The molecular basis of APC-resistance: role of coagulation factor abnormalities

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The Molecular Basis of APC-Resistance:
Role of Coagulation Factor
Abnormalities

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The Molecular Basis of APC-Resistance: Role of Coagulation Factor Abnormalities

PROEFSCHRIFT

ter verkrijging van de graad doctor
aan de Universiteit Maastricht,
op gezag van de Rector Magnificus,
Prof. mr. G.P.M.F. Mols,
volgens het besluit van het College van Decanen,
in het openbaar te verdedigen op
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CHAPTER 1

General Introduction

1.1 Introduction

Blood is a vital component of the human body, that functions as a carrier of substances like nutrients, metabolites, oxygen and waste products. It flows through the vascular system, thereby reaching all organs and tissues in our body.

When the vascular system is damaged, blood will leak out. At this point it is very important that the bleeding is stopped and tissue repair can start. Therefore, at the place of injury, blood is converted from a fluid phase into a solid phase, thereby preventing further blood loss. The process whereby blood is maintained fluid under normal conditions but is allowed to clot when and where necessary is regulated by a large variety of components present in both our plasma and the vessel wall: this delicate balance is called haemostasis.

The haemostatic balance is the result of two opposite forces: the procoagulant pathway, which promotes coagulation and results in the formation of a clot and the anticoagulant pathway, which keeps the blood fluid. Under normal circumstances haemostasis is shifted in favor of the anticoagulant pathway, allowing blood to circulate through the body. Once blood coagulation is activated, however, the haemostatic balance shifts towards the procoagulant pathway, allowing a clot to be formed at the site of injury.

1.2 Blood coagulation

Platelets

Blood coagulation is a complex process that involves cells present in the circulation (platelets) and proteins present in both blood and in the vessel wall. After vascular damage the vessel wall constricts immediately resulting in a reduction of the blood flow. This slows down the circulating platelets and makes it possible for them to adhere to the subendothelial structures (e.g. collagen, fibronectin) that have become exposed to the bloodstream. Platelet adhesion occurs primarily via receptor-mediated interactions. The receptor involved during platelet adhesion, however, may differ depending on the shear rate in the blood vessel. Overall, initially the platelet receptor glycoprotein Ib (GPIb) binds to von Willebrand factor (vWF) [1], a protein that is exposed when endothelial damage occurs, followed by expression and activation of a whole range of other receptors resulting in an immobilized platelet on the subendothelial surface [2-6]. Upon binding, platelets are activated and undergo several changes, making them procoagulant. The phospholipid composition of the platelet membrane

changes, resulting in the exposure of negatively charged phosphatidylserine (PS) on the outer leaflet of the platelet membrane [7,8]. The platelet membrane and these negatively charged phospholipids play an important role in coagulation reactions. Simultaneously the content of the α -granules and the dense bodies is released. The α -granules contain several clotting factors such as factor V, factor XIII, fibrinogen, vWF but also other proteins like platelet factor 4, whereas the dense bodies contain a variety of platelets agonists [9-11]. The activation of platelets and the effect of platelet agonists released from the dense bodies make the platelets even more procoagulant, as they express a new receptor now, integrin $\alpha_{IIb}\beta_3$. Thanks to the $\alpha_{IIb}\beta_3$ receptor, together with the released platelet agonists and the presence of fibrinogen or fibrin, activated platelets will bind and activate other platelets [12-14]. This process is called aggregation and results in the formation of a primary thrombus at the site of injury, which however is unstable. This platelet plug stops the bleeding temporarily, but for a complete and stable thrombus to be formed additional (coagulation) reactions are required.

Coagulation cascade

The coagulation cascade is a waterfall sequence of enzymatic reactions involving several different plasma proteins. The goal of this system is, once activated by a small amount of trigger, to amplify this initial signal and eventually generate a large amount of thrombin [15,16]. Thrombin, in turn, converts fibrinogen into fibrin which forms a fibrin network and thereby stabilizes the primary platelet thrombus. Such a stepwise system has the advantage that it can easily be regulated at different levels, but also has the disadvantage that when one or more components are missing, this will affect all reactions lying downstream of the missing components (e.g. hemophilia A and B).

Most enzymes involved in coagulation are vitamin K-dependent proteins, belonging to the family of serine proteases. Their catalytic domains are highly homologous. Furthermore they contain several conserved structural motifs (such as Gla-domains, EGF-like domains, Kringle domains) that are involved in interactions with other proteins and with membranes [17]. All vitamin K-dependent proteins contain a Gla-domain which mediates binding to a negatively charged (procoagulant) membrane. For this binding calcium ions are required. These proteases circulate in blood as inactive pro-enzymes, also called zymogens, that have to be activated in order to participate in the coagulation reactions. Zymogen activation occurs by limited proteolysis on a negatively charged membrane surface in the presence of calcium ions. Such a

membrane surface is provided by the activated platelets. Other coagulation factors, such as factor V and factor VIII function as non-enzymatic cofactors, increasing the catalytic efficiency of the enzyme to which they bind (factor Xa and Factor IXa respectively). These cofactors show considerable similarities in both structure and function [18].

Historically the coagulation cascade has been divided into two parts, the intrinsic pathway and the extrinsic pathway, based on the way coagulation is initiated in clotting assays performed in clinical laboratories. The extrinsic pathway is initiated following exposure of the membrane protein tissue factor to the blood after damage of the vessel wall, while the intrinsic pathway is triggered when blood comes into contact with (artificial) negatively charged surfaces such as glass. Nowadays it is generally accepted that *in vivo* initiation of coagulation is achieved by the extrinsic pathway, while the intrinsic pathway is recruited at a later stage and ensures the amplification (propagation) of the coagulation reactions *via* several feedback loops [19,20].

Figure 1: Schematic representation of the coagulation cascade

Extrinsic pathway

The extrinsic pathway plays the major role in the initiation of blood coagulation after vascular injury. Tissue factor (TF) is an integral membrane glycoprotein present in sub-endothelial tissues that functions as a receptor for factor VII (FVII) and FVIIa once the vessel wall has been damaged [21,22]. TF binds to FVII to form a one-to-one complex, thereby facilitating the activation of FVII to FVIIa by plasma serine proteases. FVIIa only has physiological activity when complexed to TF [23]. The TF-FVIIa complex will activate factor X (FX) to factor Xa (FXa), by cleavage of a single peptide bond in the heavy chain of FX. The TF-FVIIa complex is also capable of activating factor IX by limited proteolysis [24]. This reaction is particularly of importance when only a small amount of TF-FVIIa complex is generated after vascular injury. Under these conditions, the intrinsic pathway is recruited *via* factor IXa (FIXa). Next, FXa cleaves the pro-cofactor factor V (FV) by limited proteolysis, yielding the active cofactor factor Va (FVa) [25,26]. FXa forms a complex with FVa in the presence of calcium ions and a negatively charged phospholipid membrane. This complex, called the prothrombinase complex, is able to very effectively convert prothrombin into thrombin [27,28]. Thrombin is a key player in coagulation; it converts fibrinogen into fibrin, but it also activates platelets and is responsible for several important feedback reactions. For instance, thrombin is the predominant activator of the pro-cofactors factor V [29] and factor VIII, it activates factor XI [30] thereby recruiting the intrinsic pathway and it also activates factor XIII. Factor XIIIa is a transglutaminase which covalently crosslinks the monomeric and polymeric fibrin strands into a tough insoluble fibrin network that stabilizes the primary platelet plug [31].

Intrinsic pathway

The extrinsic coagulation pathway is effectively inhibited by a plasma protease inhibitor called tissue factor pathway inhibitor (TFPI) [32]. This is a factor Xa-dependent inhibitor of the TF-FVIIa complex and blocks additional thrombin formation via the extrinsic pathway. However, the small amount of thrombin generated by the extrinsic pathway can activate factor XI, thereby recruiting part of the intrinsic pathway.

Activated factor XI (FXIa) activates factor IX (FIX) to factor IXa (FIXa) that forms a complex with factor VIIIa (FVIIIa) in the presence of calcium ions and a negatively charged phospholipid membrane. The FVIIIa is derived from the feedback activation of factor VIII by thrombin generated by the extrinsic pathway. This complex, the tenase complex, will subsequently activate factor X

to factor Xa. In this way, the intrinsic pathway takes over FXa generation after the extrinsic pathway has been shut down by TFPI.

To be complete, the intrinsic pathway includes a number of reactions that will also lead to the activation of FXI. When blood comes into contact with an artificial negatively charged surface such as glass or kaolin, kallikrein will activate factor XII (FXII). Activated FXII (FXIIa), together with kallikrein and the cofactor high molecular weight kininogen (HMWK), will then activate factor XI (FXI) to factor XIa (FXIa). The physiological importance of these first reaction steps is not completely understood and it is currently thought that, under physiological conditions, FXI is predominantly activated *via* a feedback loop by thrombin, formed via the extrinsic pathway.

Regulation of coagulation

The formation of a stable plug by platelet aggregation and fibrin polymerization following vascular injury is a very rapid and important process that prevents extensive blood loss. However, since it is not desirable that the blood vessel becomes completely occluded, totally blocking further blood flow to the organs and tissues lying downstream of the occlusion, nature has provided us with several ways to effectively regulate blood coagulation.

Coagulation inhibitors

Plasma contains a number of proteins that are able to inhibit certain *serine proteases* involved in blood coagulation. Some of these inhibitors, the so-called *serpins*, are substrate analogues that rapidly form equimolar complexes with the target protease. The formation of the inhibitor-protease complex is irreversible as a covalent bond is formed between both proteins. This inactive complex is efficiently removed from the circulation.

Antithrombin (AT) is the primary inhibitor of thrombin and therefore controls the amount of fibrin formed and the numerous feedback loops involving thrombin. The rate of thrombin inhibition by AT is rather slow, but is increased several orders of magnitude by heparin, a commonly used drug for anticoagulant treatment but which is also present in small amounts in free circulation [33], or by binding of AT to heparinoids on the vascular endothelium [34]. In addition, AT is a very potent inhibitor of FXa and FIXa, a reaction that is also stimulated by heparin.

Heparin cofactor II is similar to AT in its ability to inhibit thrombin in a heparin-dependent manner. However, whereas AT is only potentiated by certain

subspecies of heparin, HCII is stimulated by a large variety of polyanions [35]. HCII also shows a higher selectivity towards thrombin as compared to AT.

α 2-macroglobulin (α 2M) is another thrombin inhibitor, but since it is not a serpin α 2M has a different mechanism of action. When thrombin cleaves α 2M via limited proteolysis in the bait region of the molecule, α 2M undergoes a large-scale conformational change, entrapping thrombin inside the molecule [36,37]. However, the active site of thrombin is not occupied by the inhibitor, which explains why the α 2M-thrombin complex retains the ability to cleave small substrates (like chromogenic substrates), but shows no proteolytic activity towards larger macromolecules (e.g. fibrinogen).

Tissue factor pathway inhibitor (TFPI) is also not a member of the serpin family, but belongs to the family of Kunitz-type inhibitors. As mentioned earlier, TFPI inhibits the TF-FVIIa complex. However, TFPI requires the presence of catalytically active FXa in order to exert its inhibitory function. So, in fact, TFPI inhibits the extrinsic pathway by a dual mechanism: it binds and inhibits FXa directly and the TFPI-FXa complex is an effective inhibitor of the TF-FVIIa complex, blocking additional FXa formation by the extrinsic pathway [32,38].

Figure 2: The most important coagulation inhibitors in plasma.

Down-regulation of coagulation is achieved by specific inhibitors and the protein C system. This schematic overview shows the most important inhibitors as black squares and indicates where each inhibitor exhibits its regulatory function.

The protein C pathway

Besides the protease inhibitors, there is another very efficient and important negative regulator of the coagulation cascade, the protein C pathway. In contrast to most coagulation inhibitors that directly inhibit a specific serine protease, the protein C pathway regulates the amount of thrombin formed by proteolytic inactivation of the cofactors of the tenase complex and the prothrombinase complex, FVIIIa and FVa respectively [39,40]. These inactivation reactions are accomplished by activated protein C (APC) and are stimulated by protein S, a non-enzymatic protein that functions as a cofactor of APC [41,42]. The inactivated forms of FVa and FVIIIa (FVI and FVIIIi respectively) have no cofactor activity anymore and are subsequently rapidly cleared from the circulation. The protein C pathway will be discussed in more detail below. The physiological importance of the protein C pathway is illustrated by the observation that deficiencies of protein C or protein S result in an increased risk for venous thrombosis [43,44].

Figure 3: The protein C pathway.

A) Inactivation of activated factor V, the cofactor of the prothrombinase complex, by APC and its cofactor protein S. B) Inactivation of activated factor VIII, the cofactor of the tenase complex, by APC and its cofactors protein S and factor V.

The fibrinolytic system

The ultimate goal of the coagulation system is to convert fibrinogen into fibrin, thereby stabilizing the primary platelet thrombus. However, once the bleeding has stopped and the vascular injury has been repaired, the thrombus should be dissolved. This process, known as fibrinolysis, is accomplished by the fibrinolytic system. This requires binding of the circulating zymogen plasminogen to the clot, conversion of plasminogen to the active protease plasmin by tissue-type plasminogen activator (t-PA) released by the endothelial cells, proteolysis of the clot, and finally inactivation of plasmin by $\alpha 2$ -antiplasmin

[45]. Of course fibrinolysis also has to be strictly regulated, which is achieved with feedback loops and specific inhibitors.

Initially, a weak ternary complex with minimal catalytic activity is formed between t-PA, fibrin and plasminogen. Both t-PA and plasminogen bind to fibrin via interaction with the carboxyl-terminal lysine residues of fibrin. The plasmin that is formed at the fibrin surface will then cleave fibrin, thereby exposing new binding sites, enabling more t-PA and plasminogen to bind to the fibrin surface. This positive feedback mechanism ensures that active plasmin is initially generated at a slow pace, increases as more fibrin is cleaved and limits the process to the surface of the clot [46].

In order to prevent early clot lysis, fibrinolysis is inhibited by a variety of specific inhibitors. $\alpha 2$ -antiplasmin ($\alpha 2$ -AP) reacts with and inactivates plasmin very rapidly, but it also interferes with plasminogen binding to fibrin [47, 48]. Therefore, it is considered to be the primary negative regulator of the fibrinolysis. Other inhibitors include thrombin activatable fibrinolysis inhibitor (TAFI), which prevents plasmin from binding to fibrin and plasminogen activator inhibitor (PAI), which controls the action of t-PA and therefore the amount of plasmin formed.

Figure 4: Schematic overview of the fibrinolytic system.

Fibrinolysis is important to dissolve the thrombus after the tissue damage has been repaired. This system is also very tightly regulated by active enzymes (white circles) and specific inhibitors (black squares). FDP, Fibrin Degradation Products.

1.3 Components of the protein C pathway

Protein C

Protein C (PC) is the key player in the protein C pathway. It is a vitamin-K-dependent protein, synthesized by the liver as a 62 kDa glycoprotein and circulates in plasma as an inactive zymogen at a concentration of approximately 65 nM [49].

Protein C is converted into activated protein C (APC) by thrombin bound to thrombomodulin (TM), an integral membrane protein constitutively expressed by endothelial cells [50,51]. When thrombin binds to TM, its substrate specificity changes and thrombin loses its procoagulant activity [52,53]. The activation of PC by thrombin is further stimulated by the endothelial protein C receptor (EPCR), bringing PC and thrombin in close proximity [54]. Since thrombin has a high affinity for TM, and TM is abundantly expressed in the microcirculation, low levels of thrombin will mainly have an antithrombotic function. However, when the procoagulant stimulus is large enough, high levels of thrombin are generated and the procoagulant activity of thrombin will prevail.

Figure 4: *Protein C is activated by the thrombin-thrombomodulin complex*

Activated protein C (APC) is a serine protease that expresses its anticoagulant activity by proteolysis of the cofactors of the tenase complex and the prothrombinase complex, FVIIIa and FVa respectively. Furthermore, APC can also proteolytically inactivate the native form of both cofactors, FVIII and FV. These reactions are dependent on the presence of phospholipids. As FV and FVIII show strong homology [18], especially concerning the APC-cleavage sites, both inactivation reactions are quite similar, and will be discussed later in more detail. Briefly, both activated cofactors are inactivated by two cleavages in the heavy chain (at Arg306 and Arg506 in FVa, and at Arg336 and Arg562 in FVIIIa), resulting in the dissociation of the A2 domain in both cases [39,40]. Since the A2-domain of FVIIIa contains most of the interaction sites for FIXa and the A2-domain of FVa contains the only PT-binding site and a few

interaction sites for FXa, it is understandable that dissociation of the A2-domains in both molecules results in a complete loss of cofactor activity.

The inactivations of both FVIIIa and FVa by APC are stimulated by protein S, a non-enzymatic cofactor of APC in the protein C pathway [41,42,55]. In addition, FV serves as a cofactor for APC in the inactivating of FVIIIa [56-58].

Protein S

Protein S (PS) is another vitamin K-dependent protein that plays an important role in the protein C pathway. It is mainly synthesized by the liver, but is also expressed in the lungs and by endothelial cells [59-61]. PS circulates in plasma as a 75 kDa glycoprotein at a total concentration of 350 nM. Approximately 60% of all PS is complexed in a 1:1 ratio with C4b binding protein (C4bBP), a protein from the complement system, leaving only about 40% free PS in circulation [62].

Protein S itself has no catalytic domain and functions as a cofactor for APC in the inactivation of FVa and FVIIIa [41,42,55]. PS exhibits this cofactor function by enhancing the binding of APC to phospholipids [63]. It was shown that PS also causes a relocation of the active site of APC [64]. This may explain the PS specific stimulation of one of the APC-cleavage sites in FVa, located closer to the membrane. How PS stimulates the inactivation of FVIIIa by APC is not known yet, but as FV and FVIII show strong homology, this may occur via similar mechanisms.

It has been shown that PS can be cleaved by thrombin and factor Xa in a specific region, called the thrombin sensitive region [65-67]. About 10-20% of all PS in plasma is present in the cleaved form. This cleaved PS is no longer a cofactor for APC [68]. In addition, it has been shown that only free PS and not PS in complex with C4bBP functions as a cofactor for APC [69,70].

Under certain circumstances protein S also exhibits an APC-independent anticoagulant activity [71]. It was shown by Heeb and coworkers that PS binds to FVa and FXa, leading to direct inhibition of thrombin formation by the prothrombinase complex and that PS competes with FXa and prothrombin for binding sites on FVa [72-74]. Later it was shown that PS can also inhibit the intrinsic activation of factor X via a specific interaction with FVIII [75,76]. For PS to express its APC-independent anticoagulant activity it is essential that PS can bind to a phospholipid surface. According to van Wijnen, the inhibition of the prothrombinase and the tenase complex by PS is a direct consequence of the occupation of the phospholipid surface by PS molecules [77].

Coagulation Factor V

General characteristics

Human factor V (FV) is primarily synthesized in the liver but also by megakaryocytes [11,78,79]. The 80-kb FV gene is located on chromosome 1 at q21-25 and consists of 25 exons and 24 introns [80-82]. Following transcription it gives rise to a 6.8-kb mature mRNA and after translation of the mRNA and translocation of the translation product to the endoplasmatic reticulum, a 330 kDa single chain glycoprotein is formed, containing 2196 amino acids. FV circulates in blood at a concentration of approximately 20 nM (~8 µg/ml). About 20-25% of total human FV is found in the α -granules of platelets [11].

Full length FV has a typical domain structure that is identical to that of FVIII [18,83]. They both consist of three homologous A-domains, two smaller homologous C-domains and a highly glycosylated B-domain. The A-domains show homology with those of ceruloplasmin [84], the C-domains are homologous to the phospholipid binding domains present in discoidins and milk fat globule proteins. FV and FVIII share ~40% amino acid homology in their A and C domains [18,81]. However, the B-domains of FV and FVIII show little homology to each other and are also poorly conserved among species [81,85]. Whereas the A and C-domains of FV extend over several exons, the B-domain of FV is encoded by a single unusually large exon, exon 13 [82].

Factor V contains 19 cysteine residues, 14 of which are involved in disulfide bridges forming several loops, while the other 5 are present as free SH-groups [86,87]. Before being secreted, the FV molecule undergoes several posttranslational modifications, including sulfation, phosphorylation and glycosylation [88-90]. One of the consequences of these posttranslational modifications is that there is heterogeneity in the plasma FV molecules [91-93]. This heterogeneity is due to partial glycosylation of the asparagine residue at position 2181 in the C2-domain of factor V, resulting in two structurally and functionally different FV isoforms, FV1 and FV2 [94,95].

Activation of factor V

Factor V circulates in plasma as a single chain protein which by itself has little or no cofactor function in the generation of thrombin by FXa. Therefore FV has to be activated to express full procoagulant activity [96]. This activation is achieved by limited proteolysis by several proteases, the most important ones *in vivo* being thrombin [29,92,97,98] and FXa [25,99]. A FV activator important

because of its use in research and in clinical laboratories is RVV-V [29,98], a serine protease present in the venom from Russell's viper .

Figure 5: Activation of factor V by thrombin

Full length FV is activated by thrombin through cleavage of 3 peptide bonds at Arg⁷⁰⁹, Arg¹⁰¹⁸ and Arg¹⁵⁴⁵. This results in the release of the B-domain and the formation of a heterodimer, consisting of a heavy chain (domains A1, A2 and a small part of the B-domain) and a light chain (domains A3, C1 and C2), non-covalently bound by a calcium ion.

Thrombin activates FV by sequential cleavage at positions Arg⁷⁰⁹, Arg¹⁰¹⁸ and Arg¹⁵⁴⁵ in the B-domain. The cleavages at positions Arg⁷⁰⁹ and Arg¹⁰¹⁸ are kinetically favored over cleavage at position Arg¹⁵⁴⁵, however, these first two cleavages only yield a partially active cofactor (~30%), while the Arg¹⁵⁴⁵ cleavage is essential for full factor Va cofactor activity [100,101]. The first two cleavages do however facilitate the final cleavage at Arg¹⁵⁴⁵ [102]. As a result, the B-domain is released and a heterodimeric FVa molecule is formed, consisting of a heavy chain (residues 1-709, Mr=105,000) and a light chain (residues 1546-2196, Mr=74,000 or 71,000) [29,92,97,98], non-covalently linked by a single calcium ion [103,104]. First reports about activation of FV by FXa indicated initial cleavage at Arg¹⁰¹⁸ followed by a second cleavage at Arg⁷⁰⁹ [25]. Later however, Thorelli and coworkers showed that FXa also cleaves FV at Arg¹⁵⁴⁵ and that there is an additional FXa-specific cleavage site at Arg¹⁷⁶⁵ [26]. A single cleavage at Arg¹⁵⁴⁵ by the activator present in Russell's viper venom is sufficient to elicit complete FVa cofactor activity [29,98]. The activation of FV by

thrombin is, in contrast to activation by FXa, independent of a negatively charged phospholipid surface [25].

The procoagulant activity of factor Va: the prothrombinase complex

Activated FV functions as a non-enzymatic cofactor of FXa in the conversion of prothrombin to thrombin [96,105,106]. This reaction takes place in a macromolecular enzyme complex, called the prothrombinase complex, which consists of the enzyme FXa and its cofactor FVa assembled on a negatively charged phospholipid surface provided *in vivo* by the activated platelets in the presence of Ca^{2+} -ions. These Ca^{2+} -ions are essential for the binding of the vitamin K-dependent proteins to the membrane.

The assembly of the prothrombinase complex is mostly driven by the non-enzymatic components, FVa and phospholipids. Activation of prothrombin by FXa in free solution, in the absence of phospholipids and FVa, is a very slow process. Addition of a negatively charged phospholipids surface alone increases the (apparent) affinity of the enzyme for its substrate several orders of magnitude [105,107,108], resulting in a decrease of the K_m . Addition of the cofactor FVa alone causes a dramatic increase of the turnover number (k_{cat}) of the enzyme, FXa [105,106,109,110].

Activating component	K_m (μM)	V_{max} ($\text{mol}^{-1} \text{s}^{-1}$)	Catalytic efficiency k_{cat} / K_m ($\text{M}^{-1} \text{s}^{-1}$)
FXa	84	0.011	131
FXa, PL	0.06	0.038	$6.3 \cdot 10^5$
FXa, FVa	34	6.22	$1.8 \cdot 10^5$
FXa, FVa, PL	0.21	32	$1.5 \cdot 10^8$

The addition of both phospholipids and FVa combines the stimulatory effect of both components by simultaneously lowering the K_m for prothrombin and increasing the maximum turnover number of the enzyme complex. This results in an overall increase of the catalytic efficiency by at least five orders of magnitude.

Inactivation of factor V and factor Va

Activated FV is effectively inactivated *via* limited proteolysis by the protein C system (see above). Activated protein C (APC) cleaves FVa in the

heavy chain at Arg³⁰⁶, Arg⁵⁰⁶ and Arg⁶⁷⁹ [39,111], leading to the dissociation of the A2-domain [112] and subsequent complete loss of procoagulant activity [113-116]. This reaction is strongly dependent on the presence of negatively charged phospholipids and calcium ions [117]. The inactivation of FVa is a biphasic process, consisting of a rapid phase, in which approximately 50 to 60% of the cofactor activity is lost, followed by a slow phase resulting in complete loss of cofactor activity. The rapid phase corresponds with the cleavage at Arg⁵⁰⁶, while the slow phase is consistent with the Arg³⁰⁶ cleavage [114,116]. The cleavage at these two sites is random, although kinetic data show a 20-fold difference in rate constants, favoring the Arg⁵⁰⁶ cleavage in the presence of phospholipids [114]. The cleavage at Arg⁶⁷⁹ only occurs upon prolonged incubation of FVa with APC and only contributes to the loss of cofactor activity in the absence of phospholipids. Intact FV is cleaved by APC at the same positions as FVa, and has an additional cleavage site at position Lys⁹⁹⁴ in the B-domain [39].

Figure 6: Inactivation of activated factor V by APC

FVa is inactivated by APC by cleavage of Arg³⁰⁶, Arg⁵⁰⁶ and Arg⁶⁷⁹. After initial cleavage at Arg⁵⁰⁶, an intermediate is formed that still has procoagulant activity and only after subsequent cleavage at Arg³⁰⁶, FV loses all cofactor activity. Cleavage at Arg³⁰⁶ immediately results in the complete loss of FVa activity. Cleavage at Arg⁶⁷⁹ is not relevant under physiological conditions.

The inactivation of FVa by APC is greatly stimulated by protein S and inhibited by FXa [41,117-119]. For a long time proteins S was thought to increase the rate of Arg³⁰⁶ cleavage about 20-fold, while no effect on the cleavage at Arg⁵⁰⁶ was observed [119]. However, Norstrom and coworkers have recently shown that protein S does stimulate both cleavages at Arg⁵⁰⁶ and Arg³⁰⁶ in a phospholipid-dependent manner (5-fold and 27-fold respectively) [120]. Differences in experimental conditions may account for part of this discrepancy. Within the prothrombinase complex FXa has been shown to selectively inhibit the APC-catalysed cleavage at Arg⁵⁰⁶, while protein S was able to counteract this effect [118,121], indicating the possibility that FXa and protein S have overlapping binding sites on FVa [121].

The anti-coagulant function of factor V

The idea that FV possesses anticoagulant activity by acting as a cofactor of APC in the inactivation of FVIIIa was first put forward by Dahlbäck and Hildebrand in 1994 [58]. They observed that the addition of normal plasma to APC-resistant plasma, was able to normalize the clotting time in a dose-dependent manner. They concluded that the APC-resistant plasma was missing an important, so far unknown cofactor of APC that was present in normal plasma. They were able to purify this cofactor and found that it was indistinguishable from FV. Further investigations evaluating the ability of factor V to stimulate the inactivation of FVIII and FVIIIa revealed that FV and protein S act as synergistic cofactors to APC while FVa does not [56,57].

The reason why FV loses its anti-coagulant activity after activation by thrombin was investigated using site-directed mutagenesis of the thrombin cleavage-sites in FV. These studies showed that thrombin-mediated cleavage at Arg709 and/or Arg1018 yielded FV molecules that were still able to function as APC cofactors, whereas cleavage at Arg1545 led to complete loss of the APC-cofactor function [122]. Moreover, B-domain deleted FV has no APC-cofactor activity. This and additional experiments led to the conclusion that the anti-coagulant activity of FV probably resides in the C-terminal region of the B-domain of FV, residues 1477-1545 being essential.

It has been proposed that FV has to be cleaved by APC at Arg⁵⁰⁶ to express its anticoagulant function [123]. This hypothesis was confirmed by the observation that FV^{R506Q}, a naturally occurring mutation in one of the APC cleavage sites, had a 10-fold reduced cofactor activity in the APC dependent FVIIIa inactivation as compared to normal FV [56]. Thorelli and coworkers showed, using recombinant FV molecules mutated at different APC cleavage sites, that all mutants in which the APC cleavage site at position Arg⁵⁰⁶ had

been removed, expressed very poor anti-coagulant cofactor activity, whereas the other APC-cleavage site mutants expressed APC cofactor activity comparable to that of normal FV. Therefore they concluded that cleavage at Arg⁵⁰⁶ by APC is essential for the expression of full anti-coagulant activity of FV, whereas Arg to Gln substitutions in the other APC cleavage sites (Arg³⁰⁶ and Arg⁶⁷⁹) did not affect the APC-cofactor function [124].

Platelet factor V

About 20% of the total FV pool resides in platelets [11,125]. The origin of this platelet FV has been a matter of debate for a long time, as megakaryocytes have been shown to express FV *in vitro* [79,126] and FV mRNA has been found in both megakaryocytes and platelets [127]. However, it is now believed that the majority of the platelet FV is derived from endocytosis of plasma FV by bone-marrow megakaryocytes [128-131].

Platelet FV is stored in the α -granules of the platelets [132,133]. It is non-covalently associated with a 155-kD membrane-associated glycoprotein, multimerin [134]. Upon activation of the platelets, FV dissociates from multimerin and is secreted from the α -granules and become exposed on the platelet surface where it takes part in the coagulation cascade as a cofactor of FXa in the prothrombinase complex. It is thought that platelet FV plays a crucial role in the initiation of thrombin formation at the site of injury. A platelet FV deficiency, known as FV Quebec [135], is associated with a severe bleeding disorder due to the absence of functional platelet FV (even in the presence of normal plasma antigen levels). The absence of platelet FV activity is associated with a quantitative deficiency of multimerin [136].

Although platelet FV derives from plasma by endocytosis, there are differences between both FV pools. It has been shown that platelet FV is composed of fragments with apparent Mr ranging between 115 kD and 330 kD, which are different from the intermediates and end products obtained during activation of plasma FV by thrombin [137]. These partially proteolysed molecules already possess significant cofactor activity that can only be increased 2 to 3-fold by treatment with factor Xa or thrombin [138, 139]. However, the end products of thrombin activated platelet FV and plasma FV were indistinguishable. Another difference between both FV pools is that activated protein C is unable to completely inactivate platelet-derived FVa, as it seems that platelets offer protection to FVa by delaying the cleavage at Arg⁵⁰⁶ [139-141].

Coagulation Factor VIII

Activated factor VIII is an important cofactor for FIXa in the activation of FX by the tenase complex [142]. The FX-generating capacity of this tenase complex is tightly regulated for instance by the protein C pathway, as FVIIIa is inactivated by APC and its cofactors, protein S and FV. Therefore factor FVIII will be briefly discussed in this chapter.

Factor VIII (FVIII) is primarily synthesized by the liver, but also by a variety of other organs, such as kidney and spleen [143]. Although it is synthesized as a large single chain protein with the same domain structure as FV (A1-A2-B-A3-C1-C2) [144], FVIII circulates in plasma as a variety of heterodimers, non-covalently bound by a metal ion [145]. This heterogeneity is due to proteolysis in the B-A3-junction and additional cleavages at several sites in the B-domain, resulting in a heavy chain with a B-domain with variable size. The plasma concentration of FVIII is about 20 times lower than that of FV, approximately 1 nM. In contrast to FV, FVIII circulates in plasma in a tight, non-covalent complex with von Willebrand Factor (vWF) [146], a large multimeric protein functioning as a carrier protein. Binding of vWF to FVIII stabilizes the FVIII molecule and prevents premature association of FVIII with thrombogenic surfaces and also binding of FVIII to FIXa.

Factor VIII can be proteolytically activated by thrombin and FIXa, although thrombin is physiologically the most important activator (see review) [147]. Thrombin cleaves FVIII at three sites in both heavy and light chain: Arg372, Arg740 and Arg1689. The heavy chain is initially cleaved at the A2-B junction (Arg740) resulting in the release of the B-domain, followed by the cleavage at the A1-A2 junction (Arg372) separating both domains in the heavy chain. This latter cleavage is required to achieve FVIIIa activity. The main purpose of the Arg1689 cleavage is to dissociate the cofactor from its carrier protein vWF. So, FVIIIa is a heterotrimer consisting of A1, A2 and A3-C1-C2 subunit [148]. The interaction between the A1 and A3-C1-C2 subunits is dependent on a metal ion, while the association of the A2 subunit with the dimer (A1/A3-C1-C2) is mediated by electrostatic interactions.

This free FVIIIa is unstable and undergoes spontaneous decay, due to the spontaneous dissociation of the A2-subunit [148]. However, when FVIIIa is incorporated in the tenase complex, the rate of spontaneous decay is reduced by about 10-fold [149]. Apart from this spontaneous loss of FVIIIa activity, APC also inactivates FVIIIa by cleavage at Arg336 and Arg562 [40]. Initial cleavage occurs at Arg562, resulting in the abolishment of a critical FIXa binding site. This cleavage site for APC is protected by FIXa, but this protective effect is counteracted by protein S [150]. The second cleavage at Arg336 leads to the dissociation of the A2-domain. A third cleavage site has been proposed at

Arg740, resulting in the release of the B-domain fragments, but this cleavage does probably not contribute to inactivation. Of course this third APC cleavage site is only relevant when FVIII is proteolysed by APC, as FVIIIa has already been cleaved at Arg740 during activation by thrombin.

1.4 APC-resistance

First observation

The term APC-resistance was first introduced by Dahlbäck and coworkers in 1993 [151]. They described one patient with venous thrombosis, who had a poor anticoagulant response when exogenous APC was added to his/her plasma (measured as a poorly prolonged clotting time after the addition of APC). It was hypothesized that in this patient a cofactor of the APC anticoagulant pathways was missing. One year later, Dahlback and Hildebrand demonstrated that the APC-resistant phenotype in these patients could be corrected for by the addition of purified FV to the APC-resistant plasma [58]. Therefore they concluded that FV was the missing cofactor for APC.

Several studies have shown that APC-resistance is an important risk factor for thrombosis, with a prevalence of 20-30% in unselected thrombotic patients and even up to 50% in patients with a family history of thrombosis. In fact, it is the most common inherited risk factor for thrombosis identified so far [152,153].

The factor V_{Leiden} mutation

One year after the original publication of Dahlback *et al*, the molecular mechanism underlying APC-resistance was elucidated [154-158]. A single point mutation in the FV gene (a G to A transition at nucleotide 1691) leads to an amino acid substitution at position 506, where an arginine is replaced by a glutamine. This amino acid substitution results in the loss of one of the important cleavage sites for APC in FV [39]. Indeed, extensive analysis of inactivation patterns and kinetic data from the inactivations of normal FVa and FVa_{Leiden} by APC revealed that an initial cleavage at position Arg⁵⁰⁶ is essential for rapid inactivation of activated FV and this cleavage is not occurring in FVa_{Leiden} [114,119,124,159,160]. Thus, the general idea at that time was that the most important reason why the FV_{Leiden} mutation caused APC-resistance

was because it affected an important APC cleavage site, thereby rendering FV_{Leiden} less susceptible to the inactivation by APC. This appeared to be a satisfactory explanation, and therefore "the missing cofactor theory" from Dahlback was somewhat forgotten. However, some groups did not give up on the APC-cofactor theory of FV. Already in 1994, Shen *et al.* showed that in a purified system FV stimulated the inactivation of FVIIIa in a dose-dependent manner when protein S was present. They therefore concluded that FV and protein S act as synergistic cofactors in the APC-mediated inactivation of FVIIIa [57]. This was later confirmed by Varadi *et al.* who also demonstrated that FV_{Leiden} was a poor cofactor [56]. Furthermore, Thorelli and coworkers demonstrated that the anticoagulant function of FV is dependent on APC-mediated cleavage of intact FV at Arg⁵⁰⁶ [124]. Together this provides a good explanation for the decreased sensitivity towards APC of a plasma containing FV_{Leiden}, because 1) the inactivation of FV_{Leiden} by APC is impaired and 2) FV_{Leiden} does not function as a cofactor of APC in the inactivation of FVIIIa.

The FV_{Leiden} mutation is found exclusively in the Caucasian population with a frequency of approximately 5% [154,161] and about 90-95% of all cases of APC-resistance are attributable to this mutation [162]. Several epidemiological studies have estimated that the risk for thrombosis, associated with the FV_{Leiden} mutation, is increased 7-fold in heterozygous carriers of the mutation and as much as 80-fold in homozygous carriers as compared to normal individuals [162-165].

Other mutations in factor V causing APC resistance

Besides the FV_{Leiden} mutation, which affects the Arg⁵⁰⁶ cleavage site of APC, two other mutations have been described affecting the APC cleavage site at Arg³⁰⁶. A mutation at nucleotide 1091, causing a G to C transition results in the substitution of Arg³⁰⁶ by a Thr, and was first described in a British patient suffering from thrombosis [166]. This patient and his mother, who also carried the mutation, were found to be APC-resistant. However, the Arg to Thr substitution at position 306, now known as FV Cambridge, was not found in a group of 585 patients with thrombotic complications, nor was it found in a group of 226 healthy individuals [166]. Unfortunately due to the rare occurrence of this mutation, good epidemiological studies are not available.

The second mutation at Arg³⁰⁶ is caused by an A to G transition at position 1090, resulting in an Arg to Gly substitution. This mutation, now known as FV Hong Kong, was first described in Chinese patients from Hong Kong, and

was shown to be quite frequent among the Hong Kong Chinese population (4.5% prevalence) [167,168]. In contrast to FV Cambridge, FV Hong Kong has not been associated with APC-resistance yet. However, Norstrom *et al.* showed that the recombinant FV Cambridge and FV Hong Kong molecules behaved identically in *in vitro* assays, both causing mild APC-resistance due to a moderately impaired APC cofactor activity [169].

Apart from the mutations affecting the APC-cleavage sites described so far, another mutation in the heavy chain of FV has been found to be associated with APC-resistance. The T to C transition at nucleotide 1250 results in the substitution of Ile³⁵⁹ by a Thr (FV Liverpool), thereby introducing a new N-linked glycosylation consensus sequence at Asn³⁵⁷ [170]. Steen *et al.* were able to show that an additional carbohydrate at this position interferes with both APC-mediated inactivation of FV Liverpool and with its APC cofactor activity [171].

The factor V R2-polymorphism

The R2-polymorphism is a common genetic variation within the FV gene, and has been associated with mild APC-resistance and possibly with an increased risk for venous thrombosis. It was first described in 1996 by Lunghi *et al.* as an A to G transition at position 4070, causing a His to Arg substitution at position 1299 in the B-domain of FV [172]. Further analysis revealed that this substitution is tightly linked to a number of other polymorphisms throughout the FV gene, which are collectively known as the R2-haplotype [173,174]. Based on the geographical distribution of the R2-haplotype and its prevalence in both the Caucasian and non-Caucasian population, Bernardi *et al.* proposed that R2-haplotype originated from ancestral mutational events dating further back than the FV Leiden mutation [173]. The overall prevalence of the R2-haplotype is estimated to be approximately 10% in the European population, but can differ greatly between populations [173,175-177].

At the phenotypic level the R2-haplotype has been associated with slightly reduced FV levels in plasma, a shift in FV1/FV2-ratio towards the more thrombogenic FV1-isoform (42/58 vs. 30/70) and mild resistance towards APC [174,178-180]. Although it has been shown that reduced FV levels and shifted isoform ratio do account for part of the APC-resistance observed in carriers of the R2-haplotype, the mechanism by which this FV variant causes APC-resistance has still not been completely elucidated [181]. The most likely explanation is that FV-R2 has a reduced APC-cofactor activity in the FVIII(a) inactivation.

