Properties of factor V in the pro- and anticoagulant pathways of blood coagulation

Citation for published version (APA):

Hoekema, L. (2000). *Properties of factor V in the pro- and anticoagulant pathways of blood coagulation*. [Doctoral Thesis, Maastricht University]. Universiteit Maastricht. <https://doi.org/10.26481/dis.20000121lh>

Document status and date:

Published: 01/01/2000

DOI:

[10.26481/dis.20000121lh](https://doi.org/10.26481/dis.20000121lh)

Document Version:

Publisher's PDF, also known as Version of record

Please check the document version of this publication:

- A submitted manuscript is the version of the article upon submission and before peer-review. There can be important differences between the submitted version and the official published version of record. People interested in the research are advised to contact the author for the final version of the publication, or visit the DOI to the publisher's website.
- The final author version and the galley proof are versions of the publication after peer review.
- The final published version features the final layout of the paper including the volume, issue and page numbers.

[Link to publication](#)

General rights

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal.

If the publication is distributed under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license above, please follow below link for the End User Agreement:

www.umlib.nl/taverne-license

Take down policy

If you believe that this document breaches copyright please contact us at:

repository@maastrichtuniversity.nl

providing details and we will investigate your claim.

Properties of factor V in the pro- and anticoagulant pathways of blood coagulation

W. F. J. VAN DER VLIET

Department of Hematology, University Hospital Groningen,
Groningen, The Netherlands
Received 15 November 1994
Accepted 15 December 1994

Factor V is a plasma protein that is involved in the pro- and anticoagulant pathways of blood coagulation. It is a procoagulant factor in the prothrombinase complex and an anticoagulant factor in the activated protein C complex.

Correspondence:

Dr W. F. J. van der Vliet
Department of Hematology
University Hospital Groningen
P.O. Box 30.001, 3000 AA Groningen, The Netherlands

Properties of factor V in the pro- and anticoagulant pathways of blood coagulation

Properties of factor V in the pro- and anti-
coagulant pathways of blood coagulation by
Lijkle Hoekema — Maastricht.
Thesis Universiteit Maastricht, 2000
ISBN 90-9013353-4

Omslag L. Hoekema
Druk Thela Thesis, Amsterdam

© L. Hoekema, Maastricht 2000

Properties of factor V in the pro- and anticoagulant pathways of blood coagulation

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit Maastricht,
op gezag van de rector magnificus Prof. Dr. A.C. Nieuwenhuijzen Kruseman,
volgens het besluit van het College van Decanen,
in het openbaar te verdedigen op
vrijdag 21 januari 2000 om 14.00 uur.

door

Lijkle Hoekema

Promotores: Prof. Dr. J. Rosing
Prof. Dr. H.C. Hemker

Co-promotor: Dr. G. Tans

Beoordelingscommissie: Prof. Dr. M.F. von Meyenfeldt (voorzitter)
Prof. Dr. B.N. Bouma (Universiteit Utrecht)
Prof. Dr. J.P.M. Geraedts
Prof. Dr. H.F.P. Hillen
Prof. Dr. J.F.M. Smits

The work presented in this thesis is financed by program grant 900-526-192 from the Dutch Organisation for Scientific Research (NWO)

Financial support by the Netherlands Heart Foundation and by the Dutch Organisation for Scientific Research (NWO) for the publication of this thesis is greatly acknowledged.

What are the two functions of the Ca^{2+} concentration? How does the Ca^{2+} concentration affect the Ca^{2+} concentration?

What are the two functions of the Ca^{2+} concentration? How does the Ca^{2+} concentration affect the Ca^{2+} concentration?

What are the two functions of the Ca^{2+} concentration?

What are the two functions of the Ca^{2+} concentration? How does the Ca^{2+} concentration affect the Ca^{2+} concentration?

What are the two functions of the Ca^{2+} concentration? How does the Ca^{2+} concentration affect the Ca^{2+} concentration?

What are the two functions of the Ca^{2+} concentration? How does the Ca^{2+} concentration affect the Ca^{2+} concentration?

What are the two functions of the Ca^{2+} concentration? How does the Ca^{2+} concentration affect the Ca^{2+} concentration?

Voor Judy

Contents

Abbreviations	viii
1 Introduction	1
2 Effects of Protein S and Factor Xa on Peptide Bond Cleavages During Inactivation of Factor Va and Factor Va ^{R506Q} by Activated Protein C	15
3 Human Factor Va ₁ and Factor Va ₂ : Properties in the Procoagulant- and Anticoagulant Pathways	33
4 An assay to quantify the two plasma isoforms of factor V	47
5 Mutations affecting post-translational processing of coagulation factor V are associated with coronary artery disease	63
6 Functional properties of factor V and factor Va encoded by the R2-FV gene	77
7 General Discussion	93
Summary	112
Samenvatting	114
List of publications	116
Curriculum Vitae	118
Dankwoord	119

Abbreviations

aPTT	activated partial thromboplastin time
AMI	acute myocardial infarction
APC	activated protein C
BCA	bicinchoninic acid
BSA	bovine serum albumin
CAD	coronary artery disease
95%CI	95% confidence interval of the mean
1,5-DNS-GGACK-factor Xa	factor Xa inhibited with 1,5-dansyl-Glu-Gly-Arg chloromethylketone
DOPS	1,2-dioleoyl- <i>sn</i> -glycero-3-phosphoserine
DNA	deoxyribonucleic acid
DOPC	1,2-dioleoyl- <i>sn</i> -glycero-3-phosphocholine
EDTA	ethylenediamine tetra acetic acid
FV	factor V
factor Va ^{R506Q}	abnormal factor Va in which Arg ⁵⁰⁶ is substituted by Gln
I2581	N-dansyl-(<i>p</i> -guanidino)-phenylalanine-piperidide hydrochloride
<i>p</i> -NPGB	<i>p</i> -nitrophenyl- <i>p</i> '-guanidino-benzoate hydrochloride
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PI	phosphatidylinositol
PS	phosphatidylserine
Pefablock® TH	N- α -(2-naphtyl-sulfonyl-glycyl)-4-amidinophenylalanine piperidide acetate salt
RVV-X	purified factor X activator from Russell's viper venom
S2238	D-Phe-(pipecolyl)-Arg-pNA
S2366	L-pyroGlu-Pro-Arg-pNA
SDS-PAGE	polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate
SEM	standard error of the mean

Chapter 1

Introduction

Introduction

The purpose of this book is to provide a comprehensive overview of the current state of research in the field of artificial intelligence (AI). This book is intended for researchers, students, and practitioners who are interested in the latest developments in AI. The book is organized into several chapters, each focusing on a different aspect of AI. The first chapter, "Introduction," provides an overview of the field and its history. The second chapter, "Machine Learning," discusses the various techniques used in machine learning, including supervised and unsupervised learning. The third chapter, "Deep Learning," focuses on the use of deep neural networks in AI. The fourth chapter, "Natural Language Processing," discusses the techniques used to process and understand human language. The fifth chapter, "Computer Vision," discusses the techniques used to enable computers to see and understand the visual world. The sixth chapter, "Robotics," discusses the use of AI in the design and control of robots. The seventh chapter, "Ethics and Society," discusses the ethical implications of AI and its impact on society. The eighth chapter, "Future of AI," discusses the potential future developments in AI and the challenges that must be overcome. The book is written in a clear and concise style, making it accessible to a wide range of readers. It is a valuable resource for anyone interested in the field of AI.

1.1 Blood coagulation

Blood is a medium which has evolved to transport numerous substances through the body. An essential requirement for a successful realization of this task is that it stays fluid. However, in the case of injury when blood threatens to escape from the damaged vascular system, blood is transformed from a fluid into a solid medium as the result of an intricate process, called blood coagulation. Thus, at one time, blood has to stay fluid and at another it must have the ability to clot very efficiently. This seemingly contradictory requirement is met by maintaining a delicate balance between processes that are able to initiate coagulation (so-called procoagulant pathways) and processes that down-regulate coagulation (so-called anticoagulant pathways).

After injury of the smaller blood vessels bleeding is stopped by vasoconstriction, but in the larger vessels the first defence mechanism is formation of a plug consisting of aggregated platelets. This fragile primary plug is consolidated into a stable haemostatic plug by deposition of insoluble, cross-linked fibrin.

Fibrin is the end-product of the coagulation cascade. This cascade consists of a series of enzymatic processes which results, via an avalanche-like process in the formation of large amounts of thrombin. Thrombin converts soluble fibrinogen into insoluble fibrin fibers.

Pertinent to the regulation of blood coagulation are the following mechanisms:

- proteins involved in coagulation circulate in blood as inactive pro-enzymes (substrates). Only after activation via limited proteolysis by another already activated coagulation factor (enzyme), they are able to exert biological activity. Since activation of coagulation factors, only occurs after a trigger, this limits the initiation of coagulation to situations in which, as result of tissue damage, blood is exposed to a triggering substance.
- most of the reactions of the blood coagulation process can only take place in the presence of a so-called procoagulant surface that acts as a collecting device for the coagulation proteins. Physiologically important surfaces are exposed by activated platelets and disrupted endothelial cell membranes. Again, these are only present after tissue damage. Essential for the function of procoagulant surfaces is that they contain negatively charged phospholipids which are the binding sites for both the enzyme and substrate. Binding of coagulation proteins to a procoagulant surface not only results in an increase of their local concentration at the site of the injury, but may also result in a more favorable orientation for mutual interactions between coagulation factors.
- several of the serine proteases involved in the activation of blood coagulation factors exert maximal activity only in the presence of a so-called cofactor, which enhances the rate of coagulation factor activation several thousand fold. Thus, via activation and inactivation of cofactors, the process of blood coagulation can be very strictly and efficiently regulated. Since factor V, the cofactor of the prothrombin activating complex, is the central protein in this thesis, its role in prothrombin activation and the regulation of its activity are more extensively described in section 1.3 of this introduction.

In summary, initiation and efficient propagation of blood coagulation is limited in space by means of the necessity for the presence of *activated* coagulation factors (enzyme and cofactor)

1.1 Blood coagulation

Figure 1.1: **Building block of coagulation system.** Many steps in blood coagulation have the following common features. Essential components are 1) a negatively charged, procoagulant phospholipid surface, 2) an activated serine protease which activates the substrate, and 3) an activated cofactor, which enhances the efficiency of the serine protease. These three components result in a stimulation of the rate of coagulation factor activation by several orders of magnitude.

and the presence of a suitable membrane surface, as depicted in figure 1.1. Only after a trigger as result from tissue damage, both requirements are fulfilled, which restricts coagulation to the site of injury.

Control of coagulation is essential, since uncontrolled coagulation would hamper the transport of vital substances to essential organs, ultimately leading to death. Restriction of the coagulation process has to be achieved both in place and in time, since it is undesirable that a small, local injury leads to systemic coagulation or to continued coagulation after bleeding has stopped. The necessity of the presence of all components of the coagulation factor activating complexes (Fig 1.1), restricts coagulation in space since not all components will be present outside the site of injury. Control of coagulation in time is achieved by the presence in plasma of direct inhibitors of thrombin and by an anticoagulant pathway, which is actually initiated by thrombin itself and acts via inactivation of cofactor proteins participating in the coagulation cascade. This mechanism of control of coagulation is discussed in section 1.2.

Initiation of coagulation

Historically, the coagulation system is divided in two separate parts, the extrinsic and the intrinsic pathway which are schematically depicted in figure 1.2. This division is mainly based on the way blood coagulation is initiated in clinical coagulation assays. The extrinsic pathway is initiated by addition of tissue factor, whereas the intrinsic pathway is initiated via contact with negatively charged surfaces, such as glass or kaolin.

It is now generally accepted that the extrinsic coagulation system is the principal pathway of *in vivo* blood coagulation. After vascular or endothelial damage, tissue factor becomes available which complexes with factor VII or factor VIIa. Tissue factor is a cofactor for factor VIIa, a serine protease which is responsible for the activation of factor IX and factor X. The extrinsic factor Xa generating pathway is rapidly down-regulated by TFPI [43], but when the trigger is strong enough, adequate amounts of thrombin will be formed for the activation of factors V and VIII. This results in additional thrombin formation. Moreover, thrombin also activates factor XI and this will result in a second burst of thrombin formation via the intrinsic pathway [14, 61, 62].

Figure 1.2: **Schematic representation of the blood coagulation cascade.** Coagulation is initiated either via the intrinsic or extrinsic pathway. The extrinsic pathway is rapidly inhibited by TFPI, but when the trigger is strong enough, sufficient amounts of thrombin will have been formed for activation of factor V, factor VIII and factor XI. This results in a second burst of thrombin formation via the intrinsic route. Light grey: inactive coagulation proteins; dark grey: activated cofactors; black: serine proteases. Thick lines represent proteolytic activation, thin lines indicate a stimulatory effect.

1.2 Down-regulation of thrombin formation

Two major systems are available to control the activity and the quantity of thrombin formed during initiation and propagation of blood coagulation.

Inhibitors circulate in plasma which can directly neutralize the activity of proteases involved in blood coagulation.

Protein C pathway which involves the lipid-dependent inactivation of the cofactors Va and VIIIa by activated protein C (APC) and its cofactor protein S.

The first system is always available to neutralize activated coagulation factors (*e.g.* thrombin), whereas the latter system is initiated by thrombin and therefore is a response to thrombin formation. An obvious advantage for having available two separate mechanisms for control of thrombin activity is the increased capability of fine-tuning the control of coagulation.

When the trigger of coagulation is below a certain threshold, the presence of the inhibitors prevents the avalanche of thrombin formation and the process stops before it has actually begun. However, when the trigger is above this threshold, the inhibitors cannot prevent large amounts of thrombin being formed. Then the protein C pathway is initiated - by thrombin itself - to prevent uncontrolled coagulation.

Plasma inhibitors of coagulation

The proteases involved in coagulation are inactivated by several circulating protease inhibitors via formation of irreversible enzyme-inhibitor complexes which are rapidly cleared from the

1.3 Factor V

circulation. Serine proteases inhibitors (serpins) bind to and block the active site of serine proteases. Examples are antithrombin III (AT-III), α_1 antitrypsin, protein C inhibitor (PCI) and α_2 macroglobulin. TFPI is a Kunitz type inhibitor which, when complexed to factor Xa, inhibits the TF/factor VIIa complex [32].

Protein C pathway

Already in 1960 a plasma protein was described with a strong anticoagulant activity [34]. This protein was rediscovered in 1976 and called protein C, which appeared to be the vitamin K-dependent proenzyme of a serine protease that inactivates factor Va [29] and factor VIIIa [13].

Protein C is activated by thrombin in the presence of thrombomodulin that is constitutively expressed by endothelial cells. When thrombin binds to thrombomodulin, a transmembrane protein, its substrate specificity is changed. Thrombin loses its procoagulant properties (activation of factors V, VIII and XI and the conversion of fibrinogen into fibrin) and becomes an anticoagulant protein which is able to convert protein C into activated protein C (APC) [16]. Because the affinity of thrombin for thrombomodulin is very high, and since there is an abundance of thrombomodulin in the microcirculation, low levels of thrombin have an antithrombotic effect. Only at higher levels of free thrombin, it can exert its procoagulant activity.

Efficient inactivation of factor Va by APC requires Ca^{2+} -ions, negatively charged membranes and the presence of protein S, another vitamin K dependent plasma protein [64]. The physiological importance of the protein C pathway is underscored by the observation that heterozygous deficiencies in either protein C [17, 18] or protein S [7, 50] are associated with venous thrombosis. The homozygous form of deficiency of either protein is associated with severe thrombotic complications at birth [36, 51]. However, when the research presented in this thesis started, the exact mechanism of the mode of action of protein S in the protein C pathway was not fully understood, since in model systems, protein S stimulated the rate of factor Va inactivation by APC only two-fold [52, 2]. In addition, protein S was shown to have an anticoagulant property independent of APC via direct inhibition of the thrombin forming complex [24, 22].

1.3 Factor V

Cofactors are indispensable for a proper response of the coagulation system to vascular damage (cf. figure 1.1) and for the control of coagulation. This section focuses on factor V, since this is the protein that is the subject of the research presented in this thesis. Factor V is the cofactor of the so-called prothrombinase complex and it enhances the efficiency of this complex to form thrombin more than thousand fold.

Factor V is a single-chain polypeptide with a molecular weight of 330 kDa, which is synthesized in the liver and megakaryocytes and circulates in blood as an inactive procofactor. In a primate model the half-life of factor V was determined to be 13 hours [42]. Approximately 25% of the factor V is present in the α -granules of platelets ($0.5 \mu\text{g}/10^8$ cells), while the remainder is present in plasma (concentration 7–10 $\mu\text{g}/\text{ml}$, corresponding to 25 nM) [56].

Figure 1.3: **Schematic representation of the structure of human factor V.** Grey arrows indicate the peptide bonds that are cleaved during factor V activation by thrombin (IIa), factor Xa (Xa), meizothrombin (MT), and Russell's viper venom (RVV-V). Cleavage sites in factor V(a) for APC are indicated by black arrows

Platelet factor V was shown to have the same properties as plasma factor V [60], which can be explained by a recent observation that platelet factor V originates from the plasma pool [6]. Local release of platelet factor V from the α -granules after platelet activation at places with vascular damage, may lead to a situation where the concentration of platelet factor V exceeds that of plasma factor V. Thus, factor V stored in platelets may be as important as plasma factor V in the modulation of haemostasis.

Single chain factor V circulates in blood as an inactive procofactor and is activated *in vivo* via limited proteolysis by thrombin [10, 27, 53, 37], factor Xa [37, 12], or by meizothrombin [54], a reaction intermediate which is formed during prothrombin activation [48]. Thrombin catalyzes cleavage of factor V at Arg⁷⁰⁹, Arg¹⁰¹⁸ and Arg¹⁵⁴⁵ in a sequential manner. Cleavage at Arg¹⁵⁴⁵ occurs last, but is crucial for full expression of factor Va activity. A fully activated factor V molecule can also be obtained by a single cleavage at Arg¹⁵⁴⁵ by a serine protease present in the venom of Russell's viper (RVV-V) [53, 27]. Activation of factor V by thrombin is, in contrast to activation by factor Xa or meizothrombin, independent of negatively charged phospholipid vesicles [37]. Combined cleavage at Arg⁷⁰⁹ and Arg¹⁰¹⁸ by factor Xa or meizothrombin has been shown to result in partial cofactor activity [28, 37, 54]. Recently it was shown, however, that factor Xa is also able to cleave factor V at Arg¹⁵⁴⁵ and yielded factor Va with full cofactor activity [55].

Activation of factor V results in major molecular rearrangements and in a loss of 50% of the amino acids. The fully activated factor V consists of two polypeptide chains, the so-called heavy and light chains, which are tightly, but non-covalently, linked to each other via a Ca²⁺-ion [19]. The heavy chain has a molecular weight of 105 kDa and consists of amino acids 1–709 (domains A1, and A2), the 'light chain' of factor Va consists of amino acids 1546–2196 (domains A3, C1 and C2, see figure 1.3) and appears in gel electrophoresis experiments as a 71/74 kDa doublet due to differences in post-ribosomal processing (see page 8).

1.3 Factor V

Activator	K_m (μM)	V_{max} IIa/min/Xa
Xa, Ca^{2+}	84	0.68
Xa, Ca^{2+} , PL (25/75, PS/PC)	0.11	2.56
Xa, Va, Ca^{2+} , PL (25/75, PS/PC)	0.14	4050
Xa, Ca^{2+} , PL (5/95, PS/PC)	1.63	0.32
Xa, Va, Ca^{2+} , PL (5/95, PS/PC)	0.06	4330

Table 1.1: Kinetic parameters of prothrombin activation. Data from Rosing *et al.* [47] and van Rijn *et al.* [58]

Interactions of factor V(a) with prothrombin and factor Xa The heavy chain of factor Va is involved in prothrombin binding [20]. After binding to phospholipids, both the heavy and light chain of factor Va contribute to the interaction with factor Xa [57, 20]. A factor Va molecule, lacking the aminoterminal part (residues Asp⁶⁸³–Arg⁷⁰⁹) of the A2 domain, demonstrates impaired binding to factor Xa and prothrombin. This suggests that the carboxy-terminal part of the heavy chain is required for optimal interaction of factor Va with factor Xa and prothrombin [1]. Using synthetic peptides, additional binding sites for factor Xa in the region 493–506 [23] and 311–325 [30] in factor Va have been postulated.

Interaction with phospholipids Both factor V and factor Va bind with high affinity ($K_d < 1$ nM) to membranes containing negatively charged phospholipids [4]. A binding site of factor Va for phospholipids is localized in the amino terminus of the light chain (A3 domain) [31]. The results presented in that paper are consistent with a hydrophobic binding interaction. More recently, analysis of recombinant deletion mutants of factor V showed that the carboxyterminal C2 domain is also essential in the binding to phospholipid vesicles [39]. Since the binding of the C2 region is influenced by ionic strength it has been hypothesized that it involves ionic interactions with the polar head groups of anionic phospholipids [39].

Thus the membrane binding properties of factor Va may be conferred by more than one region of the light chain and may involve both hydrophobic and ionic interactions.

Cofactor activity in prothrombin activation The activated form of factor V serves as a cofactor in factor Xa-catalyzed activation of prothrombin to thrombin. This so-called prothrombinase complex consists of the serine protease factor Xa, the non-enzymatic cofactor factor Va, and Ca^{2+} -ions, assembled on negatively charged phospholipid vesicles.

From Table 1.1 it is clear that factor Va is indeed an essential component of the prothrombinase complex, since it increases the rate of prothrombin activation approximately 2000 fold. The stimulatory effect of factor Va on prothrombin activation is the result of an increased catalytic activity of factor Xa [47] and a decreased K_m for prothrombin [58]. The effect of factor Va on

the K_m for prothrombin is particularly observed on phospholipid vesicles with a low content of phosphatidylserine. At physiological concentrations of prothrombin ($\sim 1.5 \mu\text{M}$) and factor Va and factor Xa ($\sim 1 \text{ nM}$), the result of enzyme, cofactor, and substrate assembly on the membrane surface is dramatic. Compared factor Xa alone, the prothrombinase complex converts prothrombin 300,000 fold more efficient. Thus factor Xa assembled into the prothrombinase complex will hydrolyze an amount of prothrombin in 1 minute that would take factor Xa alone about 6 months.

The mechanisms by which the presence of factor Va results in the changes in the efficiency of the prothrombinase complex are as yet not fully understood. It has been proposed that factor Va has multiple functions in the prothrombinase complex and accelerates the rate of prothrombin by:

1. promoting the binding of factor Xa to negatively charged phospholipids [33, 35]
2. increasing the catalytic activity (k_{cat}) of factor Xa
3. promoting the interaction of prothrombin with the prothrombinase complex

In addition, the presence of factor Va induces a shift in the pathway for prothrombin activation. In the absence of factor Va the physiologically inactive prothrombin-2 is the major product, while in the presence of factor Va the pathway of prothrombin activation alters such that meizothrombin is the sole reaction intermediate resulting in the active end-product thrombin [46].

Post-translational modification of factor V

Carbohydrate content Electrophoretic determination of the molecular weight of factor V yields $M_r = 330 \text{ kDa}$. However, based upon the cDNA sequence, the molecular weight of the polypeptide part is 249 kDa [26]. This discrepancy is partly due to the highly asymmetric structure of factor V and partly due to glycosylation: the carbohydrate content of factor V is 13% [27]. Factor V contains 37 potential N-linked oligosaccharide attachment sites, 25 of which are present in the B-domain. However, the exact number of sites that actually contain a carbohydrate group is unknown. Both O- and N-linked carbohydrate chains are present [27]. The importance of the carbohydrate groups is indicated by observations that changes in carbohydrate groups can lead to increased [21] as well as decreased [5, 49] procoagulant activity of factor V and modulate the inactivation of factor Va by APC [11].

Sulfation of tyrosine residues Factor V is sulfated at several tyrosine residues in the A2, B and A3 domain [25]. Sulfation appears to be important for the activation of factor V by thrombin and for the interaction of factor Va with the prothrombinase complex [41].

Factor V isoforms Two forms of factor V circulate in blood, which after activation yield two forms of factor Va (factor Va₁ and factor Va₂) which differ in their light chain (factor Va₁: $M_r \approx 74 \text{ kDa}$, factor Va₂: $M_r \approx 71 \text{ kDa}$) [45]. Factor Va₁ has much lower affinity for negatively charged phospholipid vesicles than factor Va₂, which results in reduced cofactor activity

1.4 APC-resistance

in prothrombin activation at membranes with a low anionic phospholipid content [45]. The difference between the two isoforms of factor V is located in the carboxy-terminal part of the C2 domain [39] and is probably due to differences in post-translational glycosylation [40].

1.4 APC-resistance

In 1993 Dahlbäck *et al.* [8] described a hereditary disorder which is associated with venous thrombosis and which is characterized by the inability of APC to effectively prolong the clotting time of patient plasma. Therefore this phenomenon was called 'APC-resistance'. In his original publication, Dahlbäck and colleagues hypothesized that in these patients a cofactor for APC was missing [8]. In a later publication this cofactor was identified as factor V [9].

About one year after the original publication of Dahlbäck it was reported [3, 15, 63, 65] that APC-resistance is caused by a single point mutation in the factor V gene which results in the replacement of Arg⁵⁰⁶ by Gln. Since this mutation was first described by Bertina [3] from Leiden, the name FV_{Leiden} is now generally accepted for the mutant molecule. Cleavage at Arg⁵⁰⁶ by APC is essential for factor Va inactivation, and since this cleavage site is no longer available in FV_{Leiden}, this provides an explanation for the decreased sensitivity of FV_{Leiden} to APC and for the association of carriership of the FV_{Leiden} mutation with thrombotic complications. As a consequence, this explanation overshadowed the missing cofactor hypothesis of Dahlbäck.

The association of FV_{Leiden} with venous thrombosis (approximately 20% of patients with a first episode of DVT is carrier of the FV_{Leiden} mutation [44]) spurred the interest for the mechanism of APC-catalyzed factor Va inactivation. APC cleavage sites in the heavy chain of factor Va (Arg³⁰⁶, Arg⁵⁰⁶, Arg⁶⁷⁹) were described [59] but the exact mechanism of APC-catalyzed factor Va inactivation could only be elucidated after purification and kinetic analysis of APC-catalyzed inactivation of factor V from homozygous carriers of the FV_{Leiden} mutation [38]. This resulted in the assignment of rate constants of inactivation to the APC cleavage sites in factor Va.

1.5 Scope of this thesis

The mechanism and kinetics of factor Va inactivation by APC were being discovered when the work that is presented in this thesis started. The mode of action of the modulators of APC-catalyzed factor Va inactivation (*e.g.* factor Xa and protein S) were, however, largely unknown. Chapter 2 describes the mechanisms by which protein S and factor Xa modulate the inactivation of both 'normal' factor Va and factor Va from a homozygous carrier of the FV_{Leiden} mutation.

The observation that the two forms of factor V (factor V₁ and factor V₂) have different affinities for phospholipid vesicles and the fact that APC-catalyzed factor Va inactivation is a lipid-dependent process, raised the question whether the affinity difference for phospholipids also

results in different sensitivities of factor Va_1 and factor Va_2 for APC. The method of analysis and assignment of rate constants to the different cleavage sites for APC in factor Va, described in chapter 2, allowed an analysis of kinetics of inactivation of the two forms of factor Va (factor Va_1 and factor Va_2) by APC. In chapter 3, the phospholipid requirements for APC-catalyzed factor Va inactivation are determined and compared with the phospholipid requirements for expression of cofactor activity in prothrombin activation.

Chapter 4 describes a method for quantifying the amounts of factor V_1 and factor V_2 in plasma. This assay is based on the differences in procoagulant properties of the two forms as described in chapter 3.

Chapter 5 reports the association of carriership of a particular factor V polymorphism (R2-polymorphism) with coronary artery disease, and describes the effect of the missense mutations linked to this polymorphism on post-translational modifications of factor V and on the relative amounts of factor V_1 and factor V_2 in plasma.

In chapter 6, the functional properties of factor V(a) from carriers of the R2-allele are compared to normal factor Va, both in model systems and in plasma. On the basis of the results presented in this chapter a biochemical explanation for the increased risk for thrombotic complications associated with carriership of the R2-polymorphism is proposed.

Finally, in chapter 7, the results described in chapters 2–6 are discussed and put in broader context. The clinical relevance of the results is outlined.

1.6 References

- [1] H.M. Bakker, G. Tans, M.L.C.G.D. Thomassen, L.Y. Yukelson, R. Ebberink, H.C. Hemker, and J. Rosing. Functional properties of human factor Va lacking the Asp683-Arg709 domain of the heavy chain. *Journal of Biological Chemistry*, 269:20662–20667, 1994.
- [2] H.M. Bakker, G. Tans, T. Janssen-Claessen, M.C. 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. *European Journal of Biochemistry*, 208(1):171–178, 1992.
- [3] 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*, 369(6475):64–67, 1994.
- [4] J.W. Bloom, M.E. Nesheim, and K.G. Mann. Phospholipid-binding properties of bovine factor V and factor Va. *Biochemistry*, 18:4419–4425, 1979.
- [5] T. Bruin, A. Sturk, J.W. Ten Cate, and M. Cath. The function of human factor V carbohydrate moiety in blood coagulation. *Eur J Biochem*, 170:305–310, 1987.
- [6] R.M. Camire, E.S. Pollak, K. Kaushansky, and P.B. Tracy. Secretable human platelet-derived factor V originates from the plasma pool. *Blood*, 92(9):3035–3041, 1998.
- [7] P.C. Comp, R.R. Nixon, M.R. Cooper, and C.T. Esmon. Familial protein S deficiency is associated with recurrent thrombosis. *Journal of Clinical Investigation*, 74(6):2082–2088, 1984.
- [8] B. Dahlbäck, 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. *Proceedings of the National Academy of Sciences USA*, 90(3):1008, 1993.
- [9] 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. *Proceedings of the National Academy of Sciences USA*, 91:1396–1400, 1994.
- [10] C.T. Esmon. The subunit structure of thrombin-activated factor V. Isolation of activated factor V, separation of subunits, and reconstitution of biological activity. *Journal of Biological Chemistry*, 254:964–973, 1979.
- [11] J.A. Fernández, T.M. Hackeng, K. Kojima, and J.H. Griffin. The carbohydrate moiety of factor V modulates inactivation by activated protein C. *Blood*, 89(12):4348–4354, 1997.
- [12] W.B. Foster, M.E. Nesheim, and K.G. Mann. The factor Xa-catalyzed activation of factor V. *Journal of Biological Chemistry*, 258:13970–13977, 1983.
- [13] C.A. Fulcher, J.E. Gardiner, J.H. Griffin, and T.S. Zimmerman. Proteolytic inactivation of human factor VIII procoagulant protein by activated human protein C and its analogy with factor V. *Blood*, 63(2):486–489, 1984.
- [14] D. Gailani and G.J. Broze Jr. Factor XI activation in a revised model of blood coagulation. *Science*, 253(5022):909–912, 1991.
- [15] J.S. Greengard, X. Sun, X. Xu, J.A. Fernandez, J.H. Griffin, and B. Evatt. Activated protein C resistance caused by Arg⁵⁰⁶ → Gln mutation in factor Va. *Lancet*, 343(8909):1361–1362, 1994.
- [16] J.H. Griffin. Blood coagulation-the thrombin paradox. *Nature*, 378:337–338, 1995.
- [17] J.H. Griffin, B. Evatt, C. Wideman, and J.A. Fernandez. Anticoagulant protein C pathway defective in majority of thrombophilic patients. *Blood*, 82(7):1989–1993, 1993.
- [18] J.H. Griffin, B. Evatt, T.S. Zimmerman, A.J. Kleiss, and C. Wideman. Deficiency of protein C in congenital thrombotic disease. *Journal of Clinical Investigation*, 68(5):1370–1373, 1981.

- [19] E.R. Guinto and C.T. Esmon. Formation of a calcium binding-site on bovine activated factor V following recombination of the isolated subunits. *Journal of Biological Chemistry*, 259:10038–10043, 1982.
- [20] E.R. Guinto and C.T. Esmon. Loss of prothrombin and factor Xa-factor Va interactions upon inactivation of factor Va by activated protein C. *Journal of Biological Chemistry*, 259:13986–13992, 1984.
- [21] J.G. Gumprecht and R.W. Colman. Roles of sialic acid on the function of bovine factor V. *Arch. Biochem Biophys*, 169:278–286, 1975.
- [22] 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. *Journal of Biological Chemistry*, 269(33):21051–21058, 1994.
- [23] 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 Science*, 5:1883–1889, 1996.
- [24] 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. *Journal of Biological Chemistry*, 268(4):2872–2877, 1993.
- [25] G.L. Hortin. Sulfation of tyrosine residues in coagulation factor V. *Blood*, 76(5):946–952, 1990.
- [26] R.J. Jenny, D.D. Pittman, J.J. Toole, R.W. Kriz, R.A. Aldape, R.M. Hewick, R.J. Kaufman, and K.G. Mann. Complete cDNA and derived amino acid sequence of human factor V. *Proceedings of the National Academy of Science USA*, 84(14):4846–4850, 1987.
- [27] W.H. Kane and P.W. Majerus. Purification and characterization of human coagulation factor V. *Journal of Biological Chemistry*, 256:1002–1007, 1981.
- [28] F.G. Keller, T.L. Ortel, M.A. Quinn-Allen, and W.H. Kane. Thrombin-catalyzed activation of recombinant human factor V. *Biochemistry*, 34:4118–4124, 1995.
- [29] W. Kisiel, W.M. Canfield, L.H. Ericsson, and E.W. Davie. Anticoagulant properties of bovine plasma protein C following activation by thrombin. *Biochemistry*, 16(26):5824–5831, 1977.
- [30] Y. Kojima, M.J. Heeb, A.J. Gale, T.M. Hackeng, and J.H. Griffin. Binding site for blood coagulation factor Xa involving residues 311–325 in factor Va. *Journal of Biological Chemistry*, 273(24):14900–14905, 1998.
- [31] S. Krishnaswamy and K.G. Mann. The binding of factor Va to phospholipid vesicles. *Journal of Biological Chemistry*, 263:5714–5723, 1988.
- [32] T. Lindhout, J. Franssen, and G. Willems. Kinetics of the inhibition of tissue factor-factor VIIa by tissue factor pathway inhibitor. *Thrombosis and Haemostasis*, 74:910–915, 1995.
- [33] T. Lindhout, J.W.P. Govers-Riemslog, P. van de Waart, H.C. Hemker, and J. Rosing. Factor Va-factor Xa interaction. Effects of phospholipid vesicles of varying composition. *Biochemistry*, 21:5494–5502, 1982.
- [34] E.F. Mammen, W.R. Thomas, and W.H. Seegers. Activation of purified prothrombin to autoprothrombin I or autoprothrombin II (platelet cofactor II or autoprothrombin II-A). *Thromb Diathes Hemorrh*, 5:218–250, 1960.
- [35] K.G. Mann, R.J. Jenny, and S. Krishnaswamy. Cofactor proteins in the assembly and expression of blood clotting enzyme complexes. *Annual Reviews in Biochemistry*, 57:915–956, 1988.
- [36] R.A. Marlar and A. Neumann. Neonatal purpura fulminans due to homozygous protein C or protein S deficiencies. *Semin-Thromb-Hemost.*, 16:299–309, 1990.

