blood*. 2001; **98**: 1889-96.

35. Kondo A, Miyamoto T, Yonekawa O, Giessing AM, Osterlund EC, Jensen ON. Glycopeptide profiling of β2-glycoprotein I by mass spectrometry reveals attenuated sialylation in patients with antiphospholipid syndrome. *J Proteomics*. 2009; **73**: 123-33.

36. Pierangeli SS, de Groot PG, Dlott J, Favaloro E, Harris EN, Lakos G, Ortel T, Meroni PL, Otomo K, Pengo V, Tincani A, Wong R, Roubey RA. 'Criteria' aPL tests: Report of a Task Force and preconference workshop at the 13th International Congress on Antiphospholipid Antibodies, Galveston, Texas, April 2010. *Lupus*. 2011; **20**: 182-90.

37. Römisch J, Feussner A, Vermöhlen S, Stöhr HA. A protease isolated from human plasma activating Factor VII independent of tissue factor. *Blood Coagul Fibrinolysis*. 1999; **10**: 471-9.

38. Stephan F, Dienava-Verdoold I, Bulder I, Wouters D, Mast AE, Te Velthuis H, Aarden LA, Zeerleder S. Tissue factor pathway inhibitor is an inhibitor of factor VII-activating protease. *J Thromb Haemost*. 2012; **10**: 1165-71.

39. Kanse SM, Declerck PJ, Ruf W, Broze G, Etscheid M. Factor VII–activating protease promotes the proteolysis and inhibition of tissue factor pathway inhibitor. *Arterioscler*

Thromb Vasc Biol. 2012; **32**: 427-33.

40. Römisch J, Feussner A, Nerlich C, Stöhr HA, Weimer T. The frequent Marburg I polymorphism impairs the pro-urokinase activating potency of the factor VII activating protease (FSAP). *Blood Coagul Fibrinolysis*. 2002; **13**: 433-41.

41. Römisch J, Vermöhlen S, Feussner A, Stöhr HA. The FVII activating protease cleaves single-chain plasminogen activators. *Pathophysiol Haemost Thromb*. 1999; **29**: 292-9.

42. Cesarman-Maus G, Hajjar KA. Molecular mechanisms of fibrinolysis. *Br J Haematol*. 2005; **129**: 307-21.

43. Stephan F, Hazelzet J, Bulder I, Boermeester M, van Till JW, van der Poll T, Wuillemin W, Aarden LA, Zeerleder S. Activation of factor VII-activating protease in human inflammation: a sensor for cell death. *Crit Care*. 2011; **15**: R110.

44. Johansson PI, Stensballe J, Rasmussen LS, Ostrowski SR. A high admission syndecan-1 level, a marker of endothelial glycocalyx degradation, is associated with inflammation, protein C depletion, fibrinolysis, and increased mortality in trauma patients. *Ann Surg*. 2011; **254**: 194-200.

45. Maroney SA, Mast AE. Tissue factor pathway inhibitor and bacterial infection. *J Thromb Haemost*. 2011; **9**: 119-21.

46. Yun TH, Cott JE, Tapping RI, Slauch JM, Morrissey JH. Proteolytic inactivation of tissue factor pathway inhibitor by bacterial omptins. *Blood*. 2009; **113**: 1139-48.

47. Hamuro T, Kido H, Asada Y, Hatakeyama K, Okumura Y, Kunori Y, Kamimura T, Iwanaga S, Kamei S. Tissue factor pathway inhibitor is highly susceptible to chymase-mediated proteolysis. *FEBS J*. 2007; **274**: 3065-77.

48. Dahlbäck B. Inhibition of protein Ca cofactor function of human and bovine protein S by C4b-binding protein. *J Biol Chem*. 1986; **261**: 12022-7.

49. Hackeng TM, Seré KM, Tans G, Rosing J. Protein S stimulates inhibition of the tissue factor pathway by tissue factor pathway inhibitor. *Proc Natl Acad Sci U S A*. 2006; **103**: 3106-11.

50. Saller F, Brisset AC, Tchaikovski SN, Azevedo M, Chrast R, Fernández JA, Schapira M, Hackeng TM, Griffin JH, Angelillo-Scherrer A. Generation and phenotypic analysis of protein S–deficient mice. *Blood*. 2009; **114**: 2307-14.

51. Bombeli T, Basic A, Fehr J. Prevalence of hereditary thrombophilia in patients with thrombosis in different venous systems. *Am J Hematol*. 2002; **70**: 126-32.