The thrombotic risk associated with the R2-haplotype is still a matter of debate. Several epidemiological studies have been performed, but as the results of these studies were contradictory, there is inconclusive evidence whether or not the R2-haplotype on its own is associated with a relevant clinical risk for venous thrombosis [182]. However, it is generally accepted that compound heterozygous carriership of the FV Leiden and the FV-R2 is indeed associated with a higher risk for venous thrombosis than heterozygous FV Leiden alone [180,183,184].

Prothrombin, the G20210A mutation and APC-resistance

In 1996 Poort and coworkers identified a mutation in the 3' untranslated region of the prothrombin gene that resulted in an increased prothrombin level in plasma [185]. This mutation, a G to A transition at position 20210, was also associated with a 3-fold increased risk for venous thrombosis when present in the heterozygous state. The association of the G20210A variant with elevated plasma prothrombin levels was confirmed by several independent studies as well as its importance as a risk factor for venous thrombosis [186-189]. In arterial thrombotic diseases however, it seems that the prothrombin mutation does not play an important role, though it has been suggested that it could be a genetic risk factor for the development of myocardial infarction [190-193]. The overall prevalence of the prothrombin mutation in Europe was estimated to be approximately 2%, although this number seemed to differ between northern and southern Europe (1.7% versus 3.0% respectively) [194]. Apparently the prothrombin variant appeared to be very rare in individuals from African and Asian origin. The first indications that elevated plasma prothrombin levels are associated with APC-resistance came from Tripodi et al., who showed that there was an inverse relationship between the prothrombin levels in plasma and the normalized APC sensitivity ratio, measured with the aPTT-based APC-resistance assay [195]. This was confirmed by Castaman and coworkers, who demonstrated that the addition of purified prothrombin to the plasmas of 90 healthy individuals resulted in a dose dependent increase in the APC-resistance of these plasmas [196]. Nowadays it is generally accepted that elevated prothrombin levels in plasma cause APC-resistance and represent a risk factor for venous thrombosis.

The exact mechanism by which increased prothrombin levels induce APC-resistance is still not completely understood. It has been proposed that the prothrombin concentration in plasma influences the anticoagulant protein C system via an immediate and dose-dependent inhibition of the APC-cofactor,

protein S [197]. Later, Smirnov *et al.* confirmed that prothrombin levels indeed had an influence on the anticoagulant function of APC, though their data showed that this was caused by the protective role of prothrombin towards the inactivation of factor Va by APC and that this process was independent of protein S [198]. They also showed that the inactivation of FVa Leiden by APC was much less sensitive to prothrombin inhibition and postulated that the discrepancy between his findings and the observations by Mitchell might be due to the use of plasma from a FV Leiden carrier, an unknown condition at that time. Finally, Norström and coworkers used recombinant factor V molecules mutated at the APC-cleavage sites unravel the mechanism of APC-resistance caused by increased prothrombin levels. From their data they concluded that prothrombin directly impaired the activity of APC and also decreased the cofactor function of protein S [199].

Scope of this thesis

APC resistance is an important risk factor for venous thrombosis. The general objective of my thesis was to get a better insight into the mechanisms leading to APC-resistance in order to elucidate how some naturally occurring mutations in coagulation factors cause APC-resistance and thereby contribute to venous thrombosis.

The FV R2-haplotype is a common genetic variation associated with a mild APC-resistance and with reduced FV levels in plasma. Yamazaki *et al.* showed that the aspartate to glycine substitution at position 2194 in the C2-domain of FV, which is part of the FV R2-haplotype, was responsible for the impaired expression of the FV R2 allele. In chapter 2 we investigated how the D2194G mutation causes a reduction in FV levels in plasma using computational methods, which were confirmed with experimental data.

Chapter 3 focuses on the APC resistance associated with impaired APC cofactor activity of FV in carriers of the FV Leiden mutation and the R2-haplotype. The data presented in this chapter indicate that the APC cofactor activity of FV in the inactivation of FVIIIa plays an important role in the regulation of thrombin formation and that impairment of this function of FV accounts for a large fraction of the APC resistance observed in carriers of the FV Leiden mutation and of the R2-haplotype.

In chapter 4 we describe and characterize the APC-resistance associated with FV Leiden pseudo-homozygosity. To this end, we compared plasmas from 7 pseudo-homozygous FV Leiden carriers with plasmas of FV

Leiden heterozygous and FV Leiden homozygous individuals. These FV Leiden genotypes were also mimicked in a model system. We conclude that the difference in APC-resistance between a FV Leiden heterozygous individual and a pseudo-homozygous FV Leiden individual is due to the APC-cofactor activity of the normal FV that is present in the plasma of heterozygotes but which is absent in the pseudo-homozygotes.

Finally, in chapter 5 we extended our knowledge about the APC-resistance associated with increased prothrombin levels in plasma. We found that the amount of protein S present in plasma is an important modulator of the degree of APC-resistance caused by elevated prothrombin levels.

References

1. E. J. Weiss, V. T. Turitto and H. R. Baumgartner. Effect of shear rate on platelet interaction with subendothelium in citrated and native blood. *J Lab Clin Med.* 1978; 92:750-764.
2. M. J. Barnes, C. G. Knight and R. W. Farndale. The collagen - platelet interaction. *Current Opinion in Hematology.* 1998; 5:314-320.
3. S. Beumer, H. F. Heijnen, M. J. IJsseldijk, E. Orlando, P. H. de Groot and J. J. Sixma. Platelet adhesion to fibronectin in flow: the importance of von Willebrand factor and glycoprotein Ib. *Blood.* 1995; 86:3452-3460.
4. J. E. Sadler. Contact - How platelets touch von Willebrand factor. *Science.* 2002; 297:1128-1129.
5. B. Savage, E. Saldívar and Z. M. Ruggeri. Initiation of platelet adhesion by arrest onto fibrinogen or translocation on von Willebrand factor. *Cell.* 1996; 84:289-297.
6. B. Savage, F. Almus-Jacobs and Z. M. Ruggeri. Specific synergy of multiple substrate-receptor interactions in platelet thrombus under flow. *Cell.* 1998; 94:657-666.
7. E. M. Bevers, P. Comfurius, J. L. M. Van Rijn, H. C. Hemker and R. F. A. Zwaal. Generation of thrombin-converting activity and the exposure of phosphatidylserine at the outer surface of platelets. *Eur J Biochem.* 1982; 122:429-436.
8. E. M. Bevers, P. Comfurius and R. F. A. Zwaal. Changes in membrane phospholipid distribution during platelet activation. *Biochim Biophys Acta.* 1983; 736:57-66.
9. J. W. N. Akkerman, G. Gorter and E. Klopogge. Kinetic analysis of alpha-granule secretion by platelets: A methodological report. *Thromb Res.* 1982; 27:59 -64.
10. H. Holmsen, K. L. Kaplan and C. A. Dangelmaier. Differential requirements for platelet responses: A simultaneous study of dense granule, alpha-granule and acid hydrolase secretion, arachidonate liberation, phosphatidylinositol turnover and phosphatidate formation. *Biochem J.* 1982; 208:9-18.
11. P. B. Tracy, L. L. Eide, E. J. Bowie and K. G. Mann. Radioimmunoassay of factor V in human plasma and platelets. *Blood.* 1982; 60(1):59-63.
12. C. Gachet. Platelet activation by ADP: the role of ADP antagonists. *Ann. Med.* 2000; 32:15-20.
13. G. R. Sambrano, E. J. Weiss, Y. W. Zheng, W. Huang and S. R. Coughlin. Role of thrombin signalling in platelets in haemostasis and thrombosis. *Nature.* 2001; 413:74-78.
14. B. Savage, M. Cattaneo and Z. M. Ruggeri. Mechanism of platelet aggregation. *Current Opinion in Hematology.* 2001; 8:270-276.
15. R. G. MacFarland. An enzyme cascade in the blood clotting mechanism, and its function as a biochemical amplifier. *Nature.* 1964; 202:498-499.
16. E. W. Davie and O. D. Ratnoff. Waterfall sequence for intrinsic blood clotting. *Science.* 1964; 145:1310-2.
17. B. Furie and B. C. Furie. Molecular basis of blood coagulation. *Cell.* 1988; 53:505-518.
18. W. H. Kane and E. W. Davie. Blood coagulation factors V and VIII: Structural and functional similarities and their relationship to hemorrhagic and thrombotic disorders. *Blood.* 1988; 71:
19. E. W. Davie, K. Fujikawa and W. Kisiel. The coagulation cascade: Initiation, maintenance, and regulation. *Biochemistry.* 1991; 30:10363-10370.
20. B. Dahlback. Blood coagulation. *Lancet.* 2000; 355:1627-1632.

21. D. S. Fair and M. J. MacDonald. Cooperative interaction between factor VII and cell surface-expressed tissue factor. *J Biol Chem.* 1987; 262:11692-11698.
22. R. Bach, R. Gentry and Y. Nemerson. Factor FVII binding to tissue factor in reconstituted phospholipid vesicles: induction of cooperativity by phosphatidylserine. *Biochemistry.* 1986; 25:4007-4020.
23. Y. Nemerson. Tissue factor: then and now. *Thromb Haemostasis.* 1995; 74:180-184.
24. V. J. Bom, V. W. van Hinsbergh, H. H. Reinalda-Poot, R. W. Mohanlal and R. M. Bertina. Extrinsic activation of human coagulation factors IX and X on the endothelial surface. *Thromb Haemost.* 1991; 66:283-291.
25. D. Monkovic and P. B. Tracy. Activation of human factor V by factor Xa and thrombin. *Biochemistry.* 1990; 29:1118-1128.
26. E. Thorelli, R. J. Kaufmann and B. Dahlback. Cleavage requirements for activation of factor V by factor Xa. *Eur J Biochem.* 1997; 273:12-20.
27. S. Krishnaswamy, W. R. Church, M. E. Nesheim and K. G. Mann. Activation of human prothrombin by human prothrombinase: influence of factor Va on the reaction mechanism. *J Biol Chem.* 1987; 262:3291-3299.
28. M. E. Nesheim and K. G. Mann. The kinetics and cofactor dependence of the two cleavages involved in prothrombin activation. *J Biol Chem.* 1983; 258:5386-5391.
29. K. Suzuki, B. Dahlback and J. Stenflo. Thrombin-catalyzed activation of human coagulation factor V. *J Biol Chem.* 1982; 257:6556-6564.
30. K. Naito and K. Fujikawa. Activation of human blood coagulation factor XI independent of FXII. Factor FXI is activated by thrombin and factor XIa in the presents of negatively charged surfaces. *J Biol Chem.* 1991; 266:7353-7558.
31. G. S. Dufner and G. A. Marbet. Factor XIII in man: a review. *Hamostaseologie.* 2002; 22:11-19.
32. R. J. Baugh, G. J. Broze and S. Krishnaswamy. Regulation of extrinsic pathway factor Xa formation by tissue factor pathway inhibitor. *J Biol Chem.* 1998; 273:4378-4386.
33. D. J. Jordan, S. Pemberton, M. Aquila, P. G. Mori, E. G. Tuddenham and D. P. O'Brien. The kinetics of hemostatic enzyme-antithrombin interactions in the presence of low molecular weight heparin. *J Biol Chem.* 1980; 255:10081-10090.
34. J. A. Marcum and R. D. Rosenberg. Anticoagulant active heparin-like molecules from vascular tissue. *Biochemistry.* 1984; 23:1730-1737.
35. F. C. Church, R. E. Treanor, G. B. Sherrill and H. C. Whinna. Carboxylate polyanions accelerate inhibition by heparin cofactor II. *Biochem Biophys Res Commun.* 1987; 148:362-368.
36. R. J. Barret and P. M. Starkey. The interaction of α 2-macroglobulin with proteases. *Biochem J.* 1973; 133:709-715.
37. L. Sottrup-Jensen. Alpha-macroglobulins: structure, shape, and mechanism of proteases complex formation. *J Biol Chem.* 1989; 264:11539-11542.
38. T. Lindhout, J. Franssen and G. M. Willems. Kinetics of the inhibition of tissue-factor/factor VIIa by the tissue factor pathway inhibitor. *Thromb Haemost.* 1995; 74:910-915.
39. M. Kalafatis, M. D. Rand and K. C. Mann. The mechanism of inactivation of human factor V and human factor Va by activated protein C. *J Biol Chem.* 1994; 269:31869-31880.
40. P. J. Fay, T. M. Smudzin and F. J. Walker. 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.
41. F. Walker. Regulation of activated protein C by a new protein. A possible function for bovine protein S. *J Biol Chem.* 1980; 255:5521-5524.

42. F. J. Walker, S. I. Chavin and P. J. Fay. Inactivation of factor VIII by activated protein C and protein S. *Arch Biochem Biophys.* 1987; 252:322-8.
43. R. A. Marlar and A. Neumann. Neonatal purpura fulminans due to homozygous protein C or protein S deficiencies. *Semin Thrombo Hemast.* 1990; 16:299-309.
44. B. Dahlback. The protein C anticoagulant system: inherited defects as basis for venous thrombosis. *Thromb Res.* 1995; 77:1-43.
45. B. Wiman and D. Collen. Molecular mechanism of physiological fibrinolysis. *Nature.* 1978; 272:549-50.
46. N. Aiko. Fibrinolysis: Its initiation and regulation. *J Protein Chem.* 1986; 5:269-275.
47. D. Collen. a2-antiplasmin inhibitor deficiency. *Lancet.* 1979; 313:1039-40.
48. B. Wiman and D. Collen. On the kinetics of the reaction between human antiplasmin and plasmin. *Eur J Biochem.* 1978; 84:573-8.
49. W. Kisiel. Human plasma protein C: isolation, characterization, and mechanism of activation by alpha-thrombin. *J Clin Invest.* 1979; 64:761-9.
50. C. T. Esmon and W. G. Owen. Identification of an endothelial cell cofactor for thrombin catalysed activation of protein C. *Proc Natl Acad Sci USA.* 1981; 78:2249-2252.
51. W. G. Owen and C. T. Esmon. Functional properties of an endothelial cell cofactor for thrombin-catalysed activation of protein C. *J Biol Chem.* 1981; 256:5532-5535.
52. J. H. Griffin. Blood coagulation - the thrombin paradox. *Nature.* 1995; 378:337-338.
53. C. T. Esmon, N. L. Esmon and K. W. Harris. Complex formation between thrombin and thrombomodulin inhibits both thrombin-catalysed fibrin formation and factor V activation. *J Biol Chem.* 1982; 257:7944-7947.
54. D. J. Stearns-Kurosawa, S. M. Kurosawa, J.S., G. L. Ferrell and C. T. Esmon. The endothelial protein C receptor augments protein C activation by the thrombin-thrombomodulin complex. *Proc Natl Acad Sci USA.* 1996; 93:10212-10216.
55. J. A. Koedam, J. C. Meijers, J. J. Sixma and B. N. Bouma. Inactivation of human factor VIII by activated protein C. Cofactor activity of protein S and protective effect of von Willebrand factor. *J Clin Invest.* 1988; 82:1236-43.
56. K. Varadi, J. Rosing, G. Tans, I. Pabinger, B. Keil and H. P. Schwarz. Factor V enhances the cofactor function of protein S in the APC-mediated inactivation of factor VIII: influence of the Factor VR506Q mutation. *Thromb Haemost.* 1996; 76(2):208-214.
57. L. Shen and B. Dahlbäck. Factor V and protein S as synergistic cofactors to activated protein C in degradation of factor VIIIa. *J Biol Chem.* 1994; 269:18735-18738.
58. B. Dahlbäck and B. Hildebrand. Inherited resistance to activated protein C is corrected by anticoagulant cofactor activity found to be a property of factor V. *Proc Natl Acad Sci USA.* 1994; 91:1396-1400.
59. D. S. Fair, R. A. Marlar and E. G. Levin. Human endothelial cells synthesize protein S. *Blood.* 1986; 67:1168-71.
60. X. He, L. Shen, A. Bjartell and B. Dahlback. The gene encoding vitamin K-dependent anticoagulant protein S is expressed in multiple rabbit organs as demonstrated by northern blotting, in situ hybridization, and immunohistochemistry. *J Histochem Cytochem.* 1995; 43:85-96.
61. J. Hoskins, D. K. Norman, R. J. Beckmann and G. L. Long. Cloning and characterization of human liver cDNA encoding a protein S precursor. *Proc Natl Acad Sci U S A.* 1987; 84:349-53.
62. J. H. Griffin, A. Gruber and J. A. Fernandez. 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.

63. B. Dahlback, B. Frohm and G. Nelsestuen. High affinity interaction between C4b-binding protein and vitamin K-dependent protein S in the presence of calcium. Suggestion of a third component in blood regulating the interaction. *J Biol Chem.* 1990; 265:16082-7.
64. S. Yegneswaran, G. M. Wood, C. T. Esmon and A. E. Johnson. Protein S alters the active site location of activated protein C above the membrane surface. A fluorescence resonance energy transfer study of topography. *J Biol Chem.* 1997; 272:25013-21.
65. G. L. Long, D. Lu, R. L. Xie and M. Kalafatis. Human protein S cleavage and inactivation by coagulation factor Xa. *J Biol Chem.* 1998; 273:11521-6.
66. G. T. Chang, L. Aaldering, T. M. Hackeng, P. H. Reitsma, R. M. Bertina and B. N. Bouma. Construction and characterization of thrombin-resistant variants of recombinant human protein S. *Thromb Haemost.* 1994; 72:693-7.
67. F. Meijer-Huizinga, K. Mertens and J. A. van Mourik. Isolation and characterization of single-chain protein S. *Thromb Haemost.* 1994; 72:408-14.
68. K. Suzuki, J. Nishioka and S. Hashimoto. Regulation of activated protein C by thrombin-modified protein S. *J Biochem (Tokyo).* 1983; 94:699-705.
69. B. Dahlback. Inhibition of protein C cofactor function of human and bovine protein S by C4b-binding protein. *J Biol Chem.* 1986; 261:12022-7.
70. M. J. Heeb and J. H. Griffin. Activated protein C-dependent and -independent anticoagulant activities of protein S have different structural requirements. *Blood Cells Mol Dis.* 2002; 29:190-199.
71. K. M. Sere, J. Rosing and T. M. Hackeng. Inhibition of thrombin generation by protein S at low procoagulant stimuli: implications for maintenance of the hemostatic balance. *Blood.* 2004; 104:3624-30.
72. T. M. Hackeng, C. van 't Veer, J. C. Meijers and B. N. Bouma. 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.
73. M. J. Heeb, R. M. Mesters, G. Tans, J. Rosing and J. H. Griffin. Binding of protein S to factor Va associated with inhibition of prothrombinase that is independent of activated protein C. *J Biol Chem.* 1993; 268:2872-7.
74. M. J. Heeb, J. Rosing, H. M. Bakker, J. A. Fernandez, G. Tans and J. H. Griffin. Protein S binds to and inhibits factor Xa. *Proc Natl Acad Sci U S A.* 1994; 91:2728-32.
75. S. J. Koppelman, T. M. Hackeng, J. J. Sixma and B. N. Bouma. 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.
76. S. J. Koppelman, C. van't Veer, J. J. Sixma and B. N. Bouma. Synergistic inhibition of the intrinsic factor X activation by protein S and C4b-binding protein. *Blood.* 1995; 86:2653-60.
77. M. van Wijnen, J. G. Stam, C. van't Veer, J. C. Meijers, P. H. Reitsma, R. M. Bertina and B. N. Bouma. The interaction of protein S with the phospholipid surface is essential for the activated protein C-independent activity of protein S. *Thromb Haemost.* 1996; 76:397-403.
78. D. B. Wilson, H. H. Salem, J. S. Mruk, I. Maruyama and P. W. Majerus. Biosynthesis of coagulation factor V by a human hepatocellular carcinoma cell line. *J Clin Invest.* 1984; 73:654-658.
79. H. C. Chiu, P. K. Schick and R. W. Colman. Biosynthesis of factor V in isolated guinea pig megakaryocytes. *J Clin Invest.* 1985; 75:339-346.
80. W. H. Kane, A. Ichinose, F. S. Hagen and E. W. Davie. Cloning of cDNAs coding for the heavy chain region and connecting region of human factor V, a blood coagulation factor with four types of internal repeats. *Biochemistry.* 1987; 26:6508-14.
81. R. J. Jenny, D. D. Pittman and J. J. Toole. Complete cDNA and derived amino acid sequence of human factor V. *Proc Natl Acad Sci USA.* 1987; 84:4846-50.

82. L. D. Cripe, K. D. Moore and W. H. Kane. Structure of the gene for human coagulation factor V. *Biochemistry*. 1992; 31:3777-85.
83. J. Gitschier, W. I. Wood, T. M. Goralka, K. L. Wion, E. Y. Chen, D. H. Eaton, G. A. Vehar, D. J. Capon and R. M. Lawn. Characterization of the human factor VIII gene. *Nature*. 1984; 312:326-30.
84. W. R. Church, R. L. Jernigan, J. Toole, R. M. Hewick, J. Knopf, G. J. Knutson, M. E. Nesheim, K. G. Mann and D. N. Fass. Coagulation factors V and VIII and ceruloplasmin constitute a family of structurally related proteins. *Proc Natl Acad Sci USA*. 1984; 81:6934-7.
85. E. R. Guinto, C. T. Esmon, K. G. Mann and R. T. MacGillivray. The complete cDNA sequence of bovine coagulation factor V. *J Biol Chem*. 1992; 267:2971-8.
86. J. Xue, M. Kalafatis and K. C. Mann. Determination of the disulfide bridges in factor Va light chain. *Biochemistry*. 1993; 32:5917-5923.
87. J. Xue, M. Kalafatis, J. R. Silveira, C. Kung and K. G. Mann. Determination of the disulfide bridges in factor Va heavy chain. *Biochemistry*. 1994; 33:13109-16.
88. G. L. Hortin. Sulfation of tyrosine residues in coagulation factor V. *Blood*. 1990; 76:946-952.
89. D. D. Pittman, K. N. Tomkinson and R. J. Kaufman. Post-translational requirements for functional factor V and factor VIII secretion in mammalian cells. *J Biol Chem*. 1994; 269:17329-17337.
90. H. P. M. Kumar, M. J. Besman, R. L. Lundblad, N. S. Jenny and K. G. Mann. Carbohydrate analysis of plasma factor V and factor VIII. *Tromb Haemost [abstract]*. 1999; suppl 82:
91. M. E. Nesheim, W. B. Foster and R. M. Hewick. Characterization of factor V activation intermediates. *J Biol Chem*. 1984; 259:3187-3196.
92. J. Rosing, H. M. Bakker, M. C. L. G. D. Thomassen, H. C. Hemker and G. Tans. Characterization of two forms of human factor Va with different cofactor activities. *J Biol Chem*. 1993; 268:21130-21136.
93. L. Hoekema, G. A. F. Nicolaes, H. C. Hemker, G. Tans and J. Rosing. Human factor Va1 and factor Va2: properties in the procoagulant and anticoagulant pathways. *Biochemistry*. 1997; 36:3331-5.
94. G. A. F. Nicolaes, B. O. Villoutreix and B. Dahlback. Partial glycosylation of Asn2181 in human factor V as a cause of molecular and functional heterogeneity. Modulation of glycosylation efficiency by mutagenesis of the consensus sequence for N-linked glycosylation. *Biochemistry*. 1999; 38:13584-91.
95. S. W. Kim, T. L. Ortel, M. A. Quinn-Allen, L. Yoo, L. Worfolk, X. Zhai, B. R. Lentz and W. H. Kane. Partial glycosylation at asparagine-2181 of the second C-type domain of human factor V modulates assembly of the prothrombinase complex. *Biochemistry*. 1999; 38:11448-54.
96. M. E. Nesheim, J. B. Taswell and K. C. Mann. The contribution of bovine factor V and factor Va to the activity of the prothrombinase. *J Biol Chem*. 1979; 254:10952-10962.
97. C. T. Esmon. The subunit structure of thrombin-activated factor V. Isolation of activated factor V, separation of subunits and reconstitution of biological activity. *J Biol Chem*. 1979; 254:964-973.
98. W. H. Kane and P. W. Majerus. Purification and characterization of human coagulation factor V. *J Biol Chem*. 1981; 256:1002-1007.
99. W. B. Foster, M. E. Nesheim and K. C. Mann. The factor Xa-catalyzed activation of factor V. *J Biol Chem*. 1983; 258:13970-13977.
100. R. Toso and R. M. Camire. Removal of B-domain sequences from factor V rather than specific proteolysis underlie the mechanism by which cofactor function is realized. *J Biol Chem*. 2004; 279:21643-21650.
101. M. Kalafatis, D. O. Beck and K. C. Mann. Structural requirements for expression of factor Va activity. *J Biol Chem*. 2003; 278:33550-33561.

102. K. A. Marquette, D. D. Pittman and R. J. Kaufman. The factor V B-domain provides two functions to facilitate thrombin cleavage and release of the light chain. *Blood*. 1995; 86:3026-3034.
103. E. R. Guinto and C. T. Esmon. Formation of a calcium-binding site on activated bovine factor V following recombination of the isolated subunits. *J Biol Chem*. 1982; 257:
104. K. W. Sorensen, G. A. F. Nicolaes, B. O. Villoutreix, T. Yamazaki, G. Tans, J. Rosing and B. Dahlback. Functional properties of recombinant factor V mutated in a potential calcium-binding site. *Biochemistry*. 2004; 43:5803-5810.
105. J. Rosing, G. Tans, J. W. P. Govers Riemslag, R. F. A. Zwaal and H. C. Hemker. The role of phospholipids and factor Va in the prothrombinase complex. *J Biol Chem*. 1980; 255:274-283.
106. S. Krishnaswamy. Prothrombinase complex assembly contribution of protein-protein and protein-membrane interactions toward complex formation. *J Biol Chem*. 1990; 265:3708-3718.
107. J. L. M. L. Rijn van, J. W. P. Govers Riemslag, R. F. A. Zwaal and J. Rosing. Kinetic studies of prothrombin activation: Effect of factor Va and phospholipids on the formation of the enzyme-substrate complex. *Biochemistry*. 1984; 23:4557-4563.
108. S. Krishnaswamy, K. C. Jones and K. G. Mann. Prothrombinase complex assembly Kinetic mechanism of enzyme assembly on phospholipid vesicles. *J Biol Chem*. 1988; 263:3823-3834.
109. M. E. Nesheim, C. Kettner, E. Shaw and K. G. Mann. Cofactor dependence of factor Xa incorporation into the prothrombinase complex. *J Biol Chem*. 1981; 256:6537-6540.
110. M. E. Nesheim, S. Eid and K. G. Mann. Assembly of the prothrombinase complex in the absence of prothrombin. *J Biol Chem*. 1981; 256:9874-9882.
111. F. J. Walker, P. W. Sexton and C. T. Esmon. The inhibition of blood coagulation by activated protein C through selective inactivation of activated factor V. *Biochim Biophys Acta*. 1979; 571:333-342.
112. K. C. Mann, M. F. Hockin, K. J. Begin and M. Kalafatis. Activated protein C cleavage of factor Va leads to dissociation of the A2-domain. *J Biol Chem*. 1997; 272:20678-20683.
113. J. Rosing and G. Tans. Coagulation factor V: an old star shines again. *Thromb Haemost*. 1997; 78:427-433.
114. G. A. F. Nicolaes, G. Tans, M. C. L. G. D. Thomassen, H. C. Hemker, I. Pabinger, K. Varadi, H. P. Schwarz and J. Rosing. Peptide bond cleavage and loss of functional activity during inactivation of factor Va and factor VaR506Q by activated protein C. *J Biol Chem*. 1995; 270:21158-21166.
115. K. Suzuki, J. Stenflo, B. Dahlback and B. Teodorsson. Inactivation of human coagulation factor V by activated protein C. *J Biol Chem*. 1983; 258:1914-1920.
116. M. F. Hockin, K. M. Cawthorn, M. Kalafatis and K. C. Mann. A model describing the inactivation of factor Va by APC: Bond cleavage, fragment dissociation, and product inhibition. *Biochemistry*. 1999; 38:6918-6934.
117. H. M. Bakker, G. Tans, T. Janssen-Claesen, M. C. L. G. D. Thomassen, H. C. Hemker, J. H. Griffin and J. Rosing. The effect of phospholipids, calcium ions and protein S on rate constants of human factor Va inactivation by activated human protein C. *Eur J Biochem*. 1992; 208:171-178.
118. S. Solymoss, M. M. Tucker and P. B. Tracy. Kinetics of inactivation of membrane-bound factor Va by activated protein C. Protein S modulates factor Xa protection. *J Biol Chem*. 1988; 263:14884-14890.
119. J. Rosing, L. Hoekema and G. A. F. Nicolaes. Effect 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; 270:27852-27858.

120. E. A. Norstrom, M. Steen, S. Tran and B. Dahlback. Importance of protein S and phospholipid for activated protein C-mediated cleavage of factor Va. *J Biol Chem.* 2003; 278:24904-24911.
121. M. J. Heeb, Y. Kojima, T. M. Hackeng and J. H. Griffin. Binding sites for blood coagulation factor Xa and protein S involving residues 493-506 in factor Va. *Protein Sci.* 1996; 5:1883-9.
122. E. Thorelli, R. J. Kaufman and B. Dahlback. The C-terminal region of the factor V B-domain is crucial for the anticoagulant activity of factor V. *J Biol Chem.* 1998; 273:16140-16145.
123. D. Lu, M. Kalafatis, K. G. Mann and G. L. Long. Comparison of activated protein C/protein S-mediated inactivation of human factor VIII and factor V. *Blood.* 1996; 87:4708-4717.
124. E. Thorelli, R. J. Kaufman and B. Dahlback. Cleavage of factor V at Arg 506 by activated protein C and the expression of anticoagulant activity of factor V. *Blood.* 1999; 93:2552-2558.
125. W. L. Nichols, D. A. Gastineau, L. A. Solberg and K. G. Mann. Identification of human megakaryocyte coagulation factor V. *Blood.* 1985; 65:1396-1406.
126. A. M. Gewirtz, M. Keefer, K. Doshi, A. E. Annamalai, H. C. Chiu and R. W. Colman. Biology of human megakaryocyte factor V. *Blood.* 1986; 67:1639-1648.
127. A. M. Gewirtz, C. Shapiro, Y. M. Shen, R. Boyd and R. W. Colman. Cellular and molecular regulation of factor V expression in human megakaryocytes. *J Cell Physiol.* 1992; 153:277-287.
128. B. A. Bouchard, J. L. Williams, N. T. Meisler, M. W. Long and P. B. Tracy. Endocytosis of plasma-derived factor V by megakaryocytes occurs via a clathrin-dependent, specific membrane binding event. *J Thromb Haemost.* 2005; 3:541-551.
129. W. R. Gould, P. Simioni, J. R. Silveira, D. Tormene, M. Kalafatis and P. B. Tracy. Megakaryocytes endocytose and subsequently modify human factor V in vivo to form the entire pool of a unique platelet-derived cofactor. *J Thromb Haemost.* 2005; 3:450-456.
130. M. C. L. G. D. Thomassen, E. Castoldi, E. J. Magdeleyns, C. Delaunoit, L. Debusscher, K. J. Van Assche and J. Rosing. Endogenous factor V synthesis in megakaryocytes contributes negligibly to the platelet factor V pool. *Haematologica.* 2003; 88:1150-1156.
131. R. M. Camire, E. S. Pollak, K. Kaushansky and P. B. Tracy. Secretable human platelet-derived factor V originates from the plasma pool. *Blood.* 1998; 92:3035-3041.
132. J. D. Wencel Drake, B. Dahlbäck, J. G. White and M. H. Ginsberg. Ultrastructural localization of coagulation factor V in human platelets. *Blood.* 1986; 68:244-249.
133. C. M. Chesney, D. D. Pifer and R. W. Colman. Subcellular localization and secretion of factor V from human platelets. *Proc Natl Acad Sci USA.* 1981; 78:5180-5184.
134. C. P. M. Hayward, E. Furmaniak-Kazmierczak, A. M. Cieutat, J. C. Moore, D. F. Bainton, M. E. Nesheim and J. G. Kelton. Factor V is complexed with multimerin in resting platelet lysates and colocalizes with multimerin in platelet a-granules. *J Biol Chem.* 1995; 270:19217-19224.
135. P. B. Tracy, A. R. Giles, K. G. Mann, L. L. Eide, H. Hoogendoorn and G. E. Rivard. Factor V (Quebec): a Bleeding diathesis associated with a qualitative platelet factor V deficiency. *J Clin Invest.* 1984; 74:1221-1228.
136. C. P. M. Hayward, G. E. Rivard, W. H. Kane, J. Drouin, S. L. Zheng, J. C. Moore and J. G. Kelton. An autosomal dominant, qualitative platelet disorder associated with multimerin deficiency, abnormalities in platelet factor V,

- thrombospondin, von willebrand factor, and fibrinogen and an epinephrine aggregation defect. *Blood*. 1996; 87:4967-4978.
137. R. W. Viskup, P. B. Tracy and K. G. Mann. The isolation of human platelet factor V. *Blood*. 1987; 69:1188-1195.
138. D. D. Monkovic and P. B. Tracy. Functional characterization of human platelet-released factor V and its activation by factor Xa and thrombin. *J Biol Chem*. 1990; 265:17132-17140.
139. W. R. Gould, J. R. Silveira and P. B. Tracy. Unique in vivo modifications of coagulation factor V produce a physically and functionally distinct platelet-derived cofactor: characterization of purified platelet-derived factor V/Va. *J Biol Chem*. 2004; 279:2383-2393.
140. R. M. Camire, M. Kalafatis, P. Simioni, A. Girolami and P. B. Tracy. Platelet-derived factor Va/VaLeiden cofactor activities are sustained on the surface of activated platelets despite the presence of activated protein C. *Blood*. 1998; 91:2818-2829.
141. R. M. Camire, M. Kalafatis, M. Cushman, R. P. Tracy, K. G. Mann and P. B. Tracy. The mechanism of inactivation of human platelet factor Va from normal and activated protein C-resistant individuals. *J Biol Chem*. 1995; 270:20794-20800.
142. P. J. Lenting, J. A. van Mourik and K. Mertens. The life cycle of coagulation factor VIII in view of its structure and function. *Blood*. 1998; 92:3983-96.
143. K. L. Wion, D. Kelly, J. A. Summerfield, E. G. Tuddenham and R. M. Lawn. Distribution of factor VIII mRNA and antigen in human liver and other tissues. *Nature*. 1985; 317:726-9.
144. J. J. Toole, J. L. Knopf, J. M. Wozney, L. A. Sultzman, J. L. Buecker, D. D. Pittman, R. J. Kaufman, E. Brown, C. Shoemaker, E. C. Orr and et al. Molecular cloning of a cDNA encoding human antihemophilic factor. *Nature*. 1984; 312:342-7.
145. L. O. Andersson, N. Forsman, K. Huang, K. Larsen, A. Lundin, B. Pavlu, H. Sandberg, K. Sewerin and J. Smart. Isolation and characterization of human factor VIII: molecular forms in commercial factor VIII concentrate, cryoprecipitate, and plasma. *Proc Natl Acad Sci U S A*. 1986; 83:2979-83.
146. R. J. Hamer, J. A. Koedam, N. H. Beeser-Visser, R. M. Bertina, J. A. Van Mourik and J. J. Sixma. Factor VIII binds to von Willebrand factor via its Mr-80,000 light chain. *Eur J Biochem*. 1987; 166:37-43.
147. P. J. Fay. Activation of factor VIII and mechanisms of cofactor action. *Blood Rev*. 2004; 18:1-15.
148. P. J. Fay, P. J. Haidaris and T. M. Smudzin. Human factor VIIIa subunit structure. Reconstruction of factor VIIIa from the isolated A1/A3-C1-C2 dimer and A2 subunit. *J Biol Chem*. 1991; 266:8957-62.
149. P. J. Fay, T. L. Beattie, L. M. Regan, L. M. O'Brien and R. J. Kaufman. Model for the factor VIIIa-dependent decay of the intrinsic factor Xase. Role of subunit dissociation and factor IXa-catalyzed proteolysis. *J Biol Chem*. 1996; 271:6027-32.
150. L. M. Regan, B. J. Lamphear, C. F. Huggins, F. J. Walker and P. J. Fay. Factor IXa protects factor VIIIa from activated protein C. Factor IXa inhibits activated protein C-catalyzed cleavage of factor VIIIa at Arg562. *J Biol Chem*. 1994; 269:9445-52.
151. B. Dahlback, M. Carlsson and P. J. Svensson. Familial thrombophilia due to a previously unrecognized mechanism characterized by poor anticoagulant response to activated protein C: prediction of a cofactor activated protein C. *Proc Natl Acad Sci USA*. 1993; 90:1004-1008.
152. P. J. Svensson and B. Dahlback. Resistance to activated protein C as a basis for venous thrombosis. *N Engl J Med*. 1994; 330:517-522.