1.6 References

-
- [37] D.D. Monkovic and P.B. Tracy. Activation of human factor V by factor Xa and thrombin. *Biochemistry*, 29:1118–1128, 1990.
- [38] G.A.F. Nicolaes, G. Tans, M.L.C.G.D. Thomassen, H.C. Hemker, K. Váradi, H.P. Schwarz, and J. Rosing. Peptide bond cleavages and loss of functional activity during inactivation of factor Va and factor Va^{R506Q} by activated protein C. *Journal of Biological Chemistry*, 270(36):21158–21166, 1995.
- [39] T.L. Ortel, M.A. Quinn-Allen, F.G. Keller, J.A. Peterson, D. Larocca, and W.H. Kane. Localization of functionally important epitopes within the second C-type domain of coagulation factor V using recombinant chimeras. *Journal of Biological Chemistry*, 269(22):15898–905, 1994.
- [40] T.L. Ortel, L. Yoo, M.A. Quin-Allen, and W.H. Kane. Partial glycosylation at asparagine-2181 of the second C-type domain of human factor V is the structural basis of the light chain doublet. *Blood*, 84:387a, 1994.
- [41] D.D. Pittman, K.N. Tomkinson, D. Michnick, U. Selighsohn, and R.J. Kaufman. Posttranslational sulfation of factor V is required for efficient thrombin cleavage and activation and for full procoagulant activity. *Biochemistry*, 33(22):6952–9, 1994.
- [42] M.D. Rand, S.R. Hanson, and K.G. Mann. Factor V turnover in a primate model. *Blood*, 86(7):2623, 1995.
- [43] L.V.M. Rao and S.I. Rapaport. Studies of a mechanism inhibiting the initiation of the extrinsic pathway of blood coagulation. *Blood*, 69:645–651, 1987.
- [44] 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*, 85:1504–1508, 1995.
- [45] 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. *Journal of Biological Chemistry*, 268(28):21130–21136, 1993.
- [46] J. Rosing and G. Tans. Meizothrombin, a major product of factor Xa-catalyzed prothrombin activation. *Thrombosis Haemostasis*, 60:355–360, 1988.
- [47] J. Rosing, G. Tans, J.W. Govers-Riemslog, R.F. Zwaal, and H.C. Hemker. The role of phospholipids and factor Va in the prothrombinase complex. *Journal of Biological Chemistry*, 255(1):274–283, 1980.
- [48] J. Rosing, R.F. Zwaal, and G. Tans. Formation of meizothrombin as intermediate in factor Xa-catalyzed prothrombin activation. *Journal of Biological Chemistry*, 261(9):4224–4228, 1986.
- [49] S. Saraswathi and R.W. Coleman. Role of galactose in bovine factor V. *Journal of Biological Chemistry*, 250:8111–8118, 1975.
- [50] H.P. Schwarz, M. Fischer, P. Hopmeier, M.A. Batard, and J.H. Griffin. Plasma protein S deficiency in familial thrombotic disease. *Blood*, 64(6):1297–1300, 1984.
- [51] U. Selighsohn, A. Berger, M. Abend, L. Rubin, D. Attias, A. Zivelin, and S.I. Rapaport. Homozygous protein C deficiency manifested by massive thrombosis in the newborn. *New England Journal of Medicine*, 310:559–562, 1984.
- [52] 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. *Journal of Biological Chemistry*, 263(29):14884–14890, 1988.
- [53] K. Suzuki, B. Dahlbäck, and J. Stenflo. Thrombin-catalyzed activation of human coagulation factor V. *Journal of Biological Chemistry*, 257(11):6556–6564, 1982.
- [54] G. Tans, G.A. Nicolaes, M.C. Thomassen, H.C. Hemker, A.J. van Zonneveld, H. Pannekoek, and

- J. Rosing. Activation of human factor V by meizothrombin. *Journal of Biological Chemistry*, 269(23):15969–15972, 1994.
- [55] E. Thorelli, R.J. Kaufman, and B. Dahlbäck. Cleavage requirements for activation of factor V by factor Xa. *European Journal of Biochemistry*, 247(1):12–20, 1997.
- [56] P.B. Tracy, L.L. Eide, E.J. Bowie, and K.G. Mann. Radioimmunoassay of factor V in human plasma and platelets. *Blood*, 60:59–63, 1982.
- [57] M.M. Tucker, W.B. Foster, J.A. Katzmman, and K.G. Mann. A monoclonal antibody which inhibits the factor Va:factor Xa interaction. *Journal of Biological Chemistry*, 258:1210–1214, 1983.
- [58] J.L.M.L. van Rijn, J.W.P. Govers-Riemslog, 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*, 23:4557–4563, 1984.
- [59] C. van 't.Veer and K.G. Mann. Regulation of tissue factor initiated thrombin generation by the stoichiometric inhibitors tissue factor pathway inhibitor, antithrombin-III, and heparin cofactor-II. *Journal of Biological Chemistry*, 272(7):4367–4377, 1997.
- [60] R.W. Viskup, P.B. Tracy, and K.G. Mann. The isolation of human platelet factor V. *Blood*, 69:1188–1195, 1987.
- [61] P.A. von dem Borne, S.J. Koppelman, B.N. Bouma, and J.C. Meijers. Surface independent factor XI activation by thrombin in the presence of high molecular weight kininogen. *Thrombosis and Haemostasis*, 72(3):397–402, 1994.
- [62] P.A. von dem Borne, J.C.M. Meijers, and B.N. Bouma. Feedback activation of factor XI by thrombin in plasma results in additional formation of thrombin that protects fibrin clots from fibrinolysis. *Blood*, 86(15):3035–3042, 1995.
- [63] J. Voorberg, J. Roelse, R. Koopman, H. Büller, 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*, 343(8912):1535–1536, 1994.
- [64] F.J. Walker. Regulation of activated protein C by a new protein. A possible function for bovine protein S. *Journal of Biological Chemistry*, 255(12):5521–5524, 1980.
- [65] B. Zöller and B. Dahlbäck. Linkage between inherited resistance to activated protein C and factor V gene mutation in venous thrombosis. *Lancet*, 343(8912):1536–1538, 1994.

Chapter 2

Effects of Protein S and Factor Xa on Peptide Bond Cleavages During Inactivation of Factor Va and Factor Va^{R506Q} by Activated Protein C

Based on: Rosing, J., Hoekema, L., Nicolaes, G.A.F., Thomassen, M.C.L.G.D., Hemker, H.C., Váradi, K., Schwarz, H.P. & Tans, G. Effects of Protein S and Factor Xa on Peptide Bond Cleavages During Inactivation of Factor Va and Factor Va^{R506Q} by Activated Protein C, *Journal of Biological Chemistry*, 270:27852–27858, 1995.

2.1 Summary

Inactivation of membrane-bound factor Va by activated protein C (APC) proceeds via a biphasic reaction that consists of a rapid and a slow phase, which are associated with cleavages at Arg⁵⁰⁶ and Arg³⁰⁶ of the heavy chain of factor Va, respectively. We have investigated the effects of protein S and factor Xa on APC-catalyzed factor Va inactivation. Protein S accelerates factor Va inactivation by selectively promoting the slow cleavage at Arg³⁰⁶ (20-fold). Factor Xa protects factor Va from inactivation by APC by selectively blocking cleavage at Arg⁵⁰⁶. Inactivation of factor Va^{R506Q}, which was isolated from the plasma of a homozygous APC-resistant patient and which lacks the Arg⁵⁰⁶ cleavage site, was also stimulated by protein S but was not affected by factor Xa. This confirms that the target sites of protein S and factor Xa involve Arg³⁰⁶ and Arg⁵⁰⁶, respectively. Factor Xa completely blocked APC-catalyzed cleavage at Arg⁵⁰⁶ in normal factor Va (1 nM) with a half-maximal effect ($K_{\frac{1}{2}Xa}$) at 1.9 nM factor Xa. Expression of cofactor activity of factor Va in prothrombin activation required much lower factor Xa concentrations ($K_{\frac{1}{2}Xa}$ = 0.08 nM). When the ability of factor Xa to protect factor Va from inactivation by APC was determined at low factor Va concentrations during prothrombin activation much lower amounts of factor Xa were required ($K_{\frac{1}{2}Xa}$ = 0.03 nM). This indicates 1) that factor Va is optimally protected from inactivation by APC by incorporation into the prothrombinase complex during ongoing prothrombin activation, and 2) that the formation of a catalytically active prothrombinase complex and protection of factor Va from inactivation by APC likely involves the same interaction of factor Xa with factor Va. In accordance with the proposed mechanisms of action of protein S and factor Xa, we observed that the large differences between the rates of APC-catalyzed inactivation of normal factor Va and factor Va^{R506Q} were almost annihilated in the presence of factor Xa and protein S. This observation may explain why, in the absence of other risk factors APC resistance, only results in a weak prothrombotic condition.

2.2 Introduction

Activated protein C (APC) plays an important role in the down-regulation of thrombin formation [13]. The anticoagulant action of APC is associated with its ability to degrade the activated cofactors of the coagulation cascade, factors Va [23, 45] and VIIIa [42, 15], by limited proteolysis. The mechanism of proteolytic inactivation of factor Va by APC is rather well documented. Human factor Va loses its cofactor activity in prothrombin activation after peptide bond cleavages in the heavy chain [22]. Sequential cleavage at Arg⁵⁰⁶ and Arg³⁰⁶ of the heavy chain appears to be the major pathway of APC-catalyzed factor Va inactivation [22, 21, 28].

Optimal expression of anticoagulant activity of APC requires the presence of negatively charged phospholipids [45, 39, 1] and the protein cofactor, protein S [1, 44, 38]. The mechanism of action of protein S in factor Va inactivation is as yet not fully understood. In reaction systems with purified coagulation, factors protein S is a weak stimulator of APC-catalyzed factor Va inactivation [1, 38]. It has been suggested [38] that under physiological conditions protein S may act by annihilating the ability of factor Xa to protect factor Va from inactivation by APC [45, 39, 8, 27].

2.3 Experimental Procedures

The importance of protein C and protein S in the regulation of hemostatic plug formation is demonstrated by the association between thrombosis and protein C or protein S deficiency [18, 34, 9]. Recently, it has been reported [10, 24, 17, 14] that a hereditary defect that results in a poor anticoagulant response to APC (APC resistance) also is a basis for familial thrombophilia. APC resistance is associated with a single point mutation in the factor V gene that results in the replacement of Arg⁵⁰⁶ by Gln [3, 16, 43, 46]. This replacement affects the peptide bond in factor Va that has to be cleaved by APC in order to obtain a rapid loss of factor Va cofactor activity [22, 21, 28].

In the present study we have determined the effects of protein S and factor Xa on apparent second order rate constants for APC-catalyzed inactivation of normal factor Va and of factor Va that was purified from the plasma of a patient who is homozygous for the mutation Arg⁵⁰⁶ → Gln. The loss of functional activity of factor Va was correlated with the effects of protein S and factor Xa on the cleavage of APC-susceptible peptide bonds in the heavy chain of factor Va by immunoblot analysis.

2.3 Experimental Procedures

Materials Hepes, Tris, bovine serum albumin, soybean trypsin inhibitor (type IS) and Russell's viper venom were purchased from Sigma. DOPC and DOPS were obtained from Avanti Polar Lipids, Pelham, AL. The micro-BCA-protein assay reagent kit was obtained from Pierce. The chromogenic substrates S2238 and S2366 were supplied by Chromogenix, Mölndal, Sweden. 1,5-DNS-GGACK was obtained from Calbiochem and *p*-NPGB was from Nutritional Biochemicals. FPLC equipment and column materials used for protein purification were purchased from Pharmacia, Uppsala, Sweden. Immobilon-P membranes were obtained from Millipore, Bedford, MA.

Proteins Human coagulation factors were isolated from fresh frozen plasma. Human prothrombin and factor X were purified according to DiScipio *et al.* [12]. Human thrombin was isolated from prothrombin activation mixtures as described by Pletcher and Nelsestuen [29]. Human factor Xa was obtained from purified factor X after activation with RVV-X and isolation from the activation mixture by affinity chromatography on soybean trypsin inhibitor-Sepharose [5]. 1,5-DNS-GGACK-factor Xa was obtained by incubating 4 μ M factor Xa with 10 μ M 1,5-DNS-GGACK till inhibition was complete. Excess 1,5-DNS-GGACK was removed on a PD-10 column (Pharmacia). RVV-X was purified from Russell's viper venom according to Schiffman *et al.* [33]. Human protein C was prepared and activated as described by Gruber *et al.* [19]. Protein S was obtained from Enzyme Research Laboratories Inc. (Swansea, United Kingdom). Human factor V, factor V^{S506Q}, factor Va, and factor Va^{R506Q} were purified as described previously [28].

Protein concentrations Protein concentrations were determined with the micro-BCA protein assay [36]. Molar thrombin and factor Xa concentrations were determined by active site titration with *p*-NPGB [7, 37]. The concentration 1,5-DNS-GGACK-factor Xa was determined with the micro-BCA protein assay calibrated with active site-titrated factor Xa. Prothrombin concentrations were determined after complete activation of prothrombin with *Echis carinatus* venom and quantification of thrombin with *p*-NPGB. The APC concentration was determined with S2366 using kinetic parameters reported by Sala *et al.* [32]. Protein S concentrations were calculated from the A_{280} using a $A_{280}^{1\%}$ of 9.5 and $M_r = 70,000$ for protein S [11]. Factor Va was quantified as described below.

Phospholipid vesicle preparations Small unilamellar phospholipid vesicles were prepared as described before [30]. Phospholipid concentrations were determined by phosphate analysis [6].

Gel electrophoretic and immunoblot analysis The purity of protein preparations was analyzed by SDS-PAGE according to Laemmli [25] and staining with Coomassie Brilliant Blue R-250. The inactivation patterns of factor Va were analyzed after SDS-PAGE on 8% acrylamide slabgels, electrophoretic transfer of proteins from the gel to Immobilon-P membranes and visualization of heavy chain fragments with a rabbit polyclonal antibody directed against the heavy chain of factor Va and goat anti-rabbit IgG conjugated with alkaline phosphatase as described by Blake *et al.* [4].

Factor Va assay Cofactor activity of factor Va was quantified by determining the rate of factor Xa-catalyzed prothrombin activation in reaction mixtures that contained a limiting amount of factor Va, 5 nM factor Xa, 50 μ M phospholipid vesicles (DOPS/DOPC, 10/90, mol/mol) and 0.5 μ M prothrombin [41]. Rates of prothrombin activation were determined with S2238 [31]. The molar factor Va concentration in the assay mixture was calculated from the rate of prothrombin activation using a turnover number of 6000 mole of prothrombin activated per minute per mole of factor Xa-Va complex [40].

Kinetic data analysis of pseudo-first order time courses of factor Va inactivation Time courses of factor Va or factor Va^{R506Q} inactivation by APC were determined by monitoring the loss of cofactor activity of factor Va as function of time. Data reported in literature [22, 21, 28] demonstrate that the loss of cofactor activity during the inactivation of membrane bound factor Va may proceed via two pathways:

2.3 Experimental Procedures

Figure 2.1: **APC cleavage sites in the heavy chain of factor Va.** The peptide bonds located at Arg³⁰⁶, Arg⁵⁰⁶ and Arg⁶⁷⁹ in the heavy chain of human factor Va are susceptible to proteolytic cleavage by APC. The molecular weights of possible cleavage products correspond to those given in other publications [21, 28].

in which factor Va_{int} is a reaction intermediate with partial cofactor activity, factor Va_i is a form of factor Va that has completely lost its cofactor activity, k_{506} is the second order rate constant for cleavage at Arg⁵⁰⁶, k_{306} is the second order rate constant for cleavage at Arg³⁰⁶ in factor Va_{int} , and k'_{306} is the second order rate constant for cleavage at Arg³⁰⁶ in native factor Va.

Under first order conditions, *i.e.* conditions at which the inactivation rate is directly proportional to the concentrations factor Va and APC, the loss of cofactor activity is described by equation 2.1 [47]:

$$Va_t = Va_0 \cdot e^{-(k_{506} + k'_{306}) \cdot t} + B \cdot Va_0 \cdot \frac{k_{506} \cdot e^{-k_{306} \cdot t}}{k_{506} + k'_{306} - k_{306}} \cdot (1 - e^{-(k_{506} + k'_{306} - k_{306}) \cdot t}) \quad (2.1)$$

in which Va_t is the cofactor activity determined at time t , Va_0 is the cofactor activity determined before APC is added, B is the cofactor activity of factor Va_{int} (expressed as fraction of the cofactor activity of native factor Va) and k_{506} , k_{306} and k'_{306} are the rate constants defined above. The rate constants and the cofactor activity of factor Va_{int} were obtained by fitting the data to equation 2.1 using non-linear least squares regression analysis. Time courses of inactivation of normal factor Va were fitted with a fixed k'_{306} , that was obtained from kinetic analysis of factor Va^{R506Q} inactivation (*cf.* ref. [28]).

In some experiments (factor Va inactivation in the presence of high factor Xa concentrations) there was a slow loss of factor Va cofactor activity in the absence of APC. In these cases the rate constant for the loss of cofactor activity in the absence of APC was determined in a separate experiment and added to k'_{306} .

Figure 2.2: Effect of protein S on APC-catalyzed inactivation of membrane-bound factor Va. Purified human factor Va (1 nM) was incubated with 0.2 nM APC and 25 μ M phospholipid vesicles (DOPS/DOPC, 10/90, mol/mol) in 25 mM Hepes (pH 7.5), 175 mM NaCl, 3 mM CaCl_2 and 5 mg/ml BSA at 37 °C in the absence (▲) or presence of 490 nM protein S (■). At the indicated time points factor Va activity was determined as described under "Experimental Procedures". The rate constants and the activity of the reaction intermediate were obtained from the best fit of the data (solid lines) to Equation 2.1 given under "Experimental Procedures" using nonlinear least-squares regression analysis.

2.4 Results

Peptide bonds in the heavy chain of human factor Va that can be cleaved by APC Recently it was reported [22] that APC is able to cleave 3 peptide bonds in the heavy chain of human factor Va that are located at Arg³⁰⁶, Arg⁵⁰⁶ and Arg⁶⁷⁹, respectively.

Figure 2.1 is a schematic representation of the heavy chain of factor Va in which we have indicated the peptide bonds that can be cleaved by APC and the molecular weights of possible cleavage products. In this paper we will refer to the rate constants associated with the indicated peptide bond cleavages as k_{306} , k_{506} and k_{679} , respectively.

Effect of protein S on APC-catalyzed inactivation of membrane-bound factor Va Inactivation of membrane-bound factor Va by APC occurred via a biphasic reaction that consisted of a rapid phase which resulted in the formation of a reaction intermediate with partial cofactor activity (40%) that was fully inactivated during the subsequent slow phase (Fig 2.2, cf. ref. [28]). It has been shown earlier [22, 21, 28] that the rapid and slow phases of inactivation are associated with cleavages in the heavy chain of factor Va at Arg⁵⁰⁶ and Arg³⁰⁶, respectively. The rate constants obtained by fitting the time course shown in Fig 2.2 to a biphasic exponential were $k_{506} = 4.2 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$, $k_{306} = 2.2 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ and a reaction intermediate with 40% cofactor activity. When the same experiment was performed in the presence of 490 nM protein S the initial phase of factor Va inactivation was hardly affected, whereas the second phase was considerably accelerated and full loss of cofactor activity was achieved after a much shorter time interval than without protein S (Fig 2.2). This indicates that protein S preferentially accelerates the slow phase of factor Va inactivation, i.e. promotes APC-catalyzed cleavage at Arg³⁰⁶. The time course of factor Va inactivation in the presence of protein S could be perfectly fitted with $k_{506} = 4.9 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$, a reaction intermediate with 40% cofactor activity (taken from the experiment without protein S) and $k_{306} = 4.1 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$. This indicates that protein S increases the rate of APC-catalyzed cleavage at Arg³⁰⁶ at least 18-fold.

2.4 Results

Figure 2.3: **Immunoblot analysis of APC-catalyzed inactivation of membrane-bound factor Va with and without protein S.** Purified human factor Va (7 nM) was incubated with 1.1 nM APC and 25 μ M phospholipid vesicles (DOPS/DOPC, 10/90, mol/mol) in 25 mM Hepes (pH 7.5), 175 mM NaCl, 3 mM CaCl_2 and 0.2 mg/ml BSA at 37°C in the absence (*panel A*) or presence of 1 μ M protein S (*panel B*). At the indicated time intervals samples from the inactivation mixture were subjected to SDS-PAGE. After transfer to Immobilon-P membranes the heavy chain and derived fragments were visualized with a polyclonal antibody directed against the heavy chain of factor Va. The positions of the molecular weight markers are indicated at left of *panels A* and *B*. The percentages cofactor activity at the indicated time points were (A) 100, 73, 56, 45, 30, 25, 23, and 0% and (B) 100, 32, 12, 6, 3, 2, 1, and 0%. Further experimental details are described under "Experimental Procedures".

Figure 2.4: **Effect of factor Xa and 1,5-DNS-GGACK-factor Xa on APC-catalyzed inactivation of membrane-bound factor Va.** Purified human factor Va (1 nM) was incubated with 0.2 nM APC and 25 μ M phospholipid vesicles (DOPS/DOPC, 10/90, mol/mol) in 25 mM Hepes (pH 7.5), 175 mM NaCl, 3 mM CaCl_2 and 5 mg/ml BSA at 37 $^\circ\text{C}$ in the absence (▲) or presence of 20 nM factor Xa (●) or 20 nM 1,5-DNS-GGACK-factor Xa (■). At the indicated time points factor Va activity was determined as described under "Experimental Procedures". The rate constants and the activity of the reaction intermediate were obtained from the best fit of the data (solid lines) to equation 2.1 given under "Experimental Procedures" using nonlinear least-squares regression analysis.

The proposed mode of action of protein S was confirmed by immunoblot analysis of heavy chain degradation during factor Va inactivation (Fig 2.3). When factor Va was inactivated by APC in the absence of protein S there was considerable accumulation of a transient reaction product with $M_r = 75,000$ and formation of a $M_r = 26,000/28,000$ doublet, which are generated as the result of rapid cleavage at Arg^{506} (Fig 2.3A). The reaction intermediate with $M_r = 75,000$ was slowly converted into products with $M_r = 45,000$ and $M_r = 30,000$ which is indicative for cleavage at Arg^{306} . The reaction product with $M_r = 30,000$ was hardly visible on the gel since it stains poorly with our polyclonal antibody against the heavy chain.

Immunoblot analysis of a time course of factor Va inactivation in the presence of protein S showed much less accumulation of the $M_r = 75,000$ intermediate (Fig 2.3B). The fact that formation of the fragments with $M_r = 26,000/28,000$ occurred at the same rate as without protein S and that the fragments with $M_r = 60,000/62,000$ and $M_r = 45,000$ were already visible at early time points (<0.5 min) supports our conclusion that protein S does not affect cleavage at Arg^{506} but promotes cleavage at Arg^{306} . This results in 1) an increased rate of factor Va inactivation by direct cleavage at Arg^{306} and 2) accelerated processing of the partially active reaction intermediate that is formed when the peptide bond at Arg^{506} is cleaved first.

Effect of Factor Xa on APC-catalyzed inactivation of membrane-bound factor Va Many laboratories have reported that factor Xa protects factor Va from inactivation by APC [45, 39, 8, 27]. We also observed that factor Xa strongly inhibited APC-catalyzed inactivation of membrane-bound factor Va (Fig 2.4). The rate constant calculated from the initial phase of the inactivation time course in the presence of factor Xa was $2.3 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$. Since this rate constant is almost equal to the k_{306} determined in the absence of factor Xa, we propose that factor Xa blocks APC-catalyzed cleavage at Arg^{506} without affecting the cleavage at Arg^{306} . The presence of the active site of factor Xa was not needed for its protective effect since a factor Xa derivative,

2.4 Results

Figure 2.5: Immunoblot analysis of APC-catalyzed inactivation of membrane-bound factor Va in the absence or presence of 1,5-DNS-GGACK-factor Xa. Purified human factor Va (7 nM) was incubated with 1.4 nM APC and 25 μ M phospholipid vesicles (DOPS/DOPC, 10/90, mol/mol) in 25 mM Hepes (pH 7.5), 175 mM NaCl, 3 mM CaCl_2 and 0.2 mg/ml BSA at 37 $^\circ\text{C}$ in the absence (A) or presence of 20 nM 1,5-DNS-GGACK-factor Xa (B). At the indicated time intervals samples from the inactivation mixture were subjected to SDS-PAGE. After transfer to Immobilon-P membranes the heavy chain and derived fragments were visualized with a polyclonal antibody directed against the heavy chain of factor Va. The positions of the molecular weight markers are indicated at left of panels A and B. The percentages of cofactor activity at the indicated time points were (A) 100, 70, 51, 40, 25, 21, 18, and 0% and (B) 100, 92, 84, 70, 59, 49, 41 and 0%. Further details are described under "Experimental Procedures".

in which the active site had been blocked with the chloromethylketone 1,5-DNS-GGACK, was equally effective in inhibiting APC-dependent inactivation (Fig 2.4, solid squares).

The selective inhibition of the cleavage at Arg^{506} was confirmed by immunoblot analysis of APC-catalyzed inactivation of membrane-bound factor Va in the absence (Fig 2.5A) and presence (Fig 2.5B) of factor Xa. Comparison of the immunoblots shows that factor Xa prevented formation of the $M_r = 75,000$ heavy chain degradation product, which is indicative for inhibition of the cleavage at Arg^{506} and which shows that APC converts factor Va directly, but slowly, into products with $M_r = 62,000/60,000$ and 45,000 by cleavage at Arg^{306} .

Effects of protein S and factor Xa on APC-catalyzed inactivation of membrane-bound factor Va^{R506Q} In factor Va^{R506Q} , isolated from the plasma of homozygous APC-resistant patients, Arg^{506} is replaced by Gln. Compared with normal factor Va, the inactivation of factor Va^{R506Q} by APC is slow and monophasic (Fig 2.6) since cleavage at position 506 cannot occur due to the mutation. The time course of factor Va^{R506Q} inactivation can be fitted with a single exponential equation derived for an inactivation reaction in which factor Va is converted via a single peptide bond cleavage ($k_{306} = 1.6 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$) into a product with negligible cofactor activity in prothrombin activation.

Figure 2.6: **Effect of protein S and factor Xa on APC-catalyzed inactivation of membrane-bound factor Va^{R506Q}.** Purified human factor Va^{R506Q} (1 nM) was incubated with 1 nM APC and 25 μ M phospholipid vesicles (DOPS/DOPC, 10/90, mol/mol) in 25 mM Hepes (pH 7.5), 175 mM NaCl, 3 mM CaCl₂ and 5 mg/ml BSA at 37 °C in the absence (Δ) or presence of 490 nM protein S (■) or 20 nM factor Xa (●). At the indicated time points factor Va activity was determined as described under "Experimental Procedures". The rate constants of inactivation were obtained from the best fit of the data (solid lines) to equation 2.1 given under "Experimental Procedures" using nonlinear least-squares regression analysis.

If, as argued above, protein S accelerates inactivation by promoting cleavage at Arg³⁰⁶ and factor Xa blocks inactivation of normal factor Va by protection of cleavage at Arg⁵⁰⁶, it is to be expected that protein S will considerably enhance the inactivation of this abnormal factor Va variant and that factor Xa will have no effect on APC-catalyzed inactivation of factor Va^{R506Q}. The data presented in Fig 2.6 indeed show that protein S accelerated the rate of inactivation 14-fold ($k_{306} = 2.2 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$) and that the inactivation of factor Va^{R506Q} was not affected by factor Xa.

In Table 2.1 we have summarized the rate constants for APC-catalyzed cleavage at Arg⁵⁰⁶ and Arg³⁰⁶ of the heavy chain of membrane-bound factor Va and factor Va^{R506Q} that were determined in the absence and presence of protein S or factor Xa.

Concentration dependence of the effects of protein S and factor Xa on APC-catalyzed inactivation of membrane-bound factor Va We have also examined the enhancement of APC-catalyzed factor Va inactivation as a function of the protein S concentration (Fig 2.7A). Time courses of factor Va inactivation by APC were determined at varying concentrations of protein S (cf. Fig 2.2) and fitted with equation 2.1, given under "Experimental Procedures". It appeared that saturating concentrations of protein S were able to increase k_{306} 20-fold and that half-maximal stimulation of APC-catalyzed cleavage at Arg³⁰⁶ required ~ 200 nM protein S. At all protein S concentrations tested the time courses of factor Va inactivation could be perfectly fitted with k_{506} values between $3 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ and $5 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$.

We have also determined the ability of factor Xa to protect factor Va from APC inactivation at varying factor Xa concentrations (Fig 2.7B). Factor Xa could completely block cleavage at Arg⁵⁰⁶ without having an effect on the slow phase of factor Va inactivation (k_{306}). Half-maximal protection of cleavage at Arg⁵⁰⁶ was observed at 1.9 nM factor Xa.

2.4 Results

	k_{506}	k_{306}
	$M^{-1}s^{-1}$	$M^{-1}s^{-1}$
Normal factor Va	4.2×10^7	2.2×10^6
+490 nM protein S	4.9×10^7	4.1×10^7
+20 nM factor Xa	$< 1 \times 10^6$	2.3×10^6
Factor Va ^{R506Q}		1.6×10^6
+490 nM protein S		2.2×10^7
+20 nM factor Xa		1.7×10^6

Table 2.1: Effects of protein S and factor Xa on rate constants for APC-catalyzed peptide bond cleavages in membrane-bound factor Va and factor Va^{R506Q}. Rate constants for APC-catalyzed inactivation of factor Va and factor Va^{R506Q} were obtained by fitting the time courses of factor Va or Va^{R506Q} inactivation presented in Figs 2.2, 2.4 and 2.6 to equation 2.1 given under "Experimental Procedures" using nonlinear least-squares regression analysis.

Figure 2.7: Effects of varying protein S and factor Xa concentrations on rate constants of APC-catalyzed cleavage at Arg³⁰⁶ and at Arg⁵⁰⁶ in membrane-bound factor Va. The rate constants k_{506} and k_{306} were obtained from time courses of inactivation of 1 nM factor Va by 0.2 nM APC on 25 μ M phospholipid vesicles (DOPS/DOPC, 10/90, mol/mol) in 25 mM Hepes (pH 7.5), 175 mM NaCl, 3 mM CaCl₂ and 5 mg/ml BSA (37 °C) in the presence of varying concentrations protein S and/or factor Xa as described at Figs 2.2 and 2.4. (A) k_{306} as function of the concentration protein S. (B) k_{506} as function of the concentration factor Xa.

Figure 2.8: Combined effects of protein S and factor Xa on APC-catalyzed inactivation of membrane-bound factor Va. Purified human factor Va (1 nM) was incubated with 0.2 nM APC and 25 μ M phospholipid vesicles (DOPS/DOPC, 10/90, mol/mol) in 25 mM Hepes (pH 7.5), 175 mM NaCl, 3 mM CaCl_2 and 5 mg/ml BSA at 37°C in the presence of 20 nM factor Xa (●), 490 nM protein S (■) or 490 nM protein S plus 20 nM factor Xa (▲). At the indicated time points factor Va activity was determined as described in the "Experimental Procedures".

Figure 2.9: APC-catalyzed inactivation of membrane-bound factor Va and factor Va^{R506Q} in the presence of protein S and Factor Xa. 1 nM purified human factor Va or factor Va^{R506Q} was incubated with 0.2 nM APC and 25 μ M phospholipid vesicles (DOPS/DOPC, 10/90, mol/mol) in 25 mM Hepes (pH 7.5), 175 mM NaCl, 3 mM CaCl_2 and 5 mg/ml BSA at 37 °C in the absence or presence of 200 nM protein S plus 20 nM factor Xa. At the indicated time points factor Va activity was determined as described under "Experimental Procedures". ●, factor Va^{R506Q}; ▲, normal factor Va; ■, factor Va^{R506Q} plus protein S and factor Xa; ○, normal factor Va plus protein S and factor Xa.

It appeared that protein S and factor Xa can exert their effects on APC-catalyzed factor Va inactivation independent of each other. At a concentration of factor Xa that completely blocked cleavage at Arg⁵⁰⁶ (20 nM), protein S was still able to accelerate factor Va inactivation 12-fold (Fig 2.8). The fact that the time course of inactivation determined in the presence of factor Xa and protein S significantly differs from that obtained in the presence of protein S alone indicates that the factor Xa protection of cleavage at Arg⁵⁰⁶ was not annihilated by protein S.

Collectively these data support our conclusion that the stimulatory effect of protein S and the protective effect of factor Xa are associated with the different steps in the pathway of APC-catalyzed factor Va inactivation *i.e.* are confined to the peptide bond cleavages at Arg⁵⁰⁶ and Arg³⁰⁶, respectively.

Inactivation of Factor Va and Factor Va^{R506Q} by APC in the presence of both factor Xa and protein S On the basis of the proposed sites of action of protein S and factor Xa one would predict that the profound differences between the inactivation rates of factor Va and factor Va^{R506Q} by APC will disappear in reaction systems that contain both protein S and factor Xa. The exper-

2.4 Results

Figure 2.10: Effects of factor Xa on factor Va activity in prothrombin activation and on the protection of factor Va from inactivation by APC. The rate constant k_{506} was obtained from time courses of inactivation of 6 pM factor Va by 0.2 nM APC determined in the presence of 25 μM phospholipid vesicles (DOPS/DOPC, 10/90, mol/mol), 1 μM prothrombin and varying concentrations of factor Xa in 25 mM Hepes (pH 7.5), 175 mM NaCl, 3 mM CaCl_2 and 5 mg/ml BSA (37 $^\circ\text{C}$). Plotted is the value of k_{506} as function of the concentration of factor Xa (●). The factor Xa dependence of expression of factor Va cofactor activity (■) was determined in the same reaction mixtures by measuring the rate of thrombin formation before APC was added.

iment presented in Fig 2.9 shows that this indeed occurs. In the absence of protein S and factor Xa, the rates of inactivation of factor Va and factor Va^{R506Q} by APC differed about 15-fold. The rate difference was almost completely annihilated in inactivation mixtures that contained protein S and factor Xa. This phenomenon is explained by the fact that factor Xa prevents APC-catalyzed cleavage at the site (Arg⁵⁰⁶) that distinguishes normal factor Va from factor Va^{R506Q} , and that protein S stimulates cleavage Arg³⁰⁶ in both normal factor Va and factor Va^{R506Q} .