52. Mahmoodi BK, Brouwer JL, Veeger NJ, van der Meer J. Hereditary deficiency of protein C or protein S confers increased risk of arterial thromboembolic events at a young age. *Circulation*. 2008; **118**: 1659-67.

53. Rojnuckarin P, Uaprasert N, Akkawat B, Settapiboon R, Nanakorn T, Intragumtornchai T. Protein C, protein S and von Willebrand factor levels correlate with bleeding symptoms: a population-based study. *Haemophilia*. 2012; **18**: 457-62.

54. Maurissen LFA, Castoldi E, Simioni P, Rosing J, Hackeng TM. Thrombin generation-based assays to measure the activity of the TFPI–protein S pathway in plasma from normal and protein S-deficient individuals. *J Thromb Haemost*. 2010; **8**: 750-8.

55. Fernandes N, Mosnier LO, Tonnu L, Heeb MJ. Zn^{2+}-containing protein S inhibits extrinsic factor X-activating complex independently of tissue factor pathway inhibitor. *J Thromb Haemost*. 2010; **8**: 1976-85.

56. Brinkman HJM, Mertens K, van Mourik JA. Proteolytic cleavage of protein S during the hemostatic response. *J Thromb Haemost*. 2005; **3**: 2712-20.

57. Pruemer J. Prevalence, causes, and impact of cancer-associated thrombosis. *Am J Health Syst Pharm*. 2005; **62**: S4-S6.

58. Falanga A, Marchetti M. Anticancer treatment and thrombosis. *Thromb Res*. 2012; **129**: 353-9.

59. Marchetti M, Diani E, ten Cate H, Falanga A. Characterization of thrombin generation potential of leukemic and solid tumor cells by the calibrated automated thrombography. *Haematologica*. 2012.

60. Vianello F, Battisti A, Cella G, Marchetti M, Falanga A. Defining the thrombotic risk in patients with myeloproliferative neoplasms. *ScientificWorldJournal*. 2011; **11**: 1131-7.

61. Panova-Noeva M, Marchetti M, Spronk HM, Russo L, Diani E, Finazzi G, Salmoiraghi S, Rambaldi A, Barbui T, ten Cate H, Falanga A. Platelet-induced thrombin generation by the calibrated automated thrombogram assay is increased in patients with essential thrombocythemia and polycythemia vera. *Am J Hematol*. 2011; **86**: 337-42.

62. Apelseth TO, Hervig T, Bruserud Ø. Current practice and future directions for optimization of platelet transfusions in patients with severe therapy-induced cytopenia. *Blood Rev*. 2011; **25**: 113-22.

63. Hitron A, Steinke D, Sutphin S, Lawson A, Talbert J, Adams V. Incidence and risk factors of clinically significant chemotherapy-induced thrombocytopenia in patients with solid tumors. *J Oncol Pharm Pract*. 2011; **17**: 312-9.

64. Rysler C, Stoffel N, Buser A, Gratwohl A, Tsakiris DA, Stern M. Effect of beta-blockers, Ca^{2+} antagonists, and benzodiazepines on bleeding incidence in patients with chemotherapy induced thrombocytopenia. *Platelets*. 2010; **21**: 77-83.

Summary

Haemostasis is a tightly regulated process and beyond the well known factors involved in the coagulation cascade there are also a multitude of other determinants that may up-regulate or down-regulate coagulation in pathological situations. In this thesis we have evaluated three potential determinants in coagulation pathophysiology: factor seven activating protease (FSAP), anti-β2-glycoprotein I (β2GPI) autoantibodies and proteolytic modification of protein S. Glycoprotein β2GPI is a highly abundant plasma protein and in the antiphospholipid syndrome (APS) in complex with anti-β2GPI autoantibodies it may create a prothrombotic phenotype. FSAP was discovered in 1996 and since its discovery numerous functions have been proposed including interactions with the haemostatic system. Although controversial, the Marburg I polymorphism in FSAP has been proposed to be associated with thromboembolic complications. Finally, while the role of protein S as a natural anticoagulant is beyond doubt, regulation of its anticoagulant activity by cleavage in the thrombin sensitive region is less recognized. Thrombography was instrumental in large part of the research described in this thesis.