153. T. Koster, F. R. Rosendaal, H. De Ronde, E. Briet, J. P. Vandenbroucke and R. M. Bertina. Venous thrombosis due to a poor anticoagulant response to activated protein C: Leiden Thrombophilia Study. *Lancet*. 1993; 342:1503-1506.
154. R. M. Bertina, B. P. Koeleman, T. Koster, F. R. Rosendaal, R. J. Dirven, H. de Ronde, P. A. van der Velden and P. H. Reitsma. Mutation in blood coagulation factor V associated with resistance to activated protein C. *Nature*. 1994; 369:64-67.
155. J. S. Greengard, X. Sun, X. Xu, J. A. Fernandez, J. H. Griffin and B. Evatt. Activated protein C resistance caused by Arg506Gln mutation in factor Va. *Lancet*. 1994; 343:1361-2.
156. X. Sun, B. Evatt and J. H. Griffin. Blood coagulation factor Va abnormality associated with resistance to activated protein C in venous thrombophilia. *Blood*. 1994; 83:3120-3125.
157. J. Voorberg, J. C. Roelse, R. Koopman, H. R. Buller, F. Berends, J. W. Ten Cate, K. Mertens and J. A. Van Mourik. Association of idiopathic venous thromboembolism with single point-mutation at Arg506 of factor V. *Lancet*. 1994; 343:1535-1536.
158. B. Zoller, P. J. Svensson, X. He and B. Dahlbäck. Identification of the same factor V gene mutation in 47 out of 50 thrombosis-prone families with inherited resistance to activated protein C. *J Clin Invest*. 1994; 94:2521-2524.
159. M. Kalafatis, R. M. Bertina, M. D. Rand and K. G. Mann. Characterization of the molecular defect in factor VR506Q. *J Biol Chem*. 1995; 270:4053-7.
160. M. J. Heeb, Y. Kojima, J. S. Greengard and J. H. Griffin. Activated protein C resistance: molecular mechanisms based on studies using purified Gln506-factor V. *Blood*. 1995; 85:3405-11.
161. D. C. Rees, M. J. Cox and J. B. Clegg. World distribution of factor V Leiden. *Lancet*. 1995; 346:1133-1134.
162. B. Dahlback. Inherited Thrombophilia: Resistance to activated protein C as a pathogenic factor of venous thromboembolism. *Blood*. 1995; 85:607-614.
163. P. M. Ridker, C. H. Hennekens, K. Lindpaintner, M. J. Stampfer, P. R. Eisenberg and J. P. Miletich. Mutation in the gene coding for coagulation factor V and the risk of myocardial infarction, stroke, and venous thrombosis in apparently healthy men. *N Engl J Med*. 1995; 332:912-917.
164. F. R. Rosendaal, T. Koster, J. P. Vandenbroucke and P. H. Reitsma. High risk of thrombosis in patients homozygous for factor V Leiden (activated protein C resistance). *Blood*. 1995; 85:1504-1508.
165. B. Manten, R. G. J. Westendorp, T. Koster, P. H. Reitsma and F. R. Rosendaal. Risk factor profiles in patients with different clinical manifestations of venous thromboembolism. A focus on the factor V Leiden mutation. *Thromb Haemost*. 1996; 76:510-513.
166. D. Williamson, K. Brown, R. Luddington, C. Baglin and T. Baglin. Factor V Cambridge: A new mutation (Arg306 to Thr) associated with resistance to activated protein C. *Blood*. 1998; 91:1140-1144.
167. R. Liang, C. K. Lee, M. S. Wat and Y. L. Kwong. Clinical significance of Arg306 mutations of factor V gene. *Blood*. 1998; 92:2599-2600.
168. W. P. Chan, C. K. Lee, Y. L. Kwong, C. K. Lam and R. Liang. A novel mutation of Arg306 of factor V gene in Hong Kong Chinese. *Blood*. 1998; 91:1135-1139.
169. E. A. Norstrom, E. Thorelli and B. Dahlback. Functional characterization of recombinant FV Hong Kong and FV Cambridge. *Blood*. 2002; 100:524-530.
170. A. D. Mumford, J. H. McVey, C. V. Morse, K. Gomez, M. Steen, E. A. Norstrom, E. G. Tuddenham, B. Dahlback and P. H. B. Bolton-Maggs. Factor V I359T: a novel mutation associated with thrombosis and resistance to activated protein C. *British Journal of Haematology*. 2003; 123:496-501.
171. M. Steen, E. A. Norstrom, A. Tholander, P. H. B. Bolton-Maggs, A. D. Mumford, J. H. McVey, E. G. Tuddenham and B. Dahlback. Functional characterization of

- factor V-Ile359Thr: a novel mutation associated with thrombosis. *Blood*. 2004; 103:3381-3387.
172. B. Lunghi, L. Iacoviello, D. Gemmati, M. G. Dilasio, E. Castoldi, M. Pinotti, G. Castaman, R. Redaelli, G. Mariani, G. Marchetti and F. Bernardi. Detection of new polymorphic markers in the factor V gene: association with factor V levels in plasma. *Thromb Haemost*. 1996; 75:45-48.
173. F. Bernardi, E. M. Faioni, E. Castoldi, B. Lunghi, G. Castaman, E. Sacchi and P. M. Mannucci. A factor V genetic component differing from factor V R506Q contributes to the activated protein C resistance phenotype. *Blood*. 1997; 90:1552-1557.
174. E. Castoldi, J. Rosing, D. Girelli, L. Hoekema, B. Lunghi, F. Mingozzi, P. Ferraresi, S. Friso, R. Corrocher, G. Tans and F. Bernardi. Mutations in the R2 FV gene affect the ratio between the two FV isoforms in plasma. *Thromb Haemost*. 2000; 83:362-365.
175. N. M. Pecheniuk, C. P. Morris, T. P. Walsh and N. A. Marsh. The factor V HR2 haplotype: prevalence and association of the A4070G and A6755G polymorphisms. *Blood Coagul Fibrinolysis*. 2001; 12:201-206.
176. H. Kostka, G. Siegert, T. Schwarz, S. Gehrisch, E. Kuhlisch, S. Schellong and W. Jaross. Frequency of polymorphisms in the B-domain of factor V gene in APC-resistant patients. *Thromb Res*. 2000; 99:539-547.
177. E. M. Faioni. Factor V HR2: an ancient haplotype out of Africa - a reason for being interested. *Thromb Haemost*. 2000; 83:358-359.
178. T. Yamazaki, G. A. F. Nicolaes, K. W. Sorensen and B. Dahlback. Molecular basis of quantitative factor V deficiency associated with the factor V R2-haplotype. *Blood*. 2002; 100:2515-2521.
179. L. Hoekema, E. Castoldi, G. Tans, D. Girelli, D. Gemmati, F. Bernardi and J. Rosing. Functional properties of factor V and factor Va encoded by the R2-gene. *Thromb Haemost*. 2001; 85:75-81.
180. M. C. de Visser, J. F. Guasch, P. W. Kamphuisen, H. L. Vos, F. R. Rosendaal and R. M. Bertina. The HR2 haplotype of factor V: effects on factor V levels, normalized activated protein C sensitivity ratios and the risk of venous thrombosis. *Thromb Haemost*. 2000; 83:577-582.
181. J. W. P. Govers-Riemslog, E. Castoldi, G. A. F. Nicolaes, G. Tans and J. Rosing. Reduced factor V concentration and altered FV1/ FV2 ratio do not fully explain R2-associated APC-resistance. *Thromb Haemost*. 2002; 88:444-449.
182. G. Castaman, E. M. Faioni, A. Tosetto and F. Bernardi. The factor V HR2 haplotype and the risk of venous thrombosis: a meta-analysis. *Haematologica*. 2003; 88:1182-1189.
183. G. Castaman, B. Lunghi, E. Missiaglia, F. Bernardi and F. Rodeghiero. Phenotypic homozygous activated protein C resistance associated with compound heterozygosity for Arg506Gln (factor V Leiden) and His1299Arg substitutions in factor V. *British Journal of Haematology*. 1997; 99:257-261.
184. E. M. Faioni, F. Franchi, P. Bucciarelli, M. Margaglione, V. De Stefano, G. Castaman, G. Finazzi and P. M. Mannucci. Coinheritance of the HR2 haplotype in the factor V gene confers an increased risk of venous thromboembolism to carriers of factor V R506Q (factor V Leiden). *Blood*. 1999; 94:3062-6.
185. S. R. Poort, F. R. Rosendaal, P. H. Reitsma and R. M. Bertina. A common genetic variation in the 3'-untranslated region of the prothrombin gene is associated with elevated plasma prothrombin levels and an increase in venous thrombosis. *Blood*. 1996; 88:3698-3703.
186. J. C. Souto, I. Coll, D. Llobet, E. del Rio, A. Oliver, J. Mateo, M. Borrell and J. Fontcuberta. The prothrombin 20210A allele is the most prevalent genetic risk factor for venous thromboembolism in the Spanish population. *Thromb Haemost*. 1998; 80:366-9.

187. R. K. Kapur, L. A. Mills, S. G. Spitzer and M. B. Hultin. A prothrombin gene mutation is significantly associated with venous thrombosis. *Arterioscler Thromb Vasc Biol.* 1997; 17:2875-9.
188. K. Brown, R. Luddington, D. Williamson, P. Baker and T. Baglin. Risk of venous thromboembolism associated with a G to A transition at position 20210 in the 3'-untranslated region of the prothrombin gene. *Br J Haematol.* 1997; 98:907-9.
189. A. Hillarp, B. Zoller, P. J. Svensson and B. Dahlback. The 20210A allele of the prothrombin gene is a common risk factor among Swedish outpatients with verified deep venous thrombosis. *Thromb Haemost.* 1997; 78:990-992.
190. V. R. Arruda, J. M. Annichino-Bizzacchi, M. S. Goncalves and F. F. Costa. Prevalence of the prothrombin gene variant (nt20210A) in venous thrombosis and arterial disease. *Thromb Haemost.* 1997; 78:1430-3.
191. R. F. Franco, S. E. Santos, J. Elion, M. H. Tavella and M. A. Zago. Prevalence of the G20210A polymorphism in the 3'-untranslated region of the prothrombin gene in different human populations. *Acta Haematol.* 1998; 100:9-12.
192. P. M. Ridker, C. H. Hennekens and J. P. Miletich. G20210A mutation in prothrombin gene and risk of myocardial infarction, stroke, and venous thrombosis in a large cohort of US men. *Circulation.* 1999; 99:999-1004.
193. P. Ferraresi, G. Marchetti, C. Legnani, E. Cavallari, E. Castoldi, F. Mascoli, D. Ardissino, G. Palareti and F. Bernardi. The heterozygous 20210 G/A prothrombin genotype is associated with early venous thrombosis in inherited thrombophilia and is not increased in frequency in arterial disease. *Arterioscler Thromb Vasc Biol.* 1997; 17:2418-2422.
194. F. R. Rosendaal, C. J. Doggen, A. Zivelin, V. R. Arruda, M. Aiach, D. S. Siscovick, A. Hillarp, H. H. Watzke, F. Bernardi, A. M. Cumming, F. E. Preston and P. H. Reitsma. Geographic distribution of the 20210 G to A prothrombin variant. *Thromb Haemost.* 1998; 79:706-8.
195. A. Tripodi, V. Chantarangkul and P. M. Mannucci. Hyperprothrombinemia may result in acquired activated protein C resistance. *Blood.* 2000; 96:3295-3296.
196. G. Castaman, A. Toso, M. Simioni, M. Ruggeri, D. Madeo and F. Rodeghiero. Phenotypic APC resistance in carriers of the A20210 prothrombin mutation is associated with an increased risk of venous thrombosis. *Thromb Haemost.* 2001; 86:804-808.
197. C. A. Mitchell, S. M. Jane and H. H. Salem. Inhibition of the anticoagulant activity of protein S by prothrombin. *J Clin Invest.* 1988; 82:2142-2147.
198. M. D. Smirnov, O. Safa, N. L. Esmon and C. T. Esmon. Inhibition of activated protein C anticoagulant activity by prothrombin. *Blood.* 1999; 94:3839-3846.
199. E. A. Norstrom, S. Tran and B. Dahlback. Prothrombin inhibition of the individual activated protein C cleavage sites in factor Va. *Thesis.* 2004;

CHAPTER 2

Theoretical and experimental study of the D2194G mutation in the C2-domain of coagulation factor V

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ABSTRACT

Coagulation factor V (FV) is a large plasma glycoprotein with functions in both pro- and anticoagulant pathways. In carriers of the so-called R2-FV haplotype, the FV D2194G mutation, in the C2 membrane binding domain, is associated with low expression levels, suggesting a potential folding/stability problem. To analyze the molecular mechanisms potentially responsible for this *in vitro* phenotype, we used molecular dynamics (MD) and continuum electrostatic calculations. Implicit solvent simulations were performed on the X-ray structure of the wild-type C2 domain and on a model of the D2194G mutant. Because D2194 is located next to a disulfide bond (S-S bond), MD calculations were also performed on S-S bond depleted structures. D2194 is part of a salt-bridge network and investigations of the stabilizing/destabilizing role of these ionic interactions were carried out. Five mutant FV molecules were created and the expression levels measured with the aim of assessing the tolerance to amino acid changes of this region of the molecule. Analysis of the MD trajectories indicated increased flexibility in some areas and energetic comparisons suggested overall destabilization of the structure due to the D2194G mutation. This substitution causes electrostatic destabilization of the domain by ~3 kcal/mol. Together these effects likely explain the lowered expression levels in R2-FV carriers.

INTRODUCTION

Coagulation factor V (FV) is the precursor of an essential pro-coagulant cofactor that accelerates FXa-catalyzed prothrombin activation. It is a large glycoprotein that is structurally and functionally homologous to factor VIII (FVIII), the two proteins sharing the domain organization A1-A2-B-A3-C1-C2. Activation of FV involves proteolytic removal of the large B domain, converting the procofactor into the fully active cofactor FVa (Nicolaes and Dahlback, 2002; Mann and Kalafatis, 2003). Interestingly, FV also has a reported anticoagulant function in the APC-mediated inactivation of FVIIIa (reviewed by Nicolaes and Dahlback, 2002). FV and FVIII interact with many proteins and bind to cell surfaces, essentially via the C2 domain. The three-dimensional (3D) structure of the C2 domains of FV/FVIII was initially predicted using a combination of threading and comparative modeling based on the structure of galactose oxidase (Villoutreix et al., 1998; Pellequer et al., 1998; Baumgartner et al., 1998). Despite very low sequence identity (~10%) between the C domains of FV/FVIII and galactose oxidase, the overall 3D fold of the predicted structures was correct as seen from comparison with recently reported X-ray structures of the isolated C2 domains of FV and FVIII (Macedo-Ribeiro et al., 1999; Pratt et al., 1999). These C domains (also called discoidin domains) exhibit a distorted jelly-roll β -barrel motif, consisting of eight major anti-parallel strands arranged in two β -sheets. The amino- and carboxy-terminal segments are clamped together by the only disulfide bond (S-S bond) present in this module (C2038-C2193). The modeled and experimental structures of the C2 domain of FV allowed for proposal of key regions involved in membrane binding (Nicolaes et al., 2000; Kim et al., 2000). This FV membrane-binding motif consists of several exposed hydrophobic side chains located essentially on loop structures (e.g., the 2060-loop), surrounded by a ring of basic residues. In human plasma, FV circulates as a mixture of two isoforms, FV1 (glycosylated) and FV2 (not glycosylated) that have slightly different molecular weights because of partial N-linked glycosylation at N2181. This leads to differences in membrane binding behavior between FV1 and FV2 (Rosing et al., 1993; Hoekema et al., 1997; Nicolaes et al., 1999). Also, glycosylation at N2181 has been proposed to modulate the FV expression levels (Nicolaes et al., 1999).

The FV R2 haplotype, firstly described in a cohort of thromboembolic patients, encompasses several genetically linked polymorphisms, that encode amino acid substitutions (of which M385T, H1299R, M1736V and D2194G are most likely linked to the haplotype) in different domains of the molecule (Lunghi et al., 1996; Bernardi et al., 1997; Castoldi et al., 2000). There is no consensus from epidemiological studies if these polymorphisms are associated with venous

or arterial thrombosis. Yet, *in vitro* studies have demonstrated that at least, the D2194G substitution in the FV C2 domain induces a substantially impaired expression due to retention of the mutant protein in the endoplasmic reticulum (ER), a place where misfolded proteins tend to be trapped and degraded (Yamazaki et al., 2002). Thus the D2194G substitution appears to affect the folding/structure/stability of the C2 domains (Yamazaki et al., 2002; Van Der Neut Kolfshoten et al., 2003) via mechanisms that are not fully understood.

It is a major challenge to establish the links between point mutations, 3D structures and disease states (Villoutreix, 2002; Bross et al., 1999; Wickner et al., 1999; Sinha and Nussinov, 2001, Hilser et al., 1998; Taverna et al., 2002). Potential impacts of mutations on protein stability, folding and function can be investigated theoretically using a single 3D conformation of the folded native protein as starting point (Wang and Moulton, 2001) or via (short/long) molecular dynamics (MD) simulations (Cregut and Serrano, 1999; Schiffer and van Gunsteren, 1996; Kazmirski et al., 1995; Rizzuti et al., 2001). When a mutation involves a charged residue that has ionic interactions with surrounding groups, some specific electrostatic computations can also be performed to investigate the stabilizing or destabilizing roles of salt bridges on protein stability (Kumar and Nussinov, 1999; Hendsch and Tidor, 1994; Lounnas and Wade 1997; Barril et al., 1998; Dong and Zhou, 2002).

To study the potential impacts of the D2194G mutation on the C2 domain of human FV, we used MD, electrostatic computations and site-directed mutagenesis. In the native 3D structure of the C2 domain, residue D2194 is partially solvent exposed, fully conserved in the FV species and located next to the C2038-C2193 disulfide bond. It is involved in electrostatic interactions with the side chains of K2101 and K2103. We hypothesized that the D2194G substitution could affect the stability of the molecule due to electrostatic destabilization and/or induce conformational/flexibility changes. Due to its position, directly juxtaposed to the disulfide bridge in the C2 domain, the D2194G mutation could also influence disulfide bond formation. We decided to run MD simulations with an implicit solvent model on the C2 domain of wild-type FV (WT-FV) and on the D2194G mutant, in the presence or the absence of the C2038-C2193 bond. Simulations without the S-S bond were carried out to examine some of the effects of this bond on the 3D structure of the domain. Electrostatic computations were also run on different C2 structures to further investigate the role of ionic interactions in the 2194 area. Several mutant proteins (D2194K, K2101E/K2103E, K2101E/K2103E/D2194K and C2038A/C2193A) were produced and the final expression levels characterized in order to assess the overall sensitivity of this region to amino acid changes. Taken together, our data indicate that aspartate at position 2194 is important for

the stability of the domain and that the above amino acid substitutions affect the stability and/or the conformation of the domain.

MATERIALS AND METHODS

Computational methods

Molecular Dynamics

Molecular dynamics simulations were carried out on the high resolution X-ray structure (Macedo-Ribeiro et al., 1999) of domain C2 of WT-FV and on a model of the D2194G mutant. The X-ray structure of the human WT-FV C2 domain (Macedo-Ribeiro et al., 1999) was taken from the Protein Data Bank (Berman et al., 2000) (entry 1czt, 1.8 Å resolution). There is no experimental structure for the D2194G mutant and we have built an initial 3D model for this domain on the basis of the WT-structure by simply removing the D2194 side chain. The N-terminal group was acetylated in all initial structures to account for the fact that this residue is linked to the C1 domain. MD simulations were performed for the WT-C2 domain and the mutant D2194G, with or without the disulfide bond C2038-C2193. The same protocol for energy minimization and molecular dynamics was used for all the relevant simulations. Calculations were performed using the program CHARMM (Brooks et al., 1983) using CHARMM 22 parameters on two Silicon Graphics (IRIX 6.5) Fuel V12 single 600-MHz processor workstations (Mountain View, CA). Interactive analysis of the results was performed in InsightII (running on SGI workstations) or with ICM (Molsoft) running on two Precision 530 Dell PC dual-2.4-GHz processors driven by Red Hat Linux 7.3.

The importance of correct accounting for solvation effects during MD simulations has been widely discussed. Because explicit treatment of solvent is very time-consuming and eventually not needed, several implicit methods have been proposed for calculation of solvation effects in proteins (Bashford and Case, 2000, Feig and Brooks, 2002). We have used in our MD simulations the Generalized Born (GB) method as implemented by Dominy and Brooks (1999) into the program CHARMM to account for the electrostatic contribution to the solvation energy (Brooks et al., 1983) (version 27.b1 or 28.b1, Accelrys). The GB approximation is based on a two-dielectric model and its mathematical form, for a molecule with arbitrary shape, has been extensively parameterized (Dominy and Brooks, 1999).

The empirical Atomic Solvation Parameter (ASP) approach as proposed by Wesson and Eisenberg (1992) with parameterization by Sharp et al. (1991)

was also applied to account for the nonpolar solvation energy. This method assumes that the nonpolar (considered in the present article to be the solvent-solvent cavity and solute-solvent van der Waals terms) solvation free energy of the solute can be decomposed into a sum of atomic free energy components that are proportional to the solvent accessible surface (SAS) of atoms of type k : $G^{\text{hydrophobic}} = \sum \sigma_k \text{SAS}_k$. Here we used $\sigma_k = 12.3 \text{ cal/mol/\AA}^2$ for the hydrophobic carbon atoms and $\sigma_k = 0.0 \text{ cal/mol/\AA}^2$ for the other atoms as suggested by several research groups (Qiu et al., 1997; Morozov et al., 2003).

The charge-charge interactions were treated using Coulomb law with a dielectric constant $\epsilon = 1$ to be consistent with the GB parameterization. The GB parameters proposed in Dominy and Brooks (1999) were used and a dielectric constant of 80 was applied to represent the highly polarizable solvent medium. For all nonbonded interactions we used a cutoff of 15 Å and a switch function.

Initial hydrogen positions were calculated using the HBUILD utility of CHARMM. Definition of the charged state for the titratable groups in the X-ray structure of WT-FV and the mutant D2194G before the MD simulations was decided after electrostatic computations of the mean proton occupancy (see below for computational details). The results indicated that all Lys, Arg, Glu, Asp, and the C-terminal group are charged while the remaining residues including His residues were found to be neutral. Only K2148 was found not to be completely charged (calculated mean proton occupancy $q = 0.4$) at pH = 7.

The initial 3D structures were energy minimized (1500 steps steepest descent method or a tolerance of energy gradient of 0.15 kcal/mol/Å) and MD simulations were then performed on the minimized structures. The temperature of the system was raised gradually from 240 to 300 K in 20-ps followed by equilibration at 300 K for 50-ps. This was further continued by a 900-ps production run of NVT-MD at 300K with the Berendsen algorithm (Berendsen et al., 1984) and the protein conformations were recorded every 1-ps. The time step was 1-fs and the SHAKE algorithm was used to eliminate the high frequency hydrogen/heavy atom bond vibrations.

Electrostatic Calculations

All electrostatic calculations (pKa, electrostatic free energies of stabilization) were performed for the WT and mutants but only on the structures possessing the S-S bond. Thus, the X-ray structure of the WT, the structures with the D2194G, D2194K, K2101E/K2103E, K2101E/K2103E/D2194K substitutions (without simulations) were selected for computations. The same calculations were also carried out on the last conformations of the MD simulations for the WT and the D2194G mutant, as well as on 30 structures

sampled from the MD trajectories from 670 to 970 ps of the production run (one structure selected every 10 ps).

Finite difference Poisson-Boltzmann energy calculations

For computation of electrostatic energies used in protonation states and electrostatic stabilization calculations, we used the program UHBD (Davis et al., 1991) that solves the Poisson-Boltzmann equation numerically on a grid by the finite difference method (Warwicker and Watson, 1982; Sharp and Honig, 1990; Yang et al., 1993). It has been previously proposed (Antosiewicz et al., 1994) that a better agreement between calculated and experimental pK_a values of titratable groups can be obtained using a higher protein dielectric constant (e.g., $\epsilon_{in}=20$). However, in this work we use $\epsilon_{in}=4$, because accurate results for evaluation of salt-bridge energetic were obtained earlier with this value (Hendsch & Tidor, 1994; Lounnas and Wade, 1997; Barril et al., 1998). The protein atomic radii and charges were based on the CHARMM 22 parameter set. The dielectric boundary between the protein and solvent was defined using the solvent accessibility surface with probe sphere of radius 1.4 Å and 500 dots per atom for generation of the surface. Initial boundary conditions were determined by calculating the potential at each boundary point using the Debye-Huckel approximation. A salt concentration of 0.15 M and salt exclusion radius of 2.0 Å were used in the finite difference Poisson-Boltzmann calculations.

In the calculation of protonation states, first the protein structures were included into a grid of 45x45x45 points with a resolution of 2.5 Å. A focusing scheme proposed previously (Yang et al., 1993; Antosiewicz et al., 1994) using four successive lattices with increasing grid resolution was applied for calculation of electrostatic potentials created by every ionized group. The last focusing run was done in a 3D grid of dimension of 20 nodes and a resolution of 0.25 Å centered at the titration site. A procedure with two focusing runs in boxes of dimension of 65 with resolution of 1.5 Å, followed by a box of dimension of 70 with a 0.3 Å resolution, was applied to calculate the electrostatic energies involved in the calculation of electrostatic stabilization of the charged triads (see below).

Protonation states

The FDPB method has been widely used for calculation of protein protonation states (Bashford and Karplus, 1990; Yang et al., 1993; Antosiewicz et al., 1994). To calculate intrinsic pK_a value (pK^{int}) of a given titratable group (Tanford and Kirkwood, 1957), (the pK^{int} is defined as the pK_a that the group would have if all other titratable groups were neutral) one unit charge at the protonation site of this group and all permanent partial charges of the protein

were taken into account.

The protonation state n of a protein with N titratable residues is characterized by the protonation state vector $\delta_n(i)$, $i=1$ to N , where $\delta_n(i)=1$ when the i th group is charged and 0 when it is neutral. The free energy of the n th protonation state ΔG^n is given by:

$$\Delta G^n = \sum_{i=1}^N \delta_n(i) \cdot \chi(i) \cdot (2.3RT(\text{pH} - \text{p}K_i^{\text{int}})) + \frac{1}{2} \sum_{\substack{i=1 \\ i \neq j}}^N \delta_n(i) \delta_n(j) \cdot \Delta G^{ij} \quad (1)$$

where $\chi(i) = -1$ or 1 for an acidic or basic group i , respectively, $\text{p}K_i^{\text{int}}$ is the intrinsic $\text{p}K_a$ value of the group i , and ΔG^{ij} is the electrostatic interaction energy between groups i and j in their charged states defined by $\delta(i)$ and $\delta(j)$. In this work the protonation states of all studied structures were obtained at pH 7.0 (Antosiewicz et al., 1994).

In the computation of electrostatic potentials ϕ_{ij} due to the charge j at the location of the charged site i , involved in pairwise interactions calculation ($\Delta G^{ij} = \gamma_i \cdot \phi_{ij}$), only a unit charge at the protonation site was included. The protonation sites were set to the atoms: C^γ of Asp, C^δ of Glu, C^ζ of Arg, N^ζ of Lys, $\text{N}^{\epsilon 2}$ of His, OH of Tyr, C atom of main-chain C-terminus. The mean proton occupancies q of titratable groups at pH 7 are computed by averaging over the protonation states sampled by the Monte Carlo method based on the ΔG^n calculations (Antosiewicz and Porschke, 1989).

Electrostatic free energy contributions to the protein stability for salt-link triads

The electrostatic free energy, $\Delta\Delta G^{\text{tot}}$, contributing to protein stability due to a triad of charged amino acid residues in a protein (WT- or mutants) was calculated relative to a reference protein hydrophobic isosteres (an isostere is defined as a residue with the same conformation and position in the protein as the original residue but neutral). The term $\Delta\Delta G^{\text{tot}}$ was calculated following the procedure proposed by Hendsch and Tidor 1994:

$$\Delta\Delta G^{\text{tot}} = \Delta\Delta G^{\text{sol}} + \Delta\Delta G^{\text{triad}} + \Delta\Delta G^{\text{pr}} \quad (2)$$

The three terms $\Delta\Delta G^{\text{sol}}$, $\Delta\Delta G^{\text{triad}}$, $\Delta\Delta G^{\text{pr}}$ were computed as differences between the energies of the protein and the protein with appropriate amino acids involved into the ionic triad replaced to their respective hydrophobic isosteres.

The desolvation electrostatic energy ΔG^{sol} of the triad in a protein was calculated as:

$$\Delta G^{\text{sol}} = \Delta G^{\text{sol}}_{\text{A}} + \Delta G^{\text{sol}}_{\text{B}} + \Delta G^{\text{sol}}_{\text{C}} \quad (3)$$

and $\Delta G^{\text{sol}}_{\text{A}}$, $\Delta G^{\text{sol}}_{\text{B}}$, and $\Delta G^{\text{sol}}_{\text{C}}$ are the electrostatic desolvation free energies of each residue of the triad A, B, C, respectively. The electrostatic desolvation energy ΔG^{sol} for taking a residue from solvent to protein environment is given following Gilson and Honig (1988).

The electrostatic energy of internal interactions in the triad ΔG^{triad} was presented as:

$$\Delta G^{\text{triad}} = \Delta G_{\text{ABC}} - \Delta G_{\text{A}} - \Delta G_{\text{B}} - \Delta G_{\text{C}} \quad (4)$$

where ΔG_{ABC} is the electrostatic free energy of the protein involving only the charges of the triad; ΔG_{A} , ΔG_{B} , ΔG_{C} are the electrostatic free energies of the protein involving the charges of only one of the triad residues A, B, or C, respectively.

The electrostatic free energy of interactions between the triad and the rest of the protein ΔG^{pr} is calculated by:

$$\Delta G^{\text{pr}} = \Delta G_{\text{prot,triad}} - \Delta G_{\text{ABC}} - \Delta G_{\text{prot}} \quad (5)$$

The three terms in Eq.5 correspond to electrostatic free energy of the protein when all protein charges are present ($\Delta G_{\text{prot,triad}}$); all charges are present excluding the charges of the triad (ΔG_{prot}), and only the charges of the triad are present (ΔG_{ABC}).

These calculations were performed for investigation of the WT triad (D2194, K2101, K2103 versus the mutant G2194, K2101, K2103). In the calculation of these electrostatic energies of stabilization, the atomic partial charges corresponding to CHARMM22 parameters were used (such charged state of the groups is in agreement with our calculated mean proton occupancies q at pH 7 for the WT and D2194G mutant). In the modeled MD structures of WT- and mutant D2194G all Lys, Arg, Glu, Asp and C-term residues were calculated to be charged and the others groups, including the His residues were computed to be neutral.

For other C2 mutant structures (D2194K, K2101E/K2103E, K2101E/K2103E/D2194K), residue E2103 in mutants K2101E/K2103E and K2101E/K2103E/D2194K, as well as residue K2101 in mutant D2194K were taken non-charged in the calculation of triad electrostatic stabilization, as computed from the mean proton occupancies.

Experimental procedures

Site-directed Mutagenesis

Site-directed mutagenesis was performed using the Quick Change Site-Directed Mutagenesis Kit (Stratagene, USA) according to the manufacturer's instructions in order to generate new variant FV recombinant molecules: D2194K, K2101E/K2103E, C2038A/C2193A. As a template we used the full-length cDNA of human FV, coding for the FV2 isoform (WT-N2181Q) described previously (Nicolaes et al., 1999). A fourth mutant was constructed by mutagenesis of D2194 into K, using the K2101E/K2103E plasmid as a template. The construction of an expression vector for recombinant FV containing the R2-associated D2194G mutation has previously been described (Yamazaki et al., 2002). Two complementary oligonucleotides (DNA Technology, Denmark) were used as mutagenic primers for each construct, the sense oligo being:

5'-GAACTCTTTGGCTGTAAGATTTACTAGAATTG-3' (D2194K),
 5'-TTGATCTACTCGAGATCGAGAAGATAACGGC-3' (K2101E/K2103E),
 5'-TGAGGTAAATGGAGCTTCCACACCCCTG-3' (C2038A) and
 5'-CTGGAACCTTTTGGCGCTGATATTTACTAG-3' (C2193A).

To ensure that during the mutagenesis no additional mutations were introduced, a small polymerase chain reaction (PCR) fragment containing the desired mutation(s) was cut out of the PCR-product by restriction endonucleases and ligated into a new template that had been cleaved by the same restriction enzymes. The presence of only the desired mutations in each of the plasmids was checked by DNA sequencing.

Transient expression of Factor V

Recombinant Factor V plasmids were transiently co-expressed with a Green Fluorescent Protein-lamin construct (GFP-lamin) in COS-1 cells using Fugene 6 Transfection Reagent (Roche Molecular Biochemicals, Indianapolis, IN). A transfection mixture was made by mixing 100 µl of OptiMem serum-free medium (OptiMem with Glutamax I; Gibco, Life Technologies, Gaithersburg, MD) with 7.5 µl Fugene 6 Transfection Reagent, 2 µg of mutated pMT2-FV and 0.5 µg pEGFP-lamin plasmid. This mixture was incubated at room temperature for 20 minutes, before adding it to ~80% confluent monolayer of COS1 cells in 2 ml Dulbecco modified Eagle medium (DMEM), supplemented with L-glutamine. Transfection was allowed to proceed for 16 hours before replacing the transfection medium with fresh DMEM, supplemented with L-glutamine, 10% fetal calf serum, 100 IU/ml penicillin and 100 µg/ml streptomycin. After 48 h

posttransfection, cells were washed with phosphate-buffered saline and after trypsinisation, 25% of the cells were harvested to quantitate the transfection efficiency by flow cytometry. The remainder of the cells was cultured for an additional 28 h in 3 ml serum-free medium supplemented with 0.1 mg/ml Albumax I (Gibco BRL; Life Technologies) and 2.5 mM CaCl_2 .

FV antigen measurements in media

Conditioned media were collected in prechilled tubes and centrifuged for 10 minutes at 200g to remove cells and cell debris. The supernatant was aliquoted and stored at -80°C . The Factor V antigen levels in the media were determined by a FV ELISA kit (Kordia, The Netherlands). ELISA standard curves were constructed with normal pooled plasma from 81 healthy individuals.

RESULTS

Overall characteristics of the MD simulations

MD simulations were performed on the C2 domain of the WT and of the D2194G mutant with or without the C2038-C2193 S-S bond. The minimization procedure that was applied to relax the structures yielded conformations with root-mean-square deviations (RMSD) for heavy atoms between the initial WT x-ray structure and the minimized structure of 0.2 Å. With regard to MD, for all studied structures, the potential energy decreased during the first 150 ps of the simulation and then stabilized. Therefore, the entire production time interval (900 ps) was considered for the MD analysis. For the wild-type, the average RMS deviations for the C α atoms relative to the x-ray structure over the last 900 ps are 1.67 Å and 1.75 Å, in the presence or absence of the S-S bridge, respectively. For the mutant D2194G with or without the disulfide bond, RMSD were 1.86 Å and 1.76 Å, respectively. Similar RMSD for the simulated structures indicate that the mutation D2194G and the removal of the disulfide bridge do not result in large structural rearrangements (see below).

Figure 1A shows plots of the potential energy calculated over the trajectories. The higher potential energy observed for both mutant forms (irrespective of the presence of the S-S bond) suggests that the D2194G mutation has destabilizing effects (see below). Figs.1B and 1C show the Coulombic and GB solvation energies of both WT forms (WT with or without S-S bond) calculated during the MD trajectories. It was previously proposed by Spassov et al. (1994) that proteins containing no disulfide bonds are optimized electrostatically as compared to molecules possessing disulfide bridges. It is interesting to note that breaking the S-S-bond in the FV C2 domain leads to lower Coulombic energies during the MD indicating optimization of electrostatic interactions. The lack of S-S bond apparently facilitates reorientation of charged residues and formation of stronger favorable charge-charge interactions. In contrast, more favorable Coulombic interactions lead to less-favorable solvation energies. This can be seen from the GB energies, which are higher for the WT when the structure does not have the S-S bond. The van der Waals interactions (not shown) were similar for all trajectories and remained stable during the MD simulations.

FIGURE 1: Energetic values obtained during the MD trajectories.
(A) Potential energy versus the time. The WT C2 domain is shown in black; WT without S-S bond, blue; D2194 mutant, red; and D2194G mutant without S-S bond, magenta. (B) Coulombic energy versus the time. The WT C2 domain is shown in black; WT without S-S bond, blue. (C) GB solvation energy versus the time. The WT C2 domain is shown in black; WT without S-S bond, blue.

Structural and flexibility differences between x-ray and simulated WT

The root mean square fluctuations (RMSF) for the C α atoms of the C2 domain per residue (sampled over 900 structures) were calculated. In Fig. 2A the fluctuations of the simulation WT structures with and without S-S bond and the B factors (C α atoms) of the C2 x-ray structure are presented.

The calculated fluctuations of simulated WT (with S-S bond) are in good agreement with the B factors. The zones of greatest flexibility in the simulated and crystal structures tend to be similar but some differences are also noted. The loop 2140-2150 is very flexible in the WT-simulated and crystal structures. The membrane binding loop (residues 2060 to 2067 including two key Trp residues 2063 and 2064) and residues 2130-2139 appear to be more flexible in the crystal than in the WT-simulated structure. Some differences between the B-factors of the crystal and calculated RMSF for the WT-simulated structures are also seen for the region 2152-2162 that is found to be more rigid in the x-ray structure.

No significant structural differences are present between the x-ray and averaged simulation structure of the WT C2 domain (not shown). Important changes occur only for the loop 2060-2067 involved in membrane binding, which appears to be flexible (i.e., it adopts two different conformations in the crystal structures) (Macedo-Ribeiro et al., 1999). Some conformational changes also take place at the level of the 2074-2080-loop, which is very flexible in both, the x-ray and simulated WT structures.

A

B **C**

FIGURE 2: Root mean square fluctuations (RMSF) computed over the last 900 ps of the MD simulations. (A) RMSF of the simulated WT structures with (black) and without (blue) S-S bond and the B factors (C^α atoms) of the C2 x-ray structure (red). (B) RMSF of the simulated mutant D2194G structures with (black) and without (dotted line) S-S bond. (C) Ribbon diagram for the averaged modeled structure of the D2194G mutant over the last 900 ps of the MD simulations color coded according to the calculated RMSF.

Structural and flexibility differences between WT and mutant D2194 with S-S bond

Fig. 2B shows the fluctuations of modeled mutant D2194G with or without the disulfide bridge. In Fig. 2C the averaged modeled structure for the D2194G mutant over the last 900 ps of the MD simulations is given color coded according to the calculated RMSF. Comparisons of the flexibilities in the modeled WT and the mutant indicate that the regions 2075-2085 and 2140-2150 are flexible in both WT and D2194G mutant (Fig. 2). The loop 2042-2053, situated nearby the mutated residue, is more flexible in the mutant structure. The 2060-2067 loop also becomes more flexible in the D2194G mutant. This is possibly caused by the increased mobility of the loop 2042-2053.

FIGURE 3: Ribbon diagram of the WT and D2194G C2 domain. The last structures obtained in the simulations (with S-S bond) are shown and key regions are labeled (see text).

Fig. 3 shows the last structure obtained from the simulation for the WT protein domain and the D2194G mutant (S-S bond present). These structures can be considered as representative of the MD simulations in the area of residue 2194 since the calculated electrostatic stabilization energies of a set of sampled MD conformations (see below) are similar to those calculated for the last MD structure. The regions showing structural changes upon the D2194G substitution (in the presence of S-S bond) involve the loop 2130-2139 and the C-terminal residues (the N-term residues remain essentially unchanged). The

D2194G mutation does not affect the dynamical properties of these two regions, which do not appear flexible in the simulated WT and D2194G mutant structures (Fig. 2), but the mutation rather causes conformational changes. In the last simulation structures (with S-S bond, Fig. 3), the loop 2130-2139 is opened in the mutant as compared to the WT, the C α of N2132 shifts by about 3 Å.

We also noted that upon mutation of D2194, the nonflexible C-terminus (Tyr2196) inserts into the structure of the domain, attempting to fill the hole created by the D2194 to G substitution, probably also trying to compensate for the missing negative charge of this mutant. These events could lead to enhanced flexibility of the loop 2042-2053 and to conformational changes of the 2130-2139 region.

Structural and flexibility differences between structures with or without disulfide bond

It is evident from Figs. 2A and 2B that the N-terminal (not charged in the simulation since it is *in vivo* connected to the C1 domain) and C-terminal regions of the WT- and mutant D2194G modeled structures become extremely mobile in the absence of the disulfide bond. As a result, important conformational changes occur at both termini. Also, the flexibility of the 2087-2093 and 2113-2121 loops in the WT is enhanced upon removal of the S-S bridge. Loss of the S-S bond also results in a decreased mobility and in structural changes of regions 2152-2162 in both the simulated WT and D2194G mutant. The observed changes in the dynamical properties of C2 domain occurring upon S-S bond disruption could influence the folding process.

Electrostatic contribution to the stability of the triad K2101-K2103-D2194/K2101-K2103-G2194

The electrostatic free energies $\Delta\Delta G^{\text{tot}}$ of the triads K2101, K2103, D2194 in the WT and K2101, K2103, G2194 in the mutant, relative to their corresponding hydrophobic isoesters were computed only for the structures possessing the S-S bond. The electrostatic stabilization of the triad, taking into account the mean proton occupancies at pH 7, calculated first on the x-ray structure and on the x-ray structure with simple D2194G substitution without simulations are given in Table 1. The same calculations were also carried out on the last conformations of the MD simulations for the WT and the D2194G mutant, as well as on 30 structures sampled from the MD trajectories from 670 to 970 ps of the production run (one structure selected every 10 ps). The averaged energies for the 30 MD conformations and the term contributing to $\Delta\Delta G^{\text{tot}}$ with standard deviations are reported in Table 1.

	$\Delta\Delta G^{\text{tot}}$		$\Delta\Delta G^{\text{sol}}$		*
	WT	mutant	WT	mutant	
X-ray	-2.13	+0.93	+1.85	+0.72	
MD: last conformation	-6.34	-4.46	+9.41	+5.48	
MD: 30conformations	-6.81+0.7*	-4.64+1.0	+9.83+1.1	+4.56+0.4	

	$\Delta\Delta G^{\text{triad}}$		$\Delta\Delta G^{\text{pr}}$		*
	WT	mutant	WT	mutant	
	-3.38	+1.23	-0.60	-1.02	
	-10.62	-0.91	-5.13	-9.03	
	-11.46+1.2	-0.68+0.5	-5.17+0.95	-8.52+0.9	

TABLE 1: Electrostatic free energy contributions of the triad to the protein stability (kcal/mol): K2101/K2103/D2194 versus K2101/K2103/G2194

Additional computations of the electrostatic stabilization of the triad 2101, 2103, 2194 and the pKa values of titratable groups were carried out for the mutants D2194K, K2101E/K2103E and K2101E/K2103E/D2194K. The stabilization contributions and mean charges at pH 7 of the triad residues are given in Table 2.

Mean charge at pH 7 of the triad:

Mutant	2101	2103	2194	$\Delta\Delta G^{\text{tot}}$
D2194K	0.00	0.98	1.00	+3.46
K2101E/K2103E/D2194K	-0.99	0.00	1.00	-0.31
K2101E/K2103E	-0.68	0.00	-0.86	+1.97

TABLE 2: Mutants D2194K, K2101E/K2103E/D2194K and K2101E/K2103E

The $\Delta\Delta G$ energies are well optimized for the structures sampled from the MD simulations, relative to the x-ray structure. The electrostatic energies obtained from averaging over 30 conformations were similar to those for the final simulations structures of the WT and the mutant and the relatively small

standard deviations confirm that the structures in the region of the mutation are very similar in the last 300 ps of the simulations.