Protection of factor Va against APC inactivation by incorporation in the prothrombinase complex Protection of factor Va from cleavage by APC appears to require much higher factor Xa concentrations ($K_{\frac{1}{2}\text{Xa}} = 1.9 \text{ nM}$) than needed for the expression of cofactor activity of factor Va in prothrombin activation ($K_{\frac{1}{2}\text{Xa}} = 0.05\text{--}0.10 \text{ nM}$, cf. refs [28, 30]). It should be emphasized, however, that expression of factor Va cofactor activity by factor Xa is determined 1) in the presence of prothrombin, a protein that may promote factor Xa-Va complex formation (cf. ref. [26]) and 2) at factor Va concentrations (0.005–0.05 nM) that are several orders of magnitude lower than the factor Va concentrations in experiments in which inactivation by APC is studied.

Therefore, we have determined in the same experiment the amounts of factor Xa required to protect factor Va from APC cleavage and to express the cofactor activity of factor Va in prothrombin activation (Fig 2.10). Under these conditions (low concentration of factor Va and prothrombin present), similar amounts of factor Xa were required to protect factor Va from inactivation by APC and to express factor Va cofactor activity in prothrombin activation ($K_{\frac{1}{2}\text{Xa}} = 0.03 \text{ nM}$ for protection, $K_{\frac{1}{2}\text{Xa}} = 0.08 \text{ nM}$ for the expression of cofactor activity). These data indicate that factor Va is optimally protected from inactivation by APC when it is incorporated into the prothrombinase complex during ongoing catalysis and that complex formation between factor Xa and factor Va at the same time expresses the cofactor activity of factor Va and protects it from inactivation by APC.

2.5 Discussion

Recently it has been reported that APC-catalyzed inactivation of factor Va proceeds via a biphasic reaction [22, 21, 28]. The first step of the inactivation process is a rapid cleavage at Arg⁵⁰⁶ in the heavy chain domain of factor Va that results in the formation of a reaction intermediate that exhibits partial cofactor activity in prothrombin activation. This reaction intermediate is subsequently fully inactivated by slow cleavage at Arg³⁰⁶. Here we report that protein S stimulates the inactivation of membrane bound factor Va 20-fold by specific acceleration of the cleavage at Arg³⁰⁶ and that factor Xa protects factor Va from inactivation by APC by blocking the cleavage site at Arg⁵⁰⁶. However, factor Xa does not completely inhibit factor Va inactivation by APC. At saturating factor Xa concentrations, factor Va inactivation still proceeds with a slow rate that is associated with APC-catalyzed cleavage at Arg³⁰⁶. This means that the latter cleavage is apparently not affected by factor Xa.

These conclusions are supported by immunoblot analysis and by experiments with Va^{R506Q} obtained from a homozygous APC-resistant patient. Immunoblots of time courses of inactivation of membrane-bound factor Va by APC, in which the heavy chain and its degradation products were visualized with a polyclonal antibody directed against the heavy chain, showed transient generation of a reaction product with $M_r = 75,000$ which is formed after rapid cleavage of the peptide bond at Arg⁵⁰⁶ by APC (Fig 2.3A). With protein S present there is much less accumulation of the $M_r = 75,000$ fragment and increased formation of products with M_r of 60,000/62,000, and 45,000 (Fig 2.3B). This is indicative for a protein S-dependent increase of the rate of cleavage at Arg³⁰⁶ which either results in increased direct factor Va inactivation or in accelerated processing of the $M_r = 75,000$ intermediate.

In the presence of factor Xa there is also no formation of the $M_r = 75,000$ reaction intermediate (Fig 2.5B). In this situation there is a slow formation of products with $M_r = 62,000/60,000$ and 45,000 which shows that factor Xa blocks cleavage at Arg⁵⁰⁶ and that inactivation of factor Va proceeds via slow cleavage at Arg³⁰⁶.

Similar information is obtained from kinetic analysis of inactivation of factor Va^{R506Q}. This abnormal factor Va molecule lacks the cleavage site at Arg⁵⁰⁶ due to a substitution of Arg⁵⁰⁶ by Gln [3, 16, 43, 46]. In line with the proposed effects of protein S and factor Xa on APC-catalyzed peptide bond cleavages in the heavy chain of factor Va we observed that protein S drastically stimulates inactivation of factor Va^{R506Q} (Fig 2.6) which has been reported to be primarily associated with cleavage at Arg³⁰⁶ [21, 28]. We further show that factor Xa does not protect factor Va^{R506Q} from inactivation by APC. This observation is explained by the fact that factor Va^{R506Q} lacks Arg⁵⁰⁶ which is the target site for factor Xa.

It is the first time that a profound effect is reported for protein S in a reaction system with purified coagulation factors. In earlier publications [1, 38] modest effects were described for protein S. The reason for this apparent discrepancy is that in the earlier studies the effect of protein S was determined on initial rates of APC-catalyzed factor Va inactivation that is a phase of the reaction that is primarily associated with cleavage at Arg⁵⁰⁶ and which according to our data is hardly affected by protein S. Here we show that protein S actually acts at the slower

step of the inactivation process, that is the cleavage of the peptide bond at Arg³⁰⁶. The presence of protein S thus ensures rapid inactivation of factor Va by accelerating direct cleavage at Arg³⁰⁶ or by promoting the conversion of a partially active reaction intermediate (factor Va cleaved at Arg⁵⁰⁶) into a product with no detectable cofactor activity. Given the known clinical consequences of protein S deficiencies it is likely that the above described contribution of protein S to factor Va inactivation is of prime physiological importance.

Titration experiments at low APC and factor Va concentrations on 25 μM DOPS/DOPC phospholipid vesicles show that protein S stimulates APC-catalyzed inactivation of factor Va with a $K_{\frac{1}{2}}$ of 200 nM and that protection by factor Xa is characterized by a $K_{\frac{1}{2}\text{Xa}}$ of 1.9 nM. We have further shown that protein S and factor Xa can exert their effects on APC-catalyzed factor Va inactivation independent of each other. At a concentration of factor Xa that completely blocks cleavage at Arg⁵⁰⁶, protein S was still able to accelerate factor Va inactivation 12-fold. These data support our conclusion that the stimulatory effect of protein S and the protective effect of factor Xa on inactivation of factor Va by APC result from different interactions with factor Va and are confined to steps that involve the peptide bond cleavages at Arg³⁰⁶ and Arg⁵⁰⁶, respectively.

In terms of physiological relevance for regulation of factor Va activity, factor Xa actually has two (procoagulant) functions: 1) factor Xa protects factor Va from inactivation by APC and 2) factor Xa brings the cofactor activity of factor Va to expression by formation of the prothrombin-activating complex. It appeared that much higher concentrations factor Xa were required for protection of factor Va from inactivation by APC ($K_{\frac{1}{2}\text{Xa}} = 1.9$ nM) than for incorporation of factor Va into a catalytically active prothrombinase complex ($K_{\frac{1}{2}\text{Xa}} = 0.08$ nM). However, prothrombinase complex formation is usually determined at low factor Va concentrations in reaction mixtures that contain prothrombin as additional component. We observed that much lower amounts of factor Xa ($K_{\frac{1}{2}\text{Xa}} = 0.03$ nM) were required to protect factor Va from inactivation by APC when it was incorporated into a prothrombinase complex that was catalyzing prothrombin activation. These data demonstrate: 1) that factor Va is most effectively protected from inactivation by APC when incorporated into the prothrombinase complex during ongoing prothrombin activation and 2) that protection of factor Va from APC cleavage and prothrombinase complex formation likely involve the same interaction of factor Xa with factor Va.

Recently, it was recognized that APC resistance alone is only a mild risk factor for thrombosis [20, 35, 2]. Considering the large differences in inactivation rates of factor Va and factor Va^{R506Q} by APC this is a somewhat puzzling observation. In accordance with the proposed effects of protein S and factor Xa we observed that the large differences in the rates of APC-catalyzed inactivation of factor Va and Va^{R506Q} were almost annihilated when factor Va and factor Va^{R506Q} were inactivated by APC in the presence of factor Xa and protein S (Fig 2.9). Since it is likely that *in vivo* regulation of factor Va activity by APC occurs in the presence of protein S and factor Xa, our observations may explain why in the absence of other genetic or environmental risk factors APC resistance only results in a relatively weak prothrombotic condition.

2.6 References

- [1] H.M. Bakker, G. Tans, T. Janssen-Claessen, M.C. 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. *European Journal of Biochemistry*, 208(1):171–178, 1992.
- [2] K.A. Bauer and J.H. Griffin. *New England Journal of Medicine*, 332:1383, 1995.
- [3] 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*, 369(6475):64–67, 1994.
- [4] M.S. Blake, K.H. Johnston, G.J. Russell-Jones, and E.C. Gotschlich. A rapid, sensitive method for detection of alkaline phosphatase-conjugated anti-antibody on Western blots. *Anal-Biochem*, 136(1):175–179, 1984.
- [5] P.E. Bock, P.A. Craig, S.T. Olson, and P. Singh. Isolation of human blood coagulation alpha-factor Xa by soybean trypsin inhibitor-sepharose chromatography and its active-site titration with fluorescein mono-p-guanidinobenzoate. *Arch. Biochem. Biophys*, 273(2):375–388, 1989.
- [6] C.J.F. Böttcher, C.M. van Gent, and C. Pries. A rapid and sensitive sub-micro phosphorus determination. *Anal. Chim. Acta.*, 24:203–207, 1961.
- [7] T. Chase, Jr. and E. Shaw. Comparison of the esterase activities of trypsin, plasmin, and thrombin on guanidinobenzoate esters. titration of the enzymes. *Biochemistry*, 8(5):2212–2224, 1969.
- [8] P.C. Comp and C.T. Esmon. Activated protein C inhibits platelet prothrombin-converting activity. *Blood*, 54(6):1272–1281, 1979.
- [9] P.C. Comp, R.R. Nixon, M.R. Cooper, and C.T. Esmon. Familial protein S deficiency is associated with recurrent thrombosis. *Journal of Clinical Investigation*, 74(6):2082–2088, 1984.
- [10] B. Dahlbäck, 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. *Proceedings of the National Academy of Sciences USA*, 90(3):1008, 1993.
- [11] R.G. DiScipio and E.W. Davie. Characterization of protein S, a gamma-carboxyglutamic acid containing protein from bovine and human plasma. *Biochemistry*, 18(5):899–904, 1979.
- [12] R.G. DiScipio, M.A. Hermodson, S.G. Yates, and E.W. Davie. A comparison of human prothrombin, factor IX (Christmas factor), factor X (Stuart factor), and protein S. *Biochemistry*, 16(4):698–706, 1977.
- [13] C.T. Esmon. The roles of protein C and thrombomodulin in the regulation of blood coagulation. *Journal of Biological Chemistry*, 264(9):4743–4746, 1989.
- [14] E.M. Faioni, F. Franchi, D. Asti, E. Sacchi, F. Bernardi, and P.M. Mannucci. Resistance to activated protein C in nine thrombophilic families: interference in a protein S functional assay. *Thrombosis and Haemostasis*, 70(6):1067–1071, 1993.
- [15] C.A. Fulcher, J.E. Gardiner, J.H. Griffin, and T.S. Zimmerman. Proteolytic inactivation of human factor VIII procoagulant protein by activated human protein C and its analogy with factor V. *Blood*, 63(2):486–489, 1984.
- [16] J.S. Greengard, X. Sun, X. Xu, J.A. Fernandez, J.H. Griffin, and B. Evatt. Activated protein C resistance caused by Arg⁵⁰⁶ → Gln mutation in factor Va. *Lancet*, 343(8909):1361–1362, 1994.
- [17] J.H. Griffin, B. Evatt, C. Wideman, and J.A. Fernandez. Anticoagulant protein C pathway defective in majority of thrombophilic patients. *Blood*, 82(7):1989–1993, 1993.

2.6 References

- [18] J.H. Griffin, B. Evatt, T.S. Zimmerman, A.J. Kleiss, and C. Wideman. Deficiency of protein C in congenital thrombotic disease. *Journal of Clinical Investigation*, 68(5):1370–1373, 1981.
- [19] A. Gruber, J.H. Griffin, L.A. Harker, and S.R. Hanson. Inhibition of platelet-dependent thrombus formation by human activated protein C in a primate model. *Blood*, 73(3):639–642, 1989.
- [20] P. Hopmeier and W. Krugluger. Factor V Leiden and thrombophilia. *New England Journal of Medicine*, 332:1381–1382, 1995.
- [21] M. Kalafatis, R.M. Bertina, M.D. Rand, and K.G. Mann. Characterization of the molecular defect in factor V^{R506Q}. *Journal of Biological Chemistry*, 270(8):4054–4057, 1995.
- [22] 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. *Journal of Biological Chemistry*, 269(50):31869–31880, 1994.
- [23] W. Kiesel, W.M. Canfield, L.H. Ericsson, and E.W. Davie. Anticoagulant properties of bovine plasma protein C following activation by thrombin. *Biochemistry*, 16(26):5824–5831, 1977.
- [24] T. Koster, F.R. Rosendaal, H. de Ronde, E. Briët, J.P. Vandenbroucke, and R.M. Bertina. Venous thrombosis due to poor anticoagulant response to activated protein C: Leiden thrombophilia study. *Lancet*, 342(8886-8887):1503–1506, 1993.
- [25] U.K. Laemmli. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227(259):680–685, 1970.
- [26] Y. Nemerson and R. Gentry. An ordered addition, essential activation model of the tissue factor pathway of coagulation: evidence for a conformational cage. *Biochemistry*, 25(14):4020–4033, 1986.
- [27] M.E. Nesheim, W.M. Canfield, W. Kiesel, and K.G. Mann. Studies of the capacity of factor Xa to protect factor Va from inactivation by activated protein C. *Journal of Biological Chemistry*, 257(3):1443–1447, 1982.
- [28] G.A.F. Nicolaes, G. Tans, M.L.C.G.D. Thomassen, H.C. Hemker, K. Váradi, H.P. Schwarz, and J. Rosing. Peptide bond cleavages and loss of functional activity during inactivation of factor Va and factor Va^{R506Q} by activated protein C. *Journal of Biological Chemistry*, 270(36):21158–21166, 1995.
- [29] C.H. Pletcher and G.L. Nelsestuen. The rate-determining step of the heparin-catalyzed antithrombin/thrombin reaction is independent of thrombin. *Journal of Biological Chemistry*, 257(10):5342–5345, 1982.
- [30] 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. *Journal of Biological Chemistry*, 268(28):21130–21136, 1993.
- [31] J. Rosing, G. Tans, J.W. Govers-Riemslog, R.F. Zwaal, and H.C. Hemker. The role of phospholipids and factor Va in the prothrombinase complex. *Journal of Biological Chemistry*, 255(1):274–283, 1980.
- [32] N. Sala, W.G. Owen, and D. Collen. A functional assay of protein C in human plasma. *Blood*, 63(3):671–675, 1984.
- [33] S. Schiffman, I. Theodor, and S.I. Rapaport. Separation from Russell's viper venom of one fraction reacting with factor X and another reacting with factor V. *Biochemistry*, 8(4):1397–1405, 1969.
- [34] H.P. Schwarz, M. Fischer, P. Hopmeier, M.A. Batard, and J.H. Griffin. Plasma protein S deficiency in familial thrombotic disease. *Blood*, 64(6):1297–1300, 1984.
- [35] P. Simioni, A. Scudeller, and A. Girolami. Factor V Leiden and thrombophilia. *New England Journal of Medicine*, 332(20):1382, 1995.

- [36] P.K. Smith, R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson, and D.C. Klenk. Measurement of protein using bicinchoninic acid. *Anal-Biochem*, 150(1):76–85, 1985.
- [37] R.L. Smith. Titration of activated bovine factor X. *Journal of Biological Chemistry*, 248(7):2418–2423, 1973.
- [38] 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. *Journal of Biological Chemistry*, 263(29):14884–14890, 1988.
- [39] K Suzuki, J. Stenflo, B. Dahlbäck, and B. Teodorsson. Inactivation of human coagulation factor V by activated protein C. *Journal of Biological Chemistry*, 258(3):1914–1920, 1983.
- [40] G. Tans, T. Janssen-Claessen, H.C. Hemker, R.F. Zwaal, and J. Rosing. Meizothrombin formation during factor Xa-catalyzed prothrombin activation. Formation in a purified system and in plasma. *Journal of Biological Chemistry*, 266(32):21864–21873, 1991.
- [41] G. Tans, J. Rosing, M.C. Thomassen, M.J. Heeb, R.F. Zwaal, and J.H. Griffin. Comparison of anticoagulant and procoagulant activities of stimulated platelets and platelet-derived microparticles. *Blood*, 77(12):2641–2648, 1991.
- [42] G.A. Vehar and E.W. Davie. Preparation and properties of bovine factor VIII (antihemophilic factor). *Biochemistry*, 19(3):401–410, 1980.
- [43] J. Voorberg, J. Roelse, R. Koopman, H. Büller, 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*, 343(8912):1535–1536, 1994.
- [44] F.J. Walker. Regulation of activated protein C by a new protein. A possible function for bovine protein S. *Journal of Biological Chemistry*, 255(12):5521–5524, 1980.
- [45] F.J. Walker, P.W. Sexton, and C.T. Esmon. The inhibition of blood coagulation by activated protein C through the selective inactivation of activated factor V. *Biochim-Biophys-Acta*, 571(2):333–342, 1979.
- [46] B. Zöller and B. Dahlbäck. Linkage between inherited resistance to activated protein C and factor V gene mutation in venous thrombosis. *Lancet*, 343(8912):1536–1538, 1994.
- [47] Fersht, A. (ed). *Enzyme Structure and Mechanism* pp 128–138, W.H. Freeman & Co., New York (1985).

Chapter 3

Human Factor Va₁ and Factor Va₂: Properties in the Procoagulant- and Anticoagulant Pathways

Based on: Hoekema, L., Nicolaes, G.A.F, Hemker, H.C., Tans, G. & Rosing, J. Human Factor Va₁ and Factor Va₂: Properties in the Procoagulant- and Anticoagulant Pathways *Biochemistry*, 36:3331-3335, 1997

3.1 Summary

Human plasma factor V is heterogeneous and yields two forms of activated factor V that bind with low (factor Va₁) and high affinity (factor Va₂) to phospholipids. The properties of factor Va₁ and factor Va₂ in the anticoagulant and procoagulant pathways were evaluated by comparing their sensitivity for inactivation by APC and their ability to act as cofactor in prothrombin activation. At low phospholipid concentrations and on membranes containing low amounts of phosphatidylserine (PS), factor Va₁ was inactivated by APC at 15-fold lower rates than factor Va₂, both in the absence and in the presence of protein S. At high phospholipid concentrations and on membranes with more than 15 mole% PS, factor Va₁ and factor Va₂ were inactivated with equal efficiency. Differences between cofactor activities of factor Va₁ and factor Va₂ in prothrombin activation were only observed on membranes with less than 7.5 mole% PS. Due to the different phospholipid requirements of APC-catalyzed factor Va inactivation and of expression of factor Va cofactor activity in prothrombin activation, the thrombin-forming capacity of factor V₁ was 7-fold higher than that of factor V₂ in a reaction system containing factor Xa, prothrombin, APC, protein S, vesicles with a phospholipid composition resembling that of activated platelets, and traces of thrombin to initiate prothrombin activation. This shows that in the process of generation, expression, and down-regulation of factor Va cofactor activity on physiological membranes, the overall procoagulant activity of factor V₁ can considerably exceed that of factor V₂.

3.2 Introduction

Inactivation of factor Va by activated protein C (APC) plays an important role in the down-regulation of thrombin formation [11]. Factor Va loses its cofactor activity in prothrombin activation after APC-catalyzed cleavage in its heavy chain domain at positions Arg⁵⁰⁶ and Arg³⁰⁶ [15, 18]. Factor Va inactivation by APC is stimulated by negatively charged phospholipids [32, 1] and protein S [1, 31, 28]. Recently, we reported that protein S promotes factor Va inactivation by specifically accelerating the slow peptide bond cleavage at Arg³⁰⁶ [23]. The physiological importance of APC and protein S is demonstrated by the association of venous thrombosis with hereditary deficiencies in protein C [12] or protein S [26, 7] and by the occurrence of familial thrombophilia in individuals that have a hereditary defect (replacement of Arg⁵⁰⁶ by Gln) that makes factor Va insensitive to proteolytic inactivation by APC (APC resistance, [8, 2]).

In 1993 we reported [22] that plasma contains two forms of factor V. Activation by thrombin yields two forms of factor Va (factor Va₁ and factor Va₂) which differ in their affinity for procoagulant membrane surfaces and which exhibit different cofactor activities in prothrombin activation. Considering the physiological importance of down-regulation of factor Va activity via the anticoagulant pathway, we have compared the procoagulant and anticoagulant properties of factors Va₁ and Va₂ in order to get insight in their contributions to *in vivo* thrombin formation. We report that under several conditions factors Va₁ and Va₂ have similar procoag-

3.3 Experimental Procedures

ulant activity whereas they considerably differ in their sensitivity for APC. In these situations, the overall procoagulant activity of factor V_1 is higher than that of factor V_2 .

3.3 Experimental Procedures

Materials Phospholipids were obtained from Avanti Polar Lipids, Pelham, AL. Small unilamellar phospholipid vesicles were prepared as described before [22]. S2238, S2366 and I2581 were supplied by Chromogenix, Mölndal, Sweden. Materials used for protein purification were purchased from Pharmacia. Human prothrombin, thrombin and factor Xa were prepared by earlier described procedures [10, 21, 5]. Human protein C was prepared and activated as reported by Gruber *et al.* [13]. Human protein S was obtained from Enzyme Research Laboratories Inc. (Swansea, UK). Human factor V_1 , factor V_2 , factor V_{a1} and factor V_{a2} were purified as described previously [22].

Protein concentrations Molar prothrombin, thrombin and factor Xa concentrations were determined as described before [6, 27]. APC concentrations were determined with S2366 using kinetic parameters reported by Sala *et al.* [25]. Protein S concentrations were calculated from the A_{280} using an $A_{280}^{1\%}$ of 9.5 and $M_r = 70,000$ for protein S [9]. Factor Va was quantified as described below.

Factor Va assay Cofactor activity of factor Va was quantified by determining the rate of factor Xa-catalyzed prothrombin activation in reaction mixtures that contained a limiting amount of factor Va, 5 nM factor Xa, 50 μ M phospholipid vesicles DOPS/DOPC, 10/90, mol/mol) and 0.5 μ M prothrombin [30]. Rates of prothrombin activation were determined with S2238 [24]. The molar factor Va concentration in the assay mixture was calculated from the rate of prothrombin activation using a turnover number of 6000 moles prothrombin activated per minute per mole of factor Xa-Va complex [29].

Inactivation of factor Va by APC Factor V_{a1} or factor V_{a2} was incubated at 37 °C with phospholipid vesicles in the absence or presence of protein S in a reaction mixture containing 25 mM Hepes (pH 7.5), 175 mM NaCl, 3 mM $CaCl_2$ and 5 mg/ml BSA and its concentration was determined in triplicate to obtain $[factor\ Va]_{t=0}$. Factor Va inactivation was started by addition of APC, and the time-dependent loss of factor Va cofactor activity was followed as described in the previous paragraph.

Curve fitting of pseudo-first order time courses of factor Va inactivation Time courses of factor V_{a1} or factor V_{a2} inactivation by APC were determined by monitoring the loss of cofactor activity of factor Va as a function of time. Data reported in literature [15, 14, 18, 23] demonstrate that the loss of factor Va cofactor activity can proceed via two pathways:

in which factor Va_{int} is a reaction intermediate with partial cofactor activity, factor Va_i is a form of factor Va that has completely lost its cofactor activity, k_{506} is the second-order rate constant for cleavage at Arg⁵⁰⁶, k_{306} is the second-order rate constant for cleavage at Arg³⁰⁶ in factor Va_{int} and k'_{306} is the second-order rate constant for cleavage at Arg³⁰⁶ in native factor Va. The rate constants k_{506} , k_{306} and k'_{306} and the cofactor activity of factor Va_{int} were obtained by fitting time courses of factor Va inactivation to equation 3.1 using nonlinear least-squares regression analysis [18, 23].

$$Va_t = Va_0 \cdot e^{-(k_{506} + k'_{306}) \cdot t} + B \cdot Va_0 \cdot \frac{k_{506} \cdot e^{-k_{306} \cdot t}}{k_{506} + k'_{306} - k_{306}} \cdot (1 - e^{-(k_{506} + k'_{306} - k_{306}) \cdot t}) \quad (3.1)$$

In this equation, k_{306} , k'_{306} and k_{506} are the rate constants for peptide bond cleavages defined above and B represents the residual cofactor activity (percent of native factor Va) of the reaction intermediate factor Va_{int}.

Cofactor activity of factor Va in prothrombin activation Factor Va₁ or factor Va₂ was incubated with 2.5 pM factor Xa and phospholipid vesicles at 37 °C in 25 mM Hepes (pH 7.5), 175 mM NaCl, 3 mM CaCl₂ and 5 mg/ml BSA. After 5 min, prothrombin was added, and 2 and 4 min later, the amount of thrombin formed was quantified as described before [24].

3.4 Results

Effect of phospholipid on inactivation of factor Va₁ and factor Va₂ by APC in the absence and presence of protein S Recently, we have shown [18] that inactivation of factor Va by APC proceeds via a biphasic reaction (*cf.* figure 3.1) in which fast cleavage at Arg⁵⁰⁶ in the heavy chain of factor Va results in the formation of an intermediate with partial cofactor activity in prothrombin activation that is fully inactivated by APC after slow cleavage at Arg³⁰⁶. We subsequently reported that protein S promotes factor Va inactivation by specifically accelerating cleavage at Arg³⁰⁶ [23]. It was also reported [22] that plasma factor V is heterogeneous and yields two forms of factor Va (Va₁ and Va₂) after activation that bind with different affinities to negatively charged membranes. In figure 3.1, it is shown that on membranes with 5 mole% phosphatidylserine, factor Va₁ was inactivated by APC at considerably lower rates than factor Va₂, both in the absence (figure 3.1A) and presence of protein S (figure 3.1B). The rate constants for APC-catalyzed cleavage at Arg⁵⁰⁶ and Arg³⁰⁶ (k_{506} and k_{306}) were about 15 times

3.4 Results

Figure 3.1: Inactivation of factor Va₁ and factor Va₂ by APC. 1.5 nM purified human factor Va₁ (■, □) or factor Va₂ (●, ○) was incubated at 37 °C with 1 nM APC (A, B) or 0.12 nM APC (C) without (■, □) or with (●, ○) 200 nM protein S in 25 mM Hepes (pH 7.5), 175 mM NaCl, 3 mM CaCl₂, 5 mg/ml BSA, and (A,B) 5 μM DOPS/DOPC (5/95, mol/mol) or (C) 50 μM DOPS/DOPC (20/80, mol/mol). At the indicated time points, factor Va activity was determined as described under Experimental Procedures. The solid lines represent a best fit of the data to equation 3.1 given under Experimental Procedures using nonlinear least-squares regression analysis.

	No protein S		With protein S	
	k_{506}	k_{306}	k_{506}	k_{306}
5 μM PS/PC (5/95)				
factor Va ₁	8.3×10^5	3.4×10^4	1.1×10^6	5.7×10^5
factor Va ₂	7.0×10^6	5.1×10^5	1.6×10^7	3.3×10^6
50 μM PS/PC (20/80)				
factor Va ₁	2.1×10^8	6.4×10^6	2.2×10^8	1.2×10^8
factor Va ₂	1.2×10^8	6.6×10^6	1.2×10^8	1.4×10^8

Table 3.1: Rate constants ($M^{-1}s^{-1}$) for APC-catalyzed peptide bond cleavages in factor Va₁ and factor Va₂. The rate constants of APC-catalyzed inactivation of factor Va were obtained by fitting the time courses of factor Va inactivation (figure 3.1) using equation 3.1 for a biphasic inactivation reaction described under Experimental Procedures.

Figure 3.2: **Effect of phosphatidylserine on rate constants of APC-catalyzed cleavage at Arg⁵⁰⁶ and Arg³⁰⁶ in factor Va₁ and factor Va₂.** 1.5 nM purified human factor Va₁ (■) or factor Va₂ (●) was incubated at 37 °C with varying amounts (0.4–60 nM) of APC in 25 mM Hepes (pH 7.5), 175 mM NaCl, 3 mM CaCl₂, 5 mg/ml BSA, and 5 μ M DOPS/DOPC vesicles with the mole percentage phosphatidylserine (DOPS) indicated in the figure. Rate constants for APC-catalyzed cleavage at Arg⁵⁰⁶ (k_{506}) and Arg³⁰⁶ (k_{306}) were obtained from the best fit of the time courses of factor Va inactivation (cf. figure 3.1) to equation 3.1 given under Experimental Procedures using nonlinear least-squares regression analysis. Plotted are k_{506} (A) and k_{306} (B) as a function of the phosphatidylserine content of the membrane.

lower for factor Va₁ than for factor Va₂ (table 3.1). When the same experiment was performed in the presence of a 10-fold higher concentration of phospholipid vesicles containing 20 mole% phosphatidylserine, inactivation was much faster, and factor Va₁ and factor Va₂ were inactivated by APC at the same rate, both in the absence and presence of protein S (figure 3.1C).

APC-catalyzed inactivation of factor Va₁ and factor Va₂ on membranes containing varying amounts of phosphatidylserine Time courses of factor Va₁ and factor Va₂ inactivation by APC (cf. figure 3.1) were determined on 5 μ M phospholipid vesicles containing varying amounts of phosphatidylserine. Since protein S had similar effects on the inactivation of both forms of factor Va, we have performed these experiments in the absence of protein S. The time courses of factor Va inactivation were fitted using an equation for a biphasic inactivation reaction (equation 3.1, cf. [18, 23]) which yielded the rate constants, k_{506} and k_{306} , for APC-catalyzed cleavage at Arg⁵⁰⁶ and Arg³⁰⁶ in the heavy chain of factor Va. Increasing the amount of phosphatidylserine in the membrane surface resulted in an increase of both k_{506} (figure 3.2A) and k_{306} (figure 3.2B) for each form of factor Va. This indicates that the two cleavages required for full inactivation of factor Va are equally affected by variation of the phosphatidylserine content of the membrane. The data further show that factor Va₁ was inactivated at considerably lower rates than factor Va₂ on membranes with less than 15 mole% phosphatidylserine. At a higher phosphatidylserine content both forms of factor Va were inactivated with similar rates, and at 30 mole% phosphatidylserine, factor Va₁ was even inactivated about 2 times faster than factor Va₂.

3.4 Results

Figure 3.3: Effect of the phospholipid concentration on rate constants of APC-catalyzed cleavage at Arg⁵⁰⁶ and Arg³⁰⁶ in factor Va₁ and factor Va₂. 1.5 nM purified human factor Va₁ (■) or factor Va₂ (●) was incubated at 37 °C with varying amounts (2.6–46 nM) of APC in 25 mM Hepes (pH 7.5), 175 mM NaCl, 3 mM CaCl₂, 5 mg/ml BSA and varying amounts of DOPS/DOPC (5/95, mole/mole). Rate constants for APC-catalyzed cleavage at Arg⁵⁰⁶ (k_{506}) and Arg³⁰⁶ (k_{306}) were obtained from the best fit of the time courses of factor Va inactivation (*cf.* figure 3.1) to equation 3.1 given under Experimental Procedures using nonlinear least-squares regression analysis. Plotted are k_{506} (A) and k_{306} (B) as a function of the phospholipid concentration

Effect of variation of the phospholipid concentration on APC-catalyzed inactivation of factor Va₁ and factor Va₂ The previous experiments have shown that factor Va₁ was inactivated by APC at considerably lower rates than factor Va₂ on 5 μM phospholipid vesicles containing 5 mole% phosphatidylserine. Since this likely reflects differences in binding affinities of factors Va₁ and Va₂ for membrane surfaces [22], it is expected that the difference in sensitivity of both forms of factor Va for APC will annihilate at higher phospholipid concentrations. The experiment presented in figure 3.3 shows that this indeed occurs. At low phospholipid concentrations, the rate constants, k_{506} (figure 3.3A) and k_{306} (figure 3.3B), for factor Va₁ inactivation were ~15-fold lower than those for factor Va₂. At increasing phospholipid concentrations, the rates of factor Va inactivation gradually converged, and at 200 μM phospholipid, the difference between the rates of factor Va₁ and Va₂ inactivation was about 2-fold.

Effect of phospholipid composition and concentration on the cofactor activity of factor Va₁ and factor Va₂ in prothrombin activation In a previous paper [22], we reported that at low concentrations of phospholipid vesicles with a low phosphatidylserine content factor Va₂ exhibits a higher cofactor activity in prothrombin activation than factor Va₁. In figure 3.4, it is shown, however, that optimal expression of factor Va cofactor activity in prothrombin activation required much less phospholipid and phosphatidylserine than APC-catalyzed factor Va inactivation (*cf.* figure 3.2 and figure 3.3). Although at low phospholipid concentrations and on membranes with a low phosphatidylserine content factor Va₂ was a more effective cofactor than factor Va₁, the cofactor activities of both forms of factor Va were optimal and similar

Figure 3.4: **Effect of the phospholipid composition and concentration on the cofactor activity of factor Va₁ and factor Va₂ in prothrombin activation.** 1.5 nM purified human factor Va₁ (■) or factor Va₂ (●) was incubated at 37 °C with 2.5 pM factor Xa in 25 mM Hepes (pH 7.5), 175 mM NaCl, 3 mM CaCl₂, 5 mg/ml BSA and (A) 5 μM DOPS/DOPC vesicles with varying mole percentages phosphatidylserine (DOPS) or (B) varying amounts of DOPS/DOPC (5/95, mole/mole) vesicles. After 5 min, prothrombin was added to a final concentration of 1 μM. The rate of prothrombin activation was determined as described under Experimental Procedures. Plotted are the rate of prothrombin activation as a function of the phosphatidylserine content of the membranes (A) or as a function of the phospholipid concentration (B).

at 5 μM phospholipid vesicles containing 10 mole% phosphatidylserine (figure 3.4A) and on 50 μM phospholipid vesicles with 5 mole% phosphatidylserine (figure 3.4B).