Chapter 1 provides a general introduction into the complexity of normal coagulation as well as the development and application of thrombography. Furthermore, for each of the in this thesis evaluated proteins we discuss the in the literature available information on their structure and function and identify several questions related to their possible role in coagulation pathophysiology. Can we isolate monoclonal anti-β2GPI antibodies from APS patients representing the actual pathogenic antibodies and can these antibodies serve as tools for a more consistent study of APS pathogenesis? What are the immunological aspects of APS in terms of antigen uptake, processing and presentation by dendritic cells? Is FSAP capable of initiating thrombin generation by activating FVII, or do other mechanisms contribute towards the procoagulant activity of FSAP? And finally, what is the effect of proteolytic modification of protein S on thrombin generation and what is the source of the thrombin sensitive region-cleaving protease?

In **Chapter 2** we employed phage display to isolate β2GPI-binding immunoglobulin single-chain variable fragments from the B-cell repertoire of two APS patients. With this approach we found four unique single-chain variable fragments which were further used to construct full length IgG1 antibodies. These antibodies were binding to β2GPI and cardiolipin. Epitope mapping revealed that two of them could bind isolated domain I of β2GPI while the other two antibodies also required the presence of domain II. Testing lupus anticoagulant activity in various coagulation assays revealed that their potency is dependent on the affinity for and presence of β2GPI. Our results show successful construction of human monoclonal anti-β2GPI antibodies that display lupus anticoagulant activity related to their affinity for β2GPI, and therefore these antibodies may serve as useful tools in further research on the pathogenesis of APS.

We explored the uptake and the subsequent presentation of β2GPI by dendritic cells in **Chapter 3**. Confocal microscopy and flow cytometry revealed that β2GPI could be efficiently and rapidly internalized by immature dendritic cells. By a novel mass spectrometry approach, a limited number of peptides were identified to be presented on MHC II class molecules after a prolonged incubation with β2GPI and subsequent maturation of dendritic cells. Finding of these predominantly domain V peptides

will promote further research on T cell recognition implicated in immune response associated with APS.

As no study has been successful in confirming the initial reported name-giving activity of FSAP, in **Chapter 4** we explored whether FSAP is capable of activating FVII. Creation of a recombinant thermolysin-activated FSAP R313Q mutant allowed researching its activity under controlled conditions. The recombinant FSAP exhibited the same reactivity towards chromogenic peptide substrates as plasma derived FSAP and retained its ability to activate single chain urokinase type plasminogen activator. However, FVII proved resistant to FSAP, and neither in western blot nor in FVII activity assay any activation of FVII could be observed. We therefore conclude that FSAP, in contrast to what its name suggests, does not play a critical role in mediating haemostasis through activation of FVII.

In **Chapter 5** we further explored involvement of FSAP in haemostasis employing thrombin generation assay. FSAP had a procoagulant effect in plasma which was restricted to conditions of sub-picomolar tissue factor concentrations. This procoagulant effect was independent of FVII activation but instead involved tissue factor pathway inhibitor (TFPI). This interaction was confirmed in kinetic experiments as rapid and tight inhibition. Furthermore, the Marburg I mutant of FSAP had a strongly decreased interaction with TFPI and a diminished procoagulant effect in thrombin generation. Our data show that FSAP may amplify thrombin generation by inhibiting TFPI at the onset of coagulation. Our data also suggest that it is highly unlikely that Marburg I polymorphism-challenged thromboembolism is due to FSAP amplified thrombin generation.

Contribution of a yet unknown platelet protease to the proteolytic modification of the anticoagulant activity of protein S in the circulation was analyzed in **Chapter 6**. We showed that only intact but not *in vivo* cleaved protein S has anticoagulant properties in plasma. Due to the variation of platelet numbers and the extent of protein S cleavage in normal population, only in individuals with pathological conditions characterised by extreme low and high platelet counts, as occurred in patients with haematological malignancies and treated with myelosuppressive drugs, a positive correlation between platelet count and levels of cleaved protein S in the circulation was observed. These data contribute new insights into the contribution of platelets in the protein S-mediated regulation of thrombin formation under normal and pathological conditions.

Finally, in **Chapter 7** we discuss the findings presented in this thesis on β2GPI autoantibodies, FSAP and proteolytic modification of protein S in view of various pathological conditions that disturb the haemostatic balance. A schematic summary show how these new determinants may contribute to coagulation pathophysiology.