In the simulated structures (30 conformations) strong favorable charge-charge interactions for the triad ($\Delta\Delta G^{\text{triad}}$) were obtained for the WT (-11.46 kcal/mol), whereas in the mutant no significant electrostatic interactions were calculated. In all structures no large differences between the simulated WT and the mutant were found for electrostatic interactions of the triad with the rest of the protein $\Delta\Delta G^{\text{pr}}$. The averaged electrostatic stabilization due to the interactions between the triad and the protein varies between -5.14 and -8.52 kcal/mol in the WT and mutant, respectively. As can be expected, the presence of three partially buried ionized amino acid residues causes larger unfavorable desolvation energy $\Delta\Delta G^{\text{sol}}$ in the WT (+9.83 kcal/mol) compared to the mutant (+4.56 kcal/mol).

The electrostatic stabilization values calculated for the triad 2101, 2103, 2194 in the mutants D2194K, K2101E/K2103E, K2101E/K2103E/D2194K demonstrate the destabilizing effect of these mutations in comparison to electrostatic stabilization energy of the triad in WT computed on the x-ray structure.

Mutagenesis

To investigate the importance of the charged triad K2101, K2103 and D2194 and of the S-S bond, several mutant FV molecules were created. These mutagenesis experiments were also performed to probe the tolerance of this region of FV to amino acid substitution. To ensure that any effects observed on expression levels of the recombinant proteins were not due to altered glycosylation of the C2 domain, which *per se* is a modulator of FV expression levels (Nicolaes et al., 1999), all triad mutations were constructed using a template that resulted in the production of only the FV2 isoform (lacking a glycan structure at position N2181). This construct will subsequently be called WT*. The effect of the presently studied FV mutations on the final levels of FV expression in COS-1 cells, which do not express endogenous FV, were quantitated by FV-ELISA. Results were corrected for variations in transfection efficiency by cotransfecting the pMT2-FV with a pEGFP-C1-lamin vector as described in the Materials and Methods section.

FIGURE 4: Transient expression of FV proteins in serum-free culture medium.

COS-1 cells were transiently transfected to express WT-FV or the FV mutants. FV antigen levels were measured in the culture media by ELISA. Bars represent the means $\pm$ standard deviations ($n = 6$). The mean value of WT-FV was 23.8 ng/35 mm well and is assigned to be 100% in this figure. This WT-FV construct was used as a template for the D/G mutation and to construct the WT* plasmid (which only expresses the FV2 isoform). There was no significant difference between the expression levels of the WT FV-plasmid and the WT* plasmid, the WT* plasmid serving as a template for the other FV-mutants (nomenclature: D2194G (D/G), D2194K (D/K), K2101E/K2103E (KK/EE), K2101E/K2103E/D2194K (DKK/KEE), and C2038A/C2193A (CC/AA)).

FV antigen level in serum-free media, conditioned for 28 hours in cells expressing the WT FV-plasmid, ranged from 17.6 ng to 26.9 ng/35 mm well ($n=6$). In conditioned media of cells transfected with the pMT2/FV-D2194G plasmid, the FV antigen level was 53% as compared to WT (Fig. 4). This is in line with previous observations by Yamazaki et al. (2002), who found that the D2194G mutation is the major determinant of low expression levels within the R2-polymorphism (Yamazaki et al., 2002). Compared to WT, a seven- to eightfold reduction in FV expression levels was observed for the mutants D2194K, K2101E/K2103E, K2101E/K2103E/D2194K and C2038A/C2193A (Fig. 4). Together these data indicate that mutations in the C2-domain of FV that interfere with the charged triad D2194, K2101 and K2103 lower the amount of FV secreted by the cells. Mutation of the S-S bridge results in decreased final FV expression. However, expression of the mutants studied was not completely inhibited by these mutations as expression levels $>13\%$ were achieved for all constructs.

DISCUSSION

Recently, it has been proposed that ~80 % of missense mutations associated with disease states are amino acid substitutions that affect the stability of the proteins by about ~1-3 kcal/mol (Wang and Moulton, 2001). Stability problems can be due to loss of hydrogen bonds, of S-S bonds, overpacking, backbone strain and/or burial of charged residues. *In silico* strategies can be used to investigate the potential roles of a mutation on, for instance, protein stability.

In the present study, MD simulations (970 ps) were executed on the C2 domain of WT FV and the D2194G mutant that is present in carriers of the FV-R2 gene. Because this mutation could potentially act on the formation of the nearby C2038-C2193 S-S bond and/or the conformation of the N-term/C-term residues, simulations of the WT and D2194G S-S bondless structures were compared. Electrostatic computations were performed to investigate the energetics of the WT K2101-K2103-D2194 triad and of several mutants. Mutagenesis experiments and secretion levels were also used to probe further the 2194 region of the FV C2 domain (mutants were: D2194G, D2194K, K2101E/K2103E, K2101E/K2103E/D2194K, C2038A/C2193A) because observed secretion efficiencies often relate to mutant stability *in vitro*. These experimental data were correlated with theoretical work while trying to include in our reasoning the fact that protein engineering methods usually probe several physical contributions at the same time (e.g., electrostatic, steric and entropic) (Lounnas and Wade, 1997) while theoretical investigations may not.

A key step after MD computations is to evaluate the accuracy of the calculations, through for instance evaluation of RMSD, potential energy versus time and others (Figs. 1 and 2). Yet, while MD simulations with implicit solvation models have been found of reasonable accuracy, some authors suggest that despite low RMSD between the simulated structures and the starting x-ray model, the conformations of the simulated molecules are of poor quality (Simonsen T., 2001). We thus investigated several structures of the simulated WT with the energy strain utility included in the ICM package (Molsoft) (Maierov and Abagyan, 1998). The simulated WT and mutant C2 domains had similar structural features as the starting high resolution x-ray structure. Secondary structure contents in the starting x-ray and simulated structures (WT or mutant) computed using the method of Kabsch and Sander (1983) were similar. Furthermore, the protein core of the WT or mutant C2 domain remained stable during the simulations performed on structures in the absence of the S-S bond, despite increased flexibility/conformational changes in some regions of the modeled structures (Figs. 2 and 3). These results as well as the low RMSD

calculated between the x-ray and modeled structures together with the ICM evaluations confirm that the implicit solvation method and simulation protocol used here are appropriate.

It has been shown that physical energy functions similar to the one used in this study can identify native-like structure from misfolded protein models and that low quality 3D structures tend to unfold during a simulation (Dominy and Brooks, 2002; Feig and Brooks, 2002). Although we did not observe unfolding of the D2194G mutant we noted that its total potential energy (with or without S-S bond) is significantly higher than the one of the WT (Fig. 1). Thus, in this specific case, investigation of the potential energy suggests overall destabilization of the protein due to the mutation.

We then analyzed trajectories obtained for the WT C2 structure (with or without S-S bond). It is known that disulfide bonds in proteins can influence the stability as well as the folding pathway and the efficiency of folding (Kowalski et al., 1998; Omura et al., 1992; Parodi 2000). Generally, loss of a native disulfide bond reduces stability and such mutant proteins may not be able to fold, secretion can be impeded or delayed. Yet, in some situations, removal of S-S bonds does not seem to affect the 3D structure of a protein nor its function. In the simulation of the WT C2 domain with or without S-S-bond, the protein core remained stable. However, the increased mobility of the two termini, in the absence of S-S bond could impair correct folding/stability of the domain and damage interaction with the C1 domain. The WT protein without S-S bond does not express well, suggesting folding/stability problems. Similar folding/stability/expressions problems were also noticed when removing S-S bond(s) in retinoschisin, a discoidin-domain containing protein (Wu and Molday, 2003).

The potential energies of the WT C2 domain (with or without S-S bond) are similar and one would not expect folding/stability problems, but S-S bond depleted 3D structures have to be analyzed with special care. It appears that removing the disulfide bond in the FV C2 domain facilitates optimization of some energy terms (thus lower the total potential energy). This hypothesis is supported by the observation that electrostatic interactions are optimized in proteins that do not contain S-S bond as compared to molecules containing S-S bonds (Spasov et al., 1994). Thus, potential energies for the WT C2 domain with or without S-S bond can not be directly correlated with expression levels. In addition, it can be noticed in the WT and D2194G simulations that the region 2152-2162 is less flexible in the structure without the S-S bond. This is consistent with previous MD simulations showing that removal of an S-S bond can decrease the flexibility of large portions of a protein (Rizzuti et al., 2001).

The protein core of the D2194G mutant with or without the S-S bond remained also stable during the simulation. However, some regions become more rigid or more flexible due to the mutation (Fig. 2). For the mutant protein without the S-S bond we observe significantly higher flexibilities within the N-term residues as compared to the WT (simulation without S-S bond). This suggests potential folding/stability problems and/or damaged interaction with the C1 domain. Also it is probable that the increased flexibility of the N-term residues due to the D2194G mutation affects the formation of the nearby S-S bond. A possible *in vivo* scenario could be that mutant molecules (either D2194G or S-S bond free structure) could be marginally stable and could equilibrate between the folded state and an ensemble of unfolded conformations. This latter population could then be recognized inside the ER, be partially retained and degraded. This hypothesis would be in fact in good agreement with the observed low expression levels of these mutants.

	2101	2103(buried)	2194
	*	*	*
hFV_C2	: ...ANNKQWLEIDLL	KIK KITAIIT.....	LELFGCDIY
bFV_C2	: ...ANNNNQWLQIDLL	KIK KITAIVT.....	LELFGCDMY
pigFV_C2	: ...ANNNNQWLQIDLL	KIK KITAIIT.....	LELFGCDIY
hFVIII_C2	: ...VNNPKEWLQVDFQ	KTM KVTGVTT.....	MEVLGCEAQ
mouseFVIII_C2	: ...VNDPKQWLQVDLQ	KTM KVTGIIT.....	LEILGCEAQ
pigFVIII_C2	: ...VSSAEEWLQVDLQ	KTV KVTGITT.....	LEVLGCEAQ

TABLE 3: Multiple sequence alignment in the area of FV residue 2194 and surrounding area

FV C2 domain residue D2194 is involved in electrostatic interactions with the side chains of K2101 and K2103. Conservation (full or partial) of ionic interaction in this region of FV or FVIII suggests that these salt-bridges could be important for the stability/structure of the C2 domain (Table 3). It has been noted that conserved salt bridges important for the stability of proteins are often buried (Schueler and Margalit, 1995) whereas in the C2 domain, the 2101-2103-2194 triad is partially exposed. However, surface salt bridges can also be important for protein stability (Strop and Mayo, 2000; Dong and Zhou, 2002). In fact, salt bridges can be stabilizing or destabilizing depending on their environment (Kumar and Nussinov, 2001; Kumar and Nussinov, 1999; Schutz and Warshel, 2001). In the present study, we assumed that the D2194G

mutation could alter the stability of the FV C2 domain. Comparison of the electrostatic free energies ($\Delta\Delta G^{\text{tot}}$) of the 2101-2103-2194 triad between the WT FV C2 domain and the D2194G mutant shows electrostatic destabilization due to the mutation by ~ 3 kcal/mol. This value is in the range of what has been observed by Wang and Moulton (2001). Therefore, we suggest that the D2194G mutation destabilizes the domain and that this modified structure could be detected by the proofreading apparatus inside the cells.

To understand better the reactions taking place at the level of residue 2194, other mutants were created *in vitro* and *in silico*. Changing the distribution of charged groups in the 2194 area could underline the importance of the salt-bridge network and could provide information about tolerance to amino acid substitutions. Computations of electrostatic energies of the 2101-2103-2194 triad in the mutants D2194K (D/K, thus 3 expected positive charges), K2101E/K2103E (KK/EE, thus 3 expected negative charges), K2101E/K2103E/D2194K (DKK/KEE, modification of the distribution of charges) calculated on the rigid structures (see Table 2) also show destabilization effects in comparison to the WT (x-ray structure). The D2194K, K2101E/K2103E, K2101E/K2103E/D2194K mutants show lower expression levels than the D2194G mutant. These experimental data correlate well with the calculated electrostatic destabilizations excluding the K2101E/K2103E/D2194K (DKK/KEE) mutant. Generally, it is expected that mutations leading to the positioning of three negatively or positively charged groups close in space will be destabilizing, as observed here in the case of the D2194K (D/K) and K2101E/K2103E (KK/EE) substitutions (Table 2). These mutants are thus predicted to be less stable than the D2194G mutant and this correlates well with the experimental expression levels. On the other hand, we initially hypothesized that the mutant K2101E/K2103E/D2194K (DKK/KEE) would form a stabilizing salt-bridge triad that could mimic the WT protein. However, the calculated proton occupancies at pH 7 show that 2103 is not charged while in the WT the three triad residues are ionized. This indicates that the distribution of charges in the K2101E/K2103E/D2194K mutant is not favorable as compared to the one of the WT. Although $\Delta\Delta G^{\text{tot}}$ suggests that the mutated triad is slightly stabilizing, apparently, in this case, additional structural features have to be integrated in the analysis.

In summary, the D2194G substitution in the C2 domain seems to electrostatically destabilize the structure, as shown by computer calculations. Low expression levels were observed for this mutant, suggesting also potential stability and/or folding problems. Our experimental and MD/electrostatic data shows that the right distribution of charges in the 2194 region of the FV C2

domain is essential to the stability and the presence of a S-S bond is required for the stability/folding of this essential coagulation protein.

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REFERENCES

- Antosiewicz, J., J.A. McCammon, and M. Gilson. 1994. Prediction of pH-dependent properties of proteins. *J. Mol. Biol.* 238: 415-436
- Antosiewicz, J., and D. Porschke. 1989. The nature of protein dipole moments: experimental and calculated permanent dipole of α -chymotrypsin. *Biochemistry* 28:10072-10078
- Barril, X., C. Aleman, M. Orozco, and F.J. Luque. 1998. Salt bridge interactions: stability of the ionic and neutral complexes in the gas phase, in solution, and in proteins. *Proteins* 32:67-79
- Bashford D., and D.A. Case. 2000. Generalized born models of macromolecular solvation effects. *Annu. Rev. Phys. Chem.* 51:129-152
- Bashford, D., and M. Karplus. 1990. The pKa's of ionizable groups in proteins: atomic detail from a continuum electrostatic model. *Biochemistry* 29:10219-10225
- Baumgartner, S., K. Hofmann, R. Chiquet-Ehrismann, and P. Bucher. 1998. The discoidin domain family revisited: new members from prokaryotes and a homology-based fold prediction. *Protein Sci.* 7:1626-1631
- Berman, H.M., J. Westbrook, Z. Feng, G. Gilliland, T.N. Bhat, H. Weissig, I.N. Shindyalov, and P.E. Bourne. 2000. The Protein Data Bank. *Nucleic Acids Res.* 28: 235-242
- Bernardi, F., E.M. Faioni, E. Castoldi, B. Lunghi, G. Castaman, E. Sacchi, and P.M. Mannucci. 1997. A factor V genetic component differing from factor V R506Q contributes to the activated protein C resistance phenotype. *Blood* 90:1552-1557
- Brooks, B.R., R.E. Bruccoleri, B.D. Olafson, D.J. States, S. Swaminathan, and M. Karplus. 1983. CHARMM: A program for macromolecular energy, minimization and dynamics calculations. *J. Comp. Chem.* 4:187-217
- Bross, P., T.J. Corydon, B.S. Andresen, M.M. Jørgensen, L. Bolund, and N. Gregersen. 1999. Protein misfolding and degradation in genetic diseases. *Hum. Mutat.* 14:186-198
- Castoldi, E., J. Rosing, D. Girelli, L. Hoekema, B. Lunghi, F. Mingozzi, P. Ferraresi, S. Friso, R. Corrocher, G. Tans, and F. Bernardi. 2000. Mutations in the R2 FV gene affect the ratio between the two FV isoforms in plasma. *Thromb. Haemost.* 83:362-365
- Cregut D., and L. Serrano. 1999. Molecular dynamics as a tool to detect protein foldability. A mutant of domain B1 of protein G with non-native secondary structure propensities. *Protein Sci.* 8:271-282
- Davis, M.E., J.D. Madura, B.A. Luty, and J.A. McCammon. 1991. Electrostatics and diffusion of molecules in solution: Simulations with the University of Houston Brownian Dynamics program. *Comput. Phys. Commun.* 62:187-197
- Dominy, B., and C. Brooks. 1999. Development of a generalized born model parametrization for proteins and nucleic acids. *J. Phys. Chem. B* 103:3765-3773
- Dominy, B., and C. Brooks. 2002. Identifying native-like protein structures using physics-based potentials. *J. Comput. Chem.* 23:147-160
- Dong, F., and H.-X. Zhou. 2002. Electrostatic contributions to T4 lysozyme stability: solvent-exposed charges versus semi-buried salt bridges. *Biophysical J.* 83:1341-

- Feig, M., and C. Brooks. 2002. Evaluating CASP4 predictions with physical energy evaluations. *Proteins* 49:232-245
- Gilson, M., and B. Honig. 1988. Calculation of the total electrostatic energy of a macromolecular system: solvation energies, binding energies, and conformational analysis. *Proteins* 4:7-18
- Hendsch, Z.S., and B. Tidor. 1994. Do salt bridges stabilize proteins? A continuum electrostatic analysis. *Protein Sci.* 3:211-226
- Hilser, V.J., D. Dowdy, T.G. Oas, and E. Freire. 1998. The structural distribution of cooperative interactions in proteins: analysis of the native state ensemble. *Proc. Natl. Acad. Sci. USA* 95:9903-9908
- Hoekema, L., G.A.F. Nicolaes, H.C. Hemker, G. Tans, and J. Rosing. 1997. Human Factor Va₁ and Factor Va₂: Properties in the Procoagulant- and Anticoagulant Pathways. *Biochemistry* 36:3331-3335
- Kabsch, W., and C. Sander. 1983. Dictionary of protein secondary structure: pattern recognition of hydrogen-bonded and geometrical features. *Biopolymers* 22:2577-2637
- Kazmirski, S.L., D.O. Alonso, F.E. Cohen, S.B. Prusiner, and V. Daggett. 1995. Theoretical studies of sequence effects on the conformational properties of a fragment of the prion protein: implications for scrapie formation. *Chem. Biol.* 2:305-315
- Kim, S.W., M.A. Quinn-Allen, J.T. Camp, S. Macedo-Ribeiro, P. Fuentes-Prior, W. Bode, and W.H. Kane. 2000. Identification of functionally important amino acid residues within the C2-domain of human factor V using alanine-scanning mutagenesis. *Biochemistry* 39:1951-1958
- Kowalski, J.M., R. Parekh, and K.D. Wittrup. 1998. Secretion efficiency in *Saccharomyces cerevisiae* of Bovine Pancreatic Trypsin Inhibitor mutants lacking disulfide bonds is correlated with thermodynamic stability. *Biochemistry* 37:1264-1273
- Kumar, S., and R. Nussinov. 1999. Salt bridge stability in monomeric proteins. *J. Mol. Biol.* 293:1241-1255
- Kumar, S., and R. Nussinov. 2001. Fluctuations in ion pairs and their stabilities in proteins. *Proteins* 43:433-454
- Lounnas, V., and R.C. Wade. 1997. Exceptionally stable salt bridges in cytochrome P450cam have functional roles. *Biochemistry* 36:5402-5417
- Lunghi, B., L. Iacoviello, D. Gemmati, M.G. Dilasio, E. Castoldi, M. Pinotti, G. Castaman, R. Redaelli, G. Mariani, G. Marchetti, and F. Bernardi. 1996. Detection of new polymorphic markers in the factor V gene: association with factor V levels in plasma. *Thromb. Haemost.* 75:45-48
- Macedo-Ribeiro, S., W. Bode, R. Huber, A. Quinn-Allen, W. Kim, G.P. Bourenkov, H. Bartunik, M.T. Stubbs, W.H. Kane, and P. Fuentes-Prior. 1999. Crystal Structures of the Membrane-Binding C2 Domain of Coagulation Factor V. *Nature* 402:434-439
- Maierov, V., and R. Abagyan. 1998. Energy strain in three-dimensional protein structures. *Fold. Des.* 3:259-269

- Mann, K.G., and M. Kalafatis. 2003. Factor V: a combination of Dr Jekyll and Mr Hyde. *Blood* 101:20-30
- Nicolaes, G.A., and B. Dahlback. 2002. Factor V and thrombotic disease: description of a janus-faced protein. *Arterioscler. Thromb. Vasc. Biol.* 22:530-538
- Nicolaes, G.A.F., B.O. Villoutreix, and B. Dahlbäck. 1999. Partial glycosylation of Asn²¹⁸¹ in human factor V as a cause of molecular and functional heterogeneity. Modulation of glycosylation efficiency by mutagenesis of the consensus sequence for N-linked glycosylation. *Biochemistry* 38:13584-13591
- Nicolaes, G.A., B.O. Villoutreix, and B. Dahlback. 2000. Mutations in a potential phospholipid binding loop in the C2 domain of factor V affecting the assembly of the prothrombinase complex. *Blood Coagul. Fibrinolysis* 11:89-100
- Omura, F., M. Otsu, and M. Kikuchi. 1992. Accelerated secretion of human lysozyme with a disulfide bond mutation. *Eur. J. Biochem.* 205:551-559
- Parodi, A.J. 2000. Role of N-oligosaccharide endoplasmic reticulum processing reactions in glycoprotein folding and degradation. *Biochem. J.* 348:1-13
- Pellequer, J.L., A.J. Gale, J.H. Griffin, and E.D. Getzoff. 1998. Homology models of the C domains of blood coagulation factors V and VIII: a proposed membrane binding mode for FV and FVIII C2 domains. *Blood Cells Mol. Dis.* 24:448-461
- Pratt, K.P., B.W. Shen, K. Takeshima, E.W. Davie, K. Fujikawa, and B.L. Stoddard. 1999. Structure of the C2 domain of human factor VIII at 1.5 Å resolution. *Nature* 402:439-442
- Rizzuti, B., L. Sportelli, and R. Guzzi. 2001. Evidence of reduced flexibility in disulfide bridge-depleted azurin: a molecular dynamics simulation study. *Biophys. Chem.* 94:107-120
- Rosing, J., H.M. Bakker, M.C. Thomassen, H.C. Hemker, and G. Tans. 1993. Characterization of two forms of human factor Va with different cofactor activities. *J. Biol. Chem.* 268:21130-21136
- Schiffer, C.A., and W.F. van Gunsteren. 1996. Structural stability of disulfide mutants of basic pancreatic trypsin inhibitor: a molecular dynamics study. *Proteins* 26:66-71
- Schueler, O., and H. Margalit. 1995. Conservation of salt bridges in protein families. *J. Mol. Biol.* 248:125-135
- Schutz, C.N., and A. Warshel. 2001. What are the dielectric "constants" of proteins and how to validate electrostatic models? *Proteins* 44:400-417
- Sharp, K.A., and B. Honig. 1990. Electrostatic interactions in macromolecules: theory and applications. *Annu. Rev. Biophys. Biophys. Chem.* 19:301-332
- Sharp, K., A. Nicholls, R. Friedman, and B. Honig. 1991. Extracting hydrophobic free energies from experimental data: Relationship to protein folding and theoretical models. *Biochemistry* 30:9686-9697
- Simonson, T. 2001. Macromolecular electrostatics: continuum models and their growing pains. *Curr. Opin. Struct. Biol.* 11:243-252
- Sinha, N., and R. Nussinov. 2001. Point mutations and sequence variability in proteins: redistributions of preexisting populations. *Proc. Natl. Acad. Sci. USA* 98:3139-3144
- Spassov, V.Z., A.D. Karshikoff, and R. Ladenstein. 1994. Optimization of the electrostatic interactions in proteins of different functional and folding type.

Protein Sci. 3:1556-1569

- Strop, P., and S.L. Mayo. 2000. Contribution of surface salt bridges to protein stability. *Biochemistry* 39:1251-1255
- Tanford, C., and J.G. Kirkwood. 1957. Theory of protein titration curves. I. General equations for impenetrable spheres. *J. Am. Chem. Soc.* 79:5333-5339
- Taverna, D.M., and R.A. Goldstein. 2002. Why are proteins so robust to site mutations? *J. Mol. Biol.* 315:479-484
- Van Der Neut Kolfschoten, M., R.J. Dirven, H.L. Vos, and R.M. Bertina. 2003. The R2-haplotype associated Asp2194Gly mutation in the light chain of human factor V results in lower expression levels of FV, but has no influence on the glycosylation of Asn2181. *Thromb. Haemost.* 89:429-437
- Villoutreix BO, Bucher P, Hofmann K, Baumgartner S, Dahlbäck B. 1998. Molecular models for the two discoidin domains of human blood coagulation factor V. *J. Mol. Model.* 4:268-275
- Villoutreix, B.O. 2002. Structural bioinformatics: methods, concepts and applications to blood coagulation proteins. *Curr. Protein Pept. Sci.* 3:341-364.
- Wang, Z., and J. Moul. 2001. SNPs, Protein Structure , and Disease. *Hum. Mutat.* 17:263-270
- Warwicker, J., and H.C. Watson. 1982. Calculation of the electric potential in the active site cleft due to α -helix dipoles. *J. Mol. Biol.* 157:671-679
- Wesson, L., and D. Eisenberg. 1992. Atomic solvation parameters applied to molecular dynamics of proteins in solution. *Protein Sci.* 1:227-235
- Wickner, S., M.R. Maurizi, and S. Gottesman. 1999. Posttranslational quality control: Folding, refolding and degrading proteins. *Science* 286:1888-1893
- Wu, W.W., and R.S. Molday. 2003. Defective discoidin domain structure, subunit assembly and ER processing of retinoschisin are primary mechanisms responsible for X-linked retinoschisis. *J. Biol. Chem.* In press.
- Yamazaki, T., G.A.F. Nicolaes, K.W. Sørensen, and B. Dahlbäck. 2002. Molecular basis of quantitative factor V deficiency associated with factor V R2 haplotype. *Blood* 100:2515-2521
- Yang, A.-S., M. Gunner, R. Sampogna, K. Sharp, and B. Honig. 1993. On the calculation of pKas in proteins. *Proteins* 15:252-265

CHAPTER 3

Impaired APC cofactor activity of factor V plays a major role in the APC resistance associated with the factor V Leiden (R506Q) and R2 (H1299R) mutations

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SUMMARY

APC resistance is a major risk factor for venous thrombosis. Factor V (FV) gene mutations like FV_{Leiden} (R506Q) and FV_{R2} (H1299R) may cause APC resistance either by reducing the susceptibility of FVa to APC-mediated inactivation or by interfering with the cofactor activity of FV in APC-catalysed FVIIIa inactivation. We quantified the APC-cofactor activity expressed by FV_{Leiden} and FV_{R2}, and determined the relative contributions of reduced susceptibility and impaired APC-cofactor activity to the APC resistance associated with these mutations. Plasmas containing varying concentrations of normal FV, FV_{Leiden} or FV_{R2} were assayed with the Immunochrom[®] APC Response test, which specifically measures the APC-cofactor activity of FV, and with the Coatest[®] APC[™] Resistance assay, which probes both the susceptibility and APC-cofactor components. FV_{R2} expressed 73% of the APC-cofactor activity of normal FV, whereas FV_{Leiden} exhibited no cofactor activity in FVIIIa inactivation. Poor susceptibility to APC and impaired APC-cofactor activity contributed equally to FV_{Leiden}-associated APC resistance, while FV_{R2}-associated APC resistance was entirely due to the reduced APC-cofactor activity of FV_{R2}. Thrombin generation assays confirmed the importance of the anticoagulant activity of FV and indicated that FV_{Leiden} homozygotes are exposed to a higher thrombotic risk than heterozygotes because their plasma lacks normal FV acting as an anticoagulant protein.

INTRODUCTION

The protein C pathway is a major anticoagulant mechanism that down-regulates the prothrombin- and intrinsic factor X (FX)-activating complexes *via* inactivation of their respective cofactors activated factors V (FVa) and VIII (FVIIIa) ¹. Cofactor inactivation occurs by limited proteolysis at amino acid positions 306, 506 and 679 in FVa ², and 336, 562 and 740 in FVIIIa ³. These reactions are catalysed by the serine protease activated protein C (APC) and stimulated by the APC-cofactor protein S. *In vitro* experiments using purified proteins have shown that FV also stimulates the APC-mediated inactivation of FVIIIa ⁴⁻⁶. In order to express full APC-cofactor activity, FV must retain (part of) the B domain ^{4,6,7} and be cleaved by APC at Arg⁵⁰⁶ ⁸. Although this anticoagulant function of FV is still poorly characterized, a recent report suggests that it plays a critical anti-thrombotic role *in vivo* ⁹.

Functional defects of the protein C pathway, due to inherited or acquired conditions, determine a plasma phenotype known as APC resistance ¹⁰, which is a prevalent and important risk factor for venous thrombosis ¹¹⁻¹³. A plasma is termed APC resistant when the addition of exogenous APC fails to prolong its clotting time in an aPTT assay ¹⁰. Since the discovery of APC resistance, several other methods have been developed to detect this condition and to study the underlying molecular mechanism.

To date, a few FV gene mutations have been identified in association with APC resistance. In principle, these mutations may cause APC resistance either by reducing the susceptibility of FVa to APC-mediated inactivation (susceptibility component) or by interfering with the APC-cofactor activity of FV in FVIIIa inactivation (APC-cofactor activity component). The FV R506Q mutation (FV_{Leiden} ¹⁴) is the major cause of hereditary APC resistance in the Caucasian population. Due to the loss of the APC-cleavage site at Arg⁵⁰⁶, FV(a)_{Leiden} is less susceptible to inactivation by APC ^{15,16} and expresses reduced APC-cofactor activity in FVIIIa inactivation ^{8,17}. The common FV gene haplotype marked by the H1299R (R2) polymorphism ^{18,19} has also been reported to cause mild APC resistance, particularly in the homozygous condition ^{19,20}. Although the underlying molecular mechanism remains unknown, it has been shown that FV(a)_{R2} is inactivated by APC at the same rate as normal FVa, whereas its cofactor activity in APC-mediated FVIIIa inactivation is impaired ²⁰. The FV R306T (FV_{Cambridge} ²¹) and FV R306G (FV_{Hong Kong} ²²) mutations, which affect the APC-cleavage site at Arg³⁰⁶, are rather rare and confer only mild APC resistance ^{23,24}. Among all these mutations, only FV_{Leiden} has been conclusively associated with an increased risk of venous thrombosis ²⁵, while FV_{R2} has been

reported to increase FV_{Leiden}-related thrombosis risk in doubly heterozygous individuals^{26,27}.

Although the relative weight of the susceptibility and APC-cofactor activity components in FV_{Leiden}-associated APC resistance has never been quantified²⁸, it is generally believed that resistance of FVa_{Leiden} to APC-mediated inactivation is the predominant underlying defect²⁹. In the present study we have dissected the relative contributions of the susceptibility and APC-cofactor activity components to the APC resistance associated with the FV_{Leiden} and FV_{R2} mutations. Our findings not only indicate that poor APC-cofactor activity of FV is a major component of APC resistance, but they also provide evidence for an important role of the anticoagulant function of FV in the *in vivo* regulation of thrombin formation.

MATERIALS AND METHODS

Plasma samples

Venous blood was drawn in 129 mM sodium citrate (9:1 vol/vol). Platelet-poor plasma was obtained by centrifugation at 3,000g (25 minutes at room temperature) followed by a second centrifugation at 20,000g (30 minutes at 4 °C), after which samples were aliquoted, snap-frozen in liquid nitrogen and stored at -80 °C until use.

A normal plasma pool was prepared by pooling the plasma of 74 healthy blood donors. Moreover, plasma was obtained from two FV_{R2} homozygotes and two FV_{Leiden} homozygotes. All blood samples were collected from consenting individuals in accordance to the Helsinki protocol. FV genotypes were ascertained by PCR amplification of genomic DNA followed by restriction analysis (R506Q and H1299R mutations). The FV_{R2}-homozygous individuals were homozygous for the whole HR2 haplotype, as determined by direct sequencing of FV exons 8, 13, 16 and 25. The demographic characteristics of the subjects who donated blood, as well as the FV, FVIII and protein S levels of all plasmas, are shown in Table 1. Congenitally FV-deficient plasma was purchased from George King Bio-Medical Inc., Overland Park, KS.

APC resistance assays

Measurement of APC resistance with the Immunochrom[®] APC Response assay

Normal pooled plasma and individual plasmas from subjects with different FV genotypes (two FV_{R2} homozygotes and two FV_{Leiden} homozygotes, see Table 1), respectively, were mixed with FV-deficient plasma in various

proportions, ranging from 2.5% to 100% sample plasma, and assayed with the Immunochrom[®] APC Response kit (Progen Biotechnik GmbH, Heidelberg, Germany). This test³⁰ specifically quantifies the ability of APC and its cofactors protein S and FV to inhibit FXa generation by the intrinsic FX-activating complex *via* inactivation of FVIIIa. Phospholipids, CaCl₂ and all relevant proteins (FIXa, FX and APC) are exogenously added, except for FVIII, protein S and FV, which are contributed by the plasma. The assay was performed as previously described³¹ and the APC-sensitivity ratio (APCsr) was expressed as the ratio of the amounts of FXa generated in the absence and in the presence of APC. A low APCsr indicates a defect in the inactivation of FVIIIa and consequent APC resistance. APCsr's were measured four times in duplicate for normal plasma, two times in duplicate for FV_{R2}-homozygous plasma and three times in duplicate for FV_{Leiden}-homozygous plasma.

Measurement of APC resistance with the Coatest[®] APC[™] Resistance assay

Dilutions of sample plasmas in FV-deficient plasma were also assayed with the Coatest[®] APC[™] Resistance kit (Chromogenix, Mölndal, Sweden), *i.e.* the classical aPTT-based APC resistance test that reflects the effect of APC on both FVa and FVIIIa inactivation. The test was performed according to the manufacturer's instructions in an ACL 300 Research coagulometer (Automated Coagulation Laboratory). The APC-sensitivity ratio (APCsr) was expressed as the ratio of the clotting times determined in the presence and absence of APC. Also in this assay a low APCsr indicates APC resistance. APCsr's were measured six times in duplicate for normal plasma, four times in duplicate for FV_{R2}-homozygous plasma and three times in duplicate for FV_{Leiden}-homozygous plasma.

Statistics

Regression lines in Figs. 1 and 2 were calculated according to the least squares method. After performing an analysis of variance (ANOVA) on the regression, the slopes of the normal FV and FV_{R2} lines were compared using the t-test for parallelism of regression lines.

Measurement of FV, FVIII and protein S levels in plasma

The FV concentration in plasma was measured *via* a prothrombinase-based chromogenic assay.³² Plasma was diluted 1:1000 in a buffer containing 25 mM Hepes (pH 7.7 at room temperature), 175 mM NaCl, 0.5% BSA, 3 mM CaCl₂ and 10 μ M phospholipid vesicles (DOPS/DOPC 20/80 mol/mol), and activated with 1 nM thrombin at 37 °C for 10 minutes. Prothrombin activation was started by the addition of 5 nM FXa and 1 μ M prothrombin (final

concentrations) and stopped after 2 minutes by the addition of (ice-cold) buffer containing EDTA (19.6 mM final). The amount of thrombin formed, which is a measure of the FVa present in the reaction mixture, was quantitated using the chromogenic substrate S2238. The FV concentration was calculated from a calibration curve constructed with known amounts of purified plasma FV.

FVIII levels were calculated from the data obtained with the Immunochrom[®] APC Response assay. Since FVIII is the limiting factor under the assay conditions, the FXa generation rate is directly proportional to the FVIII concentration in plasma. Therefore, the measurement obtained in the absence of APC reflects the amount of FVIII present in a given plasma, which was expressed as percentage of the FVIII present in pooled normal plasma determined in the same experiment.

Protein S levels were measured with an enzyme-linked immunosorbent assay (ELISA)³³ in plasma samples diluted 1:800 or 1:1600 in a buffer containing 25 mM Hepes (pH 7.7 at room temperature), 175 mM NaCl and 0.5% BSA. Serial dilutions of normal plasma were used as a reference.

Reconstitution experiments

Human FV was purified from pooled normal plasma and from the plasma of a FV_{Leiden}-homozygous individual, as described.³⁴ Purified FV was desalted on a PD-10 column (Pharmacia Biotech, Uppsala, Sweden) and the FV concentration was measured as described above. Congenitally FV-deficient plasma (George King Bio-Medical Inc., Overland Park, KS) was supplemented with purified FV to various final concentrations, ranging from 0.6 nM (2.5%) to 23 nM (100%), and the APCsr of the reconstituted plasmas was determined with the Coatest[®] APC[™] Resistance assay kit (Chromogenix, Mölndal, Sweden).

Measurement of thrombin generation

The plasma concentration of FV was varied between 10% and 120% of the normal level by adding a variable amount of purified normal FV or FV_{Leiden} to congenitally FV-deficient plasma (George King Bio-Medical Inc., Overland Park, KS). In a similar way, FV-deficient plasma was reconstituted with normal FV and/or FV_{Leiden} to simulate normal plasma (100% normal FV), FV_{Leiden}-heterozygous plasma (50% normal FV and 50% FV_{Leiden}), FV_{Leiden}-homozygous plasma (100% FV_{Leiden}) and FV_{Leiden} pseudo-homozygous³⁵ plasma (50% FV_{Leiden} only). Plasma doubly heterozygous for FV_{Leiden} and FV_{R2} was obtained by mixing FV_{Leiden}-homozygous and FV_{R2}-homozygous plasmas in equal proportions.

Thrombin generation was measured essentially as described³⁶. Plasma was mixed with a low-affinity fluorogenic substrate for thrombin (Z-Gly-Gly-Arg-

AMC, BACHEM AG, Bubendorf, Switzerland, 300 μ M final) and coagulation was initiated with a mixture containing recombinant tissue factor (TF, Dade[®] Innovin[®]), synthetic phospholipid vesicles (DOPS/DOPC/DOPE 20/60/20 mol/mol/mol) and CaCl_2 (final concentrations in plasma: $\sim$ 320 μ g/ml TF ($\sim$ 6.8 μ M), 15 μ M phospholipids and 16 mM added CaCl_2), in the absence or presence of APC (5, 10 or 20 nM, as specified below). The formation of thrombin was followed continuously in a SPECTRAmax[®] GEMINI XS fluorometer (Molecular Devices Corporation, Sunnyvale, CA), using 368 nm (excitation) and 460 nm (emission) filters. A thrombin standard (Synapse b.v., Maastricht, The Netherlands) was used to correct the raw fluorescence data for inner-filter effect and substrate consumption and to convert relative fluorescence units (RFUs) into nM thrombin. After subtraction of the signal attributable to the α_2 M-thrombin complex, the first derivative of the data was calculated to obtain the fully corrected thrombin generation curve, and the underlying area (endogenous thrombin potential, ETP) was determined according to Hemker *et al.*³⁷. Each time-course of thrombin generation was measured in duplicate.

RESULTS

APC-cofactor activity of FV_{Leiden} and FV_{R2}

The concentration of normal FV, FV_{Leiden} and FV_{R2} in plasma was varied between 2.5% and 100% by mixing normal plasma, FV_{Leiden}-homozygous plasma and FV_{R2}-homozygous plasma, respectively, with FV-deficient plasma. The APCsr of the plasma mixtures was determined with the Immunochrom[®] APC Response test, which specifically probes the APC resistance resulting from poor APC-cofactor activity of FV in FVIIIa inactivation³⁰. As shown in Fig. 1, the APCsr increased linearly with increasing concentrations of normal FV and FV_{R2}, while it remained virtually constant and independent of the FV concentration in the case of FV_{Leiden}. This indicates that normal FV and, to a lesser extent, FV_{R2} act as cofactors in APC-catalysed FVIIIa inactivation, whereas FV_{Leiden} lacks APC-cofactor activity in FVIIIa inactivation.

FIGURE 1: Effect of FV level on the APCsr determined with the Immunochrom[®] APC Response test. The FV level was varied by mixing sample plasma with FV-deficient plasma in different proportions and the APCsr was determined as described under Methods. Each point is the average of two or more duplicate experiments, with error bars representing one standard error above and below the mean. Δ , normal plasma; $\blacksquare$, FV_{R2}-homozygous plasma; $\bullet$, FV_{Leiden}-homozygous plasma.