Comparison of the procoagulant activities of factor V₁ and factor V₂ The experiments presented in the previous paragraphs show that under several conditions (*e.g.* 20 μM phospholipid vesicles containing 10 mole% phosphatidylserine) the cofactor activities of factor Va₁ and factor Va₂ in prothrombin activation were approximately similar (figure 3.4) while their sensitivities for APC were considerably different (figure 3.2 and figure 3.3). This means that in a system, in which factor V is activated by factor Xa/thrombin and in which the factor Va that is formed is allowed to express its cofactor activity in prothrombin activation in the presence of APC and protein S, factor V₁ will exhibit a higher overall procoagulant activity (*i.e.*, will yield more thrombin) than factor V₂. This was confirmed by the experiment presented in figure 3.5. In this experiment, equal amounts of factor V₁ or factor V₂ were activated by factor Xa and thrombin in reaction mixtures containing 12.5 μM vesicles with a phospholipid composition identical to that of activated platelet membranes [3], 1 μM prothrombin, 4 pM factor Xa, 2 nM APC, and 200 nM protein S at physiological ionic strength. In the case of factor V₁, this indeed resulted in the formation of much more thrombin than in the case of factor V₂ (figure 3.5). From the fact that the final level of thrombin formed with FV₂ is ~14% of that of FV₁, we conclude that the overall procoagulant activity of FV₁ under these conditions was 7-fold higher than that of FV₂.

3.5 Discussion

Figure 3.5: Comparison of procoagulant activities of factor V₁ and factor V₂ in a reaction system containing prothrombinase components, APC and protein S. 1.5 nM purified human factor V₁ (■) or factor V₂ (●) was incubated at 37 °C with 0.1 nM thrombin, 4 pM factor Xa, 1 μM prothrombin, 2 nM APC, 200 nM protein S, and 12.5 μM vesicles containing 10% brain PS, 39% brain PC, 27% brain PE, 19% brain sphingomyelin and 5% liver PI on a molar basis in 25 mM Hepes (pH 7.5), 127 mM NaCl, 3 mM CaCl₂, and 5 mg/ml BSA. The amounts of thrombin formed at the indicated time points was determined as described under Experimental Procedures.

3.5 Discussion

Efficient inactivation of factor Va by APC and protein S requires the presence of negatively charged membranes [32, 1]. The membrane presumably acts as a collecting device that binds factor Va, APC, and protein S and that promotes the assembly of an anticoagulant complex, thus accelerating factor Va inactivation by APC. In this paper, we show that one of the plasma forms of factor Va (*i.e.*, factor Va₁ is inactivated by APC at considerably lower rates than the other form of factor Va (factor Va₂). Factor Va₁ and factor Va₂ have different light chain domains [22, 20, 19] and bind with different affinities to negatively charged membranes [22]. In agreement with this phenomenon, we observed that differences in rates of APC-catalyzed inactivation of factors Va₁ and Va₂ are especially observed at low membrane concentrations and on membranes that contain less than 15 mole% of the anionic phospholipid phosphatidylserine. At high phospholipid and phosphatidylserine concentrations, factor Va₁ and factor Va₂ were inactivated at similar rates. Under these conditions, the number of available binding sites for factor Va on the membrane likely exceeds the dissociation constants of the factor Va-membrane complex of both factor Va₁ and factor Va₂. This results in a situation in which almost all factor Va₁ and Va₂ will be bound to the membrane and hence will be inactivated by APC at comparable rates.

Similar differences between APC-catalyzed inactivation of factor Va₁ and factor Va₂ were observed in the presence of the APC-cofactor protein S. This indicates that, at the chosen reaction conditions, protein S does not affect the assembly of the factor Va-APC-membrane complex. Our data confirm that protein S specifically accelerates APC-catalyzed factor Va₁ and Va₂ inactivation by increasing the rate constant of cleavage at Arg³⁰⁶ [23]. Recently, we reported [18, 23] a kinetic analysis of APC-catalyzed inactivation of a factor Va preparation that contained both factor Va₁ and factor Va₂. Careful assessment of cofactor activity in prothrombin activation showed that this preparation contained ~10% factor Va₁. Since the experiments reported in these papers were performed on 25 μM PS/PC (10/90, mol/mol) vesicles, factor Va₁

negligibly contributed to factor Va inactivation, and the given rate constants were actually those for factor Va₂.

Factor Va₁ and factor Va₂ also exhibited different cofactor activities in prothrombin activation. However, full expression of factor Va cofactor activity required much less phospholipid and phosphatidylserine than APC-catalyzed factor Va inactivation. This results in a situation in which at intermediate phospholipid conditions (*e.g.* 20 μ M phospholipid with 10 mole% phosphatidylserine) both forms of factor Va have the same cofactor activity in prothrombin activation but are inactivated by APC at considerably different rates (see also below).

When the properties of factor Va₁ and factor Va₂ are compared, it appears that their functional difference diminishes in the following order: binding affinity for negatively charged membranes [22], sensitivity for APC (this paper) and cofactor activity in prothrombin activation ([22], this paper). This can be explained by the fact that:

1. binding experiments yield true affinity differences of factor Va₁ and factor Va₂ for membranes independent of the phospholipid concentration
2. that in APC-catalyzed factor Va inactivation this difference may be (partially) compensated at higher phospholipid concentrations (binding sites $\geq K_d$)
3. that in prothrombinase measurements the presence of factor Xa [24, 17, 16] and prothrombin [4] additionally contributes to the binding of factor Va to the membrane.

Our observation that much less phospholipid and phosphatidylserine are required for full expression of cofactor activity of factor Va in prothrombin activation than for regulation of factor Va cofactor activity by APC may have physiological implications. On membranes with 5–10 mole% phosphatidylserine, which likely simulate the *in vivo* situation [3], factor Va₁ and factor Va₂ have similar cofactor activities in prothrombin activation but exhibit considerable differences in their sensitivity for APC. This means that, in contrast to the expectation, the overall procoagulant activity of factor V₁ will exceed that of factor V₂ in reaction systems containing membranes with physiologic phospholipid compositions, factor V, factor Xa, prothrombin, APC, protein S, and traces of thrombin to initiate factor Va formation. The experiment presented in figure 3.5, which was performed on membranes with the phospholipid composition of activated platelets, shows that this is indeed the case. The total amount of thrombin formed in the reaction system in which factor V was activated by factor Xa and thrombin and in which the factor Va formed was simultaneously allowed to express cofactor activity in prothrombin activation and to be down-regulated by APC and protein S, was 7-fold higher with factor V₁ than with factor V₂. This shows that, due to differences in sensitivity for APC, the overall procoagulant activity of the form of factor V that has the lowest affinity for membranes (factor V₁) was at least 7 times higher than that of the form of factor V which has the highest membrane affinity (factor V₂).

In a recent paper, Zöller *et al.* [33] reported that in approximately 5% of the APC-resistant families the Arg⁵⁰⁶-Gln mutation (FV_{Leiden}), that is usually associated with APC resistance [8, 2], is not found and suggested that apart from this mutation other, as yet unknown, genetic defects may also cause inherited APC resistance. In addition, Zöller *et al.* [33], described that heterozygous carriers of the FV_{Leiden} mutation with a history of thrombosis had lower APC

3.5 Discussion

sensitivity ratios than heterozygous family members without thrombosis. It is conceivable, that individuals with low APC sensitivities in aPTT-based assays may have ratios of circulating factor V₁ and factor V₂ that differ from that of normal individuals (normal plasma contains ~30 % factor V₁ [22]). Based on the observations reported in the present paper we are currently developing a method to quantify the amounts of factor V₁ and factor V₂ in plasma. With such a procedure we will investigate whether there is a relation between non-FV_{Leiden} related APC resistance, plasma levels of factor V₁ and V₂, and a possible risk for venous thrombosis.

3.6 References

- [1] H.M. Bakker, G. Tans, T. Janssen-Claessen, M.C. 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. *European Journal of Biochemistry*, 208(1):171–178, 1992.
- [2] 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*, 369(6475):64–67, 1994.
- [3] E.M. Bevers, P. Comfurius, and R.F. Zwaal. Changes in membrane phospholipid distribution during platelet activation. *Biochim Biophys Acta*, 736(1):57–66, 1983.
- [4] D. Billy, G.M. Willems, H.C. Hemker, and T. Lindhout. Prothrombin contributes to the assembly of the factor Va-factor Xa complex at phosphatidylserine-containing phospholipid membranes. *Journal of Biological Chemistry*, 270:26883–26889, 1995.
- [5] P.E. Bock, P.A. Craig, S.T. Olson, and P. Singh. Isolation of human blood coagulation alpha-factor Xa by soybean trypsin inhibitor-sepharose chromatography and its active-site titration with fluorescein mono-p-guanidinobenzoate. *Arch-Biochem-Biophys*, 273(2):375–388, 1989.
- [6] T. Chase, Jr. and E. Shaw. Comparison of the esterase activities of trypsin, plasmin, and thrombin on guanidinobenzoate esters. titration of the enzymes. *Biochemistry*, 8(5):2212–2224, 1969.
- [7] P.C. Comp, R.R. Nixon, M.R. Cooper, and C.T. Esmon. Familial protein S deficiency is associated with recurrent thrombosis. *Journal of Clinical Investigation*, 74(6):2082–2088, 1984.
- [8] B. Dahlbäck, 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. *Proceedings of the National Academy of Sciences USA*, 90(3):1008, 1993.
- [9] R.G. DiScipio and E.W. Davie. Characterization of protein S, a gamma-carboxyglutamic acid containing protein from bovine and human plasma. *Biochemistry*, 18(5):899–904, 1979.
- [10] R.G. DiScipio, M.A. Hermodson, S.G. Yates, and E.W. Davie. A comparison of human prothrombin, factor IX (Christmas factor), factor X (Stuart factor), and protein S. *Biochemistry*, 16(4):698–706, 1977.
- [11] C.T. Esmon. The roles of protein C and thrombomodulin in the regulation of blood coagulation. *Journal of Biological Chemistry*, 264(9):4743–4746, 1989.
- [12] J.H. Griffin, B. Evatt, T.S. Zimmerman, A.J. Kleiss, and C. Wideman. Deficiency of protein C in congenital thrombotic disease. *Journal of Clinical Investigation*, 68(5):1370–1373, 1981.
- [13] A. Gruber, J.H. Griffin, L.A. Harker, and S.R. Hanson. Inhibition of platelet-dependent thrombus formation by human activated protein C in a primate model. *Blood*, 73(3):639–642, 1989.
- [14] M. Kalafatis, R.M. Bertina, M.D. Rand, and K.G. Mann. Characterization of the molecular defect in factor V^{R506Q}. *Journal of Biological Chemistry*, 270(8):4054–4057, 1995.
- [15] 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. *Journal of Biological Chemistry*, 269(50):31869–31880, 1994.
- [16] T. Lindhout, J.W.P. Govers-Riemslog, P. van de Waart, H.C. Hemker, and J. Rosing. Factor Va-factor Xa interaction. Effects of phospholipid vesicles of varying composition. *Biochemistry*, 21:5494–5502, 1982.
- [17] M.E. Nesheim, S. Eid, and K.G. Mann. Assembly of the prothrombinase complex in the absence of prothrombin. *Journal of Biological Chemistry*, 256:9874–9882, 1981.

3.6 References

- [18] G.A.F. Nicolaes, G. Tans, M.L.C.G.D. Thomassen, H.C. Hemker, K. Váradi, H.P. Schwarz, and J. Rosing. Peptide bond cleavages and loss of functional activity during inactivation of factor Va and factor Va^{R506Q} by activated protein C. *Journal of Biological Chemistry*, 270(36):21158–21166, 1995.
- [19] T.L. Ortel, M.A. Quinn-Allen, F.G. Keller, J.A. Peterson, D. Larocca, and W.H. Kane. Localization of functionally important epitopes within the second C-type domain of coagulation factor V using recombinant chimeras. *Journal of Biological Chemistry*, 269(22):15898–905, 1994.
- [20] T.L. Ortel, L. Yoo, M.A. Quin-Allen, and W.H. Kane. Partial glycosilation at asparagine-2181 of the second C-type domain of human factor V is the structural basis of the light chain doublet. *Blood*, 84:387a, 1994.
- [21] C.H. Pletcher and G.L. Nelsestuen. The rate-determining step of the heparin-catalyzed antithrombin/thrombin reaction is independent of thrombin. *Journal of Biological Chemistry*, 257(10):5342–5345, 1982.
- [22] 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. *Journal of Biological Chemistry*, 268(28):21130–21136, 1993.
- [23] J. Rosing, L. Hoekema, G.A.F. Nicolaes, M.C.L.G.D. Thomassen, H.C. Hemker, K. Váradi, H.P. Schwarz, and G. Tans. Effects of protein S and factor Xa on peptide bond cleavages during inactivation of factor Va and factor Va^{R506Q} by activated protein C. *Journal of Biological Chemistry*, 270:27852–27858, 1995.
- [24] J. Rosing, G. Tans, J.W. Govers-Riemslog, R.F. Zwaal, and H.C. Hemker. The role of phospholipids and factor Va in the prothrombinase complex. *Journal of Biological Chemistry*, 255(1):274–283, 1980.
- [25] N. Sala, W.G. Owen, and D. Collen. A functional assay of protein C in human plasma. *Blood*, 63(3):671–675, 1984.
- [26] H.P. Schwarz, M. Fischer, P. Hopmeier, M.A. Batard, and J.H. Griffin. Plasma protein S deficiency in familial thrombotic disease. *Blood*, 64(6):1297–1300, 1984.
- [27] R.L. Smith. Titration of activated bovine factor X. *Journal of Biological Chemistry*, 248(7):2418–2423, 1973.
- [28] 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. *Journal of Biological Chemistry*, 263(29):14884–14890, 1988.
- [29] G. Tans, T. Janssen-Claessen, H.C. Hemker, R.F. Zwaal, and J. Rosing. Meizothrombin formation during factor Xa-catalyzed prothrombin activation. Formation in a purified system and in plasma. *Journal of Biological Chemistry*, 266(32):21864–21873, 1991.
- [30] G. Tans, J. Rosing, M.C. Thomassen, M.J. Heeb, R.F. Zwaal, and J.H. Griffin. Comparison of anticoagulant and procoagulant activities of stimulated platelets and platelet-derived microparticles. *Blood*, 77(12):2641–2648, 1991.
- [31] F.J. Walker. Regulation of activated protein C by a new protein. A possible function for bovine protein S. *Journal of Biological Chemistry*, 255(12):5521–5524, 1980.
- [32] F.J. Walker, P.W. Sexton, and C.T. Esmon. The inhibition of blood coagulation by activated protein C through the selective inactivation of activated factor V. *Biochim-Biophys-Acta*, 571(2):333–342, 1979.
- [33] B. Zöller, 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. *Journal of Clinical Investigation*, 94:2521–2524, 1994.

Chapter 4

An assay to quantify the two plasma isoforms of factor V

Based on: Hoekema, L., Rosing, J. & Tans, G. An assay to quantify the two plasma isoforms of factor V. (submitted for publication)

4.1 Summary

Human factor V is a heterogeneous molecule, which after activation yields two forms of factor Va (factor Va₁ and factor Va₂), which have different cofactor activities in prothrombin activation and different susceptibilities for inactivation by activated protein C (APC). In model systems which mimic the physiological situation factor V₁ appeared to be more thrombogenic than factor V₂. Recently, we reported that carriers of the so-called R2 haplotype, a polymorphism which encodes several amino acid changes in factor V, have an increased risk of coronary artery disease and that the ratio of the two isoforms of factor V in their plasma is shifted in favor of the factor V₁ form. In the present paper we report the details of the assay that we have developed to quantify the plasma levels of the two isoforms of factor V. Factor V present in highly diluted plasma samples was activated with thrombin and the factor Va generated was subsequently quantified in prothrombinase-based assay systems containing saturating amounts of factor Xa and phospholipid vesicles (assay 1) and suboptimal amounts of factor Xa and phospholipid vesicles with a low mole % phosphatidylserine (assay 2). In assay 1, factor Va₁ and factor Va₂ express the same cofactor activity in prothrombin activation. Hence, this assay quantifies the total factor V levels (factor V₁ + factor V₂) present in plasma. In assay 2 factor Va₂ has approximately an 8-fold higher cofactor activity than factor Va₁. Hence, the response in this assay becomes dependent on the relative amounts of factor V₁ and factor V₂ in the plasma sample. Using calibration curves made under both assay conditions with samples containing known concentrations of purified factor Va₁ and factor Va₂ allowed calculation of the amounts of factor V₁ and factor V₂ present in plasma samples. Plasma from healthy individuals ($n = 42$, mean age = 35 years) contained 33% factor V₁ and 67% factor V₂ (range 50%–80% factor V₂, SD=6%).

4.2 Introduction

Blood coagulation factor V is a single-chain glycoprotein with a molecular weight of 330 kDa which, due to its participation in the procoagulant and anticoagulant pathways, plays an important role in the regulation of thrombin formation. The activated form of factor V (factor Va) acts as non-enzymatic cofactor in the prothrombinase complex and accelerates factor Xa-catalyzed prothrombin activation more than 1000-fold [21, 2]. Factor Va cofactor activity is down-regulated via limited proteolysis by activated protein C [13], in a reaction that is stimulated by phospholipids [32, 26] and protein S [30, 31]. The physiological importance of the protein C pathway is illustrated by the association between venous thrombosis and deficiencies in protein C [23, 10] or protein S [6, 22]. More recently it was shown that a mutation in factor V [1, 33, 9, 29] (FV_{Leiden}) which results in impaired down-regulation via the protein C/S system [16, 20] is a major cause of familial thrombophilia [7, 18].

Factor Va is a heterodimer, consisting of a heavy and light chain, which are tightly linked via a Ca²⁺-ion [11]. On SDS-PAGE, the light chain of factor Va appears as a 74/71 kDa doublet [25, 15] which appears to be caused by the fact that there are two forms of factor V (factor V₁ and factor V₂) circulating in blood [19]. After activation these two forms have been

4.3 Experimental procedures

shown to have different affinity for phospholipid vesicles [19]. Compared with factor Va_2 , factor Va_1 has a reduced affinity for phospholipid vesicles containing a low amount of negatively charged residues [19]. As a consequence factor Va_1 is less potent as cofactor in prothrombin activation [19, 12] and is less efficiently down-regulated by APC than is factor Va_2 [12]. In model systems, which mimic the physiological situation, *i.e.* prothrombin activation in the presence of APC and protein S, approximately 7-fold more thrombin was formed with factor V_1 than with factor V_2 [12].

Factor V is not only an essential cofactor in the procoagulant pathway but also plays a major role as cofactor in the APC-catalyzed inactivation of factor VIII [24, 28, 14]. Compared to factor V_2 , factor V_1 has reduced ability to act as a cofactor in the APC-catalyzed factor VIII(a) inactivation [27]. Considering the functional differences between factor V_1 and factor V_2 , it was hypothesized that changes in the relative amounts of the two isoforms of factor V in plasma are important in regulation of *in vivo* thrombin formation, especially in the presence of APC [12]. Indeed, it was recently reported that in a selected population of patients with coronary artery disease the relative amounts of the two factor V isoforms was shifted in favor of factor V_1 [5].

The present chapter contains the details of the assay used in the earlier mentioned study [5]. This assay, which enables quantification of both the total factor V concentrations and the relative amounts of both factor V isoforms in plasma, is based on the observed differences in lipid requirement for optimal expression of cofactor activity in prothrombin activation between factor Va_1 and factor Va_2 [19, 12]. The procedure described here can provide insight in the role of the relative amounts of factor V_1 and factor V_2 in the regulation of *in vivo* thrombin formation and can determine the possible contribution of these two isoforms in development of thrombotic disorders.

4.3 Experimental procedures

Materials Hepes, Tris, bovine serum albumin (BSA) were purchased from Sigma Chemical Co., USA. Dioleoylphosphatidylserine (DOPS) and dioleoylphosphatidylcholine (DOPC) were purchased from Avanti Polar Lipids, Pelham, AL., USA. D-Phe-pipecolyl-Arg-p-nitroanilide (S2238) was supplied by Chromogenix, Mölndal, Sweden. Human prothrombin, thrombin and factor Xa were prepared by earlier described procedures [8, 17, 3]. Human factor Va_1 and factor Va_2 were purified as described before [19].

Phospholipid vesicle preparations Small unilamellar phospholipid vesicles, composed of a mixture of DOPS and DOPC (20/80, M/M or 2.5/97.5, M/M) were prepared as described before [19]. After preparation, phospholipid vesicles (0.5 mM) were aliquoted in glass tubes, snap frozen in liquid nitrogen and stored in -80°C . Phospholipid concentrations were determined by phosphate analysis [4].

Normal pool plasma collection Nine parts of blood from normal healthy volunteers ($n=78$) were collected in 1 part of 0.13M trisodium citrate ($\text{pH}=7.8$). The blood was centrifuged for 15 minutes $3000 \times g$ at room temperature and subsequently for 30 minutes at $20\,000 \times g$ at 4°C . After pooling, plasma was aliquoted, snap-frozen in liquid nitrogen and stored at -80°C . Mean age of volunteers was 37 years, standard deviation 9.5 years. Male to female ratio was 2:1.

Prothrombin activation at optimal prothrombinase conditions Factor Va was diluted to $\sim 7\text{ pM}$ in 25 mM Hepes ($\text{pH}=7.5$ at 37°C), 175 mM NaCl, 2.6 mM CaCl_2 , 5 mg/ml BSA in the presence of $10\text{ }\mu\text{M}$ DOPS/DOPC (20/80, mol/mol) vesicles. In the case of plasma samples, factor V was fully activated to factor Va by including 1 nM thrombin in the reaction mixture described above and incubating it for 10 minutes at 37°C . Prothrombin activation was then started by adding factor Xa and prothrombin to final concentrations of 5 nM and $1\text{ }\mu\text{M}$, respectively. After 2 minutes initial rates of prothrombin activation were measured as described before [21] and corrected for the prothrombin activation in the absence of factor V.

Prothrombin activation at suboptimal prothrombinase conditions Factor V is diluted to approximately 2.5 pM in 25 mM Hepes ($\text{pH}=7.5$ at 37°C), 175 mM NaCl, 2.6 mM CaCl_2 , 5 mg/ml BSA in the presence of $40\text{ }\mu\text{M}$ DOPS/DOPC (2.5/97.5 mol/mol) vesicles. In the case of plasma samples, factor V was fully activated to factor Va by including 1 nM thrombin in the reaction mixture described above and incubating it for 10 minutes at 37°C . Prothrombin activation was then started by adding factor Xa and prothrombin to final concentrations 0.5 nM and $1\text{ }\mu\text{M}$, respectively. After 5 minutes initial rates of prothrombin activation were measured as described before [21] and corrected for the prothrombin activation in the absence of factor V.

Routine determination of relative amounts of factor V_1 and factor V_2 in plasma The following buffers were used in the factor V_1 /factor V_2 assay. Buffer I: 25 mM Hepes ($\text{pH}=7.5$ at 37°C), 175 mM NaCl, 3 mM CaCl_2 , 5 mg/ml BSA; Buffer II: 50 mM Tris ($\text{pH}=7.5$ at 37°C), 175 mM NaCl, 0.5 mg/ml ovalbumin and 20 mM EDTA. The procedure that we have developed basically consists of 4 steps that are performed at 37°C .

- Step 1** Thousand-fold diluted plasma samples in buffer I (which appear to be stable for at least 4 hours on ice) were aliquoted into two tubes (A and B, optimal and sub-optimal prothrombinase conditions, respectively): A) $100\text{ }\mu\text{L}$ of a 1/1000 diluted plasma sample + $50\text{ }\mu\text{L}$ buffer I; B) $20\text{ }\mu\text{L}$ of a 1/1000 diluted plasma sample + $130\text{ }\mu\text{L}$ buffer I.
- Step 2** Activation of plasma factor V was started by adding to tube A: $80\text{ }\mu\text{L}$ of a thrombin/phospholipid/ Ca^{2+} mixture (3 nM thrombin, $31.25\text{ }\mu\text{M}$ 20/80 DOPS/DOPC vesicles, 2.6 mM CaCl_2 , and to tube B: $80\text{ }\mu\text{L}$ of a thrombin/phospholipid/ Ca^{2+} mixture (3 nM thrombin, $125\text{ }\mu\text{M}$ 2.5/97.5 DOPS/DOPC vesicles), 2.6 mM CaCl_2 .
- Step 3** After 10 minutes of activation of plasma factor V, prothrombin activation was started by adding to A): $20\text{ }\mu\text{L}$ factor Xa/prothrombin ($10\text{ }\mu\text{L}$ 125 nM factor Xa + $10\text{ }\mu\text{L}$ 25 μM prothrombin in 25 mM Hepes, $\text{pH}=7.5$, 175 mM NaCl, 5 mg/mL BSA). Final concentrations in the reaction mixture were: $\sim 10\text{ pM}$ factor Va, 5 nM factor Xa,

4.4 Results

10 μM 20/80 DOPS/DOPC vesicles, 2.6 mM CaCl_2 and 1 μM prothrombin; B) 20 μL factor Xa/prothrombin (10 μL 12.5 nM factor Xa + 10 μL 25 μM prothrombin in 25 mM Hepes[pH=7.5], 175 mM NaCl, 5 mg/mL BSA). Final concentrations in the reaction mixture were: ~ 2 pM factor Va, 0.5 nM factor Xa, 40 μM 2.5/97.5 DOPS/DOPC vesicles, 2.6 mM CaCl_2 and 1 μM prothrombin.

Step 4 After 2 minutes (tube A) or 5 minutes (tube B), prothrombin activation was stopped by diluting a reaction mixture aliquot (A: 10 μL , B 20 μL) in 490 μL of buffer II at 4 $^\circ\text{C}$. Thrombin present in the diluted and quenched reaction mixtures was quantified by A) adding 50 μL of this dilution to 150 μL buffer II in a microwell plate, B) transferring 200 μL of the dilution to microwell plate. After warming to 37 $^\circ\text{C}$, 50 μL S2238 (final concentration 235 μM) in buffer II was added and the absorbance was measured at 37 $^\circ\text{C}$ at 405/492 nm in a platereader (Reader 340 ATTC, SLT Labinstruments, Salzburg, Austria) in the kinetic mode.

Values were expressed as the rate of prothrombin activation in undiluted plasma and corrected for the rate of prothrombin activation in the absence of plasma.

4.4 Results

The rationale of the factor V_1 /factor V_2 assay In previous publications we have shown that factor V_{a1} and factor V_{a2} express similar cofactor activities when prothrombin activation is determined at high concentrations of factor Xa and on phospholipid vesicles with a high anionic lipid content. In contrast, factor V_{a2} has a much higher cofactor activity than factor V_{a1} when prothrombin is activated at low concentrations of factor Xa on phospholipid vesicles with a low anionic lipid content. These observations form the basis for the development of the factor V_1 /factor V_2 assay presented in this paper. The principle of this test is to determine the cofactor activity of factor Va in a thrombin-activated plasma sample at optimal prothrombinase conditions (assay 1 which quantifies total factor V, *i.e.* factor V_1 + factor V_2) and at suboptimal prothrombinase conditions (assay 2 which mainly quantifies factor V_2). Using a calibration curve made with known amounts factor V_{a1} and factor V_{a2} under both reaction conditions one can subsequently calculate the plasma concentrations factor V_1 and factor V_2 .

Rates of prothrombin activation at varying concentrations phospholipid vesicles The phospholipid-dependence of the cofactor activities of factor V_{a1} and factor V_{a2} reported in an earlier paper [12] formed the basis of the reaction conditions chosen for the assays under optimal conditions (phospholipid vesicles containing 20 mole% DOPS and 5 nM factor Xa) and suboptimal conditions (phospholipid vesicles containing 2.5 mole% DOPS and 0.5 nM factor Xa).

The effect of varying amounts of phospholipid vesicles on the ability of factor V_{a1} and factor V_{a2} to stimulate factor Xa-catalyzed prothrombin activation, was determined at both reaction conditions (Fig. 4.1AB). On phospholipid vesicles containing 20 mole% DOPS (Fig. 4.1A)

Figure 4.1: Rates of prothrombin activation with factor Va_1 and factor Va_2 as function of the phospholipid concentration. Factor Va_1 and/or factor Va_2 were incubated at 37°C in 25 mM Hepes (pH 7.5), 175 mM NaCl, 2.6 mM CaCl_2 , 5 mg/ml BSA A) 5 nM factor Xa, 1 μM prothrombin, containing varying amounts of DOPS/DOPC vesicles (A: 20/80, M/M or B: 2.5/97.5, M/M). Prothrombin activation was started with 5 nM factor Xa and 1 μM prothrombin (A) or 0.5 nM factor Xa and 1 μM prothrombin (B). Plotted are the rates of prothrombin activation (thrombin formed/min/ Va) as a function of the phospholipid concentration. ($\blacklozenge$) 7 pM purified human factor Va_1 ; ($\blacksquare$) 7 pM purified human factor Va_2 ; a mixture of 2.3 pM factor Va_1 and 4.7 pM factor Va_2 in buffer ($\circ$) or factor V deficient plasma ($\bullet$).

both forms of factor Va expressed the same cofactor activity in prothrombin activation. Maximal rates of prothrombin activation were already obtained at the lowest phospholipid concentration (5 μM) and no further change of the activation rate was observed at higher phospholipid concentrations. This indicates that under these conditions all factor Va (factor Va_1 as well as factor Va_2) is complexed with factor Xa and exerts maximal cofactor activity in prothrombin activation. Hence, these reaction conditions allow quantification of the total amount of factor Va (factor Va_1 + factor Va_2) in a sample.

On phospholipid vesicles with 2.5 mole% DOPS the cofactor activity of factor Va_2 was between 3-fold and 14-fold higher than that of factor Va_1 at high and low phospholipid concentrations, respectively. Thus, under these reaction conditions rates of prothrombin activation determined with mixtures of factor Va_1 and factor Va_2 will be mainly due to the fraction of factor Va_2 present. This is illustrated in Fig. 4.1B for a factor Va_1 /factor Va_2 mixture with a ratio 1:2 made in either buffer or factor V-deficient plasma.

Rates of prothrombin activation as function of the amount of factor Va present in the assay mixture Under the conditions chosen for the prothrombinase assays at optimal (10 μM 20/80 DOPS/DOPC, 5 nM factor Xa) and suboptimal conditions (40 μM 2.5/97.5 DOPS/DOPC, 0.5 nM factor Xa), the dependence of the rate of prothrombin activation as function of the factor Va concentration was determined.

At optimal phospholipid and factor Xa concentrations (Fig. 4.2A), the rate of prothrombin activation was linear with the amount of purified factor Va, or plasma factor Va in the assay, up to 150 nM prothrombin activated per minute, which corresponds to ~ 25 pM factor Va in

4.4 Results

Figure 4.2: Effect of varying amounts of factor Va_1 and factor Va_2 on the rate of prothrombin activation. Varying amounts of purified human factor Va_1 ($\blacklozenge$), factor Va_2 ($\blacksquare$) or a 1000 fold diluted normal plasma sample ($\blacktriangle$) in 25 mM Hepes, 175 mM NaCl, 3 mM $CaCl_2$ and 5 mg/ml BSA were assayed in prothrombinase mixtures containing A) 5 nM factor Xa, 1 μ M prothrombin, 10 μ M DOPS/DOPC vesicles (20/80, M/M) or B) 0.5 nM factor Xa, 1 μ M prothrombin, 40 μ M DOPS/DOPC vesicles (2.5/97.5, M/M). Plotted are the rates of prothrombin activation (thrombin formed/min) as a function of the factor Va concentration. Rates of prothrombin activation and the factor Va concentration were determined as described under the experimental procedures.

the assay mixture. Therefore, the quantification of factor Va in plasma under these reaction conditions was routinely performed in 2,500 fold diluted plasma samples (~ 10 pM factor Va in the assay mixture).

The assay under suboptimal conditions (Fig. 4.2B) shows linearity with both purified factor Va_1 and factor Va_2 up the highest factor Va concentration used. However, in the case of plasma samples the rate of prothrombin activation started to deviate from linearity at factor Va concentrations above 4 pM factor Va, which corresponds to a final plasma dilution of approximately 6000-fold. Experiments with dilutions of purified factor Va in factor V-deficient plasma showed that this inhibition is due to the interference by plasma components other than factor Va with the prothrombinase assay (data not shown). On basis of these experiments, plasma was routinely used in a 12,500 fold dilution in the suboptimal assay to circumvent interference of plasma components other than factor V with the rate of prothrombin activation.

Rates of prothrombin activation with factor V mixtures containing varying relative amounts of factor Va_1 and factor Va_2 Mixtures with a constant factor Va_2 concentration but with varying relative amounts of factor Va_1 and factor Va_2 were prepared from stock solutions of purified factor Va_1 and factor Va_2 with known concentrations. The upper curve in Fig. 4.3 shows that the rates of prothrombin activation at optimal prothrombinase conditions were constant and independent of the relative amounts of factor Va_1 and factor Va_2 . When the same mixtures were assayed at suboptimal conditions the rate of prothrombin activation increased linearly with the relative amount of factor Va_2 present in the factor Va sample.

Figure 4.3: **Cofactor activities of factor Va mixtures containing varying mole fractions factor Va₁ and factor Va₂ in prothrombin activation at optimal and suboptimal conditions** Mixtures of factor Va containing varying relative amounts of purified factor Va₁ and factor Va₂ at a constant total factor V concentration were assayed at optimal (5 nM factor Xa, 1 μ M prothrombin, 10 μ M, 20/80, M/M DOPS/DOPC vesicles, ●) and at suboptimal prothrombinase conditions (0.5 nM factor Xa, 1 μ M prothrombin, 40 μ M DOPS/DOPC vesicles, 2.5/97.5, M/M, ■). Plotted are the rates of thrombin formation (nM/min) as a function of the percentage factor V₂ in the factor V sample. Further details are described under the experimental procedures.

Figure 4.4: **Normalized rates of prothrombin activation at suboptimal prothrombinase conditions as a function of the mole fraction factor Va₂**. Normalized rates of prothrombin activation ($v_{\text{suboptimal}}/v_{\text{optimal}}$) for factor Va mixtures containing varying mole fractions factor Va₁ and factor Va₂ were calculated from the data from Fig. 4.3.