Samenvatting

Hemostase is een zeer strak gereguleerd proces. Naast de bekende stollingsfactoren, zijn er tal van andere determinanten die een opregulerend of remmend effect kunnen hebben op het stollingsproces bij diverse pathologische condities. In dit proefschrift zijn de volgende drie determinanten bestudeerd: factor zeven activerend protease (FSAP), autoantistoffen tegen β2-glycoproteïne I (β2GPI) en proteolytische modificatie van proteïne S. Glycoproteïne β2GPI komt in plasma in grote hoeveelheden voor. Complexen tussen β2GPI en autoantistoffen tegen β2GPI zijn mogelijk verantwoordelijk voor het protrombotische fenotype van patiënten met het antiphospholipid syndroom (APS). Er zijn vele biologische functies toegekend aan het in 1996 geïdentificeerde FSAP, waaronder een rol in de hemostase. Er zijn aanwijzingen alhoewel omstreden, dat het Marburg I polymorfisme in FSAP kan leiden tot tromboembolische complicaties. De rol van proteïne S als onderdeel van fysiologische antistollingsmechanismen staat zonder meer vast; minder bekend is de regulering van de antistollende activiteit van proteïne S door proteolytische modificatie. Voor de in dit proefschrift beschreven onderzoek bleek te techniek "trombografie" essentieel.

Hoofdstuk 1 is een algemene introductie en geeft inzage in de complexiteit van het stollingsproces onder normale condities. In dit hoofdstuk wordt de ontwikkeling en toepassing van trombografie beschreven. Daarnaast vindt een evaluatie plaats van de in de literatuur beschikbare informatie over structuur en functie van de in dit proefschrift bestudeerde eiwitten en werden de volgende vragen geformuleerd met betrekking tot hun mogelijke bijdrage aan een aan pathofysiologische condities gerelateerde afwijkende bloedstolling: Is het mogelijk om voor het APS syndroom representatieve pathogene anti-β2GPI antistoffen te isoleren uit patiënten met gediagnosticeerd APS en kunnen deze antilichamen vervolgens gebruikt worden in onderzoek naar de pathogenese van APS? Wat zijn de immunologische aspecten van APS als het gaat om antigeen opname, verwerking en presenteren door dendritische cellen? Kan FSAP het genereren van trombine initiëren door FVII te activeren, of zijn er andere mechanismen welke een positieve invloed hebben op de coagulerende werking van FSAP? En als laatste, welk effect heeft proteolytische modificatie van proteïne S op de generatie van trombine en wat is de herkomst van het proteïne S knippend protease?

In **Hoofdstuk 2** hebben we van twee APS patiënten met behulp van faagdisplay de β2GPI-bindende immunoglobuline enkel keten variabele fragmenten uit B-cellen geïsoleerd. Met behulp van deze methode hebben we vier unieke enkel keten variabele fragmenten gevonden welke zijn gebruikt voor het synthetiseren van IgG1 antilichamen. Deze geconstrueerde antilichamen bleken te binden aan β2GPI en cardiolipine. Twee antilichamen bonden aan geïsoleerd domein I van β2GPI terwijl twee andere antilichamen alleen binding lieten zien indien ook domein II in het construct aanwezig was. Uit verschillende stollingstest bleek dat de mate van stollingsremmende eigenschap van de geconstrueerde antistoffen afhankelijk is van de affiniteit voor en aanwezigheid van β2GPI. Samengevat laten onze resultaten zien dat we in staat zijn om verschillende humane monoklonale anti-β2GPI antilichamen te construeren welke een stollingsremmende activiteit vertonen die is gerelateerd aan de affiniteit voor β2GPI. Deze geconstrueerde antilichamen kunnen van groot belang zijn in vervolgstudies naar de pathogenese van APS.

De opname en antigeenpresentatie van β2GPI door dendritische cellen hebben we bestudeerd in **Hoofdstuk 3**. Met behulp van confocale microscopie en flowcytometrie kon worden aangetoond dat β2GPI snel en efficiënt wordt opgenomen in onrijpe dendritische cellen. Na langdurige bloodstelling aan β2GPI en uitrijping van dendritische cellen kon met behulp van een nieuwe massa spectrometrische aanpak presentatie van voornamelijk domein V peptiden van β2GPI op MHC II klasse moleculen worden aangetoond. Deze kennis is van belang in vervolgstudies naar T-cel herkenning in APS geassocieerde immuunreacties.