Taking into account the actual amount of FV present in the different plasmas (Table 1), we calculated the slopes of the lines (Δ APCsr/IU FV), which are a measure of the APC-cofactor activity of the FV contained in the respective plasma samples. The slopes obtained with FV_{R2} (Δ APCsr = 0.45/IU FV, 95% CI 0.35-0.56) and normal FV (Δ APCsr = 0.62/IU FV, 95% CI 0.55-0.69) were significantly different ($P < 0.005$) and indicated that FV_{R2} expresses 73% of the

APC-cofactor activity of normal FV. FV_{Leiden} ($\Delta APCsr = -0.14/IU$ FV, 95% CI - 0.23—0.06) expresses no APC-cofactor activity and might even slightly inhibit APC-catalysed FVIIIa inactivation.

Effect of FV concentration on the APCsr determined with the Coatest® APC™ Resistance assay

We also determined the effect of FV concentration on the APCsr measured with the Coatest® APC™ Resistance assay (Fig. 2A). Since in this test clotting is initiated *via* the intrinsic pathway, both FVIIIa and FVa inactivation by APC contribute to the test result (APCsr). Consequently, variation of the FV concentration in plasma can affect the APCsr by modifying the rates of both FVIIIa inactivation, in which FV acts as a cofactor of APC, and FVa inactivation, in which FVa is the substrate of APC.

As shown in Fig. 2A, the dependence of the APCsr on the FV concentration in plasma was different for FV_{Leiden} (closed circles) and normal FV (open triangles). Variation of the concentration of FV_{Leiden} between 2.5% and 100% did not significantly affect the APCsr, which remained constant at about 1.5. This not only confirms that FV_{Leiden} does not express APC-cofactor activity, but also shows that, under the assay conditions, the susceptibility of FVa to APC is independent of the FV concentration. In contrast, variation of the amount of normal FV from 2.5% to 100% caused the APCsr to increase from 2.3 to 3.3. Since the susceptibility of FVa is independent of the FV concentration, this increase in APCsr is attributable to an increase in the APC-cofactor activity at increasing FV concentration. At very low FV levels, where the APC-cofactor activity is negligible, the difference between the APCsr of plasmas containing normal FV and FV_{Leiden} is entirely due to the difference in the susceptibility of normal and mutant FVa for APC, which does not change when the FV concentration is increased. Thus, the difference in APCsr between plasmas containing normal FV and FV_{Leiden} at any given FV concentration can be resolved in a susceptibility component, which is constant and independent of the FV concentration, and an APC-cofactor activity component, which progressively increases at increasing FV (Fig. 2B). At 100% FV, *i.e.* in full plasma, the difference between the APCsr of normal plasma and FV_{Leiden} -homozygous plasma ($\Delta APCsr = 1.81$, Fig. 2A) is due for 49% to the fact that FVa_{Leiden} is less susceptible to APC-mediated inactivation than normal FVa, and for 51% to the fact that FV_{Leiden} expresses no APC-cofactor activity in the inactivation of FVIIIa (Fig. 2B).

FIGURE 2: Effect of FV level on the APCsr determined with the Coatest® APC™ Resistance assay. A) Plot of the APCsr as a function of FV concentration. The FV level was varied by mixing sample plasma with FV-deficient plasma in different proportions and the APCsr was determined as described under Methods. B) The difference in APCsr between normal plasma and FV_{Leiden}-homozygous plasma consists of two components: a) poor susceptibility of FV_{Leiden} to APC, quantified by the difference between the Y-axis intercepts of the APCsr plots of normal and FV_{Leiden}-homozygous plasma, and b) impaired APC-cofactor activity of FV_{Leiden} in FVIIIa inactivation, whose contribution to the APCsr is deduced by subtracting the susceptibility component from the total APCsr difference between normal and FV_{Leiden}-homozygous plasma measured in full plasma. C) Blow-up of the inset in panel A. Each point is the average of three or more duplicate experiments, with error bars representing one standard error above and below the mean. △, normal plasma; ■, FV_{R2}-homozygous plasma; ●, FV_{Leiden}-homozygous plasma.

It should be emphasized that the FV-deficient plasma used to dilute sample plasmas, in contrast to many other commercially available FV-deficient plasmas³⁸, had a normal protein S level (Table 1). This congenitally FV-deficient plasma was chosen to avoid varying the protein S levels (and thereby affect the APCsr) when mixing sample plasma and FV-deficient plasma in different proportions.

Plasma	Source	Age (yr)	Sex	% FV	% FVIII	% protein S
Normal pool	Healthy blood donors (N=74)	40.0 ($\pm$ 8.9 SD)	M/F (45/29)	100%	100%	100%
FV _{R2} homozygote	Healthy blood donor	55	M	77%	150%	132%
FV _{R2} homozygote	Coronary pathic patient	49	M	80%	82%	104%
FV _{Leiden} homozygote	Healthy blood donor	28	F	91%	76%	74%
FV _{Leiden} homozygote	Thrombotic patient	75	M	121%	155%	116%
FV-deficient plasma	Commercial (George King)	-	-	<1%	118%	113%

TABLE 1: Definition of sample plasmas

The variation of the APCsr with the concentration of FV_{R2} (Fig. 2A, closed squares) was similar to that observed with normal FV (Fig. 2A, open triangles). This is in line with the report that FV_{R2} has a minor effect on the APCsr determined with the aPTT-based APC resistance test²⁰.

Since the increase in APCsr with the FV concentration is due to APC-cofactor activity, the initial slopes of the lines in Fig. 2A can be taken as a measure of the APC-cofactor activity expressed by the FV contained in the respective plasma. Fig. 2C shows that the initial slope of the APCsr plot as a function of the FV concentration was actually lower for FV_{R2} (Δ APCsr = 1.86/IU FV, 95% CI 0.65-3.08) than for normal FV (Δ APCsr = 2.24/IU FV, 95% CI 1.36-3.13), suggesting that FV_{R2} expresses reduced APC-cofactor activity, but confidence intervals largely overlapped and the difference did not reach significance.

Since the plasma from the two FV_{Leiden}-homozygous donors might not have been representative for all FV_{Leiden} homozygotes, we repeated the Coatest experiment using FV-deficient plasma reconstituted with varying amounts of purified normal FV or FV_{Leiden}. Plasma reconstituted with normal FV exhibited a similar dependence of the APCsr on the FV concentration as normal pooled plasma, while plasma reconstituted with FV_{Leiden} behaved the same as the plasma from the FV_{Leiden}-homozygous individual, showing a constant APCsr of 1.5 (data not shown). Therefore, our results can be generalized to any homozygous carrier of FV_{Leiden}.

Effect of the APC-cofactor activity of FV on TF-initiated thrombin generation

The experiments described above indicate that impaired APC-cofactor activity of FV is an important component of FV_{Leiden}-associated APC resistance as measured with the Coatest assay. To evaluate the role of the anticoagulant

function of FV within a more physiological setting, we used a thrombin generation assay in which coagulation was initiated *via* the extrinsic pathway at a low TF concentration (6.8 pM). Control experiments performed in FVIII-deficient plasma with and without added FVIII demonstrated that the intrinsic FX-activating complex significantly contributes to FXa (and thus thrombin) generation under these experimental conditions, both in the absence and presence of APC (data not shown).

First of all, we performed a FV titration of thrombin generation in FV-deficient plasma reconstituted with purified normal FV or FV_{Leiden}. In the absence of APC (Fig. 3A,B), increasing the concentration of normal FV or FV_{Leiden} up to about 60% of the normal plasma concentration resulted in a gradual increase of the amount of thrombin formed and in a decrease of the time of onset of thrombin generation. At higher FV concentrations both the time of onset of thrombin generation and the total amount of thrombin formed did not further change. In the presence of APC (10 nM), increasing the concentration of normal FV caused an initial increase of thrombin generation (up to 60% FV), which was followed by a progressive decrease of thrombin generation (Fig. 3C). This decrease is presumably attributable to the increasing anticoagulant activity expressed by increasing amounts of normal FV. Conversely, when the same experiment was performed with FV_{Leiden}, which expresses no APC-cofactor activity, thrombin generation in the presence of APC steadily increased towards a maximum and hardly any decrease at the highest FV concentrations was observed (Fig. 3D).

FIGURE 3: Effect of FV concentration on thrombin generation. Congenitally FV-deficient plasma was reconstituted with varying amounts of purified normal FV (A,C) or FV_{Leiden} (B,D). Thrombin generation after extrinsic activation of plasma in the absence (A,B) or presence (C,D) of 10 nM APC was measured as described under Methods. ◇, 10% FV; □, 20% FV; ○, 60% FV; △, 100% FV; ◆, 120% FV.

We also simulated normal, FV_{Leiden}-heterozygous, FV_{Leiden}-homozygous and FV_{Leiden} pseudo-homozygous plasmas by reconstituting FV-deficient plasma with the appropriate amount of purified normal FV and/or FV_{Leiden}. Following initiation of coagulation with TF in the absence of APC (Fig. 4A), all plasmas yielded superimposable thrombin generation curves, except for FV_{Leiden} pseudo-homozygous plasma which showed a slightly longer lag phase due to its lower (50%) FV concentration. In the presence of 20 nM APC (Fig. 4B), strong inhibition of thrombin generation was observed in FV-deficient plasma reconstituted with normal FV (open triangles). In contrast, simulated FV_{Leiden}-homozygous (100% FV_{Leiden}, closed circles) and pseudo-homozygous (50% FV_{Leiden}, open diamonds) plasmas were fully resistant to APC, with identical ETPs in the presence and absence of APC. In FV_{Leiden}-heterozygous plasma (open circles), which differs from pseudo-homozygous plasma by the additional presence of 50% normal FV, about half as much thrombin was formed as in pseudo-homozygous plasma. This reduction in thrombin generation is likely due to the anticoagulant activity expressed by normal FV in the presence of APC.

FIGURE 4: Thrombin generation in FV-deficient plasma reconstituted with normal FV and/or FV_{Leiden}. Congenitally FV-deficient plasma was reconstituted with purified normal FV and/or FV_{Leiden} in order to simulate normal plasma (△), FV_{Leiden}-heterozygous plasma (○), FV_{Leiden}-homozygous plasma (●) and FV_{Leiden} pseudo-homozygous plasma (◇). Thrombin generation after extrinsic activation of plasma in the absence (A) and presence (B) of 20 nM APC was measured as described under Methods.

Since FV_{R2} expresses reduced APC-cofactor activity in $FVIII(a)$ inactivation (Figs. 1 and 2C), we expected that more thrombin would be generated in FV_{R2} -homozygous plasma than in normal plasma following activation in the presence of APC. As a matter of fact, the amount of thrombin formed in the plasma from a FV_{R2} -homozygous individual in the presence of APC (5 nM) was 23% of that in the absence of APC (Fig. 5B), as compared to a residual thrombin generation of 13% in normal pooled plasma (Fig. 5A). In other words, while virtually equal amounts of thrombin were formed in the absence of APC, in the presence of APC the plasma from the FV_{R2} homozygote generated 1.8 times more thrombin than normal pooled plasma. To exclude the possibility that increased thrombin generation in FV_{R2} -homozygous plasma in the presence of APC was due to plasma components other than FV, we repeated the experiment after a 5-fold dilution of both plasmas in FV-deficient plasma and verified that the difference persisted (data not shown).

FIGURE 5: Thrombin generation in plasmas containing FV_{R2} and/or FV_{Leiden} . Thrombin generation after extrinsic activation of plasma in the absence (closed symbols) and presence (open symbols) of 5 nM APC was measured as described under Methods. (A) normal pooled plasma; (B) plasma of a FV_{R2} -homozygous individual; (C) FV_{Leiden} -heterozygous plasma obtained by pooling plasma from two unrelated FV_{Leiden} -heterozygous individuals; (D) simulated FV_{Leiden}/FV_{R2} -doubly heterozygous plasma obtained by mixing plasma from a FV_{Leiden} -homozygous individual and from a FV_{R2} -homozygous individual in equal proportions.

Since it has been reported that co-inheritance of FV_{R2} enhances the APC resistance^{19,39} and the thrombotic risk^{26,27} associated with FV_{Leiden} , we also investigated the combined effect of FV_{R2} and FV_{Leiden} on thrombin generation in the presence and absence of APC. To this end, a simulated FV_{Leiden}/FV_{R2} -doubly heterozygous plasma, prepared by mixing FV_{Leiden} -homozygous and FV_{R2} -homozygous plasmas in equal proportions, was compared to pooled plasma from two FV_{Leiden} -heterozygous individuals. In the presence of 5 nM APC, residual thrombin generation (relative to thrombin generation in the same plasma triggered in the absence of APC) was 55% in FV_{Leiden} -heterozygous plasma (Fig. 5C) and 79% in FV_{Leiden}/FV_{R2} -doubly heterozygous plasma (Fig. 5D).

DISCUSSION

In the original report on APC resistance, Dahlbäck and coworkers observed that the addition of normal plasma to APC-resistant plasma corrected the abnormal phenotype and they concluded that APC resistance was due to the defect of a new APC-cofactor¹⁰, which later turned out to be FV⁴⁰. Subsequently, the ability of FV to stimulate APC-mediated inactivation of FVIIIa was proven directly in model systems containing purified proteins⁴⁻⁶. However, after the FV_{Leiden} mutation was discovered as the main cause of APC resistance¹⁴, little attention was paid to the role of FV as an APC-cofactor. The abolition of the APC-cleavage site at Arg⁵⁰⁶, which impaired the inactivation of FVa_{Leiden} in *in vitro* studies^{15,16,29,41}, appeared a satisfactory explanation for the APC resistance associated with the mutation. Only when it was reported that FV_{Leiden} is a poor cofactor in APC-catalysed FVIIIa inactivation^{8,17}, was it realized that reduced APC-cofactor activity may also contribute to FV_{Leiden}-associated APC resistance.

Here we demonstrate that FV plays a crucial role in the APC-mediated inactivation of FVIIIa in plasma and that this anticoagulant activity of FV is lost in FV_{Leiden} (Fig. 1). This result extends to the plasma milieu previous reports on the APC-cofactor activities of purified and recombinant FV and FV_{Leiden} determined in model systems of FVIIIa inactivation^{4-6,8}. Furthermore we show that, although reduced susceptibility of FVa_{Leiden} to APC-catalysed inactivation remains an important determinant of FV_{Leiden}-associated APC resistance, impaired APC-cofactor activity of FV_{Leiden} in FVIIIa inactivation accounts for about half of the difference between the APCsr of normal individuals and FV_{Leiden} homozygotes, as measured with the Coatest[®] APC resistance assay (Fig. 2B).

In order to gain an insight into the physiological role of the anticoagulant activity of FV, we measured thrombin generation in plasma after initiation of coagulation *via* the extrinsic pathway. This assay allows the evaluation of the overall efficiency of the coagulation system under conditions that are close to the *in vivo* situation⁴². To ensure that FXa formed *via* the intrinsic FX-activating complex contributes to thrombin generation, coagulation was initiated with a low TF concentration. As illustrated in Fig. 3C, increasing the concentration of normal FV in plasma triggered in the presence of APC led to an initial increase of thrombin generation, followed by a gradual decrease at FV concentrations higher than 60%. This remarkable pattern is likely explained by the dual function of FV in coagulation. At low FV concentrations the procoagulant activity of FV prevails over the anticoagulant activity, leading to progressively higher thrombin generation at increasing FV concentration. However, at a FV level of 60%, the

procoagulant activity of FV is saturated (Fig. 3A), and a further increase of the FV concentration results in increased anticoagulant activity and consequently decreased thrombin generation. This interpretation is supported by the parallel experiment conducted with FV_{Leiden} (Fig. 3D), which showed hardly any inhibition of thrombin generation in the presence of APC at high FV concentrations, thus confirming that FV_{Leiden} expresses no anticoagulant activity.

The physio-pathological relevance of the APC-cofactor activity of FV is well illustrated by the comparison of heterozygosity, homozygosity and pseudo-homozygosity³⁵ for the FV_{Leiden} mutation as shown in Fig. 4. FV-deficient plasma reconstituted with 50% purified FV_{Leiden} (which corresponds to the plasma of a FV_{Leiden} pseudo-homozygous individual) was fully resistant to APC in the thrombin generation test. However, when 50% normal FV was added to this plasma, thrombin generation in the presence of APC was reduced by half (Fig. 4B), due to the anticoagulant activity expressed by the normal FV. Conversely, the addition to FV_{Leiden} pseudo-homozygous plasma of 50% FV_{Leiden} (which does not express APC-cofactor activity) was ineffective in decreasing thrombin generation in the presence of APC (Fig. 4B). These findings may explain the respective thrombotic risks associated with heterozygosity, homozygosity and pseudo-homozygosity for the FV_{Leiden} mutation. Moreover, they suggest that the reason why FV_{Leiden} homozygotes are exposed to a higher thrombotic risk than heterozygotes is not the presence of twice as much FV_{Leiden} in their plasma, but the absence of normal FV capable of expressing anticoagulant activity. This is in line with the recent *in vivo* observation that the thrombotic lethality of FV_{Leiden} pseudo-homozygous mice with heterozygous TFPI deficiency (FV^{Q/-} TFPI^{+/-} genotype) can be corrected by transgene expression of wild-type FV⁹.

In contrast to FV_{Leiden}, the APC-cofactor activity of FV_{R2} is reduced to 73% of that of normal FV (Figs. 1 and 2C). However, since FV_{R2} is expressed at a lower level than normal FV^{18,20,39}, the overall APC-cofactor activity contained in the plasma of a typical FV_{R2} homozygote with a FV level of ~75% is actually about half of that present in normal plasma. Since it has been reported that FV_{R2} and normal FVa are inactivated at equal rates by APC, both in the presence and in the absence of protein S²⁰, reduced APC-cofactor activity in FVIIIa inactivation appears to be the only determinant of the mild APC resistance observed in carriers of the FV_{R2} haplotype. The patho-physiological consequences of the decreased anticoagulant activity of FV_{R2} are apparent in the thrombin generation assay, which shows that FV_{R2}-homozygous plasma forms almost twice as much thrombin as normal plasma in the presence of 5 nM APC (Fig. 5A,B). Although the plasma of only a single FV_{R2}-homozygous individual was used in the thrombin generation experiments, increased thrombin

formation in the presence of APC was still observed after 1:5 dilution in FV-deficient plasma, which strongly reduces the interference by plasma components other than FV. Therefore, in spite of the inconclusive results of epidemiological studies (see ref.⁴³ and references therein), our experiments provide evidence for the mild prothrombotic character of FV_{R2}, both *per se* (Fig. 5A,B) and in combination with FV_{Leiden} (Fig. 5C,D).

In conclusion, our study shows 1) that the impaired APC-cofactor activity of FV_{Leiden} and FV_{R2} in FVIIIa inactivation contributes prominently to the APC resistance associated with carriership of these mutations; and 2) that the anticoagulant activity of FV plays a pivotal role in the regulation of thrombin formation.

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REFERENCES

1. Walker FJ, Fay PJ. Regulation of blood coagulation by the protein C system. *Faseb J*. 1992;6:2561-2567.
2. 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;269:31869-31880.
3. 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;266:20139-20145.
4. 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;269:18735-18738.
5. Váradi K, Rosing J, Tans G, Schwarz HP. Influence of factor V and factor Va on APC-induced cleavage of human factor VIII. *Thromb Haemost*. 1995;73:730-731.
6. Lu D, Kalafatis M, Mann KG, Long GL. Comparison of activated protein C/protein S-mediated inactivation of human factor VIII and factor V. *Blood*. 1996;87:4708-4717.
7. Thorelli E, Kaufman RJ, Dahlbäck B. The C-terminal region of the factor V B-domain is crucial for the anticoagulant activity of factor V. *J Biol Chem*. 1998;273:16140-16145.
8. Thorelli E, Kaufman RJ, Dahlbäck B. Cleavage of factor V at Arg 506 by activated protein C and the expression of anticoagulant activity of factor V. *Blood*. 1999;93:2552-2558.
9. Westrick RJ, Sun H, Eitzman DT, et al. The factor V Leiden mutation results in the in vivo loss of a critical FV anticoagulant function [abstract]. *Blood*. 2002;100:127a.
10. Dahlbäck B, Carlsson M, Svensson PJ. Familial thrombophilia due to a previously unrecognized mechanism characterized by poor anticoagulant response to activated protein C: prediction of a cofactor to activated protein C. *Proc Natl Acad Sci U S A*. 1993;90:1004-1008.
11. Koster T, Rosendaal FR, de Ronde H, Briët E, Vandenbroucke JP, Bertina RM. Venous thrombosis due to poor anticoagulant response to activated protein C: Leiden Thrombophilia Study. *Lancet*. 1993;342:1503-1506.
12. Svensson PJ, Dahlbäck B. Resistance to activated protein C as a basis for venous thrombosis. *N Engl J Med*. 1994;330:517-522.
13. de Visser MC, Rosendaal FR, Bertina RM. A reduced sensitivity for activated protein C in the absence of factor V Leiden increases the risk of venous thrombosis. *Blood*. 1999;93:1271-1276.
14. Bertina RM, Koeleman BP, Koster T, et al. Mutation in blood coagulation factor V associated with resistance to activated protein C. *Nature*. 1994;369:64-67.
15. Nicolaes GA, Tans G, Thomassen MC, et al. Peptide bond cleavages and loss of functional activity during inactivation of factor Va and factor Va^{R506Q} by activated protein C. *J Biol Chem*. 1995;270:21158-21166.

16. Kalafatis M, Bertina RM, Rand MD, Mann KG. Characterization of the molecular defect in factor V^{R506Q}. *J Biol Chem*. 1995;270:4053-4057.
17. Váradi K, Rosing J, Tans G, Pabinger I, Keil B, Schwarz HP. Factor V enhances the cofactor function of protein S in the APC-mediated inactivation of factor VIII: influence of the factor V^{R506Q} mutation. *Thromb Haemost*. 1996;76:208-214.
18. Lunghi B, Iacoviello L, Gemmati D, et al. Detection of new polymorphic markers in the factor V gene: association with factor V levels in plasma. *Thromb Haemost*. 1996;75:45-48.
19. Bernardi F, Faioni EM, Castoldi E, et al. A factor V genetic component differing from factor V R506Q contributes to the activated protein C resistance phenotype. *Blood*. 1997;90:1552-1557.
20. Hoekema L, Castoldi E, Tans G, et al. Functional properties of factor V and factor Va encoded by the R2-gene. *Thromb Haemost*. 2001;85:75-81.
21. Williamson D, Brown K, Luddington R, Baglin C, Baglin T. Factor V Cambridge: a new mutation (Arg³⁰⁶→Thr) associated with resistance to activated protein C. *Blood*. 1998;91:1140-1144.
22. Chan WP, Lee CK, Kwong YL, Lam CK, Liang R. A novel mutation of Arg306 of factor V gene in Hong Kong Chinese. *Blood*. 1998;91:1135-1139.
23. van der Neut Kolfschoten M, Dirven RJ, Tans G, Rosing J, Vos HL, Bertina RM. The activated protein C (APC)-resistant phenotype of APC cleavage site mutants of recombinant factor V in a reconstituted plasma model. *Blood Coagul Fibrinolysis*. 2002;13:207-215.
24. Norström E, Thorelli E, Dahlbäck B. Functional characterization of recombinant FV Hong Kong and FV Cambridge. *Blood*. 2002;100:524-530.
25. Nicolaes GA, Dahlbäck B. Factor V and thrombotic disease: description of a janus-faced protein. *Arterioscler Thromb Vasc Biol*. 2002;22:530-538.
26. Faioni EM, Franchi F, Bucciarelli P, et al. Coinheritance of the HR2 haplotype in the factor V gene confers an increased risk of venous thromboembolism to carriers of factor V R506Q (factor V Leiden). *Blood*. 1999;94:3062-3066.
27. Folsom AR, Cushman M, Tsai MY, et al. A prospective study of venous thromboembolism in relation to factor V Leiden and related factors. *Blood*. 2002;99:2720-2725.
28. Dahlbäck B. The discovery of activated protein C resistance. *J Thromb Haemost*. 2003;1:206-213.
29. Heeb MJ, Kojima Y, Greengard JS, Griffin JH. Activated protein C resistance: molecular mechanisms based on studies using purified Gln⁵⁰⁶-factor V. *Blood*. 1995;85:3405-3411.
30. Váradi K, Moritz B, Lang H, et al. A chromogenic assay for activated protein C resistance. *Br J Haematol*. 1995;90:884-891.
31. Govers-Riemslog JW, Castoldi E, Nicolaes GA, Tans G, Rosing J. Reduced factor V concentration and altered FV₁/FV₂ ratio do not fully explain R2-associated APC-resistance. *Thromb Haemost*. 2002;88:444-449.
32. Hoekema L, Rosing J, Tans G. An assay to quantify the two plasma isoforms of factor V. *Thromb Haemost*. 2000;84:1066-1071.

33. 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;40:8852-8860.
34. Rosing J, Bakker HM, Thomassen MC, Hemker HC, Tans G. Characterization of two forms of human factor Va with different cofactor activities. *J Biol Chem*. 1993;268:21130-21136.
35. Simioni P, Scudeller A, Radossi P, et al. "Pseudo homozygous" activated protein C resistance due to double heterozygous factor V defects (factor V Leiden mutation and type I quantitative factor V defect) associated with thrombosis: report of two cases belonging to two unrelated kindreds. *Thromb Haemost*. 1996;75:422-426.
36. Castoldi E, Govers-Riemslog JW, Pinotti M, et al. Co-inheritance of FV Leiden enhances thrombin formation and is associated with a mild bleeding phenotype in patients homozygous for the FVII 9726+5G>A (FVII Lazio) mutation. *Blood*. 2003;102:4014-4020.
37. Hemker HC, Wielders S, Kessels H, Béguin S. Continuous registration of thrombin generation in plasma, its use for the determination of the thrombin potential. *Thromb Haemost*. 1993;70:617-624.
38. Samama MS, Gouin-Thibault I, Trossaërt M, et al. Low levels of protein S activity in factor V depleted plasma used in APC resistance test. *Thromb Haemost*. 1998;80:715-716.
39. de Visser MCH, Guasch JF, Kamphuisen PW, Vos HL, Rosendaal FR, Bertina RM. The HR2 haplotype of factor V: Effects on factor V levels, normalized activated protein C sensitivity ratios and the risk of venous thrombosis. *Thromb Haemost*. 2000;83:577-582.
40. Dahlbäck B, Hildebrand B. Inherited resistance to activated protein C is corrected by anticoagulant cofactor activity found to be a property of factor V. *Proc Natl Acad Sci U S A*. 1994;91:1396-1400.
41. Aparicio C, Dahlbäck B. Molecular mechanisms of activated protein C resistance. Properties of factor V isolated from an individual with homozygosity for the Arg506 to Gln mutation in the factor V gene. *Biochem J*. 1996;313:467-472.
42. Hemker HC, Béguin S. Phenotyping the clotting system. *Thromb Haemost*. 2000;84:747-751.
43. Castaman G, Faioni EM, Tositto A, Bernardi F. The factor V HR2 haplotype and the risk of venous thrombosis: a meta-analysis. *Haematologica*. 2003;88:1182-1189.

CHAPTER 4

Expression of the normal factor V allele modulates the APC resistance phenotype in heterozygous carriers of the factor V Leiden mutation.

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ABSTRACT

Background: Functional defects of the protein C pathway, detectable in plasma as activated protein C (APC) resistance, are a prevalent risk factor for venous thrombosis. The factor V (FV) Leiden mutation causes APC resistance by interfering with the APC-mediated inactivation of both FVa and FVIIIa. Co-inheritance of FV Leiden and quantitative FV deficiency on different alleles, a rare condition known as pseudo-homozygous APC resistance, is associated with pronounced APC resistance and 50% reduced FV levels, due to non-expression of the non-Leiden FV allele.

Objectives: The role of normal FV in modulating the APC resistance phenotype in carriers of FV Leiden was investigated in patients with pseudo-homozygous APC resistance and in model systems.

Patients/Methods: Four functional plasma assays probing both components of APC resistance (susceptibility of FVa to APC and cofactor activity of FV in FVIIIa inactivation) were employed to compare 7 clinically and genetically characterised FV Leiden pseudo-homozygotes to 30 relatives with different FV genotypes (including 12 FV Leiden heterozygotes and 7 carriers of FV deficiency) and to 32 unrelated FV Leiden homozygotes.

Results and Conclusions: All assays consistently indicated that FV Leiden pseudo-homozygotes are significantly more APC-resistant than heterozygotes and indistinguishable from homozygotes. Thrombin generation measurements in FV-deficient plasma reconstituted with purified normal FV and FV Leiden confirmed these observations and showed that the expression of the normal FV allele is an important modulator of APC resistance in FV Leiden heterozygotes. These findings provide an explanation for the higher thrombotic risk of FV Leiden pseudo-homozygotes as compared to heterozygotes.

INTRODUCTION

The protein C pathway plays a major role in the down-regulation of coagulation by proteolytically inactivating the non-enzymatic cofactors Va (FVa) and VIIIa (FVIIIa). Functional defects of this pathway result in a plasma phenotype known as activated protein C (APC) resistance, which is characterised by a relative inability of exogenous APC to prolong the clotting time in an aPTT-based assay [1]. APC resistance is the most common risk factor for venous thromboembolism.

Most cases of inherited APC resistance in the Caucasian population are due to the Arg⁵⁰⁶→Gln mutation in the factor V gene (FV Leiden) [2]. This mutation decreases the susceptibility of FVa to inactivation by APC [3,4] and impairs the ability of FV to stimulate the APC-mediated inactivation of FVIIIa [5,6]. As a consequence, FV Leiden heterozygotes experience a 7-fold increased risk of venous thrombosis and homozygotes an 80-fold increased risk as compared to non-carriers [7].

Pseudo-homozygous APC resistance is a rare condition characterised by the co-inheritance of the FV Leiden mutation and a FV null mutation on different alleles [8]. Despite genotypic heterozygosity for FV Leiden, pseudo-homozygous individuals show an APC resistance phenotype that resembles that of homozygotes. This discrepancy is due to the fact that the non-Leiden FV allele is not expressed, resulting in the exclusive presence of FV Leiden molecules in plasma [9,10].

Although most cases of pseudo-homozygous APC resistance reported in the literature are thrombotic patients [8-18], the thrombotic risk associated with this condition has been difficult to establish in epidemiological studies [19], because of its rare occurrence in the general population [9]. Based on the comparison of Kaplan-Meier thrombosis-free survival curves of probands with different FV Leiden genotypes, we have recently shown that FV Leiden pseudo-homozygotes experience the same thrombotic risk as FV Leiden homozygotes [20].

To account for this finding and to get more insight into the role of the non-Leiden allele in FV Leiden carriers, we now report a full characterisation of the APC resistance phenotype in seven pseudo-homozygous individuals and in a plasma model system.

MATERIALS AND METHODS

Patients

Seven FV Leiden pseudo-homozygous individuals (Table 1), belonging to six unrelated families, and 33 family members were recruited. Among the pseudo-homozygotes, six were consecutive thrombotic patients, while the seventh was the asymptomatic brother of one of them. Family members were normal (n=12), heterozygous for the FV Leiden mutation (n=13) or carriers of quantitative FV deficiency (n=8). Three of them (one for each genotype) were excluded from the study due to ongoing oral anticoagulant treatment following a thrombotic event. None of the remaining 30 relatives had experienced venous thrombosis. Five of the six probands [20] and three families [8-10,17] have been previously reported.

An unrelated group of 32 FV Leiden homozygotes belonging to 25 different families was also investigated. Of these, 12 were thrombotic patients (6 had deep vein thrombosis, 5 superficial vein thrombosis and 1 a transient ischaemic attack) and 20 were asymptomatic members of thrombophilic families. None of the patients included in the study was on oral anticoagulants at the time of blood collection.

Blood was drawn by venipuncture in 3.8% sodium citrate (1:9 vol/vol). Platelet-poor plasma was obtained by two centrifugation steps at 2000×g for 15 min, snap-frozen and stored at -80 °C. The study was conducted in accordance with the Helsinki protocol and informed consent was obtained from all participants.

DNA analysis

Genomic DNA was extracted from peripheral blood leukocytes. Carriership of the FV Leiden mutation was ascertained by Mnl I-restriction analysis of amplified exon 10. Screening for the mutations responsible for FV deficiency was performed by direct sequencing of FV gene exons and splicing junctions, as described [21].

aPTT-based APC resistance test

The aPTT-based APC resistance test was performed in undiluted plasma as described [22].

Thrombin generation assay (ETP-based APC resistance test)

The thrombin generation assay was performed essentially as reported [23]. Conversion of the fluorogenic substrate by the thrombin formed in plasma was followed in a Fluoroskan Ascent[®] reader (Thermo Labsystems, Helsinki,

Finland). Thrombin generation curves and the area-under-the-curve (endogenous thrombin potential, ETP) were calculated using the Thrombinoscope™ software (Synapse B.V., Maastricht, The Netherlands). All thrombin generation curves were determined at least in duplo.

For each plasma, the ratio of the ETPs obtained in the presence and absence of APC was calculated and normalised against the ratio of normal pooled plasma (normalised APC sensitivity ratio, nAPCsr) [24]. A high nAPCsr indicates APC resistance. The APC concentration in the assay (~5 nM) was aimed at reducing the ETP of normal plasma to 10% of its value without APC. In the experiment in which FV Leiden pseudo-homozygotes and homozygotes were compared (Fig. 3B), a higher APC concentration was used to enhance a possible difference between the two groups.

Data presented in Figs. 4 and 5 were obtained by performing the ETP-based assay in congenitally FV-deficient plasma (George King Bio-Medical Inc., Overland Park, KS) reconstituted with purified normal FV and/or FV Leiden. Single-chain FV was purified as previously described [4] and migrated as a single band of ~330 kD on an SDS-gel.

Assay for the measurement of FVa susceptibility to APC

The susceptibility of FVa to APC-mediated inactivation was determined with a previously described prothrombinase-based assay [25], with minor modifications. Plasma was diluted 1/1000 in a buffer containing 25 mM Hepes (pH 7.7 at room temperature), 175 mM NaCl, 3 mM CaCl₂, 5 mg/ml BSA and 21.6 μM phospholipids (DOPS/DOPC, 10/90 mol/mol), and FV was activated with 2.7 nM IIa in the presence or absence of 0.26 nM APC. After 8 minutes, the FVa present in the activation/inactivation mixtures was quantified *via* a prothrombinase-based assay in a reaction mixture containing 0.3 nM factor Xa (FXa), 1 μM prothrombin, 19.8 μM phospholipids (DOPS/DOPC, 10/90 mol/mol) and 2.6 mM CaCl₂ (final concentrations). The ratio of the FVa activities determined in the presence and absence of APC was expressed as the percentage FVa left after APC treatment (%rest). The higher the %rest, the more resistant FVa is to APC-mediated inactivation. Each sample was measured in duplo.

This assay was also used to determine the residual expression of the non-Leiden FV allele in plasma from the FV Leiden pseudo-homozygotes. FV-deficient plasma reconstituted with a fixed amount of purified FV Leiden (11.5 nM) and increasing amounts of purified normal FV (0-11.5 nM) was diluted 1/1000 in buffer to simulate dilutions of plasma from FV Leiden-heterozygous individuals with variable expression of the counterpart FV allele. Based on the %rest of these reconstituted plasmas in the prothrombinase-based APC

resistance test, a calibration curve was constructed and used to determine the normal FV/FV Leiden ratio in plasma from the pseudo-homozygotes. Each point was measured three times in duplo.

Assay for the measurement of the APC-cofactor activity of FV

The ability of FV to stimulate APC-mediated FVIIIa inactivation was measured with a home-made assay based on a test kit for the measurement of FVIII levels in plasma (Immunochrom FVIII:C, Progen Biotechnik GmbH, Heidelberg, Germany). Plasma was diluted 1:21 in the buffer provided with the kit and incubated with either buffer or APC (Kordia, Leiden, The Netherlands, ~30 nM final concentration) in the presence of phospholipids (Reagent A) for 2 min. Subsequently, the residual FVIII was activated and quantified by adding Reagent B (thrombin, factor IXa, FX and Ca^{2+}), and FXa generation was stopped after 5 min with EDTA-containing buffer. The amount of FXa generated was subsequently quantified using the chromogenic substrate S2765, and the ratio between the amidolytic activities obtained in the absence and in the presence of APC was calculated (APCsr). This method yields APCsr's comparable to those obtained with the Immunochrom[®] APC Response assay [26]. All samples were measured in duplo.

Measurement of coagulation factor concentrations in plasma

FV antigen was measured by a home-made ELISA [8]. FV activity levels were obtained from the prothrombinase-based assay (measurement without APC). FVIII activity levels were derived from the FVIII-based APC resistance test (measurement without APC). Prothrombin levels were measured with an amidolytic assay after activation of prothrombin with Ecarin (Pentapharm, Basel, Switzerland). Free protein S levels were determined by ELISA [27].

Statistics

For statistical purposes individuals were grouped according to their FV genotypes, irrespective of patient/control status. Plasma levels of coagulation factors and APC resistance values were compared between genotype groups using the Mann-Whitney-Wilcoxon test.

RESULTS

Characterisation of FV Leiden pseudo-homozygous individuals

Seven FV Leiden pseudo-homozygotes were enrolled in the study. Their demographic, genetic and clinical features are summarised in Table 1.

Diagnosis of pseudo-homozygous APC resistance was based on heterozygous carriership of the FV Leiden mutation, reduction of FV antigen and activity levels in plasma (Table 1) and pronounced APC resistance in the aPTT-based assay. Absence of normal FV in plasma was subsequently verified *via* the prothrombinase-based APC resistance assay, whose outcome (%rest) is a sensitive marker of the ratio between normal FV and FV Leiden present in plasma. Comparison of the %rest obtained in the plasmas of the pseudo-homozygotes with a calibration curve constructed with FV-deficient plasma reconstituted with 50% purified FV Leiden and varying amounts of purified normal FV (Fig. 1A) indicated that the non-Leiden allele expressed between 1.5% and 3.6% FVa activity in the different pseudo-homozygous individuals (Fig. 1B).

FIGURE 1: Determination of normal FV expression in plasma from FV Leiden pseudo-homozygotes. FV present in 1/1000-diluted plasma was activated with thrombin and subsequently inactivated with APC, and FVa activity was determined *via* a prothrombinase-based assay. The outcome of the test was expressed as residual FVa activity (%rest) after APC treatment. Each data point is the average of three duplo measurements and error bars represent the standard error of the mean. A) Calibration curve determined in FV-deficient plasma reconstituted with a fixed amount (50%) of purified FV Leiden and a variable amount (0-50%) of purified normal FV. B) Percentage expression of the non-Leiden FV allele in the FV Leiden pseudo-homozygous individuals as estimated from the %rest in the prothrombinase-based APC resistance assay.

FV deficiency was caused by frame-shift or nonsense mutations in four pseudo-homozygous individuals and by missense mutations in the other three (Table 1). After screening all family members for carriership of the FV defect identified in the respective proband, complete co-segregation between FV gene mutation and reduced FV levels was observed in all families.

APC resistance in FV Leiden pseudo-homozygotes and their relatives with different FV genotypes

APC resistance was measured in the seven FV Leiden pseudo-homozygotes and in 30 family members, among which 12 FV Leiden heterozygotes, 7 carriers of quantitative FV deficiency and 11 normal subjects (Fig. 2). Both in the aPTT-based assay (Fig. 2A) and in the ETP-based assay (Fig. 2B), plasma from FV Leiden pseudo-homozygotes was significantly more APC-resistant than that from FV Leiden heterozygotes, which in turn was more APC-resistant than plasma from non-carriers of the FV Leiden mutation. No significant difference was detected in either assay between individuals with partial FV deficiency (in the absence of FV Leiden) and their relatives with a normal FV genotype. Although the mean plasma levels of the major determinants of the APC resistance tests (FVIII, protein S and prothrombin) did not differ significantly between any of the genotype groups (data not shown), multiple regression analysis was performed to dissect the net effect of FV genotype on APC resistance. Correction for demographic parameters (age, sex and patient/control status) and plasma variables (FV, FVIII, protein S and prothrombin levels) hardly affected the observed differences in APC resistance between genotype groups, and FV genotype remained the main determinant of these differences.