4.4 Results

Theoretical background of quantification of factor V_1 and factor V_2 in plasma samples At suboptimal conditions the rate of prothrombin activation is not only dependent on the relative amounts of factor V_{a1} and factor V_{a2} in a sample (cf. Fig. 4.3) but also on the total amount of factor V_a present (cf. Fig. 4.2B). Thus, determination the rate of prothrombin activation at suboptimal conditions by itself is not sufficient for quantification of relative amounts of factor V_{a1} and factor V_{a2} . However, when the rates of prothrombin activation at suboptimal conditions are normalized with respect to total amount of factor V_a present through division by the rate of prothrombin activation at optimal conditions they become solely dependent on the relative amounts of factor V_{a1} and factor V_{a2} . Thus, the normalized rates of prothrombin activation with factor V_{a1} , factor V_{a2} and a factor V_a sample with unknown amounts of factor V_{a1} and factor V_{a2} are:

$$X = \frac{v_1}{v_2} \quad (4.1)$$

$$Y = \frac{v_3}{v_4} \quad (4.2)$$

$$R = \frac{v_5}{v_6} \quad (4.3)$$

where v_1 , v_3 and v_5 are the rates of prothrombin activation at suboptimal conditions and v_2 , v_4 and v_6 are the rates of prothrombin activation at optimal conditions for factor V_{a1} , factor V_{a2} and the sample with unknown amounts of factor V_1 and factor V_2 , respectively.

Since the rates of prothrombin activation for factor V_{a1} and factor V_{a2} are additive, a sample which contains a mixture of factor V_{a1} and factor V_{a2} will yield a rate of prothrombin activation equal to the sum of the contributions of factor V_{a1} and factor V_{a2} . Expressed as a function of the mole fractions factor V_{a2} (a) and factor V_{a1} ($1 - a$) present in a sample, the normalized rate of prothrombin activation (R) of a mixture of factor V_{a1} and factor V_{a2} can then be written as

$$R = \frac{v_5}{v_6} = (1 - a) * X + a * Y \quad (4.4)$$

which can be rearranged as:

$$a = \frac{R}{Y - X} - \frac{X}{Y - X} \quad (4.5)$$

This equation predicts that a plot of the mole fraction factor V_{a2} (a) vs R should be a straight line. Fig. 4.4 shows that plotting the data from Fig. 4.3 according to Eq. 4.5 indeed results in a straight line. Thus, when X and Y are known, the relative amounts of factor V_{a1} and factor V_{a2} in a sample can be calculated from the ratio (R) of the rates of prothrombin activation at suboptimal and optimal prothrombinase conditions using this equation.

Calibration The reaction conditions for the optimal assay are such that all factor V_a is complexed in the prothrombinase complex and that factor V_{a1} and factor V_{a2} express the same, i.e. full cofactor activity in prothrombin activation. Since all reaction components (except factor V_a) are present in excess, the rate of prothrombin activation under these reaction conditions

is not influenced by small changes in the concentrations of phospholipid, factor Xa or prothrombin.

In contrast, the conditions at the sub-optimal assay are such that not all factor Va is incorporated in the prothrombinase complex. Thus, any variation in the concentrations of the components of the prothrombinase complex will result in a change of the extent of incorporation of factor Va into the prothrombinase complex and hence, in variable rates of prothrombin activation. To minimize day to day variation, it is therefore necessary to daily calibrate the assay performed at suboptimal prothrombinase conditions. Here we describe three methods of calibration.

Method 1 is based on determination of the cofactor activities of purified factor Va₁ and factor Va₂ preparations, *i.e.* that is determination of X and Y (Eq. 4.1 and Eq. 4.2). From Eq 4.5 the relative amounts of factor V₁ and factor V₂ in a plasma sample then can be calculated. Using this method of calibration, the relative amount of factor V₂ in pooled normal plasma was determined as 67.1% (SEM=1.1 %, n=13).

In fact the determination of the amounts of factor V₁ and factor V₂ in plasma samples via the calibration curve made with factor Va₁ and factor Va₂ (Eq. 4.5) relies on the determination of only two reference points, *i.e.* X and Y . The reliability of the factor V₁-factor V₂ assay can be improved (method 2) by adding a third reference point, *i.e.* a sample with known relative amounts of factor V₁ and factor V₂ and fitting the data points (factor V₁, factor V₂ and the sample with known amounts of factor V₁ and factor V₂) to a straight line (*cf.* Fig 4.4) using nonlinear least-squares analysis. As third reference sample a normal pooled plasma can be used under the assumption that it contains 67% factor V₂ (see above). Method 3 uses the assumption that $X \rightarrow 0$ (*i.e.* compared to factor Va₂, factor Va₁ has negligible cofactor activity under suboptimal conditions). Eq. 4.5 then reduces to

$$\text{mole fraction } FV_2 = \frac{R}{Y} \quad (4.6)$$

In that case is not necessary anymore to use factor Va₁ as reference sample. If purified factor Va₂ is not available a normal plasma sample can be taken as a reference sample. Assuming that normal plasma contains 67% factor V₂, the percentage factor V₂ in an unknown plasma sample can be calculated according the formula:

$$FV_2(\%) = \frac{R_{\text{plasma}}}{R_{\text{normal plasma}}} * 67\% \quad (4.7)$$

This method introduces a systematic error as it disregards the contribution of factor Va₁ to the rate of prothrombin activation at suboptimal conditions and results in factor V₂-values that are closer to reference plasma than the actual value is. This effect is less pronounced for samples which contain relative amounts of factor Va₁ and factor Va₂ close to that present in

4.4 Results

	factor V_2 (%)	Between-assay variation (n=13) (%)	Within-assay variation (n=21) (%)
sample #1	70	4.0	4.4
sample #2	65	8.2	4.1
sample #3	47	8.5	5.8
	FV level (% nPL)		
sample #1	104	4.1	2.6
sample #2	97	4.7	3.0
sample #3	86	5.4	3.7

Table 4.1: **Reproducibility of the assay.** Between-assay and within-assay variation for the relative amounts of factor V_1 and factor V_2 were obtained using calibration method 2.

the reference plasma (pooled normal plasma). In our hands, the phospholipid concentration in the suboptimal assay is chosen such that $X = 0.06$ and $Y = 0.36$ which yields an assay ratio (R) for pooled normal plasma close to 0.3. Assuming that $X = 0$, a sample containing 50% factor V_2 yields 54% factor V_2 when calculated by method 3, and a sample with 80% factor V_2 yields a value of 77% factor V_2 .

Within- and between-assay variation Both the between-assay variation and the within-assay variation were determined using method 2, for three plasma different samples which contained amounts of factor V_2 , below (47%) and close to (65 and 70%) the percentage of factor V_2 present in pooled normal plasma.

The within-assay variation (SD/mean), which was calculated for these samples by measuring each sample 21 times on the same day, was 4.8% (Table 4.1). The between-assay variation, determined by analyzing the same three plasma samples on 13 different days, was 6.9% (Table 4.1).

The total level of factor V in a plasma is obtained from the measurement under optimal prothrombinase conditions. Expressed as percentage of the factor V level present in normal plasma the within-assay variation of the factor V_a assay was 3.1% (n=21) and the in-between assay variation was 4.7% (n=13).

Determination of relative amounts of factor V_1 and factor V_2 in the normal population To establish the range of the relative amounts factor V_1 and factor V_2 present in plasma of healthy individuals, we analyzed samples of 42 volunteers who donated blood for our normal plasma pool (22 males, 20 females, mean age = 35, SD=8). The mean factor V_2 level in these plasma samples was 67% (range 50%-80%, SD = 6%, Fig. 4.5A). No difference in %factor V_2 was observed for male and female ($P=0.85$).

Figure 4.5: **Relative amounts of factor V₁, factor V₂ and total factor V levels in plasma from healthy individuals.** Factor V₁, factor V₂ and factor V levels were determined in plasma samples from 42 volunteers who donated blood to our normal plasma pool. (A) Factor V₂ levels expressed as fraction of total factor V, i.e. factor V₂/FV. (B) Total factor V levels in the same samples expressed as the percentage of factor V present in pooled normal plasma.

4.5 Discussion

Plasma contains two forms of factor V (factor V₁ and factor V₂) [19], the relative amounts of which may be important in maintaining the haemostatic balance. This can be inferred from the fact that it was previously demonstrated that factor V₁ and factor V₂ have different characteristics in haemostasis:

1. on membranes mimicking physiological conditions, factor V₁ has a higher overall pro-coagulant activity than factor V₂, since it has reduced sensitivity to APC under these conditions [12]
2. factor V₁ has reduced ability to act as a cofactor in the factor VIII(a) inactivation by APC and protein S [27].

Therefore, an increased relative amount of factor V₁ in the circulation will likely result in increased thrombin formation, especially in the presence of APC, and hence, may be associated with hypercoagulable state and with an increased risk for thrombosis.

In this paper we present a method for quantification of factor V₁ and factor V₂ in plasma. This assay, which reliably measures the factor V₁, factor V₂, and total factor V concentrations in plasma, is based on the observation that the two forms of factor V have different cofactor activities in prothrombin activation on phospholipid vesicles with a low phosphatidylserine content [19, 12]. The use of highly diluted plasma samples, makes the assay insensitive to interference by other plasma components. Since factor V₁ and factor V₂ are quantified at suboptimal prothrombinase conditions, small variations in the concentrations of reactants will

4.5 Discussion

result in a variation in the rate of prothrombin activation and hence in the concentrations factor V_1 and factor V_2 that are calculated from the assay result. Since the rate of prothrombin activation at suboptimal prothrombinase conditions is a linear function of the relative amount of factor V_2 (Figs. 4.3 and 4.4), the factor V_1 and factor V_2 concentration in a plasma sample can be calculated from a calibration curve made with purified factor V_{a1} and factor V_{a2} .

Routine application of this assay could be improved via calibration by means of three plasma samples with known relative amounts of factor V_2 (containing low, medium and high amounts factor V_2), since diluted plasma factor V is more stable than purified factor Va and is therefore easier to handle.

The range of the relative amounts of factor V_2 in the normal population is quite small, indicating that the plasma level of the two isoforms of factor V is strictly regulated. However, the difference between the lowest value of factor V_2 determined in the normal population (50% factor V_2) and the amount of factor V_2 present in normal pooled plasma (67%) is not insignificant. In model systems that mimic physiological conditions, *i.e.* prothrombin activation in the presence of proteins from the anticoagulant pathway, factor V_1 yields 7 fold higher levels of thrombin formation than factor V_2 . Thus it can be calculated that in a plasma sample containing 50% factor V_2 , thrombin formation is expected to be 35% higher than in a plasma sample containing 67% factor V_2 . Moreover, it should be emphasized that there is a second mechanism via which a relative excess of factor V_1 could lead to increased thrombin formation. That is that compared with factor V_2 , factor V_1 is a poor cofactor in APC-catalyzed factor VIII(a) inactivation [27]. This implies that an increase of the relative amount of factor V_1 will be accompanied with impaired down-regulation of factor VIII by APC and increased thrombin formation via the intrinsic coagulation pathway.

The clinical relevance of variation of the relative amounts of plasma factor V_1 and factor V_2 for *in vivo* thrombin regulation was recently established. It was shown that a factor V gene polymorphism (R2-gene), that is associated with increased relative amount of factor V_1 in plasma, is a risk factor for arterial thrombosis [5].

With the assay described here, the contribution of an imbalance of the factor V isoforms in the development of thrombotic complications both in the venous and arterial part of the vascularity can be investigated.

4.6 References

- [1] 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*, 369(6475):64–67, 1994.
- [2] J.W. Bloom, M.E. Nesheim, and K.G. Mann. Phospholipid-binding properties of bovine factor V and factor Va. *Biochemistry*, 18:4419–4425, 1979.
- [3] P.E. Bock, P.A. Craig, S.T. Olson, and P. Singh. Isolation of human blood coagulation alpha-factor Xa by soybean trypsin inhibitor-sepharose chromatography and its active-site titration with fluorescein mono-p-guanidinobenzoate. *Arch-Biochem-Biophys*, 273:375–388, 1989.
- [4] C.J.F. Böttcher, C.M. van Gent, and C. Pries. A rapid and sensitive sub-micro phosphorus determination. *Anal. Chim. Acta.*, 24:203–207, 1961.
- [5] E. Castoldi, J. Rosing, D. Girelli, L. Hoekema, B. Lunghi, F. Mingozzi, P. Ferraresi, S. Friso, R. Corrocher, G. Tans, and F. Bernardi. Mutations affecting post-translational processing of coagulation factor V are associated with coronary artery disease. *submitted for publication*.
- [6] P.C. Comp, R.R. Nixon, M.R. Cooper, and C.T. Esmon. Familial protein S deficiency is associated with recurrent thrombosis. *Journal of Clinical Investigation*, 74(6):2082–2088, 1984.
- [7] B. Dahlbäck, 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. *Proceedings of the National Academy of Sciences USA*, 90(3):1008, 1993.
- [8] R.G. DiScipio, M.A. Hermodson, S.G. Yates, and E.W. Davie. A comparison of human prothrombin, factor IX (Christmas factor), factor X (Stuart factor), and protein S. *Biochemistry*, 16(4):698–706, 1977.
- [9] J.S. Greengard, X. Sun, X. Xu, J.A. Fernandez, J.H. Griffin, and B. Evatt. Activated protein C resistance caused by Arg⁵⁰⁶ → Gln mutation in factor Va. *Lancet*, 343(8909):1361–1362, 1994.
- [10] J.H. Griffin, B. Evatt, T.S. Zimmerman, A.J. Kleiss, and C. Wideman. Deficiency of protein C in congenital thrombotic disease. *Journal of Clinical Investigation*, 68(5):1370–1373, 1981.
- [11] E.R. Guinto and C.T. Esmon. Formation of a calcium binding-site on bovine activated factor V following recombination of the isolated subunits. *Journal of Biological Chemistry*, 259:10038–10043, 1982.
- [12] L. Hoekema, G.A. Nicolaes, H.C. Hemker, G. Tans, and J. Rosing. Human factor Va₁ and factor Va₂: Properties in the procoagulant and anticoagulant pathways. *Biochemistry*, 36(11):3331–3335, 1997.
- [13] W. Kisiel, W.M. Canfield, L.H. Ericsson, and E.W. Davie. Anticoagulant properties of bovine plasma protein C following activation by thrombin. *Biochemistry*, 16(26):5824–5831, 1977.
- [14] D.S. 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*, 87:4708–4717, 1996.
- [15] M.E. Nesheim, W.B. Foster, R. Heywick, and K.G. Mann. Characterization of factor V activation intermediates. *Journal of Biological Chemistry*, 259:3187–3196, 1984.
- [16] G.A.F. Nicolaes, G. Tans, M.L.C.G.D. Thomassen, H.C. Hemker, K. Váradi, H.P. Schwarz, and J. Rosing. Peptide bond cleavages and loss of functional activity during inactivation of factor Va and factor Va^{R506Q} by activated protein C. *Journal of Biological Chemistry*, 270(36):21158–21166, 1995.
- [17] C.H. Pletcher and G.L. Nelsestuen. The rate-determining step of the heparin-catalyzed antithrom-

4.6 References

- bin/thrombin reaction is independent of thrombin. *Journal of Biological Chemistry*, 257(10):5342–5345, 1982.
- [18] 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*, 85:1504–1508, 1995.
- [19] 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. *Journal of Biological Chemistry*, 268(28):21130–21136, 1993.
- [20] J. Rosing, L. Hoekema, G.A.F. Nicolaes, M.C.L.G.D. Thomassen, H.C. Hemker, K. Váradi, H.P. Schwarz, and G. Tans. Effects of protein S and factor Xa on peptide bond cleavages during inactivation of factor Va and factor Va^{R506Q} by activated protein C. *Journal of Biological Chemistry*, 270:27852–27858, 1995.
- [21] J. Rosing, G. Tans, J.W. Govers-Riemslog, R.F. Zwaal, and H.C. Hemker. The role of phospholipids and factor Va in the prothrombinase complex. *Journal of Biological Chemistry*, 255(1):274–283, 1980.
- [22] H.P. Schwarz, M. Fischer, P. Hopmeier, M.A. Batard, and J.H. Griffin. Plasma protein S deficiency in familial thrombotic disease. *Blood*, 64(6):1297–1300, 1984.
- [23] U. Seligsohn, A. Berger, M. Abend, L. Rubin, D. Attias, A. Zivelin, and S.I. Rapaport. Homozygous protein C deficiency manifested by massive thrombosis in the newborn. *New England Journal of Medicine*, 310:559–562, 1984.
- [24] L. Shen and B. Dahlbäck. Factor V and protein S as synergistic cofactors to activated protein C in degradation of factor VIIIa. *Journal of Biological Chemistry*, 269(29):18738, 1994.
- [25] K. Suzuki, B. Dahlbäck, and J. Stenflo. Thrombin-catalyzed activation of human coagulation factor V. *Journal of Biological Chemistry*, 257(11):6556–6564, 1982.
- [26] K. Suzuki, J. Stenflo, B. Dahlbäck, and B. Teodorsson. Inactivation of human coagulation factor V by activated protein C. *Journal of Biological Chemistry*, 258(3):1914–1920, 1983.
- [27] K. Váradi, 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 V^{R506Q} mutation. *Thrombosis and Haemostasis*, 76:208–214, 1996.
- [28] K. Váradi, J. Rosing, G. Tans, and H.P. Schwarz. Influence of factor V and factor Va on APC-induced cleavage of human factor VIII. *Thrombosis and Haemostasis*, 73(4):730–731, 1995.
- [29] J. Voorberg, J. Roelse, R. Koopman, H. Büller, 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*, 343(8912):1535–1536, 1994.
- [30] F.J. Walker. Regulation of activated protein C by a new protein. A possible function for bovine protein S. *Journal of Biological Chemistry*, 255(12):5521–5524, 1980.
- [31] F.J. Walker. Regulation of activated protein C by protein S. *Journal of Biological Chemistry*, 256:11128–11131, 1981.
- [32] F.J. Walker, P.W. Sexton, and C.T. Esmon. The inhibition of blood coagulation by activated protein C through the selective inactivation of activated factor V. *Biochim-Biophys-Acta*, 571(2):333–342, 1979.
- [33] B. Zöller and B. Dahlbäck. Linkage between inherited resistance to activated protein C and factor V gene mutation in venous thrombosis. *Lancet*, 343(8912):1536–1538, 1994.

Chapter 5

Mutations affecting post-translational processing of coagulation factor V are associated with coronary artery disease

Adapted from: Castoldi, E., Rosing, J., Girelli, D., Hoekema, L., Lunghi, B., Mingozzi, F., Ferraresi, P., Friso, S., Corrocher, R., Tans, G. & Bernardi, F. Mutations in the R2 FV gene affect the ratio between the FV isoforms in plasma, *Thrombosis and Haemostasis*, (accepted for publication)

5.1 Summary

Factor V (FV) genetic components other than FV_{Leiden} may represent potential candidate determinants of coronary artery disease (CAD). We have determined the prevalence of a particular FV allele (the "R2 gene"), which encodes several aminoacid changes in FV, in 323 patients with CAD and 323 age- and sex-matched healthy controls. Prevalence of heterozygosity or homozygosity for the R2 gene was higher ($P < 0.02$) in patients (18.6%) than in controls (12.4%). The difference was even more pronounced when patients and controls older than 60 years were compared (24.1% vs. 12.1%, OR=2.3, 95%CI 1.2–4.4, $P < 0.007$). Prevalence of the R2 gene among CAD patients who had experienced myocardial infarction (18.1%) did not differ from that of all CAD patients. The molecular basis of the association between the R2 gene and CAD was investigated at the gene and protein levels. New missense polymorphisms, among which a functional candidate (2194Asp/Gly), were identified in the R2 gene, and a relative increase of the more thrombogenic and more glycosylated FV isoform was observed in plasma of 2194Gly homozygotes. We conclude that carriership of the R2 FV gene is associated with CAD, possibly via a novel mechanism of disease based on the imbalance between the two functionally different forms of FV.

5.2 Introduction

The contribution of factor V (FV) genetic components to the development of arterial vascular disease is still a matter of debate. Several reports have failed to find an association between FV_{Leiden} [4] and myocardial infarction [25, 1, 9] or stroke [24, 18, 5], whereas others [26, 8] have found a correlation in selected populations (e.g. young female smokers).

FV genetic components, different from the FV_{Leiden} mutation, might contribute to the occurrence of the atherothrombotic disease, possibly by mechanisms not mediated by "classical" activated protein C (APC)-resistance [7]. In fact, a fair fraction of FV present in blood is stored in platelets [30], which play an important role in the atherosclerotic process [11, 12]. Moreover, both plasma and platelet FV have been shown [27] to comprise two isoforms, FV₁ and FV₂, differing in the degree of glycosylation of the carboxy-terminal C2 domain [22]. Since activated FV₁ and FV₂ have different affinities for phosphatidylserine-containing membranes [13], their relative proportions *in vivo* may act as a modulator of the hemostatic balance, particularly at the atherosclerotic plaque, where apoptotic endothelial cells are known to expose phosphatidylserine [10]. The crucial role of FV at the crossroads of procoagulant and anticoagulant pathways and its multiple molecular interactions [28] make mutations in its gene candidate determinants of arterial vascular disease.

An ancient and conserved FV gene, marked by the G (R2) allele of the RsaI polymorphism (4070A/G, R1/R2) in exon 13 [19] and encoding several aminoacid changes, has been described by our group [3]. This FV gene (called the "R2 gene" hereafter) was shown to be common in the general population (approximately 12% of the Italian population are carriers) and appeared to be associated with mild APC-resistance [3] and moderately reduced FV

levels [19]. These findings have prompted us to investigate the association of the R2 gene with the atherothrombotic vascular disease, particularly with coronary artery disease (CAD). Functional determinants of the R2-associated phenotype were sought for at the gene and protein levels.

5.3 Methods

Study population Between May 1996 and May 1998 we enrolled 323 consecutive unrelated adult patients of both sexes (mean age 59.7 ± 8.7 years; males 78.8%), recruited from those referred to the Institute of Cardiovascular Surgery of Verona. All of them were candidate to coronary artery bypass grafting, having angiographically documented severe multi-vessel CAD. At the time of the blood sampling, a complete clinical history including cardiovascular risk factors such as smoking and hypertension was collected. The diagnosis of previous (myocardial infarction (in 57% of them) was based on medical history, previous ECG and enzyme documentation, and/or on the finding of the typical sequelae of infarction on ventricular angiography. A group of 323 age- and sex-matched subjects (mean age 58.8 ± 8.7 years, Student's *t*-test vs. CAD group = n.s.; males 72.8%, χ^2 test vs. CAD group = n.s.), recruited during the same period from blood donors and healthy hospital staff members, was used as control group. Both CAD patients and controls came from the same geographical area (Northern Italy) and had a similar socio-economic background. Two additional patients, both homozygous for the R2 gene and referred for pulmonary embolism (P # BA) and cerebrovascular disease (P # DG), respectively, were selected for the molecular genetics and biochemical investigations. A healthy independently recruited subject (C # MS), homozygous for the R2 gene, and a healthy son (C # SL) of a R2-homozygous CAD patient (P # 142) were also studied. Informed consent was obtained from every subject after a full explanation of the objects of the study.

DNA analysis

PCR-amplification Thirty pairs of primers for the amplification of all exonic regions of the FV gene, were designed on the basis of the published FV sequence (GenBank, accession number Z99572). Amplification was performed in 30 PCR cycles, using 1 unit of Taq polymerase (Promega, Madison, WI):

Restriction analysis Polymorphism 4070 A/G in exon 13 was detected by RsaI-restriction, as previously described [19].

Sequencing The quest for new polymorphic markers was performed by direct sequencing. PCR-amplified FV gene exons were purified on low melting point agarose and sequenced with the Sequenase 2.0 kit (USB, Cleveland, OH), using ^{35}S as the radiolabel.

ASA-PCR Detection systems based on allele-specific amplification (ASA) were developed for the exon 4, 8 and 25 polymorphisms. Forward (F) and reverse (R) primers (nucleotide numbering according to ref. [15]) and amplification conditions (30 cycles) were as follows: exon 4, F4 5'-TTACCTTGACCACACATTCC-3' nt. 471-491, R4_C 5'-CAGGGGCCCAATCAGTCC-3' nt. 660-642, R4_A 5'-CAGGGGCCCAATCAGTCCA-3' nt. 660-642 (annealing at 57°C); exon 8, F8 5'-ATACAGGTCTCAGCATTTG-3' nt. 1213-1231, R8_G 5'-AAAATCCCATCTTCCTTCG-3' nt. 1346-1328, R8_A 5'-AAAATCCCATCTTCCTTCA nt. 1346-1328 (annealing at 51°C); exon 25, F25 5'-TTGAAGGAAATACTAATACC-3' nt. 6624-6643, R25_T 5'-ATGTTCAATTCTAGTAGATAT-3' nt. 6777-6755, R25_C 5'-TGTTC AATTCTAGTAGATAC-3' nt. 6776-6754 (annealing at 50°C). ASA-PCR products were run on 2% agarose gel and stained with ethidium bromide.

Blood collection Venous blood was drawn in sodium citrate (12.9 mmol/l) and immediately centrifuged at $2000 \times g$ for 20 minutes at 4°C. Plasma was separated, snap-frozen and stored in aliquots at -80°C.

Biochemical analyses

Separation of FVa₁ and FVa₂ by ion-exchange chromatography In order to qualitatively analyze R2 FV in plasma samples, 0.5 ml plasma was incubated with 40 nM thrombin and 22 mM added CaCl₂ for 20 min at 37°C to fully activate FV. After removal of the fibrin clot, the plasma was diluted seven-fold in 25 mM Hepes (pH 7.5 at room temperature), 50 mM NH₄Cl, 5 mM CaCl₂, 5 mg/ml BSA and subjected to ion-exchange chromatography [27] on a Mono S (HR 5/5) column (Amersham Pharmacia Biotech, Uppsala, Sweden). FVa activity eluting from the column was quantified via its ability to stimulate factor Xa-catalyzed prothrombin activation at 37°C in the presence of CaCl₂ and 40 μM phospholipid vesicles (DOPS/DOPC; 10/90; mol/mol), as described before [27].

Quantification of FV, FV₁ and FV₂ in plasma Total plasma FV was determined after complete activation of FV with thrombin (1 nM, 10 minutes at 37°C) in 1000-fold diluted plasma samples, essentially as described before [27]. FV₁ and FV₂ were quantified via an assay that is based on differences in cofactor activity of FVa₁ and FVa₂ in prothrombin activation, determined at limiting amounts [13] of FVa and activated factor X on membranes with a low phosphatidylserine content. Details of this assay will be published elsewhere.

Statistics Two by two contingency chi-squares were used to compare allele frequencies between groups. Odds ratios (OR) were calculated using the approximation of Woolf [32]; R2-homozygous and heterozygous patients were pooled for the purpose of OR estimation. The two-sample *t*-test assuming unequal variances was employed for comparing relative abundances of FV₂ in different subjects.

5.4 Results

	N	Genotypes			R2 allele frequency	P	Odds ratio* OR (95%CI)
		R1R1 n (%)	R1R2 n (%)	R2R2 n (%)			
CAD patients	323	263 (81)	55 (17)	5 (2)	0.101	0.019	1.6 (1.0–2.5)
>60 years	145	110 (76)	30 (21)	5 (3)	0.138	0.007	2.3 (1.2–4.4)
AMI	171	140 (82)	29 (17)	2 (1)	0.096		
Controls	323	283 (88)	39 (12)	1 (<1)	0.063		
>60 years	132	116 (88)	16 (12)	0	0.061		

Table 5.1: **FV genotype frequencies in patients with coronary artery disease and in normal controls.** * Odds ratios calculated versus age-matched controls

5.4 Results

Association between CAD and the R2 FV gene To evaluate the association between the occurrence of CAD and carriership of the R2 gene, R2 frequency was determined in 323 CAD patients and in an equal number of age- and sex-matched healthy controls (Table 5.1). The prevalence of heterozygosity or homozygosity for the R2 gene was higher ($P < 0.02$) in CAD patients ($R1R2 + R2R2 = 18.6\%$) than in the control group (12.4%). An OR of 1.6 (95%CI 1.0–2.5) was obtained. An increased OR was measured for patients older than 60 years who were carriers of at least one R2 gene (24.1%, OR=2.3, 95%CI 1.2–4.4, Table 5.1), when compared with healthy controls older than 60 years (12.1%). Five R2-homozygotes were detected among patients, all in the older age group.

No significant difference was observed in the prevalence of the R2 gene in patients of the two sexes (males, 19.2%, females, 16.2%), although females ($n=68$) were clearly under-represented in this study. No preferential association was observed between the R2 gene and acquired risk factors of CAD, such as hypertension, smoking habits and high total cholesterol (not shown).

The prevalence of the R1R2 or R2R2 genotypes (18.1%, Table 5.1) in patients who had experienced acute myocardial infarction (AMI) was indistinguishable from that found in all CAD patients.

When patients were screened for FV_{Leiden}, the heterozygote frequency (4%) was not different from that [20] found in the normal Italian population (2.6%) and showed no preferential distribution over the age groups. Exclusion of FV_{Leiden} carriers from the patient group did not affect the results of the statistical analysis.

Novel polymorphic markers in the FV gene and extended R2 genotypes In order to identify the mutations potentially responsible for the phenotype associated with the R2 FV gene, a

Polymorphisms						
Gene position	Exon 4		Exon 8	Exon 13	Exon 16	Exon 25
Nucleotide change	642 G/T		1328 T/C	4070 A/G (R1/R2)	5380 G/A	6755 A/G
Aminoacid change	156 Ser		385 Met/Thr	1299 His/Arg	1736 Val/Met	2194 Asp/Gly
Protein domain	A1		A2	B	A3	C2
Genotypes of R2-homozygous patients and controls						
1	P#49, P#449, P#DG, C#MS	P#142, P#BA, C#146,	GG	CC	R2R2	GG
2	P # 364		GT	CC	R2R2	GG
3	P # 417		GT	CC	R2R2	GA

Table 5.2: **FV polymorphisms and classification of genotypes in R2-homozygous patients (P#) and controls (C#).**

complete FV exon scanning was performed in three R2-homozygous subjects and two normal controls. Systematic sequencing in the FV gene led to the detection of three novel biallelic markers in exons 4, 8 and 25 respectively (Table 5.2).

All R2-homozygotes from this study ($n=6$, five CAD patients and one control), as well as three additional R2-homozygous subjects, one healthy (C # MS) and two referred for pulmonary embolism (P # BA) and cerebrovascular disease (P # DG), respectively, were genotyped (Table 5.2) for these markers, and for five previously described [19, 29, 2] missense polymorphisms (2379 A/G, 2663 G/A, 2684 G/A, 3943 C/A and 5380 G/A). This analysis indicates the presence, in R2-homozygotes, of three different genotypes (numbered 1–3 in Table 5.2), which differ only for residues 1736 (exon 16) and 2194 (exon 25) at the protein level. While all individuals turned out to be homozygous and identical for all polymorphisms located between exons 8 and 13 (Table 5.2), heterozygosity was observed for the exon 4 (P # 364 and P # 417, Table 5.2), exon 16 (P # 417, Table 5.2) and exon 25 (P # 417, Table 5.2) markers, probably as a result of recombination.

An affordable estimate of the linkage between markers was obtained by genotyping all R2-heterozygous patients ($n=55$, Table 5.1). Neither allele of the exon 4 polymorphism turned out to be preferentially associated with the R2 allele. In contrast, the exon 25 G allele (2194 Gly) was found to be tightly linked with the R2 allele (only 3 recombinations over 55 R2 genes). Combining these data with those obtained for R2-homozygotes ($n=5$, Table 5.1, 1 recombinant over 10 R2 genes), a total of 61 out of 65 R2 genes (94%) present in patients were found to be associated with the 2194 Asp to Gly (exon 25) change.

Figure 5.1: **Chromatographic separation of FV isoforms in plasma.** Ion-exchange chromatography of thrombin-activated plasma samples was performed as described under Methods. The elution profiles obtained in two R2-homozygous patients with genotype 1, P # 142 ($\blacktriangle$) and P # DG ($\bullet$), and the average of two elution profiles ($\circ$) from pooled normal plasma are reported as FVa concentration present in the column eluate.

The tight linkage between the exon 25 marker and the R2 allele makes genotype/clinical phenotype association studies based on the exon 25 marker virtually overlapping with those based on the R2 polymorphism.

Biochemical characterization of FV in selected homozygous subjects Plasmas from three R2-homozygous patients and one R2-homozygous healthy subject were treated with thrombin to activate FV and compared with a similarly treated pooled normal plasma by ion-exchange chromatography on a Mono-S column. It has previously been shown that, following activation, normal plasma FVa elutes in two well separated peaks (designated FVa₁ and FVa₂ by order of elution) which differ in M_r of the FVa light chain [27]. The elution profile of R2-homozygotes was qualitatively identical to that of normal plasma (Fig 5.1), showing two peaks which eluted at the same positions as the FVa₁ and FVa₂ forms in normal plasma [27]. However, the relative proportion of FVa₁ was increased to various degrees in the R2-homozygotes with respect to normal plasma. Since the recovery of FVa after chromatography may vary between 30 and 70%, the elution profile is not quantitatively representative. Hence, a more affordable quantification of the relative proportions of the two forms was achieved by means of a functional assay. Three CAD (P # 49, P # 142 and P # 417) and two other patients (P # BA and P # DG), all homozygous for the R2 gene, as well as one R2-homozygous (C # MS) and two R2-heterozygous (C # 4 and C # SL) healthy subjects were available for this analysis. As a reference, 19 controls without the R2 gene, and with confirmed homozygous 6755A (2194Asp) in exon 25, were studied. The relative proportions of the FV isoforms were measured and the FV₁/FV₂ ratio was calculated (Fig 5.2). Ratios turned out to be significantly ($P = 0.0002$) increased

Figure 5.2: **FV₁/FV₂ ratios in carriers of the R2-gene.** FV₁/FV₂ ratios as determined by the functional assay, in four R2-homozygous patients and one R2-homozygous control, all with genotype 1 (●), one R2-homozygous patient with genotype 3 (△), two R1/R2 heterozygous controls (◈), and 19 R1-homozygous controls (○).

in the five R2-homozygotes with genotype 1 (P # 49, P # 142, P # BA and P # DG, C # MS; mean 0.71, 95%CI 0.66–0.76), irrespectively of patient/control status, as compared to controls not carrying this gene (mean 0.37, 95%CI 0.34–0.40). The two heterozygous subjects showed intermediate ratios, as did patient P # 417 who, although R2-homozygous, is heterozygous for the exon 16 and exon 25 polymorphisms (genotype 3, Table 5.2).