Alhoewel de naam FSAP anders doet suggereren, is er nog geen algemene consensus wat betreft het activeren van FVII door FSAP. In **Hoofdstuk 4** hebben we onderzocht of FSAP werkelijk in staat is om FVII te activeren. Met behulp van een recombinant thermolysine-activeerbaar FSAP R313Q mutant was het mogelijk om de enzymatische activiteit van FSAP onder gecontroleerde omstandigheden the onderzoeken. Geactiveerd FSAP R313Q laat dezelfde reactiviteit zien voor chromogene peptidesubstraten als voor uit plasma geïsoleerd en geactiveerd FSAP. Daarnaast is het recombinante FSAP in staat enkel keten urokinase type plasminogeen activator te activeren. FVII blijkt echter resistent voor FSAP. Zowel uit western blot analyses en bepalingen van FVII activiteit bleek dat geactiveerd FSAP niet in staat was FVII the activeren. Uit dit onderzoek concluderen wij dat de activering van FVII door FSAP, in tegenstelling tot wat de naam suggereert, geen belangrijke rol speelt in de hemostase.

In **Hoofdstuk 5** werd de mogelijke functie van FSAP in de hemostase verder onderzocht met behulp van trombografie. Geactiveerd FSAP R313Q liet een procoagant effect in plasma zien, echter alleen bij sub picomolaire concentraties aan weefselfactor. Dit procoagulante effect van FSAP was onafhankelijk van de activering van FVII, maar bleek gemedieerd via weefselfactor-pathway inhibitor (TFPI). Een interactie tussen FSAP en TFPI werd bevestigd in kinetische analyses. Aanwezigheid van het Marburg I polymorfisme gaf aanleiding tot een sterk verminderende interactie met TFPI en een zeer laag procoagulant effect in de trombinegeneratie. Deze data suggereren dat FSAP de generatie van trombine kan versterken door het remmen van TFPI bij aanvang van het stollingsproces. Onze data laten ook zien dat het zeer onwaarschijnlijk is dat een door FSAP versterkte trombinegeneratie een rol speelt in een verhoogd risico op trombo-embolie bij individuen met een Marburg I polymorfisme.

De rol van een tot nu toe onbekend bloedplaatjesprotease bij de regulering van de stollingsremmende activiteit van proteïne S is bestudeerd in **Hoofdstuk 6**. We laten zien dat alleen intact en niet *in vivo* proteolytische gemodificeerd proteïne S anticoagulante eigenschappen bezit in plasma. Een correlatie tussen bloedplaatjesaantal en de mate van proteolytische modificatie van proteïne S *in vivo* kon niet worden aangetoond bij gezonde vrijwilligers. Een positieve correlatie tussen aantal plaatjes en proteïne S proteolyse werd wel gevonden in patiënten met hematologische maligniteiten. Deze resultaten dragen bij tot nieuwe inzichten in de rol van bloedplaatjes bij de proteïne S- gemedieerde regulering van de trombinegeneratie onder normale en pathologische condities.

In **Hoofdstuk 7** bediscussiëren we de in dit proefschrift gepresenteerde bevindingen met betrekking tot een mogelijke bijdrage van B2GPI auto-antilichamen, FSAP en proteolytische modificatie van proteïne S aan een verstoorde hemostase bij diverse pathologische condities. Een schematische samenvatting geeft aan op welke wijze deze nieuwe determinanten betrokken kunnen zijn in de pathofysiologie van de bloedstolling.

Kopsavilkums

Asins rece ir stingri regulēts process, kas pasargā no asins zuduma un palīdz sadziedēt asins vadu bojājumus. Papildus labi zināmajiem faktoriem, kas ir iesaistīti asins reces kaskādē, pastāv vēl daudzi citi regulējoši faktori, kas dažādās patoloģiskās situācijās asins reci var vai nu pastiprināt vai nomākt. Šajā disertācijā mēs esam izvērtējuši trīs potenciālus regulatorus asins reces patofizioloģijā: VII faktora aktivējošo proteāzi (FSAP), antivielas pret β2-glikoproteīnu I (β2GPI) un proteīna S proteolītisko modifikāciju.

Glikoproteīns β2GPI asins plazmā ir sastopams ļoti augstā koncentrācijā, tomēr tā fizioloģiskā funkcija nav skaidra. No otras puses, ir skaidri parādīts, ka β2GPI var veicināt trombozes rašanos antifosfolipīdu sindroma (APS) gadījumā, kompleksā ar anti-β2GPI antivielām. FSAP tika atklāts 1996. gadā, un kopš atklāšanas tam ir piedēvētas dažādas funkcijas, ieskaitot saistību ar asins reces sistēmu. Mārburgas I mutācijai vai polimorfismam FSAP proteīnā tiek piedēvēts paaugstināts trombembolisko komplikāciju risks, lai gan šajā jautājumā dati vēl atšķiras. Lai gan proteīna S loma kā fizioloģiskam antikoagulantam ir zināma, tomēr maz vēl skaidrs par proteīna S aktivitātes regulēšanu ar šķelšanu trombīn-jutīgajā rajonā. Šajā disertācijā aprakstītajos pētījumos liela nozīme bija trombogrāfijas mērījumiem.