To further characterise the APC resistance phenotype in FV Leiden pseudo-homozygous plasma, we also determined APC resistance with the prothrombinase-based assay (Fig. 2C), which probes the susceptibility of FVa for APC, and with the FVIII-based test, which specifically quantifies the APC-cofactor activity of FV in FVIIIa inactivation (Fig. 2D). In both tests, FV Leiden pseudo-homozygotes proved significantly more APC-resistant than heterozygotes, indicating that the higher APC resistance in FV Leiden pseudo-homozygous plasma is due both to decreased APC-susceptibility of FVa and to impaired APC-cofactor activity of FV in FVIIIa inactivation.

Interestingly, a significant difference in APC-cofactor activity was observed between normal individuals and subjects with partial FV-deficiency in the FVIII-based APC resistance test (Fig. 2D).

FIGURE 2: APC resistance in FV Leiden pseudo-homozygotes and their family members with different FV genotypes. APC resistance was determined with the aPTT-based (A), ETP-based (B), prothrombinase-based (C) and FVIII-based (D) assays as described under Methods. FVL, FV Leiden. The horizontal lines represent the means of each genotype group.

APC resistance in FV Leiden pseudo-homozygotes versus FV Leiden homozygotes

The FV Leiden pseudo-homozygous individuals were also compared to an unrelated group of 32 FV Leiden homozygotes (Fig. 3). Although the latter included both thrombotic patients ($n=12$) and asymptomatic relatives ($n=20$), all FV Leiden homozygotes were treated as a single group since APC resistance, FV, FVIII, protein S and prothrombin levels were not significantly associated with the disease status (data not shown). FV Leiden pseudo-homozygotes and homozygotes appeared virtually indistinguishable in the aPTT-based (Fig. 3A), ETP-based (Fig. 3B) and prothrombinase-based (Fig. 3C) APC resistance assays. Only in the FVIII-based test (Fig. 3D), plasma from FV Leiden pseudo-

homozygotes was significantly *more* APC-resistant than plasma from FV Leiden homozygotes. However, multiple regression analysis showed that 60% of this difference was attributable to small differences in demographic and plasma parameters between the two genotype groups, and after correction for these variables the APC resistance difference between FV Leiden homozygotes and pseudo-homozygotes was not significant anymore.

FIGURE 3: APC resistance in FV Leiden pseudo-homozygotes and in unrelated FV Leiden homozygotes. APC resistance was determined with the aPTT-based (A), ETP-based (B), prothrombinase-based (C) and FVIII-based (D) assays as described under Methods. FVL, FV Leiden. The horizontal lines represent the means of each genotype group.

ETP-based APC resistance test in simulated FV Leiden pseudo-homozygous plasma

To exclude the possibility that differences in plasma variables other than FVIII, protein S and prothrombin among the FV genotype groups could have influenced our results, we also performed a "proof-of-principle" experiment

in a plasma model. FV Leiden-heterozygous, homozygous and pseudo-homozygous plasmas as well as normal plasma were simulated by reconstituting FV-deficient plasma with purified normal FV and/or FV Leiden, and the nAPCsr of the reconstituted plasmas was determined with the ETP-based APC resistance test. While in the absence of APC all plasmas yielded virtually identical ETPs, in the presence of APC FV Leiden homozygous and pseudo-homozygous plasma generated twice as much thrombin as FV Leiden-heterozygous plasma (Fig. 4A). Consequently, after normalisation against simulated normal plasma, FV Leiden pseudo-homozygous plasma (nAPCsr 9.1) proved significantly more APC-resistant than heterozygous plasma (nAPCsr 4.6) and was actually indistinguishable from homozygous plasma (nAPCsr 9.2) (Fig. 4B).

FIGURE 4: ETP-based APC resistance assay in simulated plasmas from individuals with different FV genotypes. FV-deficient plasma was reconstituted with purified FV to mimic normal plasma (100% normal FV), FV Leiden-heterozygous plasma (50% FV Leiden and 50% normal FV), FV Leiden-homozygous plasma (100% FV Leiden) and FV Leiden pseudo-homozygous plasma (50% FV Leiden only). Thrombin generation was measured after extrinsic activation of plasma in the absence and presence of APC. A) ETP of the different plasmas in the absence (black) and presence (white) of APC; B) nAPCsr as a function of the FV genotype.

APC resistance in FV Leiden heterozygotes: effect of the counterpart FV allele

The data presented above indicate that, in heterozygous carriers of FV Leiden, complete non-expression of the counterpart FV allele significantly increases APC resistance. To further evaluate the role of the non-Leiden FV allele as a possible modulator of the APC resistance phenotype in FV Leiden heterozygotes, we simulated plasmas from FV Leiden heterozygotes with

variable expression of the counterpart FV allele and measured thrombin generation in the absence and presence of APC. When the concentration of normal FV complementing the 50% FV Leiden was varied in plasma from 0% (true pseudo-homozygosity) to 50% (perfect heterozygosity) and further increased to 60% (to mimic a slight over-expression of the normal FV allele), the ETP in the absence of APC remained constant, while the ETP in the presence of APC progressively decreased with increasing normal FV (Fig. 5A). Consequently, the nAPCsr decreased linearly (plasma became less APC-resistant) with the proportion of normal FV present in plasma (Fig. 5B).

FIGURE 5: ETP-based assay in simulated plasmas from FV Leiden heterozygotes with variable expression of the counterpart FV allele. FV-deficient plasma was reconstituted with a fixed amount (50%) of purified FV Leiden and a variable amount (0-60%) of purified normal FV, and thrombin generation was measured after extrinsic activation of plasma in the absence and presence of APC. A) ETP of the different plasmas in the absence (black) and presence (white) of APC; B) nAPCsr as a function of the relative amount of normal FV in plasma.

Code	Sex (M/F)	Age (years)	FV defect (nLaa, domain)	Type of defect	FV:Ag (%)	FV:C (%)	Clinical features	Ref.
P#1	M	58	5279A/G 1702 Tyr/Cys (A3)	Missense	44	44	Recurrent SVT since age 40 Idiopathic DVT in the right leg at age 50	[8,10,17,20]
P#12	M	42	6302 delG Stop at codon 2049 (C1)	Frameshift	51	56	SVT at age 25 Spontaneous DVT at age 35 Recurrent DVT and PE at age 37	[20]
P#13	M	54	6302 delG Stop at codon 2049 (C1)	Frameshift	60	56	Asymptomatic	[20]
P#22	F	74	3706 insTC Stop at codon 1224* (B)	Frameshift	60	51	Recurrent SVT since age 27 Spontaneous DVT in the right leg at age 63 Recurrent DVT and PE at age 66	[9,20]
P#28	F	38	4996G/A 1608 Glu/Lys (A3)	Missense	51	56	DVT in left leg at age 25 during OCT	-
P#30	F	44	5854C/T 1894 Gln/Stop (C1)	Nonsense	46	62	Bilateral SVT at age 27 during pregnancy Recurrent SVT until age 38 Spontaneous distal DVT at age 40 Severe post-thrombophlebitis syndrome	[10,20]
P#36	M	47	6509G/A 2112 Gly/Asp (C2)	Missense	59	52	Recurrent SVT since age 25	[20]

SVT, superficial vein thrombosis; DVT, deep vein thrombosis; PE, pulmonary embolism; OCT, oral contraceptive treatment; nt, nucleotide; aa, amino acid

* Erroneously reported as codon 1282 in the original publication

Table 1: FV Leiden pseudo-homozygous individuals

DISCUSSION

Several case reports describing pseudo-homozygous APC resistance, *i.e.* double heterozygosity for FV Leiden and a FV null mutation, have appeared in the literature during the last 10 years [8-18]. Pseudo-homozygous APC-resistant individuals are characterised by a discrepancy between FV Leiden genotype (heterozygous) and APC resistance phenotype (similar to that of homozygotes), accompanied by a 50% reduction of FV levels in plasma. Although most known cases are thrombotic patients, the severity of the hypercoagulable state and of the thrombotic risk associated with pseudo-homozygous APC resistance has been difficult to establish due to the rarity of the condition and a possible referral bias.

In this paper we present a detailed characterisation of the APC resistance phenotype associated with pseudo-homozygous APC resistance, based on the study of seven FV Leiden pseudo-homozygotes (Table 1) and on simulation of this condition in a plasma model. All pseudo-homozygotes included in the study were characterised by virtual absence of normal FV in plasma, as demonstrated by a) the 50% reduction of plasma FV antigen and activity levels, and b) the very low (<4%) residual expression of the non-Leiden FV allele as estimated in the prothrombinase-based APC resistance test (Fig. 1B). Quantitative FV deficiency in the seven pseudo-homozygotes was caused by a wide spectrum of molecular defects including frameshift, nonsense and missense mutations (Table 1). While the frameshift and nonsense mutations are definitely null defects [9,10], the missense mutations are compatible with normal mRNA expression [15,17] and might theoretically lead to (residual) expression of the mutated protein. However, the 1702 Tyr/Cys and 2112 Gly/Asp mutations have been reported in the homozygous state in patients with undetectable FV levels and severe bleeding [28,29], and shown to yield no protein by Western blot analysis (1702 Tyr/Cys [17]) and by expression of the recombinant molecule (2112 Gly/Asp [29]), respectively. Although the 1608 Glu/Lys mutation was shown to produce appreciable FVa activity in the conditioned medium of transfected cells [30], its expression in patient P #28 was extremely low (2.9%) and indistinguishable from that of the truly null FV defects as estimated by the prothrombinase-based APC resistance test (Fig. 1B).

Four functional plasma tests that measure different aspects of APC resistance were used to compare the 7 pseudo-homozygous individuals to 30 family members with different FV genotypes (including 12 FV Leiden heterozygotes) and to 32 unrelated FV Leiden homozygotes. All assays consistently showed that FV Leiden pseudo-homozygotes are significantly more APC-resistant than FV Leiden heterozygotes (Fig. 2) and indistinguishable from

homozygotes (Fig. 3). Since the aPTT-based and ETP-based APC resistance tests, which were performed in full plasma, are sensitive to plasma FVIII, protein S and prothrombin levels [31], inter-individual differences in the levels of these proteins may account for the variation in nAPCsr observed *within* each FV genotype group. However, since the average levels of FVIII, protein S and prothrombin did not differ significantly between any of the FV genotype groups, the observed differences in APC resistance between FV Leiden pseudo-homozygous and heterozygous individuals are largely attributable to the FV genotype, as confirmed by multiple regression analysis. The same applies to the FVIII-based APC resistance test which is sensitive to FVIII and protein S levels as well as FV levels [32]. These conclusions are confirmed by ETP measurements in simulated plasmas (Fig. 4), where the influence of plasma components other than FV is excluded by reconstitution of one and the same FV-deficient plasma with purified FV. As shown in Fig. 4B, the ETP-based nAPCsr's of FV Leiden pseudo-homozygous and homozygous plasmas are identical and twice as high as that of heterozygous plasma when potential modulators of the APC resistance phenotype are kept constant.

In plasma, the difference between FV Leiden pseudo-homozygotes and heterozygotes lies in the absence or presence of the 50% normal FV produced by the counterpart (non-Leiden) allele. This normal FV not only ensures APC-cofactor activity in FVIIIa inactivation (Fig. 2D), but it also increases the overall susceptibility of plasma FVa to APC-mediated inactivation (Fig. 2C). This explains why a progressive reduction of the relative amount of normal FV in plasma from FV Leiden heterozygotes results in increasing APC resistance (Fig. 5B). Differently, the 50% extra FV Leiden that distinguishes FV Leiden-homozygous from pseudo-homozygous plasma does not affect FVa susceptibility to APC-catalysed inactivation (Fig. 3C) nor the inactivation of FVIIIa. In fact, the difference in FVIII-based APCsr between FV Leiden homozygotes and pseudo-homozygotes (Fig. 3D) largely disappeared after correction for demographic and plasma variables, in accordance with the notion that FV Leiden does not express APC-cofactor activity in FVIIIa inactivation [23].

By comparing the thrombosis-free survival of probands with different FV genotypes, we have recently shown that FV Leiden pseudo-homozygous and homozygous individuals experience a higher thrombotic risk than heterozygotes [20]. Since APC resistance is an excellent indicator of thrombotic risk [33,34], the present data offer a functional explanation for these epidemiological observations.

In conclusion, our findings indicate that the hypercoagulable state associated with pseudo-homozygous APC resistance is indistinguishable from

that found in FV Leiden homozygotes and that APC resistance in FV Leiden carriers does not depend on the absolute amount of FV Leiden in plasma, but rather on the relative amount of normal FV.

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REFERENCES

- 1 Dahlbäck B, Carlsson M, Svensson PJ. Familial thrombophilia due to a previously unrecognized mechanism characterized by poor anticoagulant response to activated protein C: prediction of a cofactor to activated protein C. *Proc Natl Acad Sci U S A* 1993; **90**: 1004-8.
- 2 Bertina RM, Koeleman BP, Koster T, Rosendaal FR, Dirven RJ, de Ronde H, van der Velden PA, Reitsma PH. Mutation in blood coagulation factor V associated with resistance to activated protein C. *Nature* 1994; **369**: 64-7.
- 3 Kalafatis M, Bertina RM, Rand MD, Mann KG. Characterization of the molecular defect in factor VR506Q. *J Biol Chem* 1995; **270**: 4053-7.
- 4 Nicolaes GA, Tans G, Thomassen MC, Hemker HC, Pabinger I, Váradi K, Schwarz HP, Rosing J. Peptide bond cleavages and loss of functional activity during inactivation of factor Va and factor VaR506Q by activated protein C. *J Biol Chem* 1995; **270**: 21158-66.
- 5 Váradi K, Rosing J, Tans G, Pabinger I, Keil B, Schwarz HP. Factor V enhances the cofactor function of protein S in the APC- mediated inactivation of factor VIII: influence of the factor VR506Q mutation. *Thromb Haemost* 1996; **76**: 208-14.
- 6 Thorelli E, Kaufman RJ, Dahlbäck B. Cleavage of factor V at Arg 506 by activated protein C and the expression of anticoagulant activity of factor V. *Blood* 1999; **93**: 2552-8.
- 7 Rosendaal FR, Koster T, Vandenbroucke JP, Reitsma PH. High risk of thrombosis in patients homozygous for factor V Leiden (activated protein C resistance). *Blood* 1995; **85**: 1504-8.
- 8 Simioni P, Scudeller A, Radossi P, Gavasso S, Girolami B, Tormene D, Girolami A. "Pseudo homozygous" activated protein C resistance due to double heterozygous factor V defects (factor V Leiden mutation and type I quantitative factor V defect) associated with thrombosis: report of two cases belonging to two unrelated kindreds. *Thromb Haemost* 1996; **75**: 422-6.
- 9 Castoldi E, Kalafatis M, Lunghi B, Simioni P, Ioannou PA, Petio M, Girolami A, Mann KG, Bernardi F. Molecular bases of pseudo-homozygous APC resistance: the compound heterozygosity for FV R506Q and a FV null mutation results in the exclusive presence of FV Leiden molecules in plasma. *Thromb Haemost* 1998; **80**: 403-6.
- 10 Kalafatis M, Bernardi F, Simioni P, Lunghi B, Girolami A, Mann KG. Phenotype and genotype expression in pseudohomozygous factor V^{LEIDEN}: the need for phenotype analysis. *Arterioscler Thromb Vasc Biol* 1999; **19**: 336-42.
- 11 Zehnder JL, Jain M. Recurrent thrombosis due to compound heterozygosity for factor V Leiden and factor V deficiency. *Blood Coagul Fibrinolysis* 1996; **7**: 361-2.
- 12 Guasch JF, Lensen RP, Bertina RM. Molecular characterization of a type I quantitative factor V deficiency in a thrombosis patient that is "pseudo homozygous" for activated protein C resistance. *Thromb Haemost* 1997; **77**: 252-7.
- 13 Castaman G, Lunghi B, Missiaglia E, Bernardi F, Rodeghiero F. Phenotypic homozygous activated protein C resistance associated with compound heterozygosity for Arg506Gln (factor V Leiden) and His1299Arg substitutions in factor V. *Br J Haematol* 1997; **99**: 257-61.

- 14 Delahousse B, lochmann S, Pouplard C, Fimbel B, Charbonnier B, Gruel Y. Pseudo-homozygous activated protein C resistance due to coinheritance of heterozygous factor V Leiden mutation and type I factor V deficiency. Variable expression when analyzed by different activated protein C resistance functional assays. *Blood Coagul Fibrinolysis* 1997; **8**: 503-9.
- 15 Lunghi B, Castoldi E, Mingozzi F, Bernardi F, Castaman G. A novel factor V null mutation detected in a thrombophilic patient with pseudo-homozygous APC resistance and in an asymptomatic unrelated subject. *Blood* 1998; **92**: 1463-4.
- 16 Standen G, Morse C, Aslam S, Bowron A. Recurrent thrombosis in a patient with pseudohomozygous activated protein C resistance and homozygosity for MTHFR gene polymorphism C677T. *Thromb Haemost* 1999; **81**: 663-4.
- 17 Castoldi E, Simioni P, Kalafatis M, Lunghi B, Tormene D, Girelli D, Girolami A, Bernardi F. Combinations of 4 mutations (FV R506Q, FV H1299R, FV Y1702C, PT 20210G/A) affecting the prothrombinase complex in a thrombophilic family. *Blood* 2000; **96**: 1443-8.
- 18 Dargaud Y, Trzeciak MC, Meunier S, Angei C, Pellechia D, Negrier C, Vinciguerra C. Two novel factor V null mutations associated with activated protein C resistance phenotype/genotype discrepancy. *Br J Haematol* 2003; **123**: 342-5.
- 19 Castaman G, Tosetto A, Ruggeri M, Rodeghiero F. Pseudohomozygosity for activated protein C resistance is a risk factor for venous thrombosis. *Br J Haematol* 1999; **106**: 232-6.
- 20 Simioni P, Castoldi E, Lunghi B, Tormene D, Rosing J, Bernardi F. An underestimated combination of opposites resulting in enhanced thrombotic tendency. *Blood*. 2005; 106(7): 2363-5.
- 21 Scanavini D, Girelli D, Lunghi B, Martinelli N, Legnani C, Pinotti M, Palareti G, Bernardi F. Modulation of factor V levels in plasma by polymorphisms in the C2 domain. *Arterioscler Thromb Vasc Biol* 2004; **24**: 200-6.
- 22 de Ronde H, Bertina RM. Laboratory diagnosis of APC-resistance: a critical evaluation of the test and the development of diagnostic criteria. *Thromb Haemost* 1994; **72**: 880-6.
- 23 Castoldi E, Brugge JM, Nicolaes GA, Girelli D, Tans G, Rosing J. Impaired APC cofactor activity of factor V plays a major role in the APC resistance associated with the factor V Leiden (R506Q) and R2 (H1299R) mutations. *Blood* 2004; **103**: 4173-9.
- 24 Nicolaes GA, Thomassen MC, Tans G, Rosing J, Hemker HC. Effect of activated protein C on thrombin generation and on the thrombin potential in plasma of normal and APC-resistant individuals. *Blood Coagul Fibrinolysis* 1997; **8**: 28-38.
- 25 Nicolaes GA, Thomassen MC, van Oerle R, Hamulyák K, Hemker HC, Tans G, Rosing J. A prothrombinase-based assay for detection of resistance to activated protein C. *Thromb Haemost* 1996; **76**: 404-10.
- 26 Váradi K, Moritz B, Lang H, Bauer K, Preston E, Peake I, Rivard GE, Keil B, Schwarz HP. A chromogenic assay for activated protein C resistance. *Br J Haematol* 1995; **90**: 884-91.
- 27 Koenen RR, Thomassen MC, Tans G, Rosing J, Hackeng TM. Effect of oral contraceptives on the anticoagulant activity of protein S in plasma. *Thromb Haemost* 2005; **93**: 853-9.

- 28 Castoldi E, Lunghi B, Mingozzi F, Muleo G, Redaelli R, Mariani G, Bernardi F. A missense mutation (Y1702C) in the coagulation factor V gene is a frequent cause of factor V deficiency in the Italian population. *Haematologica* 2001; **86**: 629-33.
- 29 Olds R, Simioni P, Thompson E, Morgan T, Girolami A, Lane DA. Factor V Gly2112Asp, a C2-domain variant, is associated with severe deficiency and bleeding tendency. *J Thromb Haemost* 2003; **1** (Suppl. 1): P1206.
- 30 Lunghi B, Scanavini D, Castoldi E, Gemmati D, Tognazzo S, Redaelli R, Ghirarduzzi A, Ieran M, Pinotti M, Bernardi F. The factor V Glu1608Lys mutation is recurrent in familial thrombophilia. *J Thromb Haemost* 2005; **3**: 2032-8.
- 31 de Visser MC, van Hylckama Vlieg A, Tans G, Rosing J, Dahm AE, Sandset PM, Rosendaal FR, Bertina RM. Determinants of the APTT-based and ETP-based APC sensitivity tests. *J Thromb Haemost* 2005; **3**: 1488-94.
- 32 Govers-Riemsdijk JW, Castoldi E, Nicolaes GA, Tans G, Rosing J. Reduced factor V concentration and altered FV₁/FV₂ ratio do not fully explain R2-associated APC-resistance. *Thromb Haemost* 2002; **88**: 444-9.
- 33 de Visser MC, Rosendaal FR, Bertina RM. A reduced sensitivity for activated protein C in the absence of factor V Leiden increases the risk of venous thrombosis. *Blood* 1999; **93**: 1271-6.
- 34 Rodeghiero F, Tosetto A. Activated protein C resistance and factor V Leiden mutation are independent risk factors for venous thromboembolism. *Ann Intern Med* 1999; **130**: 643-50.

CHAPTER 5

Protein S levels modulate the activated protein C resistance phenotype induced by elevated prothrombin levels

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SUMMARY

Elevated plasma prothrombin levels, due to the prothrombin 20210 G/A mutation or to acquired causes, are a risk factor for venous thrombosis, partly because of prothrombin-mediated inhibition of the protein C anticoagulant pathway and consequent activated protein C (APC) resistance. We have determined the effect of plasma prothrombin concentration on the APC resistance phenotype and evaluated the role of protein S levels as a modulating variable. The effect of prothrombin and protein S levels on APC resistance was investigated in reconstituted plasma systems and in a population of healthy individuals using both the aPTT-based and the thrombin generation-based APC resistance tests. In reconstituted plasma APC resistance increased at increasing prothrombin concentration in both assays. Enhanced APC resistance was caused by the effect of prothrombin on the clotting time in the *absence* of APC in the aPTT-based test, and on thrombin formation in the *presence* of APC in the thrombin generation-based test. In plasma from healthy individuals prothrombin levels were highly correlated to protein S levels. Since prothrombin and protein S had opposite effects on the APC resistance phenotype, the prothrombin/protein S ratio was a better predictor of APC resistance than the levels of either protein alone. Prothrombin titrations in plasmas containing different amounts of protein S confirmed that protein S levels modulate the ability of prothrombin to induce APC resistance. These findings suggest that carriers of the prothrombin 20210 G/A mutation, who have a high prothrombin/protein S ratio, may experience a higher thrombosis risk than non-carriers with comparable prothrombin levels.

INTRODUCTION

Prothrombin is a 72-kDa vitamin K-dependent glycoprotein synthesised in the liver and circulating in plasma at a concentration of 1.5-2.0 μM . Structurally, it comprises a Gla domain, responsible for Ca^{2+} -dependent binding to phospholipid membranes, two kringle domains and a serine-protease domain. Prothrombin is the inactive precursor of thrombin, which plays a pivotal role in thrombus formation by converting fibrinogen to fibrin and activating coagulation factors (F) XIII, XI, VIII and V as well as platelets (1). After binding to thrombomodulin on the surface of endothelial cells, thrombin loses its affinity for these substrates and activates protein C, thereby initiating the anticoagulant pathway. Activated protein C (APC) proteolytically inactivates coagulation FVa and FVIIIa. The vitamin K-dependent APC-cofactor protein S stimulates APC-mediated FVa- and FVIIIa-inactivation (2), but also expresses anticoagulant activity in the absence of APC (3,4). Functional defects of the protein C pathway, *e.g.* associated with the FV Leiden mutation (5), determine a typical plasma phenotype known as APC resistance (6), which is a common risk factor for venous thrombosis. The hallmark of APC-resistant plasma is the impaired ability of exogenous APC to prolong the clotting time of plasma in an aPTT-based assay (6).

The 20210 G/A mutation in the 3'-untranslated region of the prothrombin gene causes a ~30% increase in plasma prothrombin levels in the heterozygous state (7). Elevated plasma levels of prothrombin, due to this mutation or to acquired conditions, are a prevalent risk factor for venous thrombosis (7). Although the molecular basis for the association between increased risk of venous thrombosis and elevated prothrombin levels is unclear, several possible explanations have been put forward (8-12). Evidence has also been provided that prothrombin interferes with the protein C anticoagulant pathway (FVa inactivation) by inhibiting APC (13) and/or protein S (14) function. This effect is already observed at physiological prothrombin concentrations and is not explained by simple competition between prothrombin and the APC/protein S complex for binding to the phospholipid surface (13,15). Accordingly, elevated plasma prothrombin levels were shown to cause APC resistance both in unselected individuals (16) and in carriers of the prothrombin 20210 G/A mutation (17).

In order to get more insight into prothrombin-induced APC resistance, we have investigated the effect of plasma prothrombin levels on the APC resistance phenotype in reconstituted plasma systems and in a population of healthy individuals using two functional APC resistance assays.

MATERIALS AND METHODS

Plasmas

Prothrombin-deficient plasma. Congenitally prothrombin-deficient plasma (FII:C ~2%) was purchased from George King Bio-Medicals Inc. (Overland Park, KS).

Individual plasmas. Blood was collected in 3.8% sodium citrate from 77 fasting healthy individuals (49 males and 28 females; mean age 41.0 years) free of medication and not using oral contraceptives. Platelet-poor plasma was prepared by centrifuging twice at 3000g for 15 min at room temperature, aliquoted and snap-frozen at -80 °C. All donors were genotyped for the FV Leiden and prothrombin 20210 G/A mutations, and FV Leiden carriers (n=3) were excluded from subsequent analyses while carriers of the prothrombin mutation (n=2) were not.

Protein S-depleted plasma. Blood was drawn in 3.2% sodium citrate from 7 fasting healthy volunteers (6 males and 1 female; mean age 38.4 years) and platelet-poor plasma was obtained as described above. After pooling the plasma, protein S-immunodepletion was performed according to Koenen *et al.* (10). This procedure simultaneously removes C4b-binding protein, a complement protein that complexes ~60% of plasma protein S. The residual protein S level in protein S-depleted plasma was 10% of normal pooled plasma as determined by a total protein S ELISA (18). The prothrombin concentration was 96% of that of normal pooled plasma.

Variation of the prothrombin and protein S concentrations in plasma

Prothrombin and prothrombin fragment 1+2 titrations (Figs. 1 and 2). Human prothrombin and prothrombin fragment 1+2 (comprising the Gla domain and the two kringle domains) were purified from normal pooled plasma as reported (19,20). The prothrombin concentration in plasma was varied between 10% and 175% by reconstituting prothrombin-deficient plasma with increasing amounts of purified prothrombin. The normal plasma prothrombin concentration (100%) was assumed to be 2.0 μ M. The titrations in Fig. 2 were performed by adding increasing amounts of purified prothrombin or prothrombin fragment 1+2, ranging from 0 to 1.2 μ M (*i.e.* 0-60% of the normal plasma concentration of prothrombin), to normal pooled plasma.

Protein S titrations (Fig. 5). Human protein S was purified from normal pooled plasma as reported (18). The concentration of free protein S in plasma was varied between 10% and 175% by supplementing protein S-depleted

plasma with increasing amounts of purified protein S. The normal plasma concentration of free protein S (100%) was assumed to be 200 nM.

Prothrombin titrations at different protein S concentrations (Fig. 6). Plasma containing 50% protein S was prepared by mixing protein S-depleted plasma with normal plasma. Plasma containing 100% protein S was normal pooled plasma. Plasma containing 150% protein S was obtained by supplementing normal pooled plasma with 50% (100 nM) purified protein S. The prothrombin concentration in these plasmas was varied between 100% and 175% by adding increasing amounts of purified prothrombin. For the double titration (Fig. 6B), normal pooled plasma was supplemented with both purified prothrombin and purified protein S in a constant molar ratio.

Measurement of APC resistance

aPTT-based APC resistance assay. The aPTT-based APC resistance test was performed in undiluted plasma using the Coatest[®] APC Resistance kit (Chromogenix, Mölndal, Sweden) in an ACL 300 Research coagulometer (Automated Coagulation Laboratory). All clotting times were measured in duplicate ($n=2$, unless otherwise stated in the figure legend) and results were expressed as normalised APC-sensitivity ratios (nAPCsr). A low nAPCsr indicates APC resistance.

Thrombin generation-based APC resistance assay. The thrombin generation assay was performed essentially as reported (21), with minor modifications. The tissue factor (TF) and phospholipid concentrations in the trigger were doubled, yielding final concentrations of ~ 13.6 pM TF (Innovin[®], DADÉ-Behring, Marburg, Germany) and 30 μ M synthetic phospholipid vesicles (DOPS/DOPC/DOPE, 20/60/20 mol/mol/mol) in the reaction mixture. Conversion of the fluorogenic substrate (Z-Gly-Gly-Arg-AMC, BACHEM AG, Bubendorf, Switzerland) by the thrombin formed in plasma was followed in a Fluoroskan Ascent[®] reader (Thermo Labsystems, Helsinki, Finland). Thrombin generation curves and the endogenous thrombin potential (ETP) (22) were calculated using the Thrombinoscope[™] software (Synapse B.V., Maastricht, The Netherlands). All thrombin generation curves were determined in duplicate ($n=2$, unless otherwise stated in the figure legend).

For each plasma a normalised APC sensitivity ratio (nAPCsr) was calculated by dividing the ratio of the ETPs obtained in the presence of APC (ETP_{+APC}) and in its absence (ETP_{-APC}) by the ratio of the ETP_{+APC} and ETP_{-APC} determined in normal pooled plasma in the same experiment. A high nAPCsr indicates APC resistance. In each experiment, the APC concentration was such as to reduce the ETP of normal pooled plasma to 10% of the ETP determined in

the absence of APC. In titration experiments, reconstituted plasma containing 100% prothrombin or protein S was used as a reference instead of normal pooled plasma.

Measurement of factor levels in plasma

Plasma prothrombin levels were measured by complete activation of prothrombin with Ecarin (Pentapharm, Basel, Switzerland) in 1500-fold diluted plasma and subsequent determination of the amidolytic activity of thrombin/meizothrombin with the chromogenic substrate S2238 (Chromogenix, Mölndal, Sweden). Free protein S levels were determined by ELISA (23) and FV levels by a prothrombinase-based assay (21). FVIII levels were measured using the Coatest® FVIII kit (Chromogenix, Mölndal, Sweden). Normal pooled plasma was used as a reference in all measurements.

Statistics

Multiple regression analysis was used to dissect the effect of prothrombin and free protein S levels on the aPTT-based and ETP-based nAPCsr in the healthy population. Prothrombin, free protein S, FV and FVIII levels were included as independent variables.

The slopes of the regression lines in Fig. 6 were compared using the t-test for parallelism of regression lines.

RESULTS

Effect of prothrombin levels on APC resistance in a reconstituted plasma system

To determine the effect of prothrombin concentration on APC resistance, we reconstituted prothrombin-deficient plasma with varying amounts of purified prothrombin (ranging from 10% to 175% of the normal plasma concentration) and measured the nAPCsr with the aPTT- and the ETP-based APC resistance tests.

In the aPTT-based assay, the nAPCsr progressively decreased (*i.e.* plasma became more APC-resistant) at increasing prothrombin concentration (Fig. 1A). Between 75% and 175% prothrombin, the graph was linear with a slope of -0.010 nAPCsr units/10% prothrombin ($p=0.001$). Interestingly, the effect of increasing prothrombin concentration on the nAPCsr was not due to a shortening of the clotting time measured in the presence of APC, which was virtually constant at prothrombin concentrations $\geq 100\%$ (-0.17 sec/10% prothrombin, clotting time at 100% prothrombin = 106.2 sec), but rather to a prolongation of the clotting time measured in the absence of APC ($+0.29$ sec/10% prothrombin, clotting time at 100% prothrombin = 33.7 sec) (Fig. 1B). This unexpected observation was confirmed by a similar experiment in which the prothrombin concentration was varied by mixing prothrombin-deficient plasma with normal plasma (data not shown), thus excluding an artefact due to a possible contaminant in the prothrombin preparation used to reconstitute prothrombin-deficient plasma.

In the ETP-based APC resistance assay, the nAPCsr increased linearly with increasing prothrombin concentration (slope 0.146 nAPCsr units/10% prothrombin, $p<10^{-5}$), again indicating that elevated prothrombin levels cause APC resistance (Fig. 1C). In this case, however, the effect of prothrombin concentration on the nAPCsr was driven by the ETP_{+APC} . In fact, although both the ETP_{-APC} and the ETP_{+APC} increased at increasing prothrombin concentration (Fig. 1D), the ETP_{-APC} was a linear function of the prothrombin concentration, while the ETP_{+APC} was a quadratic function of this concentration (Fig. 1D, inset), resulting in a linear dependence of the nAPCsr on the prothrombin level.

FIGURE 1: Effect of the prothrombin concentration on APC resistance (reconstituted plasma). Prothrombin-deficient plasma was reconstituted with increasing amounts of purified prothrombin and APC resistance was determined with the aPTT-based ($n=4$) and ETP-based ($n=2$) assays. The graphs illustrate the effect of the prothrombin (PT) concentration on: A) the aPTT-based nAPCsr; B) the clotting times measured in the absence (●) and in the presence (○) of APC; C) the ETP-based nAPCsr; and D) the ETP measured in the absence (●) and in the presence (○) of APC. Plasmas containing less than 40% prothrombin have been omitted from panels C and D because they yielded unmeasurably low ETPs in the presence of APC.

To verify whether the effect of prothrombin on the ETP was due to a specific protein-protein interaction or to mere competition for the phospholipid surface, an additional titration was performed with prothrombin fragment 1+2, which binds to phospholipids with approximately the same affinity as prothrombin. Normal pooled plasma was supplemented with increasing amounts of either prothrombin or prothrombin fragment 1+2, and thrombin generation was measured in the absence and presence of APC. While prothrombin brought about the expected increase of both the ETP_{-APC} and the ETP_{+APC} (Fig. 2A,B), prothrombin fragment 1+2 hardly affected either ETP (Fig. 2C,D). The effect of the prothrombin fragment 1+2 concentration on the ETP-

based nAPCsr (0.033 nAPCsr units/10% prothrombin fragment 1+2, $p=0.038$) was only ~20% of that of prothrombin (0.164 nAPCsr units/10% prothrombin, $p<10^{-4}$) (t-test for parallelism of regression lines: $p<10^{-5}$).

FIGURE 2: Comparison of the effects of prothrombin and prothrombin fragment 1+2 on thrombin generation measured in the absence and presence of APC. Normal pooled plasma containing ~2 μM prothrombin (100%) was supplemented with 0.4 μM (20%), 0.8 μM (40%) and 1.2 μM (60%) purified prothrombin (A,B) or prothrombin fragment 1+2 (C,D), and thrombin generation was measured in the absence (A,C) and presence (B,D) of APC two times in duplicate ($n=4$).

Relationship between prothrombin levels and APC resistance in plasma from healthy individuals

The relationship between APC resistance and the plasma prothrombin level was also investigated in a population of 74 healthy blood donors, including two carriers of the prothrombin 20210 G/A mutation. Prothrombin levels in this population ranged between 79% and 171%.

In accordance with the model experiment, a negative correlation was observed between plasma prothrombin levels and the nAPCsr determined with the aPTT-based APC resistance assay (Fig. 3A). Although the correlation did not reach significance, probably due to the small sample size, the slope of the regression line (-0.011 nAPCsr units/10% prothrombin, $p=0.216$) was similar to that obtained in the model system and did not change after correction for free protein S, FV and FVIII levels (-0.011 nAPCsr units/10% prothrombin, $p=0.296$). Like in the model experiment, the clotting time measured in the absence of APC was prolonged at high prothrombin levels ($+0.27$ sec/10% prothrombin, mean clotting time = 32.1 sec), while the clotting time measured in the presence of APC was virtually independent of the plasma prothrombin level (-0.16 sec/10% prothrombin, mean clotting time = 113.6 sec) (Fig. 3B).

FIGURE 3: Effect of the prothrombin concentration on APC resistance (population of healthy individuals). The prothrombin level and APC resistance in the aPTT-based and ETP-based assays were determined in the plasma from 74 healthy blood donors ($n=2$). The graphs illustrate the correlation between the plasma prothrombin (PT) level and: A) the aPTT-based nAPCsr; B) the clotting times measured in the absence (●) and in the presence (○) of APC; C) the ETP-based nAPCsr; and D) the ETP measured in the absence (●) and in the presence (○) of APC. Panel E shows the dependence of the ETP-based nAPCsr on the prothrombin/protein S ratio. The two carriers of the prothrombin 20210 G/A mutation are indicated in triangles.

APC resistance in the plasma from the 74 donors was also determined with the ETP-based test. In sharp contrast to the model experiment, there was hardly any correlation between the ETP-based nAPCsr and plasma prothrombin levels (Fig. 3C), although both the ETP_{-APC} and the ETP_{+APC} showed a tendency to increase with the plasma prothrombin level (Fig. 3D), as in the model system. This discrepancy is likely due to the fact that the individuals of the population differ not only in prothrombin levels but also in numerous other plasma variables that may affect the APC resistance phenotype. In fact, after correcting for free protein S, FV and FVIII levels, a positive correlation was found between the ETP-based nAPCsr and plasma prothrombin levels in the population, although the slope (0.074 nAPCsr units/10% prothrombin, $p=0.012$) was about half of that observed in the model experiment. According to the multiple regression analysis, the major confounder of the effect of prothrombin on the ETP-based nAPCsr was protein S, which had a strong negative effect on the ETP-based nAPCsr (-0.113 nAPCsr units/10% free protein S, $p<10^{-5}$). Accordingly, when the prothrombin level of each plasma was “corrected” for the corresponding protein S level by taking the prothrombin/protein S ratio, the ETP-based nAPCsr showed a much better correlation with this parameter ($r = 0.460$, $p<10^{-4}$, Fig. 3E) than with the prothrombin level alone ($r = -0.024$, n.s., Fig. 3C). Further analysis revealed that the plasma levels of prothrombin and protein S were significantly correlated ($r=0.605$, $p<0.01$) in the donor population (Fig. 4).

FIGURE 4: Correlation between plasma prothrombin and protein S levels in a population of healthy individuals. Prothrombin and free protein S levels were determined in plasma from 74 healthy blood donors as described under Methods. The two carriers of the prothrombin 20210 G/A mutation are indicated in triangles.

Effect of protein S levels on APC resistance

To verify the effect of protein S levels on APC resistance, we varied the concentration of free protein S in protein S-depleted plasma between 10% and 175% of its normal plasma concentration and determined the nAPCsr with the aPTT- and ETP-based APC resistance tests.

In the aPTT-based assay, the nAPCsr increased (plasma became less APC-resistant) at increasing protein S concentrations (Fig. 5A). At protein S concentrations $\geq 75\%$ a plateau was reached with a mild slope of 0.001 nAPCsr units/10% free protein S ($p=0.410$). As shown in Fig. 5B, the effect of protein S concentration on the aPTT-based nAPCsr was entirely driven by the clotting time measured in the presence of APC, while the clotting time measured in the absence of APC was independent of the protein S concentration.

FIGURE 5: Effect of protein S concentration on APC resistance (reconstituted plasma). Protein S-depleted plasma was reconstituted with increasing amounts of purified protein S and APC resistance was determined with the aPTT-based and ETP-based assays (n=2). The graphs illustrate the effect of protein S concentration on: A) the aPTT-based nAPCsr; B) the clotting times measured in the absence (●) and in the presence (○) of APC; C) the ETP-based nAPCsr; and D) the ETP measured in the absence (●) and in the presence (○) of APC.

Similarly, the ETP-based nAPCsr sharply decreased (APC resistance decreased) with increasing protein S concentration (Fig. 5C). For protein S levels $\geq 75\%$ the graph was linear and the slope (-0.104 nAPCsr units/10% protein S, $p=0.015$) was similar to that obtained in the population after multiple regression analysis. Like in the aPTT-based assay, the effect of protein S concentration on the ETP-based nAPCsr was entirely attributable to the measurement in the presence of APC, while the ETP_{APC} was not affected by the protein S concentration at the TF concentration used (Fig. 5D). Interestingly, in the absence of protein S, APC was totally ineffective in controlling thrombin generation (Fig. 5D).