5.5 Discussion

The present paper provides evidence in favor of the association between carriership of the R2 FV gene [19, 3] and occurrence of CAD, especially in elderly individuals. In fact, 24.1% of the patients over 60 years of age were found to be carriers of at least one R2 gene, as compared with 12.1% of healthy controls (Table 5.1). This suggests that this common FV gene may confer an increased risk (OR=2.3, $P = 0.007$) of developing CAD at older age.

It may be argued that the increased frequency of the R2 gene in CAD patients with respect to controls suggests a protective role of this genetic component against death from myocardial infarction after CAD has developed. However, failure to observe a significant increase of R2 prevalence among AMI survivors (57% of all patients), compared to the non-AMI group, does not favor such a hypothesis.

Although gradual association between low response to APC, also not dependent on FV_{Leiden}, and advanced atherosclerosis and arterial disease has been recently reported [17], the slight increase in APC-resistance predicted by the R2 gene [3] can hardly account for the increased risk

5.5 Discussion

of CAD associated with this genetic component. In accordance with previous observations [6], the more pronounced APC resistance caused by FV_{Leiden} has been confirmed in this study to play a negligible role in the occurrence of this disease.

To define the molecular bases of the R2-associated phenotype, and its potential role in CAD pathogenesis, R2 FV was characterized both at the protein and gene levels. Ion-exchange chromatography showed (Fig 5.1) that R2-encoded plasma FV comprises the same two isoforms (FV₁ and FV₂) that are typical of normal individuals, suggesting that the aminoacid changes predicted by the R2 gene do not interfere greatly with post-translational modification. Whether secretion efficiency is affected remains questionable, as R2 carriers tend to have marginally reduced FV levels [19]. On the other hand, the highly significant relative excess of the FV₁ form demonstrated by functional assays (Fig 5.2) hints at the preferential synthesis or secretion of this isoform. The similarly high FV₁/FV₂ ratio measured in the R2-homozygous healthy subject C # MS, who is 42 years old, and in the R2-homozygous patients (Fig 5.2), all of them over 60 years of age, indicates that the alteration in the relative proportions of FV forms depends solely on genotype and not on age or the patient selection criterium.

The newly found polymorphisms help to dissect the functional determinants of the phenotype-genotype relationship. Constancy of the central part (exons 8–13) of the R2 gene, combined with frequent recombination at the 5' end (exon 4) and very rare recombination at the 3' end (exon 25), defines three different genotypes (Table 5.2) in R2-homozygous subjects. Since the encoded FV molecules differ only at residues 1736 and 2194, (*i.e.* the ones affected by the exon 16 and 25 polymorphisms), possible differences in their biochemical properties are likely due to these gene variations. However, the exon 16 polymorphism is frequently found in the general population in the absence of the R2 allele, which makes it a poor candidate to account for the R2-associated phenotype. On the other hand, the finding that the FV₁/FV₂ ratio of the patient heterozygous for the exon 25 polymorphism (P # 417, Table 5.2) was consistently lower than that of all other R2-homozygotes and similar to those found in R2 heterozygotes (Fig 5.2), supports a functional role for 2194Gly.

As a matter of fact, the substitution of aspartate 2194 with a glycine causes a dramatic steric and charge change in the C2 domain, which is involved in the interaction with phospholipid membranes [21, 16]. Evidence has been provided [22] that the structural differences between FV₁ and FV₂ reside in the glycosylation of the C2 domain of the FV molecule, very likely at asparagine 2181, which is not far from the 2194Asp/Gly polymorphism. Taken together the molecular genetics and biochemical findings support the involvement of the exon 25 marker in modulating FV biosynthesis and function. However, it is conceivable that other R2-linked missense changes (830Lys, 837His, 1299Arg), in particular those located in the B domain which is extensively post-translationally modified [15, 23, 14], may also contribute to the structural and functional shaping of the R2 FV molecule.

The mechanism by which R2 FV could favor the onset of CAD remains a matter of speculation. The genetically predicted relative excess of FV₁, which might be present in platelet FV too, may well explain the observed association of CAD with carriership of the R2 gene. In *in vitro* model systems, which mimic the physiological situation, approximately seven-fold

more thrombin was formed with FV_1 than with FV_2 [13]. Furthermore, it was reported that, compared with FV_2 , FV_1 has reduced anticoagulant properties, being a poor cofactor in factor VIII(a) inactivation by APC [31]. Together, these phenomena may result in life-long increased thrombin formation and fibrin deposition in coronary arteries of carriers of the R2 haplotype.

In conclusion, this study prospectively a novel mechanism of disease, based on the imbalance between the two forms of plasmatic FV, and points out the R2 and exon 25 polymorphisms as candidate markers for prospective studies aimed at substantiating the role of the R2 FV gene in the development of CAD.

5.6 References

- [1] D. Ardissino, F. Peyvandi, P.A. Merlini, E. Colombi, and P.M. Mannucci. Factor V (Arg⁵⁰⁶ → Gln) mutation in young survivors of myocardial infarction. *Thrombosis and Haemostasis*, 75:701–702, 1996.
- [2] T.A. Bayston, H. Ireland, R.J. Olds, S.L. Thein, and D.A. Lane. A polymorphism in the human coagulation factor V gene. *Human Molecular Genetics*, 3(11):2085, 1994.
- [3] 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*, 90(4):1552–1557, 1997.
- [4] 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*, 369(6475):64–67, 1994.
- [5] A. Catto, A. Carter, H. Ireland, T.A. Bayston, H. Philippou, J. Barrett, D.A. Lane, and P.J. Grant. Factor V Leiden gene mutation and thrombin generation in relation to the development of acute stroke. *Arteriosclerosis Thrombosis and Vascular Biology*, 15:783–785, 1995.
- [6] M. Cushman, F.R. Rosendaal, B.M. Psaty, E.F. Cook, J. Valliere, L.H. Kuller, and R.P. Tracy. Factor V Leiden is not a risk factor for arterial vascular disease in the elderly: Results from the Cardiovascular Health Study. *Thrombosis and Haemostasis*, 79(5):912–915, 1998.
- [7] B. Dahlbäck, 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. *Proceedings of the National Academy of Sciences USA*, 90(3):1008, 1993.
- [8] C.J.M. Doggen, V.M. Cats, R.M. Bertina, and F.R. Rosendaal. Interaction of coagulation defects and cardiovascular risk factors: Increased risk of myocardial infarction associated with factor V Leiden or prothrombin 20210A. *Circulation*, 97(11):1037–1041, 1998.
- [9] S.T. Dunn, C.R. Roberts, E. Schechter, W.E. Moore, E.T. Lee, and J.E. Eichner. Role of factor V Leiden mutation in patients with angiographically demonstrated coronary artery disease. *Thrombosis Research*, 91(2):91–99, 1998.
- [10] P.D. Flynn, C.D. Byrne, T.P. Baglin, P.L. Weissberg, and M.R. Bennett. Thrombin generation by apoptotic vascular smooth muscle cells. *Blood*, 89(12):4378–4384, 1997.
- [11] V. Fuster, L. Badimon, J.J. Badimon, and J.H. Chesebro. The pathogenesis of coronary artery disease and the acute coronary syndromes (first part). *New England Journal of Medicine*, 326:242–250, 1992.
- [12] V. Fuster, L. Badimon, J.J. Badimon, and J.H. Chesebro. The pathogenesis of coronary artery disease and the acute coronary syndromes (second part). *New England Journal of Medicine*, 326:310–318, 1992.
- [13] L. Hoekema, G.A. Nicolaes, H.C. Hemker, G. Tans, and J. Rosing. Human factor Va: 1 and factor Va: 2: Properties in the procoagulant and anticoagulant pathways. *Biochemistry*, 36(11):3331–3335, 1997.
- [14] G.L. Hortin. Sulfation of tyrosine residues in coagulation factor V. *Blood*, 76(5):946–952, 1990.
- [15] R.J. Jenny, D.D. Pittman, J.J. Toole, R.W. Kriz, R.A. Aldape, R.M. Hewick, R.J. Kaufman, and K.G. Mann. Complete cDNA and derived amino acid sequence of human factor V. *Proceedings of the National Academy of Science USA*, 84(14):4846–4850, 1987.
- [16] M. Kalafatis, M.D. Rand, and K.G. Mann. Factor Va-membrane interaction is mediated by two regions located on the light chain of the cofactor. *Biochemistry*, 33(2):486–493, 1994.

- [17] S. Kiechl, A. Muigg, P. Santer, M. Mitterer, G. Egger, M. Oberhollenzer, F. Oberhollenzer, A. Mayr, A. Gasperi, W. Poewe, and J. Willeit. Poor response to activated protein C as a prominent risk predictor of advanced atherosclerosis and arterial disease. *Circulation*, 99(5):614–619, 1999.
- [18] W.T. Longstreth, F.R. Rosendaal, D.S. Siscovick, H.L. Vos, S.M. Schwartz, B.M. Psaty, T.E. Raghunathan, T.D. Koepsell, and P.H. Reitsma. Risk of stroke in young women and two prothrombotic mutations: Factor V Leiden and prothrombin gene variant (G20210A). *Stroke*, 29(3):577–580, 1998.
- [19] 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. *Thrombosis and Haemostasis*, 75:45–48, 1996.
- [20] P.M. Mannucci, F. Duca, F. Peyvandi, L. Tagliabue, G. Merati, I. Martinelli, and M. Cattaneo. Frequency of factor V Arg⁵⁰⁶ → Gln in Italians. *Thrombosis and Haemostasis*, 75:694, 1996.
- [21] T.L. Ortel, D. Devore-Carter, M. Quinn-Allen, and W.H. Kane. Deletion analysis of recombinant human factor V. Evidence for a phosphatidylserine binding site in the second C-type domain. *Journal of Biological Chemistry*, 267(6):4189–98, 1992.
- [22] T.L. Ortel, L. Yoo, M.A. Quin-Allen, and W.H. Kane. Partial glycosylation at asparagine-2181 of the second C-type domain of human factor V is the structural basis of the light chain doublet. *Blood*, 84:387a, 1994.
- [23] D.D. Pittman, K.N. Tomkinson, D. Michnick, U. Seligsohn, and R.J. Kaufman. Posttranslational sulfation of factor V is required for efficient thrombin cleavage and activation and for full procoagulant activity. *Biochemistry*, 33(22):6952–9, 1994.
- [24] R.D. Press, X.Y. Liu, N. Beamer, and B.M. Coull. Ischemic stroke in the elderly. Role of the common factor V mutation causing resistance to activated protein C. *Stroke*, 27(1):44–48, 1996.
- [25] 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. *New England Journal of Medicine*, 332(14):912–917, 1995.
- [26] F.R. Rosendaal, D.S. Siscovick, S.M. Schwartz, R.K. Beverly, B.M. Psaty, W.T. Longstreth, T.E. Raghunathan, T.D. Koepsell, and P.H. Reitsma. Factor V Leiden (resistance to activated protein C) increases the risk of myocardial infarction in young women. *Blood*, 89(8):2817–2821, 1997.
- [27] 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. *Journal of Biological Chemistry*, 268(28):21130–21136, 1993.
- [28] J. Rosing and G. Tans. Coagulation factor V: An old star shines again. *Thrombosis and Haemostasis*, 78 (1):427–433, 1997.
- [29] N.L. Shen, S.T. Fan, J. Pyati, R. Graff, R.J. LaPolla, and T.S. Edgington. The serine protease cofactor factor V is synthesized by lymphocytes. *Journal of Immunology*, 150(7):2992–3001, 1993.
- [30] P.B. Tracy, L.L. Eide, E.J. Bowie, and K.G. Mann. Radioimmunoassay of factor V in human plasma and platelets. *Blood*, 60:59–63, 1982.
- [31] K. Váradi, 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 V^{R506Q} mutation. *Thrombosis and Haemostasis*, 76:208–214, 1996.

5.6 References

- [32] B. Woolf. On estimating the relation between blood group and disease. *American Journal of Human Genetics*, 19:251, 1955.

Chapter 6

Functional properties of factor V and factor Va encoded by the R2-FV gene

Based on: Hoekema, L., Castoldi, E., Tans, G. Girelli, D., Gemmati, D., Bernardi, F. & Rosing, J. Functional properties of factor V and factor Va encoded by the R2-gene, submitted for publication

6.1 Summary

Carriership of the factor V (FV) gene marked by the R2-haplotype, a polymorphism encoding several amino acid changes in FV, is associated with mild resistance to activated protein C (APC) and with an increased risk of coronary artery disease and venous thrombosis. We compared the functional properties of normal FV(a) and R2-FV(a) from carriers of the R2-gene in model systems and in plasma. FV and R2-FV were equally well activated by thrombin and expressed identical cofactor activities in prothrombin activation. Rate constants of APC-catalyzed inactivation of FVa and R2-FVa were similar both with and without protein S. However, significant differences were observed between haemostatic parameters determined in plasma from homozygous carriers of the R2-gene ($n=5$) and age-matched non-carriers ($n=19$). Plasma from R2-carriers contained significantly lower FV levels, increased FVIII levels and the ratio of the two FV isoforms (FV₁ and FV₂) was shifted in favor of FV₁. The FV₂/FV₁ ratio was 1.4 (95%CI=1.3–1.5) in homozygous carriers of R2 and 2.8 (95%CI=2.5–3.1) in controls ($P < 0.00001$). In an APC resistance test which quantifies the cofactor activity of FV in APC-catalyzed FVIII(a) inactivation, homozygous R2-carriers had significantly lower ($P < 0.00001$) APC sensitivity ratios (APC-sr=1.54, 95%CI=1.48–1.60) than controls (APC-sr=2.17, 95%CI=2.05–2.28). This indicates that R2-FV has reduced cofactor activity in APC-catalyzed FVIII(a) inactivation. The changes of the relative amounts of FV₁ and FV₂ in carriers of the R2-gene will result in increased thrombin formation in the presence of APC and may provide a mechanistic explanation for the increased thrombotic risk associated with the R2-haplotype.

6.2 Introduction

Down-regulation of the activity of blood coagulation factors Va and VIIIa via the protein C/protein S system plays an important role in the modulation of thrombin formation [31, 10]. The physiological importance of the protein C pathway is shown by the observations that deficiencies in either protein C [12, 34] or protein S [33, 6] and resistance against the anticoagulant action of APC [7, 45, 3, 17, 28] (APC resistance) are associated with familial thrombophilia. In the majority of cases, APC-resistance is due to a mutation in the FV gene (FV_{Leiden}) which results in a FV molecule, often called FV_{Leiden}, in which Arg⁵⁰⁶ is replaced by Gln [3, 11, 44, 45]. Since Arg⁵⁰⁶ is a prime target site for cleavage of FVa by APC, factor Va_{Leiden} is less efficiently down-regulated by APC [16, 23, 30]. In addition, it has been reported that the ability of FV to serve as cofactor in APC-dependent inactivation of FVIII and FVIIIa [35, 43] is strongly diminished in the case of FV_{Leiden} [42, 38]. Recently, a biallelic polymorphism (A4070G, R1/R2) has been discovered in exon 13 of the FV gene. The G (R2) allele of this polymorphism, which predicts the His¹²⁹⁹→Arg substitution in the B-domain of FV, is invariably linked to a series of other FV missense polymorphisms (R2-haplotype) [2], and is associated with mild APC-resistance [2] and with moderately reduced plasma FV levels [21]. Recently, it has been reported that carriership of the R2-gene appears to be associated with increased risk of venous thrombosis [1] and coronary artery disease [5]. To gain more insight into the mechanisms responsible for the

6.3 Experimental Procedures

increased risk of thrombotic disease we compared the functional properties of FV present in plasma from individuals with different R2 genotypes, in normal pooled plasma and in plasma from age-matched controls without the R2 allele.

6.3 Experimental Procedures

Materials Dioleoylphosphatidylserine (DOPS) and dioleoylphosphatidylcholine (DOPC) were purchased from Avanti Polar Lipids, Pelham, AL., USA. Small unilamellar phospholipid vesicles were prepared as described before [29]. D-Phe-pipecolyl-Arg-*p*-nitroanilide (S2238), L-pyroGlu-Pro-Arg-pNA (S2366), and kits for the aPTT-based APC resistance test (Coatest® APC™) were supplied by Chromogenix, Mölndal, Sweden. N- α -(2-naphtyl-sulfonyl-glycyl)-4-amidinophenylalanin piperidide acetate salt (Pefablock® TH) was supplied by Pentapharm AG, Basel, Switzerland. The Immunochrom® APC response kit was a kind gift of Dr. H.P. Schwarz and Dr. K. Váradi (Baxter AG, Vienna). At present, this kit is not commercially available. Materials used for protein purification were purchased from Pharmacia, Uppsala, Sweden. Human prothrombin, thrombin and FXa were prepared by earlier described procedures [9, 27, 4]. Human APC and human protein S were preparations from Enzyme Research Laboratories Inc. (Swansea, UK) supplied by Kordia, Leiden, The Netherlands. Human factor Va₁ and factor Va₂ were purified as described before [29].

Blood collection Nine parts of venous blood from consenting volunteers was collected in one part sodium citrate (129 mmol/l) and immediately centrifuged at $2000 \times g$ for 20 minutes at 4 °C. Plasma was separated, snap-frozen and stored in aliquots at -80 °C until analysis. Pooled normal plasma was prepared from plasma of healthy volunteers (40 females not using OC and 50 males, mean age 35 years).

Carriers of the R2-gene and controls Homozygous carriers of the R2-gene were identified [5] among patients with coronary artery disease ($n=3$, age > 60 years, MM, GL, BZ), patients with cerebrovascular disease ($n=1$, age 59 years, DG), thrombotic patients who suffered an episode of pulmonary embolism ($n=1$, age 76, BA) and controls ($n=1$, age 42 years, MS). Included in this study were also a healthy R1/R2-heterozygous son (age 30 years, SL) of a R2 homozygote with coronary artery disease (GL) and a healthy R1R2 heterozygous individual (age 64 years, FT). The population of controls used in this study consisted of healthy individuals from the same geographic region who belonged to the same age group as the patients with thrombosis (mean age 60, range 54–64 years). For all controls the absence of the FV_{Leiden} mutation and of the R2-genotype was confirmed by DNA-analysis [5]. Informed consent to participation in the study was obtained from all individuals.

Time course of FV activation by thrombin Plasma was diluted 1000-fold in 25 mM Hepes (pH 7.5 at 37 °C), 175 mM NaCl, 2 mM CaCl₂, 20 μ M phospholipid vesicles (DOPS/DOPC

10/90 mol/mol) and 5 mg/ml BSA. FV activation was started with thrombin (final concentration 0.5 nM). At indicated times, aliquots were taken in which FVa was quantified by measuring its ability to stimulate FXa-catalyzed prothrombin activation at 37 °C in a reaction mixture containing 25 mM Hepes (pH 7.5), 175 mM NaCl, 2 mM CaCl₂, 5 mg/ml BSA, 5 nM FXa, 20 μM phospholipid vesicles (DOPS/DOPC 10/90 mol/mol), 1 μM prothrombin and 2 μM Pefablock TH. The reversible thrombin inhibitor Pefablock TH was present to prevent further activation of FV [36]. Thrombin formation was quantified with S2238 [32]. From the rate of prothrombin activation thus determined, the percentage of FV activated at each time point was calculated (the level obtained after complete activation of FV was taken as 100%). The second-order rate constant of FV activation was obtained by fitting the data to a single exponential equation using nonlinear least-squares analysis.

FXaVa complex formation The ability of FVa to form a complex with FXa and express co-factor activity in prothrombin activation was determined by measuring rates of prothrombin activation at a fixed, limiting amount of plasma FVa (~1.5 pM) and varying FXa concentrations. FV present in plasma, diluted 1000-fold in 25 mM Hepes, 175 mM NaCl, 5 mg/ml BSA, 3 mM CaCl₂, was activated with 0.5 nM thrombin at 37 °C. Rates of prothrombin activation were determined at 37 °C in a reaction mixture containing 25 mM Hepes, 175 mM NaCl, 5 mg/ml BSA, 2 mM CaCl₂, 2 μM phospholipid vesicles (DOPS/DOPC 5/95 mol/mol), 1 μM prothrombin, 1.5 pM plasma FVa (final plasma dilution 15,000-fold) and varying FXa concentrations (0.1–10 nM). Prothrombin activation was quantified with S2238 [32]. The apparent K_d for dissociation of the FXaVa complex ($K_{\frac{1}{2}FXa}$) and the prothrombin-converting activity of this complex ($V_{Xa \rightarrow \infty}$) were obtained by fitting the data to the equation for a single-site binding isotherm (hyperbola) using nonlinear least-squares analysis.

Kinetic parameters of prothrombin activation Plasma was diluted 1000-fold in 25 mM Hepes, 175 mM NaCl, 3 mM CaCl₂, 5 mg/ml BSA and FV present was activated with 0.5 nM thrombin at 37 °C. Initial rates of prothrombin activation were measured at 37 °C with S2238 [32] in a reaction mixture containing 25 mM Hepes (pH 7.5), 175 mM NaCl, 2 mM CaCl₂, 5 mg/ml BSA, 20 μM phospholipid vesicles (DOPS/DOPC 5/95 mol/mol), 2 nM FXa, ~1.5 pM plasma FVa (final plasma dilution 15,000 fold) and varying prothrombin concentrations (0.06–1 μM). The kinetic parameters (K_m for prothrombin and V_{max} of thrombin formation) were obtained by fitting the data to the Michaelis-Menten equation using nonlinear least-squares analysis.

FVa inactivation by APC FV present in plasma, diluted 1000-fold in 25 mM Hepes (pH 7.5), 175 mM NaCl, 3 mM CaCl₂, 5 mg/ml BSA, 40 μM phospholipid vesicles (DOPS/DOPC, 10/90, mol/mol) with or without 200 nM protein S, was activated at 37 °C with 0.5 nM thrombin. The amount of FVa was determined in triplicate by transfer of small aliquots to reaction mixtures containing 25 mM Hepes (pH 7.5), 175 mM NaCl, 3 mM CaCl₂, 5 mg/ml BSA, 40 μM phospholipid vesicles (DOPS/DOPC, 10/90, mol/mol), 5 nM FXa, and 0.5 μM prothrombin [37].

6.3 Experimental Procedures

From the rate of prothrombin activation determined in these mixtures [32] the FVa concentration present at time zero was calculated. Inactivation of FVa was started by addition of 0.21 nM APC (without protein S) or 0.16 nM APC (with protein S). Rate constants of APC-catalyzed cleavage at Arg⁵⁰⁶ and Arg³⁰⁶ were determined from the time courses of FVa inactivation as described before [23, 30]

aPTT-based APC-resistance measurements The aPTT-based APC resistance test was performed in undiluted plasma or in plasma diluted 5-fold in FV-deficient plasma as described by the supplier (Chromogenix, Mölndal, Sweden). Clotting times were determined on an ACL 300R coagulation analyzer (Instrumentation Laboratory, Milan, Italy). The normalized APC sensitivity ratio (nAPC-sr) was defined as the ratio of the clotting times (aPTT) determined in the presence and absence of APC normalized by division by the ratio determined in the normal plasma pool [8].

Determination of FVIII levels and sensitivity to APC via APC-catalyzed FVIII inactivation Sensitivity of FVIII to APC was measured with the Immunochrom APC response reagent kit [41] following the manufacturer's instructions. This assay is based on the capacity of APC to limit generation of FXa by the tenase complex, via inactivation of FVIII/FVIIIa in plasma. The APC sensitivity ratio was calculated as the ratio of FXa generated in plasma without and with APC. FVIII levels were obtained from the activities determined in the absence of APC and were expressed as percentage of FVIII present in the normal plasma pool.

Quantification of FV, FV₁ and FV₂ in plasma FV₁ and FV₂ were determined via an assay which is based on quantification of the differences in cofactor activity of factors Va₁ and Va₂ in prothrombin activation, determined at limiting amounts of FVa, FXa and phospholipid vesicles with a low phosphatidylserine content (*cf.* Hoekema *et al.* [15]). Details of this assay will be published elsewhere. FV present in plasma samples, that were diluted 2500-fold [assay 1] or 12500-fold [assay 2] in 25 mM Hepes (pH 7.5), 175 mM NaCl, 5 mg/ml BSA, 2.64 mM CaCl₂ and 10 μ M DOPS/DOPC (20/80 mol/mol) [assay 1] or 40 μ M DOPS/DOPC (2.5/97.5 mol/mol) [assay 2], was activated by incubation at 37 °C with 1 nM thrombin during 10 min. The FVa generated was subsequently quantified by measuring initial rates of thrombin formation [32] in prothrombinase-based assay systems containing saturating amounts of FXa (5 nM), prothrombin (1 μ M) and phospholipid vesicles (10 μ M DOPS/DOPC 20/80, M/M) [assay 1] and suboptimal amounts of FXa (0.5 nM), prothrombin (1 μ M) and phospholipid vesicles (40 μ M DOPS/DOPC, 2.5/97.5, M/M) [assay 2]. In assay 1, FVa₁ and FVa₂ express the same cofactor activity in prothrombin activation. Hence, this assay quantifies the total FV level (FV₁ + FV₂) in plasma. In assay 2, FVa₂ has approximately an 8-fold higher cofactor activity than FVa₁. Therefore, the response in this assay depends on the relative amounts of FV₁ and FV₂ in the plasma sample. From calibration curves, made with known concentrations of purified FVa₁ and FVa₂, the amounts of FV₁ and FV₂ present in plasma samples can be calculated. The total

Gene position	Exon 8	Exon 13	Exon 13	Exon 13	Exon 25
Protein domain	A2	B	B	B	C2
Amino acid position	385	830	837	1299	2194
R1 alleles (normal)	Met/Thr	Arg/Lys	Arg/His	His	Asp
R2 allele	Thr	Lys	His	Arg	Gly ^a

Table 6.1: **FV amino acid polymorphisms and definition of R2-FV.** Plasma from the following carriers of the R2 allele were used in this study: BA, DG, GL, MM, MS (R2-homozygous, genotype R2/R2); BZ (R2-homozygous, but heterozygous for the Asp/Gly mutation at amino acid 2194, genotype R2/R2^{*}); SL and FT (heterozygous, genotype R1/R2). Data from refs. [2, 5].

a. An Asp is encoded at this position in 6% of R2-genes

amount of FV (FV₁ plus FV₂) present in plasma was expressed as a percentage of FV in pooled normal plasma and FV₁ and FV₂ levels were expressed as the ratio FV₂/FV₁.

Statistical analysis The statistical significance of differences between coagulation parameters determined in plasma from carriers of the R2 polymorphism and controls was assessed with the Student's *t*-test assuming unequal variance.

6.4 Results

Genotypes of carriers of the R2-gene The R2-haplotype, which marks the R2-gene, is characterized by a series of linked mutations in the FV gene. Table 6.1 summarizes the genotypes of the carriers of the R2-gene studied in this investigation. The plasma samples used were: a normal plasma pool, plasma from homozygous carries of the R2-gene (genotype R2/R2: BA, DG, GL, MM, MS), an individual homozygous for the R2-gene but heterozygous for the Asp²¹⁹⁴/Gly²¹⁹⁴ mutation (genotype R2/R2^{*}: BZ), two heterozygotes with genotype R1/R2 (SL, FT), and plasma from 19 age-matched controls from the same geographic region who did not carry the R2-gene.

Functional properties of R2-FV(a) In order to gain insight into the effects of the amino acid changes encoded by the R2-gene on the functional properties of R2-FV(a), *i.e.* on the activation of FV by thrombin, the expression of cofactor activity of FVa in prothrombin activation and the susceptibility of FVa to APC, were studied in model systems containing purified proteins. In these experiments plasma samples, diluted more than thousand-fold, were used as a source of FV(a). Highly diluted plasma samples were used to exclude effects of other plasma components on the functional properties of FV(a). The fact that the kinetic parameters determined for FV(a) in diluted normal plasma were virtually the same as those of purified FV(a) (see below) and were very close to literature values[23, 30, 29, 36, 22], in our opinion validates the kinetic approach and indicates that other plasma proteins indeed did not interfere with the analysis.

6.4 Results

	Procoagulant pathway			Anticoagulant Pathway			
	$k_{\text{activation}}$ ($\text{M}^{-1}\text{s}^{-1}$)	$K_{\frac{1}{2}\text{Xa}}$ (nM)	$K_{m,PT}$ (μM)	- protein S k_{506} ($\text{M}^{-1}\text{s}^{-1}$)	k_{306} ($\text{M}^{-1}\text{s}^{-1}$)	+ protein S k_{506} ($\text{M}^{-1}\text{s}^{-1}$)	k_{306} ($\text{M}^{-1}\text{s}^{-1}$)
R2-carriers	$9.3 \pm 0.1 \times 10^6$	0.57 ± 0.02	0.29 ± 0.01	$3.5 \pm 0.2 \times 10^7$	$1.5 \pm 0.1 \times 10^6$	$4.7 \pm 0.1 \times 10^7$	$1.3 \pm 0.1 \times 10^7$
Normal plasma	$9.3 \pm 0.1 \times 10^6$	0.48 ± 0.02	0.28 ± 0.003	$4.0 \pm 0.2 \times 10^7$	$1.3 \pm 0.1 \times 10^6$	$4.9 \pm 0.1 \times 10^7$	$1.2 \pm 0.1 \times 10^7$
Purified FV	9.2×10^6	0.42	0.31	4.5×10^7	1.9×10^6	4.9×10^7	1.4×10^7

Table 6.2: **Functional properties of normal FV(a) and R2-FV(a).** Functional properties of normal FV(a) and R2-FV(a) were determined in highly diluted plasma samples as described under "Materials and Methods" and in the legends to Fig 6.1 and Fig 6.2. Numbers given are average values ($\pm$ S.E.M.) for FV(a) present in pooled normal plasma and in plasma from carriers of the R2-gene ($n=6$).

Activation of FV and cofactor activity of FVa Figure 6.1A shows the time-courses of activation of FV and R2-FV by thrombin. The activation of FV in a highly diluted plasma pool of normal individuals and in plasma samples from carriers of the R2-gene appeared to be indistinguishable. The second-order rate constants of thrombin-catalyzed activation of FV and R2-FV, calculated from the activation time-courses were the same as those determined for purified FV (Table 6.2).

Figure 6.1B,C demonstrates that the FVa formed in the different plasma samples expressed similar cofactor activities in prothrombin activation. The cofactor activities of FVa were assessed in assay systems which contained phospholipid vesicles with low phosphatidylserine content, conditions which optimally reflect effects of FVa on the assembly of the prothrombinase complex via direct interactions with FXa [18, 40] and prothrombin [40, 39]. The FXa concentrations required to obtain half-maximal rates of prothrombin activation at limiting amounts of FVa ($K_{\frac{1}{2}\text{Xa}}$) and the K_m for prothrombin ($K_{m,PT}$), were obtained from titrations with FXa (Fig 6.1B) and prothrombin (Fig 6.1C), respectively, and are summarized, together with those of purified FV, in Table 6.2. The $K_{\frac{1}{2}\text{Xa}}$ for R2-FVa ($K_{\frac{1}{2}\text{Xa}} = 0.53\text{--}0.62$ nM) was slightly higher than that for normal FVa ($K_{\frac{1}{2}\text{Xa}} = 0.48$ nM) and the K_m for prothrombin obtained for R2-FVa ($K_{m,PT} = 0.28\text{--}0.31$ μM) was almost the same as the K_m for normal FVa ($K_{m,PT} = 0.27\text{--}0.28$ μM).

Inactivation of FVa by APC in the absence and presence of protein S Time courses of inactivation of FVa and R2-FVa by APC were determined both in the absence (Fig 6.2A) and in the presence (Fig 6.2B) of a suboptimal concentration [30] of protein S (200 nM). The rate constants for APC-catalyzed cleavage at Arg⁵⁰⁶ (k_{506}) and Arg³⁰⁶ (k_{306}), calculated from the time courses of FVa inactivation (*cf.* Nicolaes *et al.*, ref. [23] and Rosing *et al.*, ref. [30]) are summarized in Table 6.2. It appears that FVa and R2-FVa were equally susceptible to inactivation

Figure 6.1: **Procoagulant properties of normal FV and R2-FV.** (A) Time courses of activation of normal FV and R2-FV by thrombin were determined in highly diluted plasma samples. At the indicated time points the amount of FVa generated was quantified with a prothrombinase-based FVa assay. (B) After complete activation of FV, the apparent affinity of FVa for FXa was determined by measuring the rate of activation of 1 μ M prothrombin on 2 μ M DOPS/DOPC vesicles (5/95 mol/mol) at a limiting concentration FVa and varying FXa concentrations. (C) Kinetic parameters of prothrombin activation were determined by measuring the rate of prothrombin activation on 20 μ M DOPS/DOPC vesicles (5/95 mol/mol) at 2 nM FXa, 1.5 pM plasma FVa and varying prothrombin concentrations. ◆, normal plasma pool; ▲, MM; △, SL; ●, GL; ○, DG; ■, BA; □, BZ. Further experimental details are given under "Materials and Methods". Rate constants of thrombin-catalyzed FV activation, the $K_{1/2Xa}$ and the K_m for prothrombin are summarized in Table 6.2.

by APC and that protein S stimulated APC-catalyzed inactivation of normal FVa and R2-FVa to the same extent.

Plasma levels of FVIII, FV, FV₁ and FV₂ The amounts of FVIII (expressed as % of a normal plasma pool) present in plasma from carriers of the R2-gene and of age-matched controls are summarized in Fig 6.3A. The FVIII levels determined in plasma from the five homozygous R2 carriers (mean FVIII = 156%, 95%CI = 145%–166%) were significantly higher ($P < 0.0001$) than those present in plasmas of the age-matched controls (mean FVIII = 112%, 95%CI = 100%–124%). The two heterozygous carriers of R2 (genotype R1/R2) had lower FVIII (81%). Plasma levels of FV, expressed as % of pooled normal plasma, were moderately but significantly ($P < 0.01$) decreased in carriers of the R2-allele (98%, 95%CI = 88%–107%) as compared to the age-matched controls (119%, 95%CI = 113%–125%) and there were no obvious differences between the plasmas from individuals with the different genotypes (Fig 6.3B). Recently, we reported that the relative amounts of FV₁ and FV₂ in plasma from carriers of the R2-gene considerably differ from that in plasma from R2-free controls [5]. Since it is pertinent to the discussion about the molecular basis of the increased risk of thrombotic disease in carriers of the R2-gene, these data are also shown in the present paper (Fig 6.3C). The FV₂/FV₁ ratio in the plasmas of the two heterozygous individuals (genotype R1/R2) as well as in plasma from the carrier of genotype R2/R2*, who is heterozygous for the Asp²¹⁹⁴→Gly mutation, were in the lower range of the controls (Fig 6.3C). In marked contrast, the FV₂/FV₁ ratio in plasmas of the homozygotes with genotype R2/R2 (FV₂/FV₁ ratio = 1.4, 95%CI = 1.3–1.5) was significantly lower (P_i 0.00001) than in control plasmas (FV₂/FV₁ ratio = 2.8, 95%CI = 2.5–3.1).