1. nodaļa sniedz vispārēju ievadu par normālās asins reces sarežģīto mehānismu, kā arī par trombīna veidošanās mērīšanas attīstību un pielietojumu. Par katru šajā disertācijā apskatīto proteīnu ir dots literatūras apskats par to uzbūvi un funkcijām, kā arī uzstādīti darba jautājumi par šo proteīnu nozīmi asinsreces patofizioloģijā. Vai ir iespējams radīt monoklonālas anti-β2GPI antivielas, kas darbības ziņā varētu atveidot APS pacientos esošās patogēnās antivielas? Vai šīs antivielas var izmantot, lai veiktu izsmeļošāku APS patoģenēzes izpēti? Pētot APS no imunoloģiskā viedokļa – kā notiek antigēnu uzņemšana, pārstrāde un prezentācija uz dendrītiskajām šūnām? Vai FSAP spēj uzsākt trombīna aktivēšanas kaskādi, aktivējot VII faktoru? Vai tomēr citi mehānismi ir pamatā FSAP asins reci stimulējošajai aktivitātei? Un visbeidzot – kā trombīna ģenerāciju ietekmē proteīna S proteolītiskā šķelšana un kāda ir trombīn-jutīgā rajona šķeļošās proteāzes izcelsme?

2. nodaļā mēs izmantojām fāgu displeju, lai iegūtu β2GPI atpazīstošu imunoglobulīnu vienas ķēdes variablos fragmentus no divu APS pacientu B-limfocītiem. Šādā veidā mēs ieguvām četrus unikālus vienas ķēdes variablos fragmentus, kurus tālāk izmantojām, lai radītu pilna garuma IgG1 antivielas. Šīs antivielas reaģēja ar β2GPI un kardiolipīnu. Meklējot iegūto antivielu epitopus, atklājās, ka divas no antivielām spēja atpazīt izolētu β2GPI domēnu I, bet otrām divām antivielām bija nepieciešama arī domēna II klātbūtne. Lupus antikoagulanta aktivitātes mērījumi dažādos asinsreces testos parādīja, ka antivielu efektivitāte ir atkarīga no to afinitātes un β2GPI klātbūtnes. Kopsavilkumā mūsu rezultāti parāda, ka ir iespējams veiksmīgi radīt cilvēka monoklonālās anti-β2GPI antivielas ar lupus antikoagulanta aktivitāti, kas ir saistīta ar to afinitāti pret β2GPI, un tāpēc šīs antivielas var izmantot kā materiālus tālākā APS patoģenēzes izpētē.

3. nodaļā mēs izpētījām β2GPI uzņemšanu un sekojošu prezentāciju uz dendrītiskajām šūnām. Konfokālā mikroskopija un plūsmas citometrija atklāja, ka β2GPI tika ātri un efektīvi uzņemts nenobriedušās dendrītiskajās šūnās. Izmantojot jaunu masas spektrometrijas pieeju, mēs identificējām vairākus peptīdus, kas bija prezentēti uz MHC II klases molekulām pēc tam, kad dendrītiskās šūnas tika ilgstoši

inkubētas ar β2GPI un pēc tam nobriedinātas. Šo, pārsvarā V domēna, peptīdu atrašana dod stimulu tālākai izpētei, kā T-limfocītu reakcija uz prezentētajiem peptīdiem noved pie auto-imūnās atbildes, kas izraisa APS.

Līdz šim vēl nevienā pētījumā nav izdevies apstiprināt sākotnējo novērojumu, kas deva vārdu VII faktora aktivējošajai proteāzei. Tāpēc **4. nodaļā** mēs apskatījām, vai FSAP ir spējīgs aktivēt VII faktoru (FVII). Šim nolūkam tika radīts rekombinants $FSAP^{R313Q}$ mutants, kas aktivējams ar bakteriālo proteāzi termolizīnu, tādējādi dodot iespēju pētīt proteīna darbību kontrolējamos apstākļos. Rekombinantais FSAP izrādīja tādu pašu reaktivitāti pret hromogēnajiem peptīdu substrātiem kā no asins plazmas iegūtais FSAP un saglabāja spēju aktivēt single chain urokināzes tipa plazminogēna aktivatoru. Diemžēl FSAP neiedarbojās uz FVII, un ne imunoblotā, ne FVII aktivitātes mērījumos nebija iespējams novērot FVII aktivēšanu. Tādēļ mēs secinājām, ka FSAP, par spīti savam nosaukumam, asins reci nepastiprina caur FVII aktivēšanu.