Modulation of prothrombin-induced APC resistance by protein S

The data presented above indicate that prothrombin and protein S have antagonistic effects on the APC resistance measured with the ETP-based assay. To investigate the combined effects of both proteins on the ETP-based nAPCsr, we performed prothrombin titrations in plasmas containing 50%, 100% and 150% protein S, respectively (Fig. 6A). In all three cases, APC resistance increased linearly with increasing prothrombin concentration, but the slopes of the lines were significantly different (t-test for parallelism of regression lines: $p < 10^{-4}$). The effect of prothrombin concentration on the ETP-based nAPCsr was maximal at 50% protein S (0.193 nAPCsr units/10% prothrombin, $p < 10^{-6}$) and gradually decreased when the protein S concentration was increased to 100% (0.154 nAPCsr units/10% prothrombin, $p < 10^{-6}$) and 150% (0.113 nAPCsr units/10% prothrombin, $p < 10^{-4}$).

FIGURE 6: Antagonistic effects of prothrombin and protein S levels on plasma APC resistance. A) Effect of the prothrombin (PT) concentration on the ETP-based nAPCsr of plasmas containing 50% (○), 100% (■) and 150% (△) protein S (ProtS). Plasmas containing different protein S concentrations were prepared as described under Methods and the prothrombin concentration was increased by the addition of purified prothrombin. To enable quantitative comparison of the effects of prothrombin on the nAPCsr, residual thrombin formation in the presence of APC was set at 10% in plasmas containing 50%, 100% and 150% protein S by using different APC concentrations. The slopes of the regression lines were significantly different ($p < 10^{-4}$). B) Normal pooled plasma was supplemented either with prothrombin only (■) or with prothrombin and protein S in a constant molar ratio (□) and APC resistance was measured with the ETP-based assay. The slopes of the regression lines were significantly different ($p < 10^{-5}$). All nAPCsr were determined in duplicate ($n=2$).

The correlation between prothrombin and protein S levels in the plasma from the healthy donors (Fig. 4) suggests that, in most individuals with high prothrombin levels, APC resistance will be counteracted by concomitantly high protein S levels. This predicts that carriers of the prothrombin 20210 G/A

mutation, in whom the higher prothrombin level is unopposed by protein S, will have higher APC resistance than non-carriers with comparable prothrombin levels. To test this hypothesis in a model experiment, we compared two series of plasmas containing increasing concentrations of prothrombin, ranging from 100% to 160%, in which the protein S level was either kept constant or increased proportionally to the prothrombin level. As shown in Fig. 6B, in both cases the ETP-based nAPCsr increased linearly at increasing prothrombin concentrations, but the effect of prothrombin on APC resistance was significantly ($p < 10^{-5}$) more pronounced in plasma in which only the prothrombin level was increased (0.169 nAPCsr units/10% prothrombin, $p < 10^{-5}$) than in plasma in which prothrombin and protein S levels were increased in parallel (0.105 nAPCsr units/10% prothrombin, $p < 10^{-4}$).

DISCUSSION

The molecular mechanism underlying the association between high plasma levels of prothrombin and increased risk of venous thrombosis is still a matter of debate. Since the normal plasma concentration of prothrombin (1.5-2.0 μM) largely exceeds the K_m for prothrombin activation by the prothrombinase complex (210 nM) determined in model systems (24), the procoagulant advantage conferred by an increase of the plasma prothrombin concentration is not straightforward. Recently, evidence has been provided that at least part of the prothrombotic effect of high prothrombin levels might be due to inhibition of APC-mediated FVa inactivation (13-15) and consequent APC resistance (16,17). Since hyperprothrombinemia is a prevalent condition in the general population (7), it may represent a major cause of APC resistance not associated with the FV Leiden mutation (25).

In this study, we have investigated the influence of plasma prothrombin concentration on the APC resistance phenotype using both the aPTT-based and the ETP-based APC resistance tests. Since these assays are sensitive to different plasma variables (26), they provide complementary information on the molecular basis of APC resistance. Both assays indicated that APC resistance increases at increasing prothrombin levels in plasma (Fig. 1A,C), but the underlying mechanism was different in the two cases.

In the aPTT-based test, the effect of prothrombin levels on APC resistance was mainly mediated by the measurement in the absence of APC, at least for prothrombin concentrations $\geq 100\%$. In fact, since in this range the relative effect of prothrombin concentration on the clotting time determined in the absence of APC (+0.27 sec/10% prothrombin relative to a mean clotting time of 32.1 sec) was 6 times larger than that on the clotting time determined in the presence of APC (-0.16 sec/10% prothrombin relative to a mean clotting time of 113.6 sec), ~86% of the effect of prothrombin on the aPTT-based nAPCsr is attributable to prolongation of the clotting time in the absence of APC.

Differently, in the ETP-based APC resistance test, the effect of prothrombin on the nAPCsr was driven by the measurement in the presence of APC. Our findings in reconstituted plasma confirm and extend earlier reports that thrombin generation in the absence of APC (8) and the ETP-based nAPCsr (27) are significantly higher in carriers of the prothrombin 20210 G/A mutation than in normal controls. Moreover, the inability of prothrombin fragment 1+2 to enhance the ETP_{+APC} (Fig. 2D) indicates that the inhibitory effect of prothrombin on the protein C pathway is not merely due to competition for the phospholipid surface, but mediated by specific protein-protein interactions.

In the aPTT-based assay no difference was observed between the results obtained in the plasma model (Fig. 1A,B) and in the population (Fig. 3A,B). In the physiological range of prothrombin levels (75%-175%), the dependence of the nAPCsr on the prothrombin concentration in plasma reconstituted with purified prothrombin was linear and yielded the same slope (-0.010 nAPCsr units/10% prothrombin) as in the population ($-0.011/10\%$ prothrombin). Correction for free protein S, FV and FVIII levels did not alter the slope ($-0.011/10\%$ prothrombin).

In contrast, a marked difference between the experiments conducted in reconstituted plasma (Fig. 1C,D) and in the population (Fig. 3C,D) was observed in the ETP-based APC resistance assay. The experiments performed in reconstituted plasma indicated a linear increase of the ETP-based nAPCsr at increasing prothrombin levels (Fig. 1C) with a slope of 0.146 nAPCsr units/10% prothrombin. Differently, the population experiment (Fig. 3C) showed no correlation between the ETP-based nAPCsr and prothrombin levels. This discrepancy turned out to be due to the confounding effect of protein S, whose plasma levels were highly correlated to those of prothrombin in the population (Fig. 4). Correction for free protein S levels restored the expected positive correlation between ETP-based nAPCsr and prothrombin concentration (Fig. 3E), although the slope (0.074 nAPCsr units/10% prothrombin) was not the same as in the model experiment, probably because not all assay determinants (26) were included in the multiple regression analysis. While free protein S levels in the physiological range hardly affected the aPTT-based nAPCsr (Fig. 5A), they showed a strong inverse correlation with the ETP-based nAPCsr, with comparable slopes in the model system (-0.104 nAPCsr units/10% free protein S) and in the population (-0.113 nAPCsr units/10% free protein S). The relative protein S-insensitivity of aPTT-based APC resistance tests has already been observed both *in vitro* (28) and in protein S-deficient patients (29). The superior sensitivity of the ETP-based assay to protein S and prothrombin levels might derive from the different coagulation trigger (TF instead of kaolin) and/or assay end-point (ETP instead of clotting). The trigger determines the relative contributions of the extrinsic and intrinsic coagulation pathways, and hence of APC-dependent FVa and FVIIIa inactivation, to the assay end-point. Thus, possible differences in protein S requirements between FVa and FVIIIa inactivation may result in differential effects of protein S and prothrombin (which presumably exerts its action via protein S) on the nAPCsr determined with the two assays. With respect to the assay end-point, it should be noted that clotting occurs long before most of the thrombin is generated and therefore only probes the initiation phase of thrombin generation, while the ETP is mainly determined by the propagation and termination phases. The ability to probe the termination

phase of thrombin generation likely accounts for the higher sensitivity of the ETP-based test to defects of the anticoagulant systems (antithrombin and the protein C pathway).

Taken together, our data indicate that prothrombin and protein S have antagonistic effects on the APC resistance phenotype. This makes the ratio of their levels in plasma a more accurate predictor of APC resistance than prothrombin or protein S levels alone (Fig. 3E and Fig. 6). Since prothrombin and protein S are both vitamin K-dependent proteins mainly synthesised in the liver and probably share the same biosynthetic machinery within the hepatocyte, their levels show a high degree of correlation in the general population (30,31). Therefore, most individuals with a high prothrombin level will also have a relatively high protein S level, which will mitigate prothrombin-induced APC resistance. However, in carriers of the prothrombin 20210 G/A mutation, which selectively affects prothrombin levels (7), the prothrombin/protein S ratio will be shifted in favour of prothrombin and result in enhanced APC resistance. Based on these considerations, we expect prothrombin 20210 G/A carriers to have higher APC resistance (and possibly a higher thrombosis risk) than non-carriers with comparable prothrombin levels. Of course, this prediction will have to be verified in epidemiological studies.

In conclusion, protein S levels modulate the APC resistance phenotype induced by high prothrombin levels. This phenomenon may account for the increased thrombosis risk associated with genetic and acquired conditions which perturb the prothrombin/protein S ratio in plasma, such as carriership of the prothrombin 20210 G/A mutation and oral contraceptive use.

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REFERENCES

1. Dahlbäck B. Blood coagulation. *Lancet* 2000; 355: 1627-32.
2. Walker FJ, Fay PJ. Regulation of blood coagulation by the protein C system. *Faseb J* 1992; 6: 2561-7.
3. Heeb MJ, Mesters RM, Tans G, et al. Binding of protein S to factor Va associated with inhibition of prothrombinase that is independent of activated protein C. *J Biol Chem* 1993; 268: 2872-7.
4. Hackeng TM, van 't Veer C, Meijers JC, et al. 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.
5. Bertina RM, Koeleman BP, Koster T, et al. Mutation in blood coagulation factor V associated with resistance to activated protein C. *Nature* 1994; 369: 64-7.
6. Dahlbäck B, Carlsson M, Svensson PJ. Familial thrombophilia due to a previously unrecognized mechanism characterized by poor anticoagulant response to activated protein C: prediction of a cofactor to activated protein C. *Proc Natl Acad Sci U S A* 1993; 90: 1004-8.
7. Poort SR, Rosendaal FR, Reitsma PH, et al. A common genetic variation in the 3'-untranslated region of the prothrombin gene is associated with elevated plasma prothrombin levels and an increase in venous thrombosis. *Blood* 1996; 88: 3698-703.
8. Kyrle PA, Mannhalter C, Béguin S, et al. Clinical studies and thrombin generation in patients homozygous or heterozygous for the G20210A mutation in the prothrombin gene. *Arterioscler Thromb Vasc Biol* 1998; 18: 1287-91.
9. Wolberg AS, Monroe DM, Roberts HR, et al. Elevated prothrombin results in clots with an altered fiber structure: a possible mechanism of the increased thrombotic risk. *Blood* 2003; 101: 3008-13.
10. Koenen RR, Tans G, van Oerle R, et al. The APC-independent anticoagulant activity of protein S in plasma is decreased by elevated prothrombin levels due to the prothrombin G20210A mutation. *Blood* 2003; 102: 1686-92.
11. 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.
12. Colucci M, Binetti BM, Tripodi A, et al. Hyperprothrombinemia associated with prothrombin G20210A mutation inhibits plasma fibrinolysis through a TAFI-mediated mechanism. *Blood* 2004; 103: 2157-61.
13. Smirnov MD, Safa O, Esmon NL, et al. Inhibition of activated protein C anticoagulant activity by prothrombin. *Blood* 1999; 94: 3839-46.
14. Mitchell CA, Jane SM, Salem HH. Inhibition of the anticoagulant activity of protein S by prothrombin. *J Clin Invest* 1988; 82: 2142-7.
15. Norström EA, Tran S, Dahlbäck B. Prothrombin inhibition of the individual activated protein C cleavage sites in factor Va. PhD thesis University of Lund (Sweden); 2004.
16. Tripodi A, Chantarangkul V, Mannucci PM. Hyperprothrombinemia may result in acquired activated protein C resistance. *Blood* 2000; 96: 3295-6.

17. Castaman G, Tosoetto A, Simioni M, et al. Phenotypic APC resistance in carriers of the A20210 prothrombin mutation is associated with an increased risk of venous thrombosis. *Thromb Haemost* 2001; 86: 804-8.
18. Seré KM, Janssen MP, Willems GM, et al. Purified protein S contains multimeric forms with increased APC-independent anticoagulant activity. *Biochemistry* 2001; 40: 8852-60.
19. Di Scipio RG, Hermodson MA, Yates SG, et al. A comparison of human prothrombin, factor IX (Christmas factor), factor X (Stuart factor), and protein S. *Biochemistry* 1977; 16: 698-706.
20. Govers-Riemslog JW, Speijer H, Zwaal RF, et al. The effects of bovine prothrombin fragment 1 and fragment 1.2 on prothrombin activation. *Thromb Res* 1985; 38: 375-88.
21. Castoldi E, Brugge JM, Nicolaes GA, et al. Impaired APC cofactor activity of factor V plays a major role in the APC resistance associated with the factor V Leiden (R506Q) and R2 (H1299R) mutations. *Blood* 2004; 103: 4173-9.
22. Hemker HC, Wielders S, Kessels H, et al. Continuous registration of thrombin generation in plasma, its use for the determination of the thrombin potential. *Thromb Haemost* 1993; 70: 617-24.
23. Koenen RR, Thomassen MC, Tans G, et al. Effect of oral contraceptives on the anticoagulant activity of protein S in plasma. *Thromb Haemost* 2005; 93: 853-9.
24. Rosing J, Tans G, Govers-Riemslog JW, et al. The role of phospholipids and factor Va in the prothrombinase complex. *J Biol Chem* 1980; 255: 274-83.
25. Taralunga C, Gueguen R, Visvikis S, et al. Phenotypic sensitivity to activated protein C in healthy families: importance of genetic components and environmental factors. *Br J Haematol* 2004; 126: 392-7.
26. de Visser MC, van Hylckama Vlieg A, Tans G, et al. Determinants of the APTT-based and ETP-based APC sensitivity tests. *J Thromb Haemost* 2005; 3: 1488-94.
27. Curvers J, Thomassen MC, Rimmer J, et al. Effects of hereditary and acquired risk factors of venous thrombosis on a thrombin generation-based APC resistance test. *Thromb Haemost* 2002; 88: 5-11.
28. De Ronde H, Bertina RM. Laboratory diagnosis of APC-resistance: A critical evaluation of the test and the development of diagnostic criteria. *Thromb Haemostas* 1994; 72: 880-6.
29. Grand'Maison A, Bates SM, Johnston M et al. "ProC Global": a functional screening test that predicts recurrent venous thromboembolism. *Thromb Haemost* 2005; 93: 600-4.
30. Souto JC, Almasy L, Blangero J, et al. Genetic regulation of plasma levels of vitamin K-dependent proteins involved in hemostasis: results from the GAIT Project. *Genetic Analysis of Idiopathic Thrombophilia*. *Thromb Haemost* 2001; 85: 88-92.
31. van Hylckama Vlieg A, Callas PW, Cushman M, et al. Inter-relation of coagulation factors and d-dimer levels in healthy individuals. *J Thromb Haemost* 2003; 1: 516-22.

CHAPTER 6

General Discussion

The protein C anticoagulant pathway plays an important role in the regulation of thrombin formation and in the maintenance of the haemostatic balance [1]. Activated protein C, the key player in this regulatory system, is a serine protease that proteolytically inactivates the cofactors of the intrinsic and extrinsic coagulation pathways, FVIIIa and FVa, respectively. In these inactivation reactions, protein S serves as a cofactor for APC. FV that has been cleaved by APC at Arg⁵⁰⁶ acts as an additional cofactor of APC in the inactivation of FVIIIa [2, 3], while FVa has no APC-cofactor activity. The addition of APC to plasma *in vitro* causes a prolongation of the clotting time. In some cases, however, plasma is resistant towards this regulatory function of APC, a phenotype known as APC-resistance [4] which is associated with an increased risk for venous thrombosis.

APC-resistance is most often caused by a single point mutation in the FV gene, resulting in the substitution of Arg⁵⁰⁶ by a Gln (FV_{Leiden}) [5]. Since Arg⁵⁰⁶ marks one of the APC-cleavage sites on FV(a) [6], FV_{Leiden} is less susceptible to inactivation by APC [7, 8]. As FV needs to be cleaved at Arg⁵⁰⁶ in order to stimulate the inactivation of FVIII(a) by APC [3], FV_{Leiden} also expresses reduced anticoagulant activity in the inactivation of FVIII(a) [9]. Other mutations in the FV gene can also cause APC-resistance, e.g. the R2-haplotype [10-12]. In this case, however, the susceptibility of FVa-R2 to APC is not affected and only the APC-cofactor activity of FV-R2 is reduced [13].

Since FV(a) is both a substrate and a cofactor of APC [14], mutations in the FV gene can cause APC-resistance by reducing the susceptibility of FVa to APC-mediated inactivation and/or by interfering with the APC-cofactor activity of FV in FVIIIa inactivation. Although APC-resistance was originally defined as a deficiency in the APC-cofactor function of FV, the discovery that the main underlying defect, i.e. FV_{Leiden}, causes the loss of a cleavage site for APC, led researchers to mainly focus on the mechanism of APC-mediated inactivation of FVa, which is now well-characterized [7, 8, 15-17].

In this thesis we have focused on the less well characterised APC-cofactor activity of FV by investigating 1) the role of impaired APC-cofactor activity in the APC-resistance phenotype associated with FV_{Leiden}; 2) the physiological role of the APC-cofactor activity of FV in the regulation of thrombin formation; 3) the consequences of the deficiency of this activity on thrombin generation and thrombosis risk, as illustrated by two examples: a) the rare condition known as FV_{Leiden} pseudo-homozygosity, and b) the FV R2-haplotype. Finally, we have also looked at the effect of prothrombin levels on APC-resistance.

Quantification of the anticoagulant activity of FV and FV_{Leiden}

Our first objective was to quantify the contribution of APC-cofactor activity of normal FV and FV_{Leiden} to the APC-resistance phenotype in plasma. Using an assay that specifically measures the anticoagulant activity of FV we found that normal FV stimulated the inactivation of FVIII(a) by APC in a dose-dependent manner, while FV_{Leiden} had no such stimulatory effect. Thus, normal FV possesses APC-cofactor activity that increases linearly with increasing FV concentration, whereas FV_{Leiden} completely lacks anticoagulant activity. This is not in complete agreement with earlier publications about the APC-cofactor activity of FV [2, 9], because Varadi *et al.* reported that it was only in the absence of protein S that FV_{Leiden} had no APC-cofactor activity, while in the presence of protein S FV_{Leiden} still had a detectable anticoagulant cofactor activity [9]. This discrepancy between the data presented by Váradi *et al.* and our observations can be caused by differences in the assays used to measure the APC-cofactor activity of FV_{Leiden}, or by the fact that Váradi *et al.* used purified proteins (FVIII, protein S and FV_{Leiden}), while we used homozygous FV_{Leiden} plasma mixed with FV deficient plasma as protein source. However, despite these differences, it is clear that normal FV, in synergy with protein S, functions as a cofactor for APC in the inactivation of FVIII(a), while FV_{Leiden} is severely deprived of this anticoagulant function and probably has no APC-cofactor activity at all in plasma.

To verify to what extent the impaired (or complete loss of) APC-cofactor activity of FV_{Leiden} contributes to APC-resistance, we set out to dissect the relative contribution of the susceptibility and the APC-cofactor components to APC-resistance associated with the FV_{Leiden} mutation. To this end we used the classical aPTT-based APC-resistance assay, whose outcome (the APCsr) depends on both APC-mediated FVa inactivation (susceptibility component) as well as FVIIIa inactivation (APC-cofactor activity component). Since FV_{Leiden} has no APC-cofactor activity, the APCsr of a FV_{Leiden} plasma will only reflect the susceptibility of FVa_{Leiden} to APC. Using this assay we found that increasing concentrations of FV_{Leiden} in FV-deficient plasma did not change the APCsr which led us to the conclusion that the susceptibility component is independent of the FV level. In contrast, when we varied the concentration of normal FV in plasma, the APCsr increased with increasing FV levels. As the susceptibility component is independent of the FV concentration, this increase must be due to an increase in the APC-cofactor activity of normal FV with increasing FV concentrations. Assuming that at very low FV concentrations the APC-cofactor activity of normal FV is negligible, the difference between the APCsr's of normal FV and FV_{Leiden} when the FV level is close to zero represents the difference in susceptibility between both FV molecules. From these experiments we

conclude that the difference in APCsr between plasmas containing normal FV or FV_{Leiden} at any given FV concentration is composed of a susceptibility component, which is independent of the FV concentration, and an APC-cofactor activity component, which increases with increasing FV. In full plasma containing 100% FV, the difference in APC-resistance observed between normal plasma and plasma from FV_{Leiden} homozygous carrier is for ~50% attributable to the fact that FVa_{Leiden} is less susceptible to inactivation by APC and for ~50% due to the fact that FV_{Leiden} does not express any APC-cofactor activity in the inactivation of FVIIIa.

It is still generally believed that the reduced susceptibility of FVa_{Leiden} towards APC is the main reason why FV_{Leiden} causes APC-resistance. However, our data show that reduced susceptibility of FVa_{Leiden} accounts for only half of the APC-resistance measured in the aPTT-based assay while the other half is in fact caused by the impaired APC-cofactor activity of FV_{Leiden} in the down-regulation of FVIII(a). These findings imply that impairment of the anticoagulant activity of FV should be taken into account when interpreting results obtained with the classical aPTT-based APC-resistance assay, which is still often used in clinical settings. It should be stressed that, depending on the setup and reaction conditions, other APC-resistance assays may show different contributions of susceptibility and cofactor component to the APC-resistant phenotype and therefore, care should be taken in extrapolating our results to other clinical APC-resistance assays.

The physiological relevance of the APC-cofactor activity of FV

In the aPTT-based assay coagulation is initiated in a non-physiological way, by exposing plasma to a contact activator (kaolin). Therefore, the fact that impaired APC-cofactor activity accounts for 50% of the APC-resistance associated with FV_{Leiden} as measured with the aPTT-based assay does not necessary mean that the anticoagulant activity of FV plays a role in vivo. To gain more insight in this, we evaluated the importance of the APC-cofactor activity of FV in a more physiological setting by using the thrombin generation assay [18]. The amount of thrombin formed after extrinsic activation with low TF concentrations was measured in plasmas with varying FV or FV_{Leiden} concentrations, both in the absence and presence of APC. In the absence of APC the amount of thrombin formed increased with increasing FV concentrations and reached a maximum at ~60% FV, after which it remained constant. There were no differences between normal FV and FV_{Leiden} in the amount of thrombin formed at any FV concentration. As the measurement without APC reflects the procoagulant activity of FV, these data indicate that there is no difference between the procoagulant activity of FV and FV_{Leiden}.

In the presence of APC however, titrating normal FV above 60% caused a decrease in amount of thrombin formed, while increasing the FV_{Leiden} concentrations beyond 60% had no further effect on the amount of thrombin generated. In fact, in the presence of APC, plasma containing 100% FV_{Leiden} produced three times more thrombin than plasma with 100% normal FV. As the reaction conditions (triggering with low TF) were chosen in such a way that the FVIIIa activity was the rate limiting step, this difference originates from the fact that normal FV has APC-cofactor activity in the inactivation of FVIII(a) whereas FV_{Leiden} does not. Furthermore, the fact that under these conditions the amount of thrombin formed was dependent on the FVIII concentration also indicates that thrombin generation in the presence of APC is indeed affected by FVIII(a) inactivation. So, at low FV concentrations the procoagulant activity of FV prevails, leading to a gradual increase of the amount of thrombin formed. However, at a concentration of approximately 50% to 60% FV, the procoagulant activity of FV is saturated and a further increase in FV concentration causes an increase in anticoagulant activity, leading to a decrease in the thrombin generation. These findings further suggest that carriership of FV deficiency (~50% FV levels) might be associated with a hypercoagulable state, because FV procoagulant activity is already maximal, while FV anticoagulant activity is only half-maximal.

These experiments illustrate that the APC-cofactor activity of FV plays an important role in the regulation of thrombin formation under physiological conditions. There appears to be a difference between the inactivation reactions of normal FV and FV_{Leiden} in a purified system and in plasma. Kinetic analysis in model systems has revealed that normal FVa is inactivated 20-times more efficiently by APC as compared to FVa_{Leiden}, due to the difference in susceptibility between both FV molecules [7, 8]. However, Rosing *et al.* showed that the presence of protein S and FXa during the inactivation of FVa or FVa_{Leiden} *in vitro*, a situation that may be representative for the *in vivo* condition, greatly reduces the differences between the inactivation rates of normal FVa and FVa_{Leiden} [16]. This is due to the fact that FXa can protect FVa from cleavage at Arg⁵⁰⁶ by APC, leaving only the Arg³⁰⁶ cleavage site available for APC, just like in the case of FV_{Leiden}. Therefore, one might expect that the protection of FVa by FXa and the stimulation of APC-cleavage at Arg³⁰⁶ by protein S, will result in only a small difference between inactivation of normal FVa and FVa_{Leiden} by APC *in vivo*. However, in plasma there still is a considerable difference between the inactivations of both FV molecules. These differences can be explained by the importance of the APC-cofactor activity of FV, of which FV_{Leiden} has been deprived. It might be possible that the Arg506Gln mutation has little clinical relevance for the regulation of FVa activity by APC *in*

vivo, and that the absence of APC-cofactor activity of FV is the major cause for the APC-resistance associated with FV_{Leiden}. Of course it should be emphasized that FXa only protects the 506-cleavage site when the FXa concentration exceeds the FVa concentration, a situation which is not necessarily true under physiological conditions.

FV_{Leiden} pseudo-homozygosity and APC-resistance

The patho-physiological relevance of the anticoagulant activity of FV is well illustrated by comparison of APC-resistance (and thrombotic risk) of pseudo-homozygous, homozygous and heterozygous FV_{Leiden} carriers with normal individuals. FV_{Leiden} pseudo-homozygosity is a plasma phenotype that arises from coinheritance of the FV_{Leiden} mutation and a null mutation on different FV alleles, resulting in 50% FV_{Leiden} (and no normal FV) in plasma. It has been associated with APC-resistance and it is considered a risk factor for thrombosis, although it has not been well characterized due to its rare occurrence in the population.

We have characterized plasmas from individuals, who were heterozygous, homozygous or pseudo-homozygous for the FV_{Leiden} mutation, using 4 different APC-resistance assays, also providing a detailed characterization of the APC-resistant phenotype associated with FV_{Leiden} pseudo-homozygosity. In all APC-resistance assays used, the pseudo-homozygous individuals were significantly more resistant towards APC than heterozygous FV_{Leiden} carriers. In fact, they were found to be indistinguishable from the homozygous individuals, indicating that the extra FV_{Leiden} molecules present in true homozygous plasma do not affect the susceptibility of the plasma to APC, nor do they affect the inactivation of FVIIIa by APC. The difference in APC-resistance between the pseudo-homozygotes and the heterozygotes can therefore be fully attributed to the 50% normal FV that distinguishes heterozygotes from pseudo-homozygous FV_{Leiden} carriers. This normal FV increases the overall susceptibility of FVa in plasma, as shown in the prothrombinase-based assay, and also serves as a cofactor in the inactivation of FVIIIa by APC, as shown in the FVIII-based assay. In other words, the difference in APC-resistance between the various FV_{Leiden} genotypes is not determined by the amount of FV_{Leiden} molecules in their plasma, but by the presence or absence of normal FV that possesses anticoagulant activity in the inactivation of FVIII(a). It is not the poor susceptibility of FVa_{Leiden} that causes problems in carriers of the FV_{Leiden} mutation, but the impaired APC-cofactor activity.

To exclude the possibility that plasma components other than the ones we measured (PT, FVIII, FV, PS) contributed to the observed differences

between the FV_{Leiden} genotypes, we repeated the ETP-based assay in a plasma model system. FV-deficient plasma was reconstituted with purified normal FV and/or FV_{Leiden} to mimic the various FV_{Leiden} genotypes, and thrombin generation was measured in the absence and presence of APC. In the absence of APC all simulated plasmas gave the same thrombin generation curves, indicating that the FV_{Leiden} genotype had no effect on the amount of thrombin generated in the absence of APC. However, in the presence of APC the pseudo-homozygous FV_{Leiden} plasma, containing only 50% purified FV_{Leiden} , was very resistant to APC; it generated 9 times more thrombin than plasma reconstituted with 100% normal FV. When an additional 50% purified FV_{Leiden} was added to this plasma, now simulating a homozygous plasma with 100% FV_{Leiden} , there was no change in the amount of thrombin generated in the presence of APC. This indicates that the 50% extra FV_{Leiden} molecules present in a homozygous FV_{Leiden} plasma, have no additional effect on the APC-resistance observed for the pseudo-homozygous FV_{Leiden} plasma. On the other hand, when 50% normal FV was added to the pseudo-homozygous FV_{Leiden} plasma, to mimic plasma from a heterozygous FV_{Leiden} carrier, the amount of thrombin formed was reduced by half. This clearly demonstrates the presence of anticoagulant activity in the 50% normal FV that we added and its function as a cofactor for APC. This also shows that the anticoagulant activity of FV determines how much thrombin is formed. These results might help explain the differences in thrombotic risk observed between the various FV_{Leiden} genotypes [19].

Pseudo-homozygosity for FV_{Leiden} can be seen as an extreme form of FV_{Leiden} heterozygosity, where the normal FV allele is not expressed. To evaluate the importance of the expression level of the normal FV allele in FV_{Leiden} heterozygotes, we performed a thrombin generation experiment in FV deficient plasma reconstituted with 50% FV_{Leiden} to which increasing amounts of purified normal FV were added. So, starting from the simulated pseudo-homozygous FV_{Leiden} plasma, we gradually increased the amount of normal FV finally reaching a plasma resembling a FV_{Leiden} heterozygote. The more normal FV we added to the FV_{Leiden} pseudo-homozygous plasma, the less thrombin was generated in the presence of APC and thus the less APC-resistant the plasma became. This implies that the expression level of the non-Leiden allele in FV_{Leiden} heterozygous individuals has an important influence on the APC-resistance of their plasma. Furthermore it shows that FV_{Leiden} pseudo-homozygous individuals are more resistant to APC than FV_{Leiden} heterozygous individuals.

Since APC-resistance is an indicator of thrombotic risk, our APC-resistance data suggest that FV_{Leiden} pseudo-homozygotes have a high thrombosis risk, higher than heterozygous FV_{Leiden} carriers and comparable to

that of homozygotes. However, until recently little was known about the APC-resistance phenotype and the thrombotic risk associated with FV_{Leiden} pseudo-homozygosity. There are several case reports available about thrombotic patients characterized as being pseudo-homozygous for the FV_{Leiden} mutation [20-28], but epidemiological studies assessing the thrombotic risk associated with FV_{Leiden} pseudo-homozygosity are lacking, due to the rare occurrence of this condition in the population. The case reports present in literature tend to estimate the thrombotic risk to be comparable to that of homozygous FV_{Leiden} carriers, based on the early age of onset of thrombosis and the severity of their condition. In contrast to this and also in contrast with our results obtained in the plasma model system, the only epidemiological study available until recently estimated the thrombotic risk associated with FV_{Leiden} pseudo-homozygosity to be comparable to that of heterozygous FV_{Leiden} carriers [29]. Recently however, comparison of thrombosis-free survival curves for the different FV_{Leiden} genotypes showed that FV_{Leiden} pseudo-homozygotes are exposed to the same thrombotic risk as homozygous FV_{Leiden} carriers [30].

FV-R2 and APC-resistance

The R2-haplotype is a set of genetically linked polymorphisms in the FV-gene that also has been associated with moderate APC-resistance [10-12, 31]. Since none of the polymorphisms affect an APC-cleavage site or a known functional region of the FV molecule, the mechanism of R2-associated APC-resistance is unclear. Hoekema *et al.* showed that R2-FVa is equally well inactivated by APC as normal FVa, both in the absence and presence of protein S, and that plasma of FV R2-homozygous individuals contains reduced APC-cofactor activity [13]. This means that the APC-resistance associated with the R2-haplotype is not caused by a reduced susceptibility of R2-FVa towards APC, but is due to the reduced anticoagulant activity of R2-FV. There are three possible explanations for the reduced APC-cofactor activity measured in FV-R2 plasma: 1) reduced FV levels, associated with the R2-haplotype, result in less FV to function as a cofactor, 2) the relative increase in the FV₁ isoform, which expresses less cofactor activity, and 3) a possible role of the mutations themselves in reducing the APC-cofactor activity of R2-FV. Since the reduced levels and shifted isoform ratio only account for part of R2-associated APC-resistance [32], R2-FV itself must be a poor cofactor due to the mutations. We have quantified the specific APC-cofactor activity of FV-R2 to be 73% of that of normal FV. After taking into account that the FV levels are reduced in R2-plasmas, the APC-cofactor activity present in plasma from FV R2 homozygotes is about half of that present in normal plasma. As a consequence, plasma from a homozygous R2-carrier forms almost twice as much thrombin in the presence

of APC as compared to normal plasma. Also, in combination with FV_{Leiden}, simulated FV_{Leiden}/FV_{R2}-double heterozygous plasma produced more thrombin in the presence of APC as compared to simulated FV_{Leiden} heterozygous plasma, confirming the reports that coinheritance of these mutations enhances the APC-resistant phenotype associated with FV_{Leiden} heterozygosity [10, 12].

Whether or not the R2-haplotype is associated with a relevant clinical risk for venous thrombosis is still a matter of debate. As APC-resistance *per se* is a risk factor for thrombosis [33] and we and other investigators have clearly shown that the R2-haplotype causes APC-resistance, one would conclude that the R2-haplotype is a risk factor for thrombosis. However, the results from epidemiological studies are contradictory and there is inconclusive evidence to prove whether or not the R2-haplotype is a risk factor for venous thrombosis [31]. Nevertheless, it is generally accepted that compound heterozygous carriership of the FV_{Leiden} and the FV-R2 is indeed associated with a higher risk for venous thrombosis than heterozygosity for FV_{Leiden} alone [34-36]. This can be explained by several facts that have been presented in this thesis. Our observations 1) that the expression level of the non-Leiden allele in heterozygous FV_{Leiden} carriers is an important determinant in the associated APC-resistance, 2) that the R2-haplotype indeed causes a reduction in the FV levels, and furthermore, 3) that R2-FV itself has reduced anticoagulant cofactor activity towards APC provide a good explanation why compound heterozygosity for FV_{Leiden} and FV-R2 is associated with a higher risk for venous thrombosis than heterozygosity for FV_{Leiden} alone.

Already when Lunghi *et al.* first described the R2-haplotype, they found that it was associated with a reduction of the FV levels in plasma [37]. Reduced FV concentration in plasma accounts for part of the APC-resistance caused by the R2-haplotype [32]. However, the molecular mechanism underlying the FV reduction in plasma has been unclear for a long time. Recently, Yamazaki *et al.* tackled this problem using an *in vitro* expression system to study the expression of recombinant FV molecules mimicking the R2-haplotype [38]. They found that the Asp2194Gly mutation was the major determinant of the low expression levels observed in the FV-R2 carriers. Further analysis revealed that the Asp2194Gly mutant had an impaired transport within the cell, from the endoplasmatic reticulum (ER) to the Golgi complex. As misfolded proteins are retained and trapped in the ER by the quality control system, eventually leading to the intracellular degradation of these proteins [39], Yamazaki *et al.* proposed that this was the reason for the reduced expression level associated with the Asp2194Gly mutation. These conclusions were confirmed by Van Der Neut Kolfshoten and co-workers [40].

Based on these observations, we tried to elaborate further on the mechanism by which the Asp2194Gly mutation affects the folding/structure/stability of the C2-domain in the FV-R2 haplotype. The crystal structure of the C2-domain of human FV [41] shows that the Asp2194 is involved in electrostatic interactions with the side chains of the lysine residues at positions 2101 and 2103. We hypothesized that these electrostatic interactions could bring the Cys2038 in the N-terminal part of the C2-domain in close proximity of the Cys2193 at the C-terminal end of the C2-domain, potentially facilitating the formation of a disulfide bridge, and thereby stabilizing the C2-domain. The Asp2194Gly mutation would abolish these electrostatic interactions, no longer facilitating the disulfide bridge formation. In other words, the Asp2194Gly mutation would affect the stability and/or induce conformational changes in the C2-domain, leading to the retention of the misfolded proteins in the ER, eventually resulting in a reduced secretion.

To study the role of Asp2194 in the stabilization of the C2-domain of FV, molecular dynamics simulations, electrostatic computations and the *in vitro* expression of recombinant molecules were used. The total potential energy in the Asp2194Gly mutant was significantly higher compared to the wild-type (WT), suggesting overall destabilization of the protein due to the mutation. In the simulation of the WT C2-domain with or without the disulfide bond, the protein core remained stable, but in the absence of the disulfide bond there was an increased mobility of both C- and N-termini. *In vitro* this resulted in the reduced expression of the WT protein without the disulfide bridge, probably due to an impairment of the correct folding of the protein. The protein core of the Asp2194Gly mutant also remained stable during the simulation with or without the disulfide bond, but the mutant protein without the disulfide bond showed significantly higher flexibilities in the N-terminal residues compared to the simulation of the WT without the disulfide bond. This is most likely due to the absence of the electrostatic interactions between Asp2194-Lys2101-Lys2103 in the Asp2194Gly mutant. Indeed, comparison of the electrostatic free energies of this triad between the WT FV C2-domain and the Asp2194Gly variant, indicated an electrostatic destabilization due to the mutation. Together, these data suggest that the Asp2194Gly mutation in R2-FV does indeed destabilize the C2-domain. This unstable structure can be detected by the quality control system of the cell, causing retention and degradation of the protein inside the cell, eventually leading to a reduced R2-FV level in plasma.

We also expressed mutations affecting the charges in this Asp2194-Lys2101-Lys2103 triad to get more insight into the importance of this salt bridge network. When all charges in the triad were made positive or negative, the final expression levels of these mutants were even lower than for the Asp2194Gly

mutant. Thus, these mutations, resulting in three positively or negatively charged groups in close proximity of each other destabilize the structure. These experimental data correlated well with the calculated electrostatic destabilizations. We also reversed all charges in the triad and hypothesized that this should also form a stabilizing salt bridge. However, this was not the case; the expression level of this mutant was similar to that of the mutants with repulsive charges; whereas computation of electrostatic energies suggested a slight stabilizing effect. This shows that this region is not tolerant for amino acid substitutions. Indeed, a multiple sequence alignment of FV and FVIII indicated that this area is well conserved among species, suggesting that this salt bridge triad can be important for the stability and/or the structure of the C2-domain.

Prothrombin levels and APC-resistance

An elevated prothrombin level in plasma, due to the G20210 mutation or to other causes, is a risk factor for venous thrombosis [42-46]. At least part of the risk is thought to be due to the fact that high prothrombin levels induce APC-resistance [47, 48]. The exact mechanism by which high prothrombin concentrations induce APC-resistance is not understood, although several reports have shown that increased prothrombin levels interfere with the function of the anticoagulant protein C pathway [49-51].

In chapter 5, we have further investigated the effect of elevated plasma prothrombin levels on APC-resistance, using two different APC-resistance assays (the aPTT-based assay and the ETP-based assay), in a plasma model system (prothrombin-deficient plasma reconstituted with purified prothrombin) as well as in a population of healthy individuals. Both assays showed that in the plasma model system, increasing prothrombin concentrations cause APC-resistance in a dose-dependent manner. However, there was a striking difference between both assays in the mechanism by which APC-resistance arose, probably due to the fact that the two assays are influenced by different determinants. In the aPTT-based assay the increased APC-resistance with increasing prothrombin concentration was mainly due to the prolongation of the clotting times in the absence of APC, while the clotting times in the presence of APC remained virtually constant in the physiological range of prothrombin concentration (70-150% PT). This has also been observed by Smirnov *et al.* and is most probably due to the competition between prothrombin and other coagulation factors for the phospholipid surface [50]. In the ETP-based assay on the other hand, the increased APC-resistance due to increasing prothrombin levels was mainly caused by the measurement in the presence of APC. Thrombin generation, both with and without APC increased with increasing prothrombin levels, but the ETP with APC increased to a greater extent, leading

to an overall increase in APC-resistance. This is in line with previous findings that the amount of thrombin generated in the absence of APC and the APC-resistance is significantly higher in carriers of the prothrombin mutation as compared to a control population [52, 53].