6.4 Results

Figure 6.2: **Inactivation of normal FVa and R2-FVa by APC and protein S.** FV, present in a highly diluted plasma sample, was activated with thrombin in a reaction mixture containing 25 mM Hepes (pH 7.5 at 37°C), 175 mM NaCl, 3 mM CaCl₂, 5 mg/ml BSA, 40 μ M phospholipid vesicles (DOPS/DOPC 10/90 mol/mol) without (A) or with 200 nM protein S (B). FVa inactivation was started with 0.21 nM APC (A) or 0.16 nM APC (B). At the indicated time points the remaining FVa activity was determined as described under "Materials and Methods". The rate constants of inactivation (summarized in Table 6.2) were obtained by nonlinear least-squares analysis as described before [23, 30]. ♦, normal plasma pool; ▲, MM; △, SL; ●, GL; ○, DG; ■, BA; □, BZ.

Figure 6.3: **Plasma levels of FVIII, FV, FV₁ and FV₂.** The plasma levels of FVIII, FV and FV₁ and FV₂ were determined as described under "Materials and Methods". FVIII (A) and FV levels (B) are expressed as percentage of that present in a normal plasma pool. FV₂ and FV₁ were expressed as the FV₂/FV₁ ratio (C) determined in each individual plasma. ♦, controls (genotype R1/R1), ●, homozygous carriers of the R2-allele (genotype R2/R2); ○, heterozygous carriers of the R2-allele (genotype R1/R2); □, genotype R2/R2* (BZ). In the population of homozygous carriers of the R2-allele (●) one individual was included (MS) for who no FVIII level and APC-sr (Fig 6.4) were determined.

Figure 6.4: **APC-sr of plasma from carriers of the R2-polymorphism and controls.** APC-sr were determined with (A) the aPTT-based APC resistance test, (B) the aPTT-based resistance test in which subject plasma is diluted 5-fold in FV-deficient plasma and (C) with the Immunochrom APC response kit, an APC resistance test which quantifies the cofactor activity of FV in APC-catalyzed FVIII(a) inactivation. ◆, controls (genotype R1/R1), ●, homozygous carriers of the R2-allele (genotype R2/R2); ○, heterozygous carriers of the R2-allele (genotype R1/R2); □, genotype R2/R2* (BZ).

Resistance to APC Normalized APC sensitivity ratios (nAPC-sr) were determined in plasma from carriers of the R2-allele and in 19 age-matched controls with the aPTT-based APC resistance test in both undiluted plasma (Fig 6.4A) and in plasma diluted 5-fold in FV-deficient plasma (Fig 6.4B). The assay performed in undiluted plasma showed minor differences between the nAPC-sr of the R2-plasmas and controls (Fig 6.4A), but nAPC-sr determined in plasma diluted in FV-deficient plasma (Fig 6.4B) were significantly lower ($P < 0.001$) in carriers of the R2-gene (nAPC-sr = 0.96, 95%CI = 0.93–1.00) than in the controls (nAPC-sr = 1.06, 95%CI = 1.03–1.09). APC-sr were also determined with the Immunochrom APC response kit (Fig 6.4C), an APC-resistance test which quantifies the ability of FV to act as cofactor in APC-dependent inactivation of FVIII(a) [43, 41]. This assay revealed a large difference ($P < 0.00001$) between homozygous R2 carriers with genotype R2/R2 (APC-sr = 1.54, 95%CI = 1.48–1.60) and the controls (APC-sr = 2.17, 95%CI = 2.05–2.28). The APC-sr of homozygous carriers of the R2-allele (genotype R2/R2) were only slightly higher than those of heterozygous carriers of FV_{Leiden} using the same assay (APC-sr = 1.38, 95%CI = 1.34–1.42, data not shown). The APC-sr values determined for the two heterozygous R1R2 individuals (genotype R1R2) and the individual with genotype R2/R2* were in the range of those obtained in the control population.

Correlations between APC-sr, FV and FVIII levels It has been reported that high plasma levels of FVIII cause a decrease of the APC-sr in the aPTT-based APC resistance test [14] and that, compared with FV₂, FV₁ is a poor cofactor in APC-catalyzed FVIIIa inactivation [42]. Therefore, we have tested whether correlations existed between the nAPC-sr and the plasma levels of

6.4 Results

	APC-sr		
	R1	R2	R1+R2
FVIII	$r = 0.78^{***}$	$r = 0.95^{**}$	$r = 0.82^{***}$
FV	$r = 0.30$	$r = 0.48$	$r = 0.58^{**}$
FV ₂	$r = 0.69^{**}$	$r = 0.81^*$	$r = 0.84^{***}$
FVIII/FV ₂	$r = 0.80^{***}$	$r = 0.86^{**}$	$r = 0.89^{***}$

Table 6.3: **Correlations between APC-sr and levels of FVIII, FV, FV₂ and FVIII/FV₂ ratios.** Pearson correlation coefficients and *P*-values were calculated for the APC-sr determined with the Immunochrom APC response kit as a function of the levels of FVIII (% of normal plasma), FV (% of normal plasma), FV₂ (% relative to total FV present in subject plasma) and the FVIII/FV₂ ratio in which FVIII was expressed as % of normal plasma and FV₂ as % of total FV present in subject plasma. Correlations are calculated for controls without R2 allele (R1, *n*=19), carriers of the R2 allele (R2, *n*=7) and for all subjects under study (controls and R2-carriers; R1+R2, *n*=26). * *P* < 0.05; ** : *P* < 0.005; *** : *P* < 0.0005

Figure 6.5: **Correlation between the APC-sr determined with the Immunochrom APC response kit, FVIII and FV₂.** The regression lines given are calculated for (A) the APC-sr determined with the Immunochrom APC response kit as function of the FVIII/FV₂ ratio and (B) levels of FVIII as function of FV₂ determined in plasma from controls (○) and carriers of the R2-allele (●).

FVIII, FV, FV₂ and the FVIII/FV₂ ratio (Table 6.3). nAPC-sr determined with the aPTT-based APC resistance tests showed rather poor correlations ($r < 0.5$) with the amounts of FVIII, FV and FV₂ (data not shown). However, the correlations between the APC-sr determined with the Immunochrom APC response kit and the plasma levels of FVIII, FV, FV₂ (Table 6.1), and with the FVIII/FV₂ ratio (Table 6.3, Fig 6.5A) were significant for both controls and carriers of the R2 allele (except for FV) and for all subjects under study. There also appeared to be an association between the plasma levels of FV₂ and FVIII (Fig 6.5B). Individuals with low plasma concentrations FV₂ had high levels of FVIII ($P < 0.005$).

6.5 Discussion

The R2-FV gene is associated with slightly decreased levels of FV₂ [21] and moderate APC resistance [2]. Recently, it was reported that carriers of the R2-gene have 2–3 fold increased risks of venous thrombosis [1] and coronary artery disease [5]. In order to get more insight into the molecular basis of this increased risk of thrombotic disease we have compared the functional properties of FV and R2-FV in model systems and in plasma.

Our experiments in model systems indicate that FV and R2-FV have similar properties in the procoagulant pathway and are equally susceptible to direct down-regulation via the protein C pathway. Considerable differences were observed, however, between a number of haemostatic parameters determined in plasma from carriers of the R2-gene and age-matched controls. Our data confirm that the R2-gene is associated with moderately reduced levels of FV and mild APC resistance. The latter phenomenon is particularly observed when the aPTT-based APC resistance test is performed on plasma samples diluted in FV-deficient plasma. This indicates that the increased resistance to APC established with the aPTT-based APC resistance test is due to R2-FV and is not caused by other plasma variables. The impaired response to APC in carriers of the R2-polymorphism is even more pronounced in an assay that quantifies the cofactor activity of FV in the inactivation of FVIIIa by APC (the Immunochrom APC response kit). In this APC resistance test, homozygous carriers of the R2-gene (genotype R2/R2) have APC-sr that are in the range of carriers of FV_{Leiden}. This suggests that R2-FV, like FV_{Leiden} [42, 38] has a reduced ability to act as a cofactor in APC-catalyzed FVIII(a) inactivation. Our observations further reveal that homozygous carriers of the R2-gene with genotype R2/R2 have higher plasma levels of FVIII and that the ratio of the two isoforms of FV, FV₁ and FV₂, is shifted in favor of FV₁. Since differences between FV₁ and FV₂ appear to be due to different degrees of glycosylation in the C2 domain of the light chain region of FV [25, 26, 24], we proposed that the decreased relative amounts of FV₂ present in plasma from carriers of the R2-gene are caused by the amino acid substitution (Asp²¹⁹⁴→Gly) [5], which is not far from the putative glycosylation site at Asn²¹⁸¹. This hypothesis is supported by the fact that the homozygous carrier of the R2-gene who is heterozygous for the amino acid substitution in the C2 domain (genotype R2/R2*) has a factor FV₂/FV₁ ratio in the range of heterozygous carriers of R2 (genotype R1/R2). Theoretically, the relative reduction of FV₂ in carriers of the R2-gene (20%) has to be accompanied by small changes in the procoagulant activity of FVa [29, 15] and in susceptibility to APC [15]. Indeed, the ability of R2-FVa to promote the prothrombinase complex formation, was slightly reduced ($K_{\frac{1}{2}Xa} \sim 0.57$ nM) as compared with normal FVa ($K_{\frac{1}{2}Xa} \sim 0.48$ nM). No differences were observed, however, in sensitivity for APC (Fig 6.3AB, Table 6.2). Due to experimental limitations (dilution of plasma and interference by plasma components), APC-catalyzed FVa inactivation could only be analyzed under reaction conditions (40 μ M DOPS/DOPC, 10/90 M/M) at which FVa₁ and FVa₂ exhibit marginal differences in susceptibility for APC [15]. The differences in FV₂/FV₁ ratio, *i.e.* reduced levels of FV₂, may explain the lower APC-sr obtained with the Immunochrom APC response kit. This APC resistance test quantifies the ability of FV to promote APC-mediated FVIII(a) inactivation [41] and it has been shown that, compared with FV₂, the cofactor activity of FV₁ is substantially reduced [42]. It has been reported that the B domain of FV plays an important role in the expression of cofactor activity

6.5 Discussion

of FV in APC-mediated FVIIIa inactivation [42, 38]. Hence, we cannot exclude the possibility that besides the Asp²¹⁹⁴→Gly mutation, missense mutations in other regions of R2-FV may contribute to APC resistance.

As yet, limited information is available on the molecular mechanism by which FV enhances APC-catalyzed FVIII inactivation. Good correlations between the APC-sr determined with the Immunochrom APC resistance test and the plasma levels of FVIII, FV₂ and the FVIII/FV₂ ratio (Fig 6.5A, Table 6.3) are present in the control group, in carriers of the R2 allele as well as in the group containing both controls and R2-carriers. This indicates that the correlations are not artificially created by mixing two distinct groups. The best correlation is observed between APC-sr and the FVIII/FV₂ ratio, which indicates that the actual anticoagulant effect of FV in FVIII inactivation by APC is mainly determined by the FVIII/FV₂ ratio.

It is more difficult to explain why carriers of the R2-gene have higher levels of FVIII. Since the non-activated cofactors, FV and FVIII, are also susceptible to proteolysis by APC [20] and low levels of APC are circulating in plasma [13], it is tempting to speculate that steady state inactivation of FVIII by APC influences the plasma FVIII concentration. In that case, individuals with the R2-gene may have higher levels of plasma FVIII due to impaired FVIII(a) inactivation by circulating APC (Fig 6.4C). This hypothesis is supported by the observation that low levels of plasma FV₂, the actual cofactor in APC-catalyzed FVIII inactivation [42], are associated with high levels of FVIII (Fig 6.5B) and by a recent report [19] which indicated that carriers of FV_{Leiden}, which like R2-FV is a poor cofactor in FVIII inactivation [42, 38], have increased FVIII levels.

Finally, our observations may also provide a mechanistic explanation for the increased risk of venous [1] and arterial thrombosis [5] associated with carriership of the R2-gene. We have previously shown [15] that in model systems which mimic physiological conditions (*i.e.* activation of FV, expression of cofactor activity in prothrombin activation and down-regulation of FVa by the protein C/protein S system), FV₁ gives rise to a 7-fold higher thrombin generation than FV₂. We further demonstrated that FV₁ has a much lower cofactor activity in APC-mediated FVIII(a) inactivation than FV₂ [42]. Taken together, these phenomena may explain why carriers of the R2-gene, for whom it is shown in this chapter that the ratio of FV₂/FV₁ is shifted in favor of FV₁ and that they are more resistant to APC in a FVIII(a) based APC resistance test, are exposed to a higher risk of thrombotic disease [1, 5].

Acknowledgments We wish to thank Dr. H.P. Schwarz and Dr. K. Váradi from Baxter AG (Vienna, Austria) for providing us the Immunochrom® APC response kit.

6.6 References

- [1] 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. *Thrombosis and Haemostasis*, 82(2):193–197, 1999.
- [2] 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*, 90(4):1552–1557, 1997.
- [3] 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*, 369(6475):64–67, 1994.
- [4] P.E. Bock, P.A. Craig, S.T. Olson, and P. Singh. Isolation of human blood coagulation alpha-factor Xa by soybean trypsin inhibitor-sepharose chromatography and its active-site titration with fluorescein mono-p-guanidinobenzoate. *Arch-Biochem-Biophys*, 273:375–388, 1989.
- [5] E. Castoldi, J. Rosing, D. Girelli, L. Hoekema, B. Lunghi, F. Mingozzi, P. Ferraresi, S. Friso, R. Corrocher, G. Tans, and F. Bernardi. Mutations affecting post-translational processing of coagulation factor v are associated with coronary artery disease. *submitted for publication*.
- [6] P.C. Comp, R.R. Nixon, M.R. Cooper, and C.T. Esmon. Familial protein S deficiency is associated with recurrent thrombosis. *Journal of Clinical Investigation*, 74(6):2082–2088, 1984.
- [7] B. Dahlbäck, 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. *Proceedings of the National Academy of Sciences USA*, 90(3):1008, 1993.
- [8] H. de Ronde and R.M. Bertina. Laboratory diagnosis of APC-resistance: A critical evaluation of the test and the development of diagnostic criteria. *Thrombosis and Haemostasis*, 72(6):880–886, 1994.
- [9] R.G. DiScipio and E.W. Davie. Characterization of protein S, a gamma-carboxyglutamic acid containing protein from bovine and human plasma. *Biochemistry*, 18(5):899–904, 1979.
- [10] P. H. Fay, T. M. Smudzins, and F. J. Walker. Activated protein C-catalyzed inactivation of human factor VIII and factor VIIIa. *Journal of Biological Chemistry*, 266(30):20139–20145, 1991.
- [11] J.S. Greengard, X. Sun, X. Xu, J.A. Fernandez, J.H. Griffin, and B. Evatt. Activated protein C resistance caused by Arg⁵⁰⁶ → Gln mutation in factor Va. *Lancet*, 343(8909):1361–1362, 1994.
- [12] J.H. Griffin, B. Evatt, T.S. Zimmerman, A.J. Kleiss, and C. Wideman. Deficiency of protein C in congenital thrombotic disease. *Journal of Clinical Investigation*, 68(5):1370–1373, 1981.
- [13] A. Gruber and J.H. Griffin. Direct detection of activated protein C in blood from human subjects. *Blood*, 79:2340–2348, 1992.
- [14] C.M.A. Henkens, V.J.J. Bom, and J. van der Meer. Lowered APC-sensitivity ratio related to increased factor VIII-clotting activity. *Thrombosis and Haemostasis*, 74:1198–1199, 1995.
- [15] L. Hoekema, G.A. Nicolaes, H.C. Hemker, G. Tans, and J. Rosing. Human factor Va: 1 and factor Va: 2: Properties in the procoagulant and anticoagulant pathways. *Biochemistry*, 36(11):3331–3335, 1997.
- [16] M. Kalafatis, R.M. Bertina, M.D. Rand, and K.G. Mann. Characterization of the molecular defect in factor V^{R506Q}. *Journal of Biological Chemistry*, 270(8):4054–4057, 1995.
- [17] T. Koster, F.R. Rosendaal, H. de Ronde, E. Briët, J.P. Vandenbroucke, and R.M. Bertina. Venous thrombosis due to poor anticoagulant response to activated protein C: Leiden thrombophilia study. *Lancet*, 342(8886-8887):1503–1506, 1993.

6.6 References

- [18] T. Lindhout, J.W.P. Govers-Riemslog, P. van de Waart, H.C. Hemker, and J. Rosing. Factor Va-factor Xa interaction. Effects of phospholipid vesicles of varying composition. *Biochemistry*, 21:5494-5502, 1982.
- [19] G.D.O. Lowe, A. Rumley, M. Woodward, E. Reid, and J. Rumley. Activated protein C resistance and FV^{R506Q} mutation in a random population sample. *Thrombosis and Haemostasis*, 81:918-924, 1999.
- [20] D.S. 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*, 87:4708-4717, 1996.
- [21] 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. *Thrombosis and Haemostasis*, 75:45-48, 1996.
- [22] D.D. Monkovic and P.B. Tracy. Activation of human factor V by factor Xa and thrombin. *Biochemistry*, 29:1118-1128, 1990.
- [23] G.A.F. Nicolaes, G. Tans, M.L.C.G.D. Thomassen, H.C. Hemker, K. Váradi, H.P. Schwarz, and J. Rosing. Peptide bond cleavages and loss of functional activity during inactivation of factor Va and factor Va^{R506Q} by activated protein C. *Journal of Biological Chemistry*, 270(36):21158-21166, 1995.
- [24] G.A.F. Nicolaes, B.O. Villoutreix, and B. Dahlbäck. 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 : in press*, 1999.
- [25] T.L. Ortel, D. Devore-Carter, M. Quinn-Allen, and W.H. Kane. Deletion analysis of recombinant human factor V. Evidence for a phosphatidylserine binding site in the second C-type domain. *Journal of Biological Chemistry*, 267(6):4189-98, 1992.
- [26] T.L. Ortel, M.A. Quinn-Allen, F.G. Keller, J.A. Peterson, D. Larocca, and W.H. Kane. Localization of functionally important epitopes within the second C-type domain of coagulation factor V using recombinant chimeras. *Journal of Biological Chemistry*, 269(22):15898-905, 1994.
- [27] C.H. Pletcher and G.L. Nelsestuen. The rate-determining step of the heparin-catalyzed antithrombin/thrombin reaction is independent of thrombin. *Journal of Biological Chemistry*, 257(10):5342-5345, 1982.
- [28] 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*, 85:1504-1508, 1995.
- [29] 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. *Journal of Biological Chemistry*, 268(28):21130-21136, 1993.
- [30] J. Rosing, L. Hoekema, G.A.F. Nicolaes, M.C.L.G.D. Thomassen, H.C. Hemker, K. Váradi, H.P. Schwarz, and G. Tans. Effects of protein S and factor Xa on peptide bond cleavages during inactivation of factor Va and factor Va^{R506Q} by activated protein C. *Journal of Biological Chemistry*, 270:27852-27858, 1995.
- [31] J. Rosing and G. Tans. Coagulation factor V: An old star shines again. *Thrombosis and Haemostasis*, 78 (1):427-433, 1997.
- [32] J. Rosing, G. Tans, J.W. Govers-Riemslog, R.F. Zwaal, and H.C. Hemker. The role of phospho-

- lipids and factor Va in the prothrombinase complex. *Journal of Biological Chemistry*, 255(1):274–283, 1980.
- [33] H.P. Schwarz, M. Fischer, P. Hopmeier, M.A. Batard, and J.H. Griffin. Plasma protein S deficiency in familial thrombotic disease. *Blood*, 64(6):1297–1300, 1984.
- [34] U. Seligsohn, A. Berger, M. Abend, L. Rubin, D. Attias, A. Zivelin, and S.I. Rapaport. Homozygous protein C deficiency manifested by massive thrombosis in the newborn. *New England Journal of Medicine*, 310:559–562, 1984.
- [35] L. Shen and B. Dahlbäck. Factor V and protein S as synergistic cofactors to activated protein C in degradation of factor VIIIa. *Journal of Biological Chemistry*, 269(29):18738, 1994.
- [36] G. Tans, G.A. Nicolaes, M.C. Thomassen, H.C. Hemker, A.J. van Zonneveld, H. Pannekoek, and J. Rosing. Activation of human factor V by meizothrombin. *Journal of Biological Chemistry*, 269(23):15969–15972, 1994.
- [37] G. Tans, J. Rosing, M.C. Thomassen, M.J. Heeb, R.F. Zwaal, and J.H. Griffin. Comparison of anticoagulant and procoagulant activities of stimulated platelets and platelet-derived microparticles. *Blood*, 77(12):2641–2648, 1991.
- [38] E. Thorelli, R.J. Kaufman, and B. Dahlbäck. The C-terminal region of the factor V B-domain is crucial for the anticoagulant activity of factor V. *Journal of Biological Chemistry*, 273(40):16140–16145, 1998.
- [39] P. van de Waart, H.C. Hemker, and T. Lindhout. Interaction of prothrombin with factor Va-phospholipid complexes. *Biochemistry*, 23:2838–2842, 1984.
- [40] J.L.M.L. van Rijn, J.W.P. Govers-Riemslog, 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*, 23:4557–4563, 1984.
- [41] K. Váradi, B. Moritz, H. Lang, K. Bauer, E. Preston, I. Peake, G.E. Rivard, B. Keil, and H.P. Schwarz. A chromogenic assay for activated protein C resistance. *British Journal of Haematology*, 90:884–891, 1995.
- [42] K. Váradi, 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 V^{R506Q} mutation. *Thrombosis and Haemostasis*, 76:208–214, 1996.
- [43] K. Váradi, J. Rosing, G. Tans, and H.P. Schwarz. Influence of factor V and factor Va on APC-induced cleavage of human factor VIII. *Thrombosis and Haemostasis*, 73(4):730–731, 1995.
- [44] J. Voorberg, J. Roelse, R. Koopman, H. Büller, 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*, 343(8912):1535–1536, 1994.
- [45] B. Zöller and B. Dahlbäck. Linkage between inherited resistance to activated protein C and factor V gene mutation in venous thrombosis. *Lancet*, 343(8912):1536–1538, 1994.

Chapter 7

General Discussion

7.1 APC-catalyzed inactivation of factor V and factor VIII

The APC is a serine protease that is responsible for the inactivation of factor V and factor VIII. It is a member of the trypsin family of serine proteases. The APC is a heterodimeric protein composed of a catalytic subunit (A2) and a cofactor subunit (A1). The catalytic subunit is responsible for the inactivation of factor V and factor VIII, while the cofactor subunit is responsible for the regulation of the catalytic subunit. The APC is a key component of the fibrinolytic system and is involved in the regulation of blood coagulation. The APC is a serine protease that is responsible for the inactivation of factor V and factor VIII. It is a member of the trypsin family of serine proteases. The APC is a heterodimeric protein composed of a catalytic subunit (A2) and a cofactor subunit (A1). The catalytic subunit is responsible for the inactivation of factor V and factor VIII, while the cofactor subunit is responsible for the regulation of the catalytic subunit. The APC is a key component of the fibrinolytic system and is involved in the regulation of blood coagulation.

The APC is a serine protease that is responsible for the inactivation of factor V and factor VIII. It is a member of the trypsin family of serine proteases. The APC is a heterodimeric protein composed of a catalytic subunit (A2) and a cofactor subunit (A1). The catalytic subunit is responsible for the inactivation of factor V and factor VIII, while the cofactor subunit is responsible for the regulation of the catalytic subunit. The APC is a key component of the fibrinolytic system and is involved in the regulation of blood coagulation. The APC is a serine protease that is responsible for the inactivation of factor V and factor VIII. It is a member of the trypsin family of serine proteases. The APC is a heterodimeric protein composed of a catalytic subunit (A2) and a cofactor subunit (A1). The catalytic subunit is responsible for the inactivation of factor V and factor VIII, while the cofactor subunit is responsible for the regulation of the catalytic subunit. The APC is a key component of the fibrinolytic system and is involved in the regulation of blood coagulation. The APC is a serine protease that is responsible for the inactivation of factor V and factor VIII. It is a member of the trypsin family of serine proteases. The APC is a heterodimeric protein composed of a catalytic subunit (A2) and a cofactor subunit (A1). The catalytic subunit is responsible for the inactivation of factor V and factor VIII, while the cofactor subunit is responsible for the regulation of the catalytic subunit. The APC is a key component of the fibrinolytic system and is involved in the regulation of blood coagulation.

7.1 APC-resistance

The discovery in 1993 by Dahlbäck *et al.* of venous thrombosis patients whose plasma was resistant to APC in an aPTT-based clotting assay revived the interest in the anticoagulant protein C system and blood coagulation factor V. The inheritance pattern found for APC-resistance spurred the quest for an explanation at the DNA-level and very soon after Dahlbäck's publication, four groups independently reported that a G to A substitution at position 1691 in the factor V gene was associated with APC-resistance [7, 82, 26, 78]. This substitution predicts a Arg→Gln replacement in the heavy chain domain of factor V. Since Arg⁵⁰⁶ is a cleavage site for APC it was generally accepted APC-resistance is caused by a direct effect of the reduced sensitivity of factor V^{R506Q} (factor V_{Leiden}) to APC. However, APC-resistance, can also occur in the absence of the factor V_{Leiden} mutation and without a clear inheritance pattern. This so-called acquired APC-resistance, has been linked with use of oral contraceptives [30, 48], lupus anticoagulants [20], and pregnancy [15].

Risk of carriership of factor V_{Leiden}

The risk for venous thrombosis associated with APC-resistance was determined in a case-control study and showed that in 20% of the patients with a first episode of deep vein thrombosis, APC-resistance is observed [38]. This number increases to approximately 40% when only idiopathic thrombotic complications are included. This makes APC-resistance the most frequent cause of hereditary venous thrombosis in the Western population. The risk increase associated with the factor V_{Leiden} mutation is about 7–8 fold for heterozygous carriers and for homozygotes it is approximately 80 fold [59]. The latter may be an overestimation since the majority of homozygous factor V_{Leiden} cases in this study were females, part of whom developed thrombosis due to the combination of carriership of factor V_{Leiden} and oral contraceptive use [74].

The risk increase for VTE in carriers of the factor V_{Leiden} mutation presented above is impressive, but in recent years it was recognized that the absolute risk for thrombosis is not dramatic [32, 65, 5]. Moreover, the majority of heterozygous carriers and even a substantial number of the homozygous carriers do not develop thrombosis [83]. Kaplan-Meier thrombosis-free survival curves obtained in a population-based study showed that at age 65, 94.3% of the carriers of the factor V_{Leiden} mutation, compared to 98.5% of controls had not developed venous thrombosis [58]. The risk for thrombosis associated with factor V_{Leiden} is especially increased when another risk factor is present. This 'second-hit' risk factor can either be hereditary or acquired. Examples of hereditary risk factors are (partial) deficiencies in coagulation factors of the protein C anticoagulant pathway [35, 36, 1] or AT-III deficiency [72]. Acquired risk factors, which amplify the thrombotic risk of carriers of factor V_{Leiden}, include pregnancy [9, 29], immobilization [21], surgery [69] or oral contraceptive use [74, 29, 8]. In summary, it is clear that factor V_{Leiden} is a risk factor for thrombosis, but that the risk increase is rather mild, especially in the absence of other risk factors.

Haplotype analysis of carriers of the factor V_{Leiden} mutation shows that it is very likely that all alleles originate from the same individual in which the original Arg⁵⁰⁶→Gln substitution

7.2 APC-catalyzed inactivation of factor Va and factor Va_{Leiden}

occurred, suggesting that this was a single event [81, 14] which took place about 21,000 to 34,000 years ago [81]. This is after the evolutionary divergence of Africans from non-Africans and of Caucasoid from Mongoloid subpopulations, explaining why the factor V_{Leiden} mutation is virtually absent outside the Caucasian population [54, 51, 24, 56].

There has to be a positive selection criterium for the factor V_{Leiden} as the frequency is increased, in only 1000 generations, from 1 individual to approximately 4% in the Western population. It is unknown what positive selection criteria underlie the high prevalence of this potential harmful mutation in the general population. Direct effect of the factor V_{Leiden} mutation on reproductive efficiency is a negative one, as it is associated with recurrent fetal loss [25, 11]. Potential advantages of a slight hypercoagulable state could involve reduced loss of blood accompanying severe injury or delivery. Indeed, Lindqvist *et al.* showed that female carriers of the factor V_{Leiden} mutation have reduced intrapartum blood loss compared to non-carriers [41].

7.2 APC-catalyzed inactivation of factor Va and factor Va_{Leiden}

Before the discovery of APC-resistance/factor V_{Leiden}, already a substantial amount of knowledge was available on the protein C system and the role of APC in the down-regulation of factor Va [79, 68, 28, 47, 66, 4]. Studies in model systems showed that APC-catalyzed loss of cofactor activity of factor Va in prothrombin activation is accompanied by multiple cleavages in the factor Va heavy chain [34, 33], and that factor Va inactivation by APC is enhanced by the presence of negatively charged phospholipid vesicles [79, 68] and is modulated by factor Xa and protein S [66, 4]. After the elucidation of the molecular defect in factor V_{Leiden}, many laboratories initiated studies on the mechanism of inactivation of factor Va and factor Va_{Leiden}. Purification of factor V(a) from homozygous carriers of the factor V_{Leiden} mutation was an essential first step towards elucidation of this mechanism. After a qualitative description of the APC-catalyzed inactivation of factor Va and factor Va_{Leiden} in model systems by Kalafatis *et al.* [33], a detailed kinetic analysis was presented by our laboratory [46]. Rate constants of inactivation of both factor Va and factor Va_{Leiden} were obtained by fitting the factor Va inactivation curves to an equation that is based on a model which allows two random pathways of inactivation. This model is presented in figure 7.1 and shows that APC-catalyzed inactivation of membrane-bound factor Va can occur via two pathways. Pathway I proceeds via initial cleavage at Arg⁵⁰⁶ yielding a factor Va intermediate which exhibits partial cofactor activity in prothrombin activation. Subsequent cleavage at Arg³⁰⁶ in this intermediate results in a fully inactive product. Pathway II involves a direct cleavage at Arg³⁰⁶, which immediately leads to an inactive product. From the rate constants assigned to the cleavages it is clear that cleavage at Arg⁵⁰⁶ is ~20-fold faster than at Arg³⁰⁶ which indicates that ~95% of the factor Va molecules are inactivated via pathway I [46].

It is hard to understand why factor Va_{Leiden}, in which pathway I is not available, is associated with only a moderate risk increase for thrombosis. A possible answer can be found via analysis of the APC-catalyzed inactivation of factor Va and factor Va_{Leiden} in the presence of the *in vivo* modulators, factor Xa and protein S presented in chapter 2 of this thesis.

It was found that protein S specifically accelerates the second, slow phase of the inactivation reaction, which is associated with cleavage at Arg³⁰⁶. Protein S also appeared to stimulate cleavage at Arg³⁰⁶ in factor Va_{Leiden}. Our observation that protein S hardly influences cleavage at Arg⁵⁰⁶ was later corroborated by a study by Egan *et al.* using recombinant factor Va molecules. They analyzed APC-catalyzed inactivation of a factor Va molecule in which Arg³⁰⁶ was substituted by alanine and therefore is unable to be cleaved by APC at position 306. In the presence of protein S, the rate of factor Va^{R306A} inactivation, was the same as wild-type factor Va, which confirms that the effects of protein S are confined to cleavage at Arg³⁰⁶.

In chapter 2 it is also shown that factor Xa specifically blocks APC cleavage at Arg⁵⁰⁶ in normal factor Va and has no effect on the rate of inactivation of factor Va_{Leiden}, which indicates that the effect of factor Xa is confined to Arg⁵⁰⁶. This was confirmed by immunoblot analysis of the heavy chain degradation products of normal factor Va in the presence of factor Xa which showed primarily the products of initial cleavage at Arg³⁰⁶. Total blocking of cleavage at Arg⁵⁰⁶ can be achieved, with a half-maximum effect at 1.9 nM factor Xa. Factor Va is very efficiently protected from cleavage at Arg⁵⁰⁶ during ongoing prothrombin activation, since in this case the factor Xa concentration required for half-maximum protection is very low ($K_{1/2Xa} = 0.03$ nM).

In earlier publications [66, 4] effects of protein S and factor Xa in APC-catalyzed factor Va inactivation were observed, but the effects were not assigned to specific APC cleavage sites. It was observed that in the presence of factor Xa, the rate of APC-catalyzed factor Va was lower. When protein S was also present, the rate of inactivation normalized again, whereas in the absence of factor Xa no effect of protein S was observed. Therefore it was hypothesized that protein S abrogates the protective effect of factor Xa on factor Va inactivation [66]. In these studies, however, only initial rates of factor Va inactivation were measured. Since protein S affects specifically the second phase of factor Va inactivation, this explains why previously no effect of protein S alone was observed.

The combined effects of factor Xa and protein S on factor Va inactivation by APC are depicted in figure 7.2 and predict that factor Va and factor Va_{Leiden} will be almost equally susceptible to APC in the presence of protein S and factor Xa. Factor Xa will prevent cleavage at Arg⁵⁰⁶ in normal factor Va, whereas APC is incapable to cleave at Arg⁵⁰⁶ in factor V_{Leiden}. Thus in both factor Va molecules pathway I is eliminated. Protein S specifically accelerates cleavage at Arg³⁰⁶ ~20-fold, which ensures an efficient overall inactivation of both forms of factor Va. When factor Va_{Leiden} and normal factor Va are inactivated by APC in the presence of protein S and factor Xa, the differences in the rates of inactivation were indeed completely annihilated. Since, *in vivo* inactivation of factor Va likely occurs in the presence of protein S and factor Xa, these observations may explain why carriership of factor V_{Leiden} is only a moderate risk factor for thrombotic complications in the absence of other risk factors.

These observations in model systems using purified proteins were confirmed by unpublished data obtained from experiments in extrinsically triggered plasma, in the presence of APC. Measured under these conditions, the peak value of generated factor Va is much higher in plasma from a homozygous carrier of the factor V_{Leiden} mutation, than in pooled normal plasma. However, when the peak value of factor Va is reached, the subsequent rates of inactivation are the

7.2 APC-catalyzed inactivation of factor Va and factor Va_{Leiden}

Figure 7.1: Schematic representation of APC-catalyzed inactivation of membrane bound factor Va. From the rate constants given for the different cleavages it can be seen that inactivation of factor Va will predominantly occur via pathway I.