5. nodaļā, izmantojot trombogrāfijas mērījumus, mēs tālāk pētījām, kā FSAP ir iesaistīts asins reces procesā. Asins plazmā FSAP izrādīja asins reci stimulējošu efektu, tomēr šis efekts bija novērojums tikai zem pikomolārām audu faktora koncentrācijām. FSAP prokoagulantais mehānisms neietvēra FVII aktivēšanu, bet tā vietā iesaistīja audu faktora mehānisma inhibitoru (TFPI/AFMI). Enzīmu kinētikas eksperimenti parādīja, ka šī mijiedarbība izpaužas kā ātra un cieša inhibīcija. FSAP ar Mārburgas I mutāciju daudz vājāk mijiedarbojās ar TFPI, turklāt tam bija samazināts efekts uz trombīna veidošanos. Mūsu rezultāti liecina, ka FSAP var pastiprināt trombīna aktivācijas kaskādi, nomācot TFPI kavējošo darbību tās iesākumā. Mūsu rezultāti arī liek apšaubīt teoriju, ka FSAP Mārburgas I mutācija ietekmē trombīna ģenerāciju un izraisa tromboembolismu .

6. nodaļā mēs analizējām, kā līdz šim nezināma asins plātnīšu proteāze šķeļ proteīnu S un modulē tā asinsreci kavējošo (antikoagulanto) darbību. Mēs parādījām, ka tikai neskartam, bet ne *in vivo* šķeltam proteīnam S piemīt antikoagulantas īpašības asins plazmā. Tā kā veseliem cilvēkiem asins plātnīšu skaits un proteolizētā proteīna S līmenis var spēcīgi variēt, tad tikai indivīdos ar ārkārtīgi augstu vai zemu asins plātnīšu skaitu, piemēram, pacientos ar hematoloģiskiem audzējiem vai pacientos, kas ārstēti ar mielosupresīviem medikamentiem, varēja novērot pozitīvu korelāciju starp asins plātnīšu skaitu un proteīna S proteolīzi plazmā. Šie dati dod jaunu ieskatu par asins plātnīšu ieguldījumu trombīna ģenerācijas kontrolēšanā ar proteīnu S normālos un patoloģiskos apstākļos.

Visbeidzot, **7. nodaļā** mēs apskatām šajā disertācijā aprakstītos atklājumus par anti-β2GPI antivielām, VII faktora aktivējošo proteāzi un proteīna S proteolītisko modifikāciju kopā ar dažādiem patoloģiskajiem apstākļiem, kas izjauc asins reces līdzsvaru. Shematisks kopsavilkums parāda, kā šie jaunie regulatori dod ieguldījumu asins reces patofizioloģijā.

Curriculum vitae

Ilze Dienava-Verdoold was born on 6th of November, 1982 in Riga, Latvia. A bronze medal in the Republic Olympiad in Biology in 2000 led to the educational camp "Alfa" for school subject olympiad winners which gave the first boost of choosing the path of science. In 2001 she received her secondary education diploma from the Valka Secondary School 1. In the same year she entered the Riga Stradiņš University in the Dentistry program, but two years later, in 2003, she transferred to the University of Latvia to study Biology. The first feeling of laboratory was experienced in the Laboratory of Animal Physiology in Institute of Biology, Salaspils, Latvia, under the guidance of Dr. N. Bērziņa. The second internship leading to the Bachelor thesis project "Comparison of various expression systems for expression of recombinant proteins" was performed in the Laboratory of Protein Chemistry, in Latvian Biomedical Research and Study Centre, Riga, Latvia, supervised by Dr. I. Petrovskis. For the further studies she acquired the European Social Fund grant to study Master of Research in Biomedical Sciences in the University of Ulster and she graduated in 2006 with distinction. Under supervision of Prof. Dr. A.P. McHale she performed research resulting in the Master thesis "Electroporative loading of erythrocytes for drug delivery". In September 2007 she started her research for the PhD thesis in the Department of Plasma Proteins in Sanquin Research, Amsterdam guided by Prof. Dr. K. Mertens, Dr. B. de Laat and Dr. H.J.M. Brinkman. The results of this research have been described in this thesis.