There was no difference between the results obtained with the aPTT-based assay in the plasma model system and the population. However, in the ETP-based assay there was a discrepancy between the model system and the population. Whereas in the model system APC-resistance increased with increasing PT concentrations, it appeared that in the population APC-resistance decreased with increasing prothrombin concentrations. Further analysis revealed that this was due to confounding by protein S. Prothrombin levels correlated very well with the protein S levels, and increasing levels of protein S lead to a decrease in APC-resistance. Furthermore, a protein S titration in protein S-deficient plasma showed that the APC-resistance measured with the ETP-based assay decreases at increasing protein S levels. Thus, prothrombin and protein S have opposite effects on the APC-resistance measured with the ETP-based assay, as was also reported by de Visser and coworkers [54]. After correction for protein S levels in the population, the APC-resistance indeed increased with increasing prothrombin levels.

Based on these observations we propose that the protein S levels in plasma play an important role in modulating the severity of the APC-resistance caused by elevated prothrombin concentrations. In other words, the APC-resistance observed in a plasma due to increased plasma prothrombin levels is not only dependent on the prothrombin concentration itself, but also on the protein S level in that plasma. This is strengthened by our observation that the APC-resistance in a plasma with high prothrombin and high protein S was less severe than in a plasma with high prothrombin and normal or reduced protein S levels. Therefore, the prothrombin/protein S ratio in plasma might be a better predictor of APC-resistance associated with that plasma than the prothrombin level alone.

As prothrombin and protein S both are vitamin K-dependent proteins mainly synthesized by the liver, their plasma concentrations show a high degree of correlation [43, 55]. This led us to the hypothesis that the APC-resistance will be less severe in plasmas with high prothrombin levels in the absence of the prothrombin mutation (G20210A) than in plasmas with elevated prothrombin levels in the presence of the G20210A mutation, because in the first case the protein S levels will also be elevated and to a certain degree counteract the effect of the high prothrombin, while in the latter case the protein S concentrations remain normal. Of course this remains to be proven in a

population study with carriers and non-carriers of the prothrombin G20210A mutation.

Conclusions

In conclusion, the data presented in this thesis indicate that the anticoagulant (APC-cofactor) activity of FV is a very important component in the regulation of thrombin formation. They provide evidence that the APC-resistance associated with the FV_{Leiden} mutation is not entirely due the reduced susceptibility of FVa_{Leiden} towards inactivation by APC, but that the loss of APC-cofactor activity due to the Leiden mutation accounts for at least half of the APC-resistance observed in homozygous FV_{Leiden} carriers.

We have shown that the APC-cofactor activity of FV also plays a pivotal role in the regulation of the amount of thrombin formed under physiological conditions. In fact, it is this anticoagulant activity of FV that eventually determines how much thrombin will be formed. Although the FV_{Leiden} mutation affects both the inactivation of FVa_{Leiden} by APC as well as the anticoagulant function of FV_{Leiden}, it is not only the poor susceptibility of FVa_{Leiden} that causes problems in carriers of the FV_{Leiden} mutation, but there is also a contribution of the impaired APC-cofactor activity. The reason why homozygous FV_{Leiden} carriers have a greater thrombotic risk compared to heterozygotes is not because they have more FV_{Leiden} in their plasma, but because they do not have any normal FV in their plasma that expresses anticoagulant cofactor activity towards APC.

Furthermore, we have provided a detailed characterization of the APC-resistance phenotype in FV_{Leiden} pseudo-homozygotes. In particular, we have shown that, in a plasma model system as well as in a population, plasma from FV_{Leiden} pseudo-homozygotes is significantly more APC-resistant compared to plasma from FV_{Leiden} heterozygotes but is indistinguishable from FV_{Leiden} homozygous plasma. The difference in APC-resistance between the various FV_{Leiden} genotypes is not determined by the amount of FV_{Leiden} molecules in their plasma, but by the presence or absence of normal FV. This normal FV not only increases the overall susceptibility of FVa towards APC in plasma, but also serves as a cofactor in the inactivation of FVIIIa by APC.

The importance of the APC-cofactor activity of FV is also demonstrated by the APC-resistance associated with the R2-haplotype. We have shown that the impaired anticoagulant activity of FV, together with the reduced plasma FV levels, results in an increased thrombin generation in the presence of APC. Also, we have provided insight into the mechanism through which the Asp2194Gly mutation in the R2-haplotype destabilizes the C2-domain of FV, resulting in reduced plasma FV levels.

Finally, we have evaluated the effect of elevated plasma prothrombin levels on APC-resistance. Protein S levels in plasma proved to be an important modulator of the APC-resistance associated with elevated plasma prothrombin levels. As prothrombin and protein S levels in plasma are highly correlated, the ratio of these two components might provide a more accurate estimation of the APC-resistance in a given plasma than the prothrombin level alone.

References

1. B. Dahlback. Blood coagulation. *Lancet*. 2000; 355:1627-32.
2. L. Shen and B. Dahlback. Factor V and protein S as synergistic cofactors to activated protein C in degradation of factor VIIIa. *J Biol Chem*. 1994; 269:18735-8.
3. E. Thorelli, R. J. Kaufman and B. Dahlback. Cleavage of factor V at Arg 506 by activated protein C and the expression of anticoagulant activity of factor V. *Blood*. 1999; 93:2552-8.
4. B. Dahlback, M. Carlsson and P. J. Svensson. Familial thrombophilia due to a previously unrecognized mechanism characterized by poor anticoagulant response to activated protein C: prediction of a cofactor to activated protein C. *Proc Natl Acad Sci U S A*. 1993; 90:1004-8.
5. R. M. Bertina, B. P. Koeleman, T. Koster, F. R. Rosendaal, R. J. Dirven, H. de Ronde, P. A. van der Velden and P. H. Reitsma. Mutation in blood coagulation factor V associated with resistance to activated protein C. *Nature*. 1994; 369:64-7.
6. M. Kalafatis, M. D. Rand and K. G. Mann. The mechanism of inactivation of human factor V and human factor Va by activated protein C. *J Biol Chem*. 1994; 269:31869-80.
7. G. A. Nicolaes, G. Tans, M. C. Thomassen, H. C. Hemker, I. Pabinger, K. Varadi, H. P. Schwarz and J. Rosing. Peptide bond cleavages and loss of functional activity during inactivation of factor Va and factor VaR506Q by activated protein C. *J Biol Chem*. 1995; 270:21158-66.
8. M. Kalafatis, R. M. Bertina, M. D. Rand and K. G. Mann. Characterization of the molecular defect in factor VR506Q. *J Biol Chem*. 1995; 270:4053-7.
9. K. Varadi, J. Rosing, G. Tans, I. Pabinger, B. Keil and H. P. Schwarz. Factor V enhances the cofactor function of protein S in the APC-mediated inactivation of factor VIII: influence of the factor VR506Q mutation. *Thromb Haemost*. 1996; 76:208-14.
10. F. Bernardi, E. M. Faioni, E. Castoldi, B. Lunghi, G. Castaman, E. Sacchi and P. M. Mannucci. A factor V genetic component differing from factor V R506Q contributes to the activated protein C resistance phenotype. *Blood*. 1997; 90:1552-7.
11. M. Alhenc-Gelas, V. Nicaud, S. Gandrille, P. van Dreden, J. Amiral, M. L. Aubry, J. N. Fiessinger, J. Emmerich and M. Aiach. The factor V gene A4070G mutation and the risk of venous thrombosis. *Thromb Haemost*. 1999; 81:193-7.
12. M. C. de Visser, J. F. Guasch, P. W. Kamphuisen, H. L. Vos, F. R. Rosendaal and R. M. Bertina. The HR2 haplotype of factor V: effects on factor V levels,

- normalized activated protein C sensitivity ratios and the risk of venous thrombosis. *Thromb Haemost.* 2000; 83:577-82.
13. L. Hoekema, E. Castoldi, G. Tans, D. Girelli, D. Gemmati, F. Bernardi and J. Rosing. Functional properties of factor V and factor Va encoded by the R2-gene. *Thromb Haemost.* 2001; 85:75-81.
 14. G. A. Nicolaes and B. Dahlback. Factor V and thrombotic disease: description of a janus-faced protein. *Arterioscler Thromb Vasc Biol.* 2002; 22:530-8.
 15. M. J. Heeb, Y. Kojima, J. S. Greengard and J. H. Griffin. Activated protein C resistance: molecular mechanisms based on studies using purified Gln506-factor V. *Blood.* 1995; 85:3405-11.
 16. J. Rosing, L. Hoekema, G. A. Nicolaes, M. C. Thomassen, H. C. Hemker, K. Varadi, H. P. Schwarz and G. Tans. 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.
 17. C. Aparicio and B. Dahlback. Molecular mechanisms of activated protein C resistance. Properties of factor V isolated from an individual with homozygosity for the Arg506 to Gln mutation in the factor V gene. *Biochem J.* 1996; 313 (Pt 2):467-72.
 18. H. C. Hemker, R. Al Dieri and S. Beguin. Thrombin generation assays: accruing clinical relevance. *Curr Opin Hematol.* 2004; 11:170-5.
 19. F. R. Rosendaal, T. Koster, J. P. Vandenbroucke and P. H. Reitsma. High risk of thrombosis in patients homozygous for factor V Leiden (activated protein C resistance). *Blood.* 1995; 85:1504-8.
 20. P. Simioni, A. Scudeller, P. Radossi, S. Gavasso, B. Girolami, D. Tormene and A. Girolami. "Pseudo homozygous" activated protein C resistance due to double heterozygous factor V defects (factor V Leiden mutation and type I quantitative factor V defect) associated with thrombosis: report of two cases belonging to two unrelated kindreds. *Thromb Haemost.* 1996; 75:422-6.
 21. J. L. Zehnder and M. Jain. Recurrent thrombosis due to compound heterozygosity for factor V Leiden and factor V deficiency. *Blood Coagul Fibrinolysis.* 1996; 7:361-2.
 22. J. F. Guasch, R. P. Lensen and R. M. Bertina. Molecular characterization of a type I quantitative factor V deficiency in a thrombosis patient that is "pseudo homozygous" for activated protein C resistance. *Thromb Haemost.* 1997; 77:252-7.
 23. B. Delahousse, S. Iochmann, C. Pouplard, B. Fimbel, B. Charbonnier and Y. Gruel. Pseudo-homozygous activated protein C resistance due to coinheritance of heterozygous factor V Leiden mutation and type I factor V deficiency. Variable expression when analyzed by different activated protein C resistance functional assays. *Blood Coagul Fibrinolysis.* 1997; 8:503-9.

24. G. Castaman, B. Lunghi, E. Missiaglia, F. Bernardi and F. Rodeghiero. Phenotypic homozygous activated protein C resistance associated with compound heterozygosity for Arg506Gln (factor V Leiden) and His1299Arg substitutions in factor V. *Br J Haematol.* 1997; 99:257-61.
25. E. Castoldi, M. Kalafatis, B. Lunghi, P. Simioni, P. A. Ioannou, M. Petio, A. Girolami, K. G. Mann and F. Bernardi. Molecular bases of pseudo-homozygous APC resistance: the compound heterozygosity for FV R506Q and a FV null mutation results in the exclusive presence of FV Leiden molecules in plasma. *Thromb Haemost.* 1998; 80:403-6.
26. B. Lunghi, E. Castoldi, F. Mingozzi, F. Bernardi and G. Castaman. A novel factor V null mutation detected in a thrombophilic patient with pseudo-homozygous APC resistance and in an asymptomatic unrelated subject. *Blood.* 1998; 92:1463-4.
27. M. Kalafatis, F. Bernardi, P. Simioni, B. Lunghi, A. Girolami and K. G. Mann. Phenotype and genotype expression in pseudohomozygous factor VLEIDEN : the need for phenotype analysis. *Arterioscler Thromb Vasc Biol.* 1999; 19:336-42.
28. G. Standen, C. Morse, S. Aslam and A. Bowron. Recurrent thrombosis in a patient with pseudohomozygous activated protein C resistance and homozygosity for MTHFR gene polymorphism C677T. *Thromb Haemost.* 1999; 81:663-4.
29. G. Castaman, A. Tosetto, M. Ruggeri and F. Rodeghiero. Pseudohomozygosity for activated protein C resistance is a risk factor for venous thrombosis. *Br J Haematol.* 1999; 106:232-6.
30. P. Simioni, E. Castoldi, B. Lunghi, D. Tormene, J. Rosing and F. Bernardi. An underestimated combination of opposites resulting in enhanced thrombotic tendency. *Blood.* 2005; 106(7): 2363-5
31. G. Castaman, E. M. Faioni, A. Tosetto and F. Bernardi. The factor V HR2 haplotype and the risk of venous thrombosis: a meta-analysis. *Haematologica.* 2003; 88:1182-9.
32. J. W. Govers-Riemsag, E. Castoldi, G. A. Nicolaes, G. Tans and J. Rosing. Reduced factor V concentration and altered FV1/FV2 ratio do not fully explain R2-associated APC-resistance. *Thromb Haemost.* 2002; 88:444-9.
33. M. C. de Visser, F. R. Rosendaal and R. M. Bertina. A reduced sensitivity for activated protein C in the absence of factor V Leiden increases the risk of venous thrombosis. *Blood.* 1999; 93:1271-6.
34. E. M. Faioni, F. Franchi, P. Bucciarelli, M. Margaglione, V. De Stefano, G. Castaman, G. Finazzi and P. M. Mannucci. Coinheritance of the HR2 haplotype in the factor V gene confers an increased risk of venous thromboembolism to carriers of factor V R506Q (factor V Leiden). *Blood.* 1999; 94:3062-6.

35. G. Castaman, M. Ruggeri, A. Toso and F. Rodeghiero. Heterogeneity of activated protein C resistance phenotype in subjects with compound heterozygosity for HR2 haplotype and FV Leiden mutation (R506Q) in factor V gene. *Thromb Haemost.* 2000; 84:357-8.
36. A. R. Folsom, M. Cushman, M. Y. Tsai, N. Aleksic, S. R. Heckbert, L. L. Boland, A. W. Tsai, N. D. Yanez and W. D. Rosamond. A prospective study of venous thromboembolism in relation to factor V Leiden and related factors. *Blood.* 2002; 99:2720-5.
37. B. Lunghi, L. Iacoviello, D. Gemmati, M. G. Dilasio, E. Castoldi, M. Pinotti, G. Castaman, R. Redaelli, G. Mariani, G. Marchetti and F. Bernardi. Detection of new polymorphic markers in the factor V gene: association with factor V levels in plasma. *Thromb Haemost.* 1996; 75:45-8.
38. T. Yamazaki, G. A. Nicolaes, K. W. Sorensen and B. Dahlback. Molecular basis of quantitative factor V deficiency associated with factor V R2 haplotype. *Blood.* 2002; 100:2515-21.
39. E. Chevet, P. H. Cameron, M. F. Pelletier, D. Y. Thomas and J. J. Bergeron. The endoplasmic reticulum: integration of protein folding, quality control, signaling and degradation. *Curr Opin Struct Biol.* 2001; 11:120-4.
40. M. van der Neut Kolfschoten, R. J. Dirven, H. L. Vos and R. M. Bertina. The R2-haplotype associated Asp2194Gly mutation in the light chain of human factor V results in lower expression levels of FV, but has no influence on the glycosylation of Asn2181. *Thromb Haemost.* 2003; 89:429-37.
41. S. Macedo-Ribeiro, W. Bode, R. Huber, M. A. Quinn-Allen, S. W. Kim, T. L. Ortel, G. P. Bourenkov, H. D. Bartunik, M. T. Stubbs, W. H. Kane and P. Fuentes-Prior. Crystal structures of the membrane-binding C2 domain of human coagulation factor V. *Nature.* 1999; 402:434-9.
42. S. R. Poort, F. R. Rosendaal, P. H. Reitsma and R. M. Bertina. A common genetic variation in the 3'-untranslated region of the prothrombin gene is associated with elevated plasma prothrombin levels and an increase in venous thrombosis. *Blood.* 1996; 88:3698-703.
43. J. C. Souto, I. Coll, D. Llobet, E. del Rio, A. Oliver, J. Mateo, M. Borrell and J. Fontcuberta. The prothrombin 20210A allele is the most prevalent genetic risk factor for venous thromboembolism in the Spanish population. *Thromb Haemost.* 1998; 80:366-9.
44. R. K. Kapur, L. A. Mills, S. G. Spitzer and M. B. Hultin. A prothrombin gene mutation is significantly associated with venous thrombosis. *Arterioscler Thromb Vasc Biol.* 1997; 17:2875-9.
45. A. Hillarp, B. Zoller, P. J. Svensson and B. Dahlback. The 20210 A allele of the prothrombin gene is a common risk factor among Swedish outpatients with verified deep venous thrombosis. *Thromb Haemost.* 1997; 78:990-2.

46. K. Brown, R. Luddington, D. Williamson, P. Baker and T. Baglin. Risk of venous thromboembolism associated with a G to A transition at position 20210 in the 3'-untranslated region of the prothrombin gene. *Br J Haematol.* 1997; 98:907-9.
47. A. Tripodi, V. Chantarangkul and P. M. Mannucci. Hyperprothrombinemia may result in acquired activated protein C resistance. *Blood.* 2000; 96:3295-6.
48. G. Castaman, A. Toso, M. Simioni, M. Ruggeri, D. Madeo and F. Rodeghiero. Phenotypic APC resistance in carriers of the A20210 prothrombin mutation is associated with an increased risk of venous thrombosis. *Thromb Haemost.* 2001; 86:804-8.
49. C. A. Mitchell, S. M. Jane and H. H. Salem. Inhibition of the anticoagulant activity of protein S by prothrombin. *J Clin Invest.* 1988; 82:2142-7.
50. M. D. Smirnov, O. Safa, N. L. Esmon and C. T. Esmon. Inhibition of activated protein C anticoagulant activity by prothrombin. *Blood.* 1999; 94:3839-46.
51. E. Norstrom, S. Tran and B. Dahlback. Prothrombin inhibition of the individual activated protein C cleavage sites in factor Va. *Thesis.* 2004;
52. P. A. Kyrle, C. Mannhalter, S. Beguin, A. Stumpflen, M. Hirschl, A. Weltermann, M. Stain, B. Brenner, W. Speiser, I. Pabinger, K. Lechner and S. Eichinger. Clinical studies and thrombin generation in patients homozygous or heterozygous for the G20210A mutation in the prothrombin gene. *Arterioscler Thromb Vasc Biol.* 1998; 18:1287-91.
53. J. Curvers, M. C. Thomassen, J. Rimmer, K. Hamulyak, J. van der Meer, G. Tans, F. E. Preston and J. Roseng. Effects of hereditary and acquired risk factors of venous thrombosis on a thrombin generation-based APC resistance test. *Thromb Haemost.* 2002; 88:5-11.
54. M. C. de Visser, A. Hylckama Vlieg, G. Tans, J. Roseng, A. E. Dahm, P. M. Sandset, F. R. Rosendaal and R. M. Bertina. Determinants of the APTT- and ETP-based APC sensitivity tests. *J Thromb Haemost.* 2005; 3:1488-94.
55. A. Van Hylckama Vlieg, P. W. Callas, M. Cushman, R. M. Bertina and F. R. Rosendaal. Inter-relation of coagulation factors and d-dimer levels in healthy individuals. *J Thromb Haemost.* 2003; 1:516-22.

CHAPTER 7

Summary / Samenvatting

List of publications

Curriculum vitae

Dankwoord

Summary

Blood coagulation proceeds through a complex series of enzymatic reactions in which protease zymogens are activated via limited proteolysis, eventually leading to the generation of thrombin, the central enzyme in coagulation. Adequate regulation of the amount of thrombin formed is necessary to prevent both bleeding and thrombotic disorders. The protein C pathway is a major anticoagulant mechanism that plays an important role in the regulation of thrombin formation. Activated protein C (APC), a vitamin K-dependent protein, inactivates coagulation factors FVIIIa and FVa. Protein S serves as a cofactor of APC during these inactivation reactions. It has been shown that FV also expresses APC-cofactor activity in the inactivation of FVIII(a). The addition of APC to plasma causes a prolongation of the clotting time, but the plasma of some individuals is insensitive to this regulatory function of APC, a phenotype known as APC-resistance. APC-resistance is associated with an increased risk for venous thrombosis.

APC-resistance is most often caused by a mutation in the FV gene which causes the substitution of an Arg by a Gln at position 506 (FV_{Leiden}). This mutation abolishes one of the APC-cleavage sites on FV(a) which makes FVa_{Leiden} is less susceptible to inactivation by APC. Furthermore, as FV has to be cleaved at Arg⁵⁰⁶ to serve as a cofactor for APC in the inactivation of FVIII(a), this mutation also reduces the anticoagulant activity of FV_{Leiden}. Apart from the FV_{Leiden} mutation, several other FV gene mutations also cause APC-resistance, such as the R2-haplotype. The polymorphisms that define the R2-haplotype do not affect the susceptibility of FVa-R2 towards inactivation by APC, but they specifically reduce the APC-cofactor activity of FV-R2.

Since FV(a) is both a substrate and a cofactor for APC, mutations in the FV gene can cause APC resistance either by reducing the susceptibility of FVa to APC-mediated inactivation (FV_{HongKong} and FV_{Cambridge}) or by interfering with the APC cofactor activity of FV in FVIIIa inactivation (R2-haplotype) or by affecting both FV functions at the same time (FV_{Leiden}). This thesis mainly focuses on the cofactor activity of FV in the inactivation of FVIII(a) by APC and its role in APC-resistance.

Chapter 2 illustrates the molecular mechanism by which the amino acid substitution at position 2194 in the FV molecule, changing an aspartate into a glycine, is the main contributor to the reduced FV levels in plasma of carriers of the R2-haplotype. Molecular dynamics simulations and electrostatic computations indicated that the Asp2194Gly mutation causes an overall destabilization of the FV C2-domain by disturbing the electrostatic interactions

within the triad Asp2194-Lys2101-Lys2103. Under normal conditions (no mutation) these electrostatic interactions facilitate the formation of the disulfide bridge between Cys²⁰³⁸ and Cys²¹⁹³ located at the N-terminal and the C-terminal parts of the C2-domain respectively, thereby stabilizing the molecule. The replacement of the negatively charged aspartate by a neutral glycine disturbs the correct folding of the C2-domain, resulting in the retention and degradation of the FV molecule in the ER and therefore a reduced secretion in plasma. These *in silico* data were confirmed by experimental data using recombinant molecules, showing that any mutation affecting this electrostatic triad (Asp2194-Lys2101-Lys2103) greatly reduces the level of secretion.

In **chapter 3** we have focused on the anticoagulant activity of FV in the inactivation of FVIII(a) by APC and its role in APC-resistance. First, we have quantified the relative contributions of reduced susceptibility of FVa towards inactivation by APC and impaired APC-cofactor activity of FV to FV_{Leiden}-associated APC-resistance. We have shown that the impaired anticoagulant activity of FV_{Leiden} accounts for half of the APC-resistance measured with the classical aPTT-based assay, an assay commonly used in clinical settings. Furthermore, we have demonstrated the physiological importance of the APC-cofactor activity of FV by means of thrombin generation measurements in plasmas with different FV concentrations triggered with tissue factor in the presence of APC. For FV levels between 60% and 120%, the amount of thrombin formed in plasma *decreased* at *increasing* FV concentration, due to the anticoagulant activity expressed by normal FV. In contrast, similar increasing concentrations of FV_{Leiden} (which does not express APC-cofactor activity) did not influence thrombin generation in the presence of APC. Finally, we have shown that FV-R2 expresses ~73% of the APC-cofactor activity of normal FV. This reduced APC-cofactor activity of FV-R2 in plasma from a homozygous R2-carrier results in the generation of twice as much thrombin in the presence of APC as compared to normal plasma.

The patho-physiological relevance of the anticoagulant activity of FV is further illustrated by a rare prothrombotic condition known as pseudo-homozygous APC-resistance (**chapter 4**). Pseudo-homozygotes are heterozygous carriers of FV Leiden whose counterpart (non-Leiden) FV allele is not expressed due to a null mutation. To characterize the APC-resistance phenotype associated with FV_{Leiden} pseudo-homozygosity, we have compared FV_{Leiden} pseudo-homozygotes, homozygotes and heterozygotes using 4 different APC-resistance assays which probe FVa inactivation, FVIIIa inactivation or both. FV_{Leiden} pseudo-homozygotes were more APC-resistant

than heterozygotes and were comparable to homozygotes in all assays. These findings can be explained by the fact that FV_{Leiden} has no APC-cofactor activity and the normal FV present in heterozygous plasma acts as a cofactor for APC in the inactivation of FVIII(a). These results were confirmed in a plasma model system, where the different FV genotypes were mimicked by reconstituting FV-deficient plasma with purified normal FV and/or FV_{Leiden}. Furthermore, we showed that when normal FV is titrated into pseudo-homozygous plasma, the APC-resistance of this plasma progressively decreases presumably due to the APC-cofactor activity expressed by the added normal FV. This can once again be explained by the fact that by increasing the concentration of normal FV in the pseudo-homozygous plasma, we increase the amount of FV expressing anticoagulant activity, resulting in a decrease of the APC-resistance. We concluded that the expression level of the non-Leiden allele in heterozygous FV_{Leiden} carriers is an important determinant of the APC-resistant phenotype of these individuals.

Finally, in **chapter 5** we investigated the relationship between plasma prothrombin levels and APC-resistance. In prothrombin-deficient plasma reconstituted with purified prothrombin, APC-resistance increased with increasing prothrombin concentrations, both in the aPTT-based and in the thrombin generation-based APC-resistance assays. In a population of healthy individuals the same trend was observed in the aPTT-based but not in the thrombin generation-based APC-resistance assay. This discrepancy was due to the strong correlation between the plasma levels of prothrombin and protein S in the population. Whereas high prothrombin on the one hand increases APC-resistance, this is counteracted by the high protein S level. Thus, the protein S levels in plasma play an important role in modulating the severity of the APC-resistance caused by elevated prothrombin levels. Prothrombin titrations in plasmas containing different amounts of protein S confirmed the ability of protein S to modulate the APC-resistance induced by elevated prothrombin levels. Therefore, the prothrombin/protein S ratio might provide a more accurate prediction of the APC-resistance in a given plasma than the prothrombin level alone. These findings suggest that carriers of the prothrombin 20210 G/A mutation, since they have a higher prothrombin/protein S ratio than non-carriers, may experience a higher thrombosis risk than non-carriers with comparable prothrombin levels.

Samenvatting

Het stollen van bloed is een complex gebeuren, waarbij via enzymatische reacties de inactive stollingsfactoren in het bloed worden geactiveerd. De vorming van trombine is een centraal gebeuren in deze stollingscascade. Daarom is een goede regulatie van de hoeveelheid trombine dat gevormd wordt noodzakelijk voor het behoud van een hemostatisch evenwicht. Het proteïne C systeem is een belangrijk anticoagulant mechanisme wat een rol speelt bij deze regulatie. Geactiveerd proteïne C (APC) is een vitamine-K-afhankelijk eiwit dat de cofactoren van zowel het intrinsiek (FVIIIa) als het extensiek systeem (FVa) inactieveert. Bij deze inactiveringen fungeert proteïne S als cofactor van APC. Bovendien heeft men aangetoond dat FV ook APC-cofactor activiteit bezit in de inactivering van FVIII(a). In sommige gevallen blijkt het plasma resistent te zijn tegenover de regulerende werking van APC. Dit wordt APC-resistentie genoemd. APC-resistentie wordt geassocieerd met een verhoogd risico op veneuze thrombose.

APC-resistentie wordt vaak veroorzaakt door een puntmutatie in het FV gen, die leidt tot de substitutie van een arginine op positie 506 door een glutamine. Deze mutatie wordt aangeduid als FV_{Leiden}. Aangezien één van de APC-splitsingsplaatsen hierdoor wordt getroffen, is FVa_{Leiden} minder gevoelig voor inactivatie door APC. Aangezien FV ook gesplitst dient te worden door APC bij Arg⁵⁰⁶ om als cofactor van APC te kunnen fungeren bij de inactivatie van FVIII(a), leidt deze mutatie ook tot een gereduceerde anticoagulante activiteit van FV. Naast de FV_{Leiden} mutatie zijn er ook nog andere mutaties in het FV gen mogelijk die APC-resistentie veroorzaken, zoals bijvoorbeeld het R2-haplotype. Het R2-haplotype omvat een reeks van mutaties die altijd samen voorkomen. Deze mutaties hebben echter geen effect op de inactivatie van FVa-R2 door APC, maar treffen specifiek de cofactor activiteit van FV-R2 in de FVIIIa inactivering.

Het FV(a) in plasma kan dus zowel een substraat zijn van APC, alsook fungeren als een cofactor voor APC. Mutaties in het FV gen kunnen dus APC-resistentie veroorzaken ofwel door de inactivering van FVa te beïnvloeden, ofwel door de cofactor activiteit van FV bij de inactivering van FVIII(a) te reduceren. In dit proefschrift hebben wij ons voornamelijk gericht op de APC-cofactor activiteit van FV en zijn rol in APC-resistentie.

Hoofdstuk 2 beschrijft experimenten om te achterhalen waarmee een aminozuur verandering op positie 2194 van het FV molecule, waarbij een asparaginezuur in een glycine verandert, de hoofdoorzaak is voor de gereduceerde FV concentratie in het plasma van mensen met het R2-haplotype. Moleculair dynamische en electrostatische computer simulaties geven aan dat de Asp2194Gly mutatie leidt tot een algemene destabilisering van het C2 domein van FV. Dit wordt veroorzaakt door het feit dat deze mutatie

de electrostatische interacties tussen Asp2194-Lys2101-Lys2103 verstoort. Onder normale omstandigheden (geen mutatie) zorgen deze interacties ervoor dat het N-terminaal en het C-terminaal deel van het C2-domein in elkaars buurt komen, waardoor de vorming van een disulfide brug tussen Cys2038 en Cys2193 bevorderd wordt. Dit resulteert in een stabilisatie van het eiwit. Aangezien deze Asp2194Gly mutatie de electrostatische interacties teniet doet, wordt de correcte vouwing van het C2-domein bemoeilijkt. Dit resulteert in de retentie en degradatie van incorrect gevouwen FV moleculen in het endoplasmatisch reticulum en leidt uiteindelijk tot een verminderde FV expressie. Deze computer data werden bevestigd door middel van experimenten met recombinante eiwitten, waarmee ook aangetoond werd dat niet alleen de Asp2194Gly mutatie maar om het even welke mutatie die de electrostatische interacties tussen Asp2194-Lys2101-Lys2103 treft, leidt tot een sterk verminderde expressie van het FV molecule.

In **hoofdstuk 3** hebben we ons geconcentreerd op de anticoagulante activiteit van FV in de inactivering van FVIII(a) door APC en het belang hiervan voor APC-resistentie. Hoewel het nu algemeen aanvaard is dat de gereduceerde inactivering van FV_{Leiden} door APC en de verminderde APC-cofactor activiteit van FV_{Leiden} beide bijdragen tot de waargenomen APC-resistentie, is de relatieve bijdrage van beide componenten nooit bepaald. Wij hebben aangetoond dat de verminderde anticoagulante werking van FV_{Leiden} voor de helft verantwoordelijk is voor de APC-resistentie, waargenomen met een klassieke aPTT-based APC-resistentie assay, een test die veel gebruikt wordt in klinische laboratoria. Verder hebben we het belang van de APC-cofactor activiteit van FV laten zien door trombine generatie metingen in plasmas met verschillende FV concentraties, in aan en afwezigheid van APC. In aanwezigheid van APC leidt een *verhoging* van de FV concentratie (boven 60% van de normale hoeveelheid in plasma) tot een *vermindering* van de hoeveelheid gevormd trombine. Dit geeft aan dat 60% FV genoeg is voor een optimaal procoagulante werking en dat een verdere toename van de FV concentratie resulteert in een toename van anticoagulante eigenschappen van FV. Bij FV_{Leiden}, dat geen APC-cofactor activiteit heeft, gaf een dergelijk experiment geen vermindering in trombine generatie te zien. We hebben ook laten zien dat de APC-resistentie geassocieerd met de R2-haplotype veroorzaakt wordt door een verminderde APC-cofactor activiteit van FV-R2. Hierdoor wordt, in aanwezigheid van APC, in het plasma van een homozygote drager van het R2-polymorfisme twee keer zoveel trombine gevormd als in normaal plasma.

De patho-fysiologische betekenis van de anticoagulante activiteit van FV werd geïllustreerd in **hoofdstuk 4**, door het vergelijken van plasma van pseudo-homozygote, homozygote en heterozygote FV_{Leiden} dragers met plasma

van normale individuen. Met behulp van vier verschillende APC-resistentie testen hebben we een gedetailleerde karakterisatie verricht van het APC-resistente fenotype geassocieerd met deze verschillende FV_{Leiden} genotypes. Op basis van de resultaten kunnen we besluiten dat pseudo-homozygoten en homozygoten van de FV_{Leiden} mutatie even resistent zijn voor APC, terwijl heterozygote FV_{Leiden} dragers beduidend minder resistent zijn voor inactivatie door APC. Deze resultaten worden verklaard door het feit dat FV_{Leiden} geen APC-cofactor activiteit vertoont en dat normaal FV wel als cofactor voor APC fungeert bij de inactivering van FVIII(a). Dit komt volledig overeen met de model experimenten uitgevoerd in hoofdstuk 3, waarbij deze verschillende FV_{Leiden} genotypes gesimuleerd werden door FV deficient plasma te reconstitueren met gezuiverd FV_{Leiden} en/of normaal FV. Verder hebben bijkomende experimenten aangetoond dat wanneer we FV, van nature een procoagulant eiwit, titreren in een pseudo-homozygoot plasma, de APC-resistentie van dit plasma geleidelijk afneemt. Deze waarneming impliceert dat de mate waarmee het non-Leiden allel tot expressie komt in een heterozygote FV_{Leiden} drager in belangrijke mate bijdraagt tot de individuele APC.

Tot slot, in **hoofdstuk 5** hebben we APC-resistentie bestudeerd die geassocieerd is met verhoogde protrombine concentraties in plasma. De aPTT-based test bevestigde dat de APC-resistentie in een plasma toeneemt met toenemende protrombine concentraties, zowel in een model systeem als in een populatie gezonde donoren. Hetzelfde was waar voor het model systeem, gemeten met de ETP-based test. Er was echter een discrepantie met de ETP-based test tussen de resultaten van het model systeem en de metingen in de populatie gezonde individuen. Maar na correctie voor de protein S concentraties in de populatie, werden gelijkaardige resultaten bekomen als in het model systeem. Hieruit kunnen we concluderen dat protein S het effect dat verhoogde protrombine heeft op de APC-resistentie tegenwerkt. Met andere woorden, de protein S concentratie in plasma speelt een belangrijke rol bij het moduleren van de APC-resistentie, veroorzaakt door verhoogde protrombine levels. Dit werd bevestigd wanneer we protrombine titreerden in plasmas met verschillende concentraties protein S. Aangezien de protrombine en protein S concentraties in plasma sterk gecorreleerd zijn, geeft de protrombine/protein S ratio een juister voorspelling van de APC-resistentie dan de protrombine concentratie alleen. Deze bevindingen suggereren ook dat dragers van de protrombine mutatie (G20210A), die een hogere prothrombine/protein S ratio hebben, een hogere risico voor thrombose hebben dan niet-dragers met vergelijkbare protrombine concentraties.

List of publications

Miteva MA, Brugge JM, Rosing J, Nicolaes GAF, Villoutreix BO. Theoretical and experimental study of the D2194G mutation in the C2 domain of coagulation factor V. *Biophysical Journal*. 2004; 86: 488-498

Elisabetta Castoldi, Jeroen M. Brugge, Gerry A. F. Nicolaes, Domenico Girelli, Guido Tans, and Jan Rosing. Impaired APC cofactor activity of factor V plays a major role in the APC resistance associated with the factor V Leiden (R506Q) and R2 (H1299R) mutations. *Blood*. 2004;103: 4173-4179

Brugge JM, Simioni P, Bernardi F, Tormene D, Lunghi B, Tans G, Rosing J, Castoldi E. Expression of the normal factor V allele modulates APC resistance phenotype in heterozygous carriers of the factor V Leiden mutation. *J Thromb Haemost*, 2005; Issue 12: 2695-2702

Brugge JM, Tans G, Rosing J, Castoldi E. Protein S levels modulate the activated protein C resistance phenotype induced by elevated prothrombin levels. *Thromb Haemost*, 2006; *in press*

Accepted abstracts

XIXth Congress of the International Society on Thrombosis and Haemostasis, Birmingham, UK, July 2003

Brugge J, Yamazaki T, Tans G, Dahlback B, Rosing J, Nicolaes GAF. Functional characterization of recombinant factor V molecules mimicking the R2-haplotype. *Journal of thrombosis and Haemostasis* volume 1; supplement 1 July: OC258

Castoldi E, Brugge J, Nicolaes GAF, Tans G, Rosing J. Impaired anticoagulant activity of factor V accounts for a large fraction of the APC resistance associated with the FV Leiden (R506Q) and R2 (H1299R) mutations. *Journal of thrombosis and Haemostasis* 2003; supplement 1 July: OC256

XXth Congress of the International Society on Thrombosis and Haemostasis, Sidney, Australia, August 2005

Brugge JM, Simioni P, Bernardi F, Tormene D, Lunghi B, Tans G, Pagnan A, Rosing J Castoldi E. Thrombin generation and activated protein C resistance in factor V Leiden pseudo-homozygous patients. *Journal of thrombosis and Haemostasis* volume 3; supplement 1: P1240

Curriculum vitae

De auteur van dit proefschrift werd geboren op 31 juli 1976 te Casablanca (Marokko). Na het behalen van het diploma voor het algemeen secundair onderwijs in 1994 aan het Koninklijk Atheneum te Keerbergen (Belgium), werd in het najaar van 1995 gestart met de opleiding Biologie aan de Rijksuniversiteit van Gent (RUG). Na het behalen van zijn kandidaatsdiploma Biologie in 1998, besloot Jeroen verder te gaan met de opleiding Biotechnologie aan deze universiteit. Hierin slaagde hij met grote onderscheiding, waarna op 5 juli 2000 de titel 'Licentiaat in de Biotechnologie' verkregen werd. In januari 2001 begon hij vervolgens als assistent in opleiding (AIO) aan de Universiteit van Maastricht binnen het Cardiovasculair Onderzoeksinstituut (CARIM), bij de vakgroep Biochemie van Prof. Dr. J. Rosing. Hier heeft hij ruim 4 jaar gewerkt aan een project over APC-resistentie, hetgeen tot dit proefschrift heeft geleid.

The author of this thesis was born on the 31st of July 1976 in the hospital of Casablanca (Morocco). After graduating from the Koninklijk Atheneum in Keerbergen (Belgium) in 1994, Jeroen began to study Biology at the University of Ghent in the autumn of 1995. After receiving his Bachelor degree in Biology in 1998, he decided to continue to study Biotechnology at the same university. Here, he graduated with honors and obtained his Masters degree in Biotechnology on the 5th of July 2000. In January 2001 he started as a PhD student at the University of Maastricht, within the Cardiovascular Research Institute Maastricht (CARIM) at the department of Biochemistry of Professor Dr. J. Rosing. There he has worked for over 4 years on a project about APC-resistance, the results of which are described in this thesis.

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Rory, de DJ van ons lab, kamergenoot tijdens cursussen en congressen en een uitstekende wetenschapper. We hebben samen veel plezier beleefd tijdens de cursussen in Amsterdam, Lunten, Papendaal en Houthalen. Die auto van jou, waar je na het tanken nog een scheut loodoplossing in moest gooien, vergeet ik nooit. Jij hebt mij "cultuur" bijgebracht; toen ik hier begon, wist in nauwelijks wat smartlappen waren. Maar na 3 jaar met jou het lab gedeeld te hebben, was zelfs ik een beetje droevig na het overlijden van jouw grote idool, André Hazes.

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