Figure 7.2: Schematic representation of APC-catalyzed inactivation of membrane bound factor Va in the presence of protein S and factor Xa. Cleavage at Arg⁵⁰⁶ is inhibited by factor Xa, protein S ensures an efficient overall inactivation.

same for both forms of factor Va. This indicates that in plasma, normal factor Va and factor Va^{R506Q} are equally susceptible to APC as predicted by studies in model systems. Taking into account that the total amount of factor Va formed during coagulation will be the result of activation of factor V during initiation and propagation of the initial trigger and of inactivation of factor Va activity once it has been formed, these data suggest that the *in vitro* expression of APC-resistance is the result of impaired down-regulation of factor Va_{Leiden} during the early phase of tissue-factor induced coagulation.

The mechanisms of activation of factor V in the early phase of coagulation, before significant amounts of thrombin have formed may well be different from that later on since other activators of factor V may be of physiological importance. Obviously, generation of factor Xa will precede that of thrombin and factor V activated by factor Xa may well have different sensitivities for APC. This could also be true for factor V activated by tissue factor/factor VIIa [63] or meizothrombin [70], which is an intermediate in the conversion of prothrombin into thrombin and is formed during clotting [10].

The situation where thrombosis develops is probably different from the situation where coagulation is initiated after injury. In the latter case, coagulation is efficiently triggered which results in an adequate response and high levels of activated coagulation proteins of both the pro- and anticoagulant pathways. Thrombosis, on the other hand, probably develops as a consequence of a small imbalance, over a relatively long period of time, between procoagulant and anticoagulant pathways, which results in a slight prothrombotic condition. When coagulation lingers on, and only low amounts of activated coagulation factors are present, it is very well possible that factor Va is not efficiently protected by factor Xa from inactivation by APC. Therefore, the findings reported in chapter 2 do not rule out that a direct reduced sensitivity of factor V_{Leiden} to APC contributes to the development of thrombotic complications in carriers of this mutation.

7.3 Factor Va₁ and factor Va₂

In 1993 it was described that plasma contains two forms of factor V, which after activation yield factor Va₁ and factor Va₂ (numbered in the order of elution from a Mono S column) [61]. On phospholipid vesicles with a low content of negatively charged residues, factor Va₁ has reduced ability to act as a cofactor in prothrombin activation compared to factor Va₂. This is due to a lower affinity of factor Va₁ for phospholipid vesicles [61].

In chapter 3 we addressed the question whether the lower affinity for phospholipid vesicles results in different properties in another phospholipid-dependent process, *i.e.* factor Va inactivation by APC. Indeed, large differences were observed between the rates of inactivation of factor Va₁ and factor Va₂ by APC in the presence of suboptimal phospholipid vesicle concentration or composition. At higher phospholipid concentrations or on membranes with a high content of negatively charged phospholipids, the differences between factor Va₁ and factor Va₂ disappeared.

7.4 Factor V as cofactor in factor VIII inactivation

The phospholipid requirement for expression of procoagulant cofactor activity of factor Va in prothrombin activation vastly differed from that required for down-regulation of factor Va activity by APC. It appeared that conditions can be found where the cofactor activity of factor Va₁ is identical to that of factor Va₂, but at which factor Va₁ is much less efficiently inactivated by APC. This predicts that under these conditions, factor Va₁ will give rise to higher levels of thrombin formation than factor Va₂.

Indeed we showed that in a model system in which factor V is activated, expresses its cofactor activity in prothrombin activation and is down-regulated by APC and protein S in the presence of phospholipid vesicles which mimic the concentration and composition of activated platelets, factor V₁ yields 7-fold higher levels of thrombin formation compared to factor V₂.

Variability of the relative amounts of factor V₁ and factor V₂ present in plasma might be of physiological importance and contribute to the differences in response to APC and could be an explanation for APC-resistance in the absence of the factor V_{Leiden} mutation. Therefore we developed an assay to quantify the amounts of the factor V isoforms in plasma (chapter 4).

7.4 Factor V as cofactor in factor VIII inactivation

The description by Bertina [7] and coworkers that the molecular basis of APC-resistance is a defect in the ability of factor Va to be down-regulated by APC was surprising given the data available on APC-resistance:

1. In Dahlbäck's original publication [16] an experiment was described in which the reduced sensitivity to APC of a plasma sample normalized upon dilution in normal plasma. This led to the hypothesis that a cofactor of APC was missing in the plasma of APC-resistant individuals. In a later publication it was described that this cofactor was factor V and that this cofactor activity is defective in the case of APC-resistance [17].
2. Dr. Váradi and coworkers were developing an assay based on the ability of APC to inactivate factor VIII in a diluted plasma sample [75] and were able to detect APC-resistance in this assay. This indicated that in plasma of APC-resistant individuals, *i.e.* carriers of the factor V_{Leiden} mutation, the APC-catalyzed factor VIIIa inactivation is impaired.

Very soon after the initial report of the molecular defect in factor V associated with APC-resistance, several other laboratories confirmed this observation [82, 26, 78]. As a consequence, the missing cofactor hypothesis [16, 17] as explanation for the increased risk of thrombosis associated with APC-resistance attracted much less attention.

However, in the meantime it had been shown that factor V has cofactor activity in the APC-mediated inactivation of factor VIIIa [64, 77] and that factor V and protein S act synergistically in stimulating factor VIIIa inactivation [64, 76]. Thrombin-activated factor Va does not possess cofactor activity and this cannot be restored upon addition of the isolated B-domain [76]. Essential for the expression of the cofactor activity is treatment of the unactivated factor V with APC in the presence of phospholipid vesicles [43]. The importance of cleavage at Arg⁵⁰⁶ by

APC in factor V for the expression of cofactor activity in APC-mediated factor VIIIa inactivation is demonstrated by the observation that purified factor V from a homozygous carrier of the factor V_{Leiden} mutation has strongly diminished cofactor activity [76]. This observation was recently confirmed by means of recombinant factor V molecules with the Arg⁵⁰⁶ → Gln mutation [71]. As yet, limited information is available on the exact mechanisms by which factor V enhances the APC-mediated inactivation of factor VIII, nor is clear why factor V₁ has strongly reduced cofactor activity in this reaction compared to factor V₂ [76].

7.5 Factor V polymorphisms

The crucial role of factor V in both the procoagulant and anticoagulant pathways of blood coagulation make mutations in its gene candidate determinants of thrombotic disease. Detailed analysis of factor V encoded by genes that are associated with an increased risk for thrombotic complications may help to unravel the molecular mechanisms by which factor V is involved in control of blood coagulation. The discussion below focuses on two recently discovered factor V polymorphisms.

R2-gene

In chapter 5 we analyzed the contribution of a particular factor V-gene (R2-gene) in the development of coronary artery disease (CAD). Given the high frequency of this polymorphism in the general population (~10%, ref. [6] and chapter 5) and the reported risks for this polymorphism, a considerable number of cases of CAD will be contributable to carriership of the R2-gene.

To gain insight in the phenotypic effects of the R2-gene we analyzed the properties of factor V(a) encoded by the R2-gene and compared it to factor V from normal pooled plasma (chapters 5 and 6). Procoagulant activity of R2-factor V and normal factor V were compared in model systems containing purified proteins and using highly diluted plasma samples as a source of factor V. Factor V and R2-factor V were equally well activated by thrombin and subsequently expressed similar cofactor activities in prothrombin activation as expressed by their apparent affinity for factor Xa ($K_{1/2Xa}$) and the K_m -value for prothrombin. These data indicate that the reduced sensitivity to APC of plasma from carriers of the R2-gene [6] cannot be explained by an increased procoagulant activity of R2-factor V. Moreover, we showed that R2-factor Va and normal factor Va are equally susceptible to APC in the presence as well as in the absence of protein S.

Several differences were observed in plasma of carriers of the R2-gene when compared to plasma of age-matched controls. Plasma of homozygous carriers of the R2-gene contained higher levels of factor VIII, slightly reduced factor V levels and the ratio of the two factor V isoforms was altered in favor of factor V₁. Moreover, in an APC resistance test which quantifies

7.5 Factor V polymorphisms

the cofactor activity of factor V in APC-catalyzed factor VIII(a) inactivation, homozygous R2-carriers were significantly more resistant to APC than controls. This indicates that R2-factor V has reduced cofactor activity in APC-catalyzed factor VIII(a) inactivation.

One amino acid substitution encoded by the R2-gene is of particular importance for the phenotype of R2-factor V. The Asp²¹⁹⁴→Gly mutation is very close to the site that is hypothesized to be differentially glycosylated in factor V₁ and factor V₂ [49, 50] and very likely changes the balance of the two forms in plasma. This hypothesis is supported by the observation that one patient, who is a homozygous carrier of the R2-gene, but is heterozygous at the Asp→²¹⁹⁴Gly site, and thus is only at this position identical to R1/R2 heterozygotes, has the same FV₂/FV₁ ratio as R1/R2 heterozygotes. Thus, the Asp²¹⁹⁴→Gly mutation results in a change of the ratio of the two factor V isoforms in favor of factor V₁. This form is less sensitive to down-regulation by APC and is more thrombogenic in model systems under conditions which mimic the physiological situation (chapter 3). Moreover, this form has strongly reduced cofactor activity in APC-mediated factor VIII(a) inactivation [76]. These changes in plasma of carriers of the R2-gene will lead to increased thrombin formation in the presence of APC and may well explain the increased risk for thrombotic complications associated with carriership of the R2-gene (ref. [2] and chapter 5).

Current data raise the question why, in contrast to the R2-gene, an association for factor V_{Leiden} with arterial thrombosis was either not observed [55, 53, 19] or only observed in a selected patient population [60, 18], whereas R2-factor V and factor V_{Leiden} both have reduced cofactor activity in APC-mediated factor VIII(a) inactivation (chapter 6, refs. [76, 71]), and both result in increased thrombin formation due to reduced sensitivity to APC. One difference between the two factor V polymorphisms is that increased thrombogenicity of factor V₁ was demonstrated in the presence of factor Xa and protein S, whereas in the presence of these proteins no differences in rates of APC-catalyzed inactivation of normal factor Va and factor Va_{Leiden} were observed. In addition, the change in relative amounts of factor V₁ and factor V₂ may be of particular importance in the development of arterial thrombosis since apoptotic vascular smooth muscle cells present at an atherosclerotic plaque expose limited amounts of phosphatidylserine [22]. These conditions optimally express the differences between factor V₁ and factor V₂ in overall procoagulant activity in the presence of APC. Moreover, it is likely that factor V₁ and factor V₂, like factor Va₁ and factor Va₂, differ in their affinity for phospholipid vesicles. Therefore, the differences in cofactor activity in APC-mediated factor VIII(a) inactivation could be more pronounced at a phospholipid surface with a limiting amount of phosphatidylserine. Thus the mechanisms by which the anticoagulant cofactor activity of factor V is impaired in R2-factor V and factor V_{Leiden} are probably different. This may explain the differences in risk for cardiovascular disease associated with factor V_{Leiden} and R2-factor V.

It is interesting to note that the patient with genotype R2/R2* (homozygous R2, but heterozygous at position 2194) has a FV₂/FV₁ ratio similar to that of all-over heterozygous individuals (genotype R1/R2), but the APC-sr value obtained in the Immunchrom APC-response kit is different from R1/R2 heterozygotes. This indicates that R2/R2*-factor V has lower cofactor activity in APC-mediated factor VIII inactivation than factor V from heterozygous carriers and

Figure 7.3: **Properties of factor V in pro- and anticoagulant pathways.** Factor V acts as a cofactor in the APC-catalyzed factor VIII(a) inactivation in synergy with protein S. Activated factor V is an essential cofactor in prothrombin activation is down-regulated by APC. Increased levels of factor V₁ result in increased thrombin formation due to impaired anticoagulant cofactor activity and reduced sensitivity of factor Va₁ to APC.

is comparable to that of R2/R2-homozygotes. Although it is highly speculative to draw conclusions from data obtained from one individual, these results hint towards a contribution of other mutations in linkage with the R2-gene to the decreased anticoagulant cofactor activity of R2-factor V.

The altered ratio of the two isoforms of factor V due to the R2-linked mutation at Asp²¹⁹⁴ is an appealing rationale for the association of carriership of the R2-gene with an increased risk for arterial (chapter 5) and venous thrombosis [2], although it could be argued that the changes in amounts of factor Va₁ and factor Va₂ in homozygous carriers of the R2-gene are not dramatically different from those in healthy controls. However, using a 7-fold higher thrombin generating capacity of FV₁ compared to FV₂ in the presence APC (chapter 3), the shift from a FV₂ ratio from 2.8 in controls to 1.4 in R2-homozygotes will result in 35% higher level of thrombin formation in plasma of R2-homozygous individuals. The reduced cofactor activity of FVa₁ in APC-mediated factor VIII(a) inactivation would further increase the level of thrombin formation (*cf.* Fig 7.3). This increase in thrombin formation could be of physiological significance as in the recent years data has become available which indicate that even minor changes in haemostatic parameters that stay well within the normal range can be associated with an increased risk for thrombotic complications. Examples are a mutation in the prothrombin gene which leads to ~20% higher plasma prothrombin levels [52] and the moderate changes in coagulation parameters which may be responsible for the occurrence of thrombosis during pregnancy [13] or the use of oral contraceptives [67, 44].

Moreover R2-factor V has reduced cofactor activity in APC-mediated factor VIII(a) inactivation, which can (at least partly) be attributed to the altered FV₂/FV₁ ratio. Thus an decreased

7.5 Factor V polymorphisms

FV_2/FV_1 ratio has a procoagulant effect on two consecutive steps in blood coagulation (cf. Fig 7.3) which will lead to substantially increased thrombin formation in the presence of APC. It would be interesting to establish the physiological importance of an decreased FV_2/FV_1 ratio by determining whether a change in this ratio, independent of the R2-gene, is associated with an increased risk for venous or arterial thrombosis. An altered FV_2/FV_1 could be a 'second hit' risk factor for venous thrombosis and may be an explanation for the variability of expression of thrombotic complications observed for carriers of the factor V_{Leiden} mutation.

The higher levels of factor VIII observed in plasma from homozygous carriers of the R2-gene could be explained by the reduced inactivation of factor VIII(a). Since low levels of APC are circulating in plasma [27] and cleavage at Arg⁵⁰⁶ by APC in factor V results in a molecule with cofactor activity in APC-mediated factor VIII(a) inactivation [43], it is likely that factor VIII is constitutively down-regulated by the protein C system in synergy with factor V and protein S. Since factor V_2 is the form of factor V that has the actual cofactor activity [76], and since the relative amount of this form is decreased in homozygous carriers of the R2-gene, this may explain the higher levels of factor VIII. Moreover, we have shown that a significant correlation exists between plasma levels of factor VIII and factor V_2 , both in patients and controls which indicates that the correlation between factor VIII and factor V_2 levels is not limited to carriers of the R2-gene.

Factor V_{Leiden} also has reduced cofactor activity in factor VIIIa inactivation [76, 71], which is even more pronounced than for R2-factor V. Therefore, one would expect that carrier-ship of the factor V_{Leiden} mutation results in higher plasma factor VIII levels, which could be checked by analysis of factor VIII levels of carriers of the factor V_{Leiden} mutation from a patient- [38] or population- [57] based study. Evidence for higher levels of factor VIII associated with factor V_{Leiden} is provided by Nichols and colleagues [45] who showed that severity of haemophilia A (i.e. levels of factor VIII) is influenced by the factor V_{Leiden} mutation. They described two hemophilia A genotypes and compared of each genotype a hemophilia patient homozygous for the factor V_{Leiden} mutation with a patient carrying a normal factor V gene. For both factor VIII genotypes, the patients with the factor V_{Leiden} mutation had higher levels of factor VIII.

Additional proof is provided by analysis of the data from Arbini *et al.* [3], who showed that the frequency of factor V_{Leiden} mutation is low in severe haemophilia A patients. By definition, severe haemophilia A patients have less than 1% of the normal level of factor VIII. If, as argued above, the factor V_{Leiden} mutation results in higher levels of factor VIII, one would indeed expect a very low prevalence of the factor V_{Leiden} mutation in a population selected for very low factor VIII levels. More recent evidence is provided by the group of Lowe, who showed that in a population based study, FV_{Leiden} carriers indeed have higher levels of plasma factor VIII [42].

The effect of the factor V_{Leiden} mutation on plasma factor VIII levels is not restricted to haemophilia A patients. Rodeghiero and Tosetto [58] observed a very good correlation between APC-sensitivity ratios and plasma levels factor VIII, both in patients with venous thromboembolism and in healthy controls. The observation that this correlation also exists in healthy controls

could indicate that the efficiency of the protein C system directly influences factor VIII levels independent of whether the factor V_{Leiden} mutation is present or not.

High levels of factor VIII are assumed to be an independent risk factor for deep-vein thrombosis [37] and result in reduced sensitivity to APC in an aPTT based clotting assay [31, 39]. Based on these observations the risk for thrombosis of acquired or inherited APC-resistance (i.e. factor V_{Leiden}) determined in epidemiological studies is often stratified by plasma levels of factor VIII [73, 58]. However, since it cannot be excluded that high levels of factor VIII are the result of plasma APC-resistance instead of leading to APC-resistance, the adjustment for factor VIII levels may result in an underestimation of the risk for thrombotic complications associated with non-factor V_{Leiden} APC-resistance.

Mutations at Arg³⁰⁶

Recently, point mutations at another APC-cleavage site in factor Va were described. In Hong-Kong Chinese, an Arg³⁰⁶ → Gly substitution was observed in two patients with venous thromboembolism as well as in one healthy control [12]. The only plasma sample that was available for analysis, was from a patient with previous DVT and had an APC-sr in the normal range as determined in an 'undiluted' aPTT assay. In the same issue of *Blood*, Williamsom and colleagues described another mutation at Arg³⁰⁶, resulting in a Arg → Thr substitution [80].

The clinical importance of the replacement of Arg³⁰⁶ is, as yet, not firmly established. The Arg³⁰⁶ → Thr mutation was found once in a carefully selected sample of patients with venous thrombosis and APC-resistance in the absence of factor V_{Leiden} [80]. Although the family history of the index patient showed a strong tendency for thrombosis, the patient's mother who was also heterozygous carrier of this mutation had not developed thrombosis despite 2 pregnancies. The Arg³⁰⁶ → Gly mutation was rather frequently observed in Hong Kong Chinese (~4%), both in thrombotic patients and controls [12, 40]. Moreover, neither the Arg³⁰⁶ → Thr nor Arg³⁰⁶ → Gly was observed in 104 relatively young patients with venous thrombosis, whereas one case of each mutation was observed in the 208 controls [23]. Thus, present data do not identify mutations at Arg³⁰⁶ in factor V to be important identifiers of the risk for thrombosis. In the light of the data presented in chapter 2, this is somewhat surprising since these data hint at an important role for cleavage at Arg³⁰⁶ for the down-regulation of factor Va activity. In this respect it is interesting to note that expression of the anticoagulant cofactor activity of factor V in factor VIII inactivation requires APC-catalyzed cleavage at Arg⁵⁰⁶ in factor V [76, 71]. Since this cleavage is still possible in factor V molecules mutated at Arg³⁰⁶, the lack of manifestations of thrombotic complications in carriers of these mutations might be explained by normal factor VIII inactivation.

The question whether the Arg³⁰⁶ → Gly or Arg³⁰⁶ → Thr mutation leads to APC-resistance in a aPTT based assay is as yet unanswered. Of the almost 10 samples with a mutation at Arg³⁰⁶ as determined by DNA-analysis, thus far only 3 were analyzed on APC-resistance. Two of these individuals, a son and his mother, carried the factor V Cambridge (Arg³⁰⁶ → Thr) mutation and their plasmas were APC-resistant, both in a diluted and undiluted assay. It needs to be noticed,

however, that APC-resistance was a selection criterium in this study and as a consequence the existence of carriers of the factor V Cambridge mutation without APC-resistance cannot be excluded in this study setup. The only sample with the Arg³⁰⁶ → Gly that was analyzed for APC-resistance had a value in the normal range.

It would be interesting to see whether either of these two mutations results in APC-resistance in a test based on extrinsic activation [62]. In particular, the result in the extrinsically triggered assay could be more dependent on direct inactivation of factor Va by APC, whereas an intrinsically triggered assay is expected to be more sensitive to the cofactor activity of factor V in APC-catalyzed factor VIII inactivation. It is likely that this cofactor activity of factor V is not affected by a mutation at Arg³⁰⁶, since cleavage at Arg³⁰⁶ by APC is possible. This hypothesis can be checked via direct measurement of the cofactor activity in APC-catalyzed factor VIII(a) inactivation of Arg³⁰⁶ mutated factor V [75].

Given the central role of factor V in blood coagulation and the number of investigators that focuses on the genetics of factor V, it is likely that in the near future more mutations in the factor V gene will be observed that are associated with an abnormal coagulation phenotype, both in the venous and arterial part of the vascular system. Unraveling the biochemical mechanisms of these associations will be an fascinating challenge.

7.6 References

- [1] M. Aiach, D. Borgel, P. Gaussem, J. Emmerich, M. Alhenc-Gelas, and M. S. Gandrille. Protein C and protein S deficiencies. *Seminars in Hematology*, 34 (3):205–216, 1997.
- [2] 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. *Thrombosis and Haemostasis*, 82(2):193–197, 1999.
- [3] A.A. Arbini, P.M. Mannucci, and K.A. Bauer. Low prevalence of the Factor V Leiden mutation among "severe" hemophiliacs with a "milder" bleeding diathesis. *Thrombosis and Haemostasis*, 74:1255–1258, 1995.
- [4] H.M. Bakker, T. Tans, G. Janssen-Claessen, M.C. 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. *European Journal of Biochemistry*, 208(1):171–178, 1992.
- [5] K.A. Bauer and J.H. Griffin. *New England Journal of Medicine*, 332:1383, 1995. copy present (with entry 1337).
- [6] 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*, 90(4):1552–1557, 1997.
- [7] 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*, 369(6475):64–67, 1994.
- [8] K.W. Bloemenkamp, F.R. Rosendaal, F.M. Helmerhorst, H.R. Büller, and J.P. Vandenbroucke. Enhancement by factor V Leiden mutation of risk of deep-vein thrombosis associated with oral contraceptives containing a third-generation progestagen. *Lancet*, 346(8990):1593–1596, 1995.
- [9] M.I. Bokarewa, K. Bremme, and M. Blomback. Arg⁵⁰⁶ → Gln mutation in factor V and risk of thrombosis during pregnancy. *British Journal of Haematology*, 92(2):473–478, 1996.
- [10] E.G. Bovill, R.P. Tracy, T.E. Hayes, R.J. Jenny, F.H. Bhushan, and K.G. Mann. Evidence that meizothrombin is an intermediate product in the clotting of whole blood. *Arteriosclerosis Thrombosis and Vascular Biology*, 15:754–758, 1995.
- [11] B. Brenner, H. Mandel, N. Lanir, J. Younis, H. Rothbart, G. Ohel, and Z. Blumenfeld. Activated protein C resistance can be associated with recurrent fetal loss. *British Journal of Haematology*, 97 (3):551–554, 1997.
- [12] 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*, 91(4):1135–1139, 1998.
- [13] P. Clark, J. Brennard, J.A. Conkie, F. McCall, I.A. Greer, and I.D. Walker. Activated protein C sensitivity, protein C, protein S and coagulation in normal pregnancy. *Thrombosis and Haemostasis*, 79:1166–1170, 1998.
- [14] M.J. Cox, D.C. Rees, J.J. Martinson, and J.B. Glegg. Evidence for a single origin of factor V Leiden. *British Journal of Haematology*, 92:1022–1025., 1996.
- [15] A.M. Cumming, R.C. Tait, S. Fildes, A. Yoong, S. Keeney, and C.R.M. Hay. Development of resistance to activated protein C during pregnancy. *British Journal of Haematology*, 90:725–727, 1995.
- [16] B. Dahlbäck, M. Carlsson, and P.J. Svensson. Familial thrombophilia due to a previously unrecognized mechanism characterized by poor anticoagulant response to activated protein C: Prediction

7.6 References

- of a cofactor to activated protein C. *Proceedings of the National Academy of Sciences USA*, 90(3):1008, 1993.
- [17] 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. *Proceedings of the National Academy of Sciences USA*, 91:1396–1400, 1994.
- [18] C.J.M. Doggen, V.M. Cats, R.M. Bertina, and F.R. Rosendaal. Interaction of coagulation defects and cardiovascular risk factors: Increased risk of myocardial infarction associated with factor V Leiden or prothrombin 20210A. *Circulation*, 97(11):1037–1041, 1998.
- [19] S.T. Dunn, C.R. Roberts, E. Schechter, W.E. Moore, E.T. Lee, and J.E. Eichner. Role of factor V Leiden mutation in patients with angiographically demonstrated coronary artery disease. *Thrombosis Research*, 91(2):91–99, 1998.
- [20] S. Ehrenforth, K.P. Radtke, and I. Scharrer. Acquired activated protein C-resistance in patients with lupus anticoagulants. *Thrombosis and Haemostasis*, 74:797–798, 1995.
- [21] V. Eschwege and A. Robert. Strikes in French public transport and resistance to activated protein C. *Lancet*, 347(8995):206, 1996.
- [22] P.D. Flynn, C.D. Byrne, T.P. Baglin, P.L. Weissberg, and M.R. Bennett. Thrombin generation by apoptotic vascular smooth muscle cells. *Blood*, 89(12):4378–4384, 1997.
- [23] R.F. Franco, F.H. Maffei, D. Lourenco, V. Morelli, I.A. Thomazini, C.E. Piccinato, M.H. Tavella, and M.A. Zago. Factor V Arg³⁰⁶ → Thr (Factor V Cambridge) and factor V Arg³⁰⁶ → Gly mutations in venous thrombotic disease. *British Journal of Haematology*, 103(3):888–890, 1998.
- [24] H. Fujimura, J. Kambayashi, M. Monden, H. Kato, and T. Miyata. Coagulation factor V Leiden mutation may have a racial background. *Thrombosis and Haemostasis*, 74:1381–1382, 1995.
- [25] E. Grandone, M. Margaglione, D. Colaizzo, M. Daddedda, G. Cappucci, G. Vecchione, N. Scianname, G. Pavone, and G. Diminno. Factor V Leiden is associated with repeated and recurrent unexplained fetal losses. *Thrombosis and Haemostasis*, 77 (5):822–824, 1997.
- [26] J.S. Greengard, X. Sun, X. Xu, J.A. Fernandez, J.H. Griffin, and B. Evatt. Activated protein C resistance caused by Arg⁵⁰⁶ → Gln mutation in factor Va. *Lancet*, 343(8909):1361–1362, 1994.
- [27] A. Gruber and J.H. Griffin. Direct detection of activated protein C in blood from human subjects. *Blood*, 79:2340–2348, 1992.
- [28] E.R. Guinto and C.T. Esmon. Loss of prothrombin and factor Xa-factor Va interactions upon inactivation of factor Va by activated protein C. *Journal of Biological Chemistry*, 259:13986–13992, 1984.
- [29] M. Hellgren, P.J. Svensson, and B. Dahlbäck. Resistance to activated protein C as a basis for venous thrombolism associated with pregnancy and oral contraceptives. *American Journal of Obstetrics and Gynecology*, 173:210–213, 1995.
- [30] C.M. Henkens, V.J. Bom, A.J. Seinen, and J. Van der Meer. Sensitivity to activated protein C; influence of oral contraceptives and sex. *Thrombosis and Haemostasis*, 73(3):402–404, 1995.
- [31] C.M.A. Henkens, V.J.J. Bom, and J. van der Meer. Lowered APC-sensitivity ratio related to increased factor VIII-clotting activity. *Thrombosis and Haemostasis*, 74:1198–1199, 1995.
- [32] P. Hopmeier and W. Krugluger. Factor V Leiden and thrombophilia. *New England Journal of Medicine*, 332:1381–1382, 1995.
- [33] M. Kalafatis, R.M. Bertina, M.D. Rand, and K.G. Mann. Characterization of the molecular defect in factor V^{R506Q}. *Journal of Biological Chemistry*, 270(8):4054–4057, 1995.
- [34] 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. *Journal of Biological Chemistry*, 269(50):31869–31880, 1994.
- [35] B.P. Koeleman, P.H. Reitsma, C.F. Allaart, and R.M. Bertina. Activated protein C resistance as an additional risk factor for thrombosis in protein C-deficient families. *Blood*, 84:1031–1035, 1994.
- [36] B.P.C. Koeleman, D. Vanrumpt, K. Hamulyák, P.H. Reitsma, and R.M. Bertina. Factor V Leiden: An additional risk factor for thrombosis in protein S deficient families? *Thrombosis and Haemostasis*, 74:580–583, 1995.
- [37] T. Koster, A.D. Blann, E. Briët, J.P. Vandenbroucke, and F.R. Rosendaal. Role of clotting factor VIII in effect of Von Willebrand factor on occurrence of deep-vein thrombosis. *Lancet*, 345(8943):152–155, 1995.
- [38] T. Koster, F.R. Rosendaal, H. de Ronde, E. Briët, J.P. Vandenbroucke, and R.M. Bertina. Venous thrombosis due to poor anticoagulant response to activated protein C: Leiden thrombophilia study. *Lancet*, 342(8886–8887):1503–1506, 1993.
- [39] M.A. Laffan and R. Manning. The influence of factor VIII on measurement of activated protein C resistance. *Blood Coagulation and Fibrinolysis*, 7(8):761–765, 1996.
- [40] R. Liang, C.K. Lee, M.S. Wat, Y.L. Kwong, C.K. Lam, and H.W. Liu. Clinical significance of Arg³⁰⁶ mutations of factor V gene. *Blood*, 92(7):2599–2600, 1998.
- [41] P.G. Lindqvist, P.J. Svensson, B. Dahlbäck, and K. Marsal. Factor V Q(506) mutation (activated protein C resistance) associated with reduced intrapartum blood loss—a possible evolutionary selection mechanism. *Thrombosis and Haemostasis*, 79(1):69–73, 1998.
- [42] G.D.O. Lowe, A. Rumley, M. Woodward, E. Reid, and J. Rumley. Activated protein C resistance and FV^{R506Q} mutation in a random population sample. *Thrombosis and Haemostasis*, 81:918–924, 1999.
- [43] D.S. 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*, 87:4708–4717, 1996.
- [44] J.R. Newton. Classification and comparison of oral contraceptives containing new generation progestagens. *Human Reproduction Update*, 1:231–263, 1995.
- [45] W.C. Nichols, K. Amano, P.M. Cacheris, M.S. Figueiredo, K. Michaelides, R. Schwaab, L. Hoyer, R.J. Kaufman, and D. Ginsburg. Moderation of hemophilia A phenotype by the factor V^{R506Q} mutation. *Blood*, 88(4):1183–1187, 1996.
- [46] G.A.F. Nicolaes, G. Tans, M.L.C.G.D. Thomassen, H.C. Hemker, K. Váradi, H.P. Schwarz, and J. Rosing. Peptide bond cleavages and loss of functional activity during inactivation of factor Va and factor Va^{R506Q} by activated protein C. *Journal of Biological Chemistry*, 270(36):21158–21166, 1995.
- [47] B. Odegaard and K. Mann. Proteolysis of factor Va by factor Xa and activated protein C. *Journal of Biological Chemistry*, 262(23):11233–11238, 1987.
- [48] O. Olivieri, S. Friso, F. Manzato, A. Guella, F. Bernardi, B. Lunghi, D. Girelli, M. Azzini, G. Brocco, C. Russo, and R. Corrocher. Resistance to activated protein C in healthy women taking oral contraceptives. *British Journal of Haematology*, 91:465–470, 1995.
- [49] T.L. Ortel, M.A. Quinn-Allen, F.G. Keller, J.A. Peterson, D. Larocca, and W.H. Kane. Localization of functionally important epitopes within the second C-type domain of coagulation factor V using recombinant chimeras. *Journal of Biological Chemistry*, 269(22):15898–905, 1994.
- [50] T.L. Ortel, L. Yoo, M.A. Quin-Allen, and W.H. Kane. Partial glycosylation at asparagine-2181 of the second C-type domain of human factor V is the structural basis of the light chain doublet. *Blood*, 84:387a, 1994.

7.6 References

-
- [51] G. Pepe, O. Rickards, O.C. Vanegas, T. Brunelli, A.M. Gori, B. Giusti, M. Attanasio, D. Prisco, G.F. Gensini, and R. Abbate. Prevalence of factor V Leiden mutation in non- european populations. *Thrombosis and Haemostasis*, 77:329–331, 1997.
- [52] 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*, 88:3698–3703, 1996.
- [53] R.D. Press, X.Y. Liu, N. Beamer, and B.M. Coull. Ischemic stroke in the elderly. Role of the common factor V mutation causing resistance to activated protein C. *Stroke*, 27(1):44–48, 1996.
- [54] D.C. Rees, M. Cox, and J.B. Clegg. World distribution of factor V Leiden. *Lancet*, 346:1133–1134, 1995.
- [55] 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. *New England Journal of Medicine*, 332(14):912–917, 1995.
- [56] P.M. Ridker, J.P. Miletich, C.H. Hennekens, and J.E. Buring. Ethnic distribution of factor V Leiden in 4047 men and women. Implications for venous thromboembolism screening. *Journal of the American Medical Association*, 277(16):1305–1307., 1997.
- [57] F. Rodeghiero and A. Tosetto. The epidemiology of inherited thrombophilia: The VITA project. videnza thrombophilia and atherosclerosis project. *Thrombosis and Haemostasis*, 78:636–640, 1997.
- [58] F. Rodeghiero and A. Tosetto. Activated protein C resistance and factor V Leiden mutation are independent risk factors for venous thromboembolism. *Annals of Internal Medicine*, 130:643–650, 1999.
- [59] 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*, 85:1504–1508, 1995.
- [60] F.R. Rosendaal, D.S. Siscovick, S.M. Schwartz, R.K. Beverly, B.M. Psaty, W.T. Longstreth, T.E. Raghunathan, T.D. Koepsell, and P.H. Reitsma. Factor V Leiden (resistance to activated protein C) increases the risk of myocardial infarction in young women. *Blood*, 89(8):2817–2821, 1997.
- [61] 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. *Journal of Biological Chemistry*, 268(28):21130–21136, 1993.
- [62] J. Rosing, G. Tans, G.A.F. Nicolaes, M.C.L.G.D. Thomassen, R. van Oerle, P.M.E.N. van der Ploeg, P. Heijnen, K. Hamulyák, and H.C. Hemker. Oral contraceptives and venous thrombosis: Different sensitivities to activated protein C in women using second and third generation oral contraceptives. *British Journal of Haematology