List of publications

Journal articles (peer-reviewed)

Dienava-Verdoold I, Boon-Spijker MG, de Groot PG, Brinkman HJM, Voorberg J, Mertens K, Derksen RHWM, de Laat B. Patient-derived monoclonal antibodies directed towards β2-glycoprotein I display lupus anticoagulant activity. *Journal of Thrombosis and Haemostasis*. 2011; **9**(4): 738-47.

Stavenuiter F, **Dienava-Verdoold I**, Boon-Spijker MG, Brinkman HJM, Meijer AB, Mertens K. Factor Seven Activating Protease (FSAP): does it activate Factor VII? *Journal of Thrombosis and Haemostasis*. 2012; **10**(5): 859-66.

Dienava-Verdoold I, Marchetti MR, Te Boome LC, Russo L, Falanga A, Koene HR, Mertens K, Brinkman HJ. Platelet-mediated proteolytic down regulation of the anticoagulant activity of protein S in individuals with haematological malignancies. *Thrombosis and Haemostasis*. 2012; **107**(3): 468-76.

Stephan F, **Dienava-Verdoold I**, Bulder I, Wouters D, Mast AE, Te Velthuis H, Aarden LA, Zeerleder S. Tissue factor pathway inhibitor is an inhibitor of factor VII-activating protease. *Journal of Thrombosis and Haemostasis*. 2012; **10**(6): 1165-71.

van Breevoort D, van Agtmaal EL, Dragt BS, Gebbinck JK, **Dienava-Verdoold I**, Kragt A, Bierings R, Horrevoets AJ, Valentijn KM, Eikenboom JC, Fernandez-Borja M, Meijer AB, Voorberg J. Proteomic screen identifies IGFBP7 as a novel component of endothelial cell-specific Weibel-Palade bodies. *Journal of Proteome Research*. 2012; **11**(5): 2925-36.

Conference contributions (oral presentations)

Boon-Spijker M, **Dienava I***, de Groot P, Voorberg J, Mertens K, de Laat B, Derksen R. Patient-derived monoclonal antibodies directed towards β2-glycoprotein I display LAC activity. *International Society on Thrombosis and Haemostasis, XXIIth Congress, Boston, USA, 2009*; *Journal of Thrombosis and Haemostasis*. 2009; **7**(Suppl 2): abstract #OC-MO-111.

Conference contributions (poster presentations)

Dienava-Verdoold I, Boon-Spijker M, de Groot P, Brinkman HJM, Voorberg J, Mertens K, de Laat B, Derksen RWHM. Patient-derived monoclonal antibodies directed towards β2-glycoprotein I display LAC activity. *1st joint meeting GTH & NVTH, Nürnberg, Germany, 2010*. *Hämostaseologie*. 2010; **30**(1) abstract #P04-07.

Dienava I, Meijer AB, van Alphen F, Mertens K, de Laat B. Von Willebrand factor mediates attachment of platelets to macrophages under high shear. *Biennial Sanquin Science Day, Amsterdam, The Netherlands, 2008*. (Poster award)

Acknowledgements

"The light works," he said, indicating the window, "the gravity works," he said, dropping a pencil on the floor. "Anything else we have to take our chances with."

— Douglas Adams, Dirk Gently's Holistic Detective Agency

The "anything else" is often the case in research. As anyone will know, there's many things which on any given day will give unexpected results. Sometimes it will lead to new areas to explore, sometimes – to re-evaluation of the previous findings. That I have navigated safely through this maze to the finished thesis, is also a merit of my supervisors. And therefore I would like to express my gratitude to Koen for providing constructive criticism and setting good scientific standards, to Herm-Jan for giving support and encouragement in the second half of my PhD time, and to Bas for supplying enthusiasm in the beginning of my PhD.

Of course, acknowledgements should go also to all the people who gave the technical support throughout my PhD: to our secretaries, to the safe-keepers of the various research facilities, to the librarian providing hard-to-get articles, and to all the anonymous donors of the "buisjesdonor" system.

I'm also thanking to all my colleagues for company, help and a few good laughs together. Even though I was working on my project mostly on my own, it was important to share lab and office with you!

Liels paldies manam uzticamajam atbalstītāju pulkam Latvijā un ārpus Latvijas! Sevišķi liels paldies manai ģimenei par atbalstu, pat ja ne vienmēr man izdevās paskaidrot, ko es īsti daru!

Graag wil ik mijn schoonfamilie bedanken voor hun ondersteuning en liefde welke ze mij gegeven hebben.

And, not the least, Remco, you've been with me throughout all times good and bad, cheering and supporting, and therefore an enormous "thank you" goes to you.

Just accidentally dropped the pencil. Yes, the gravity still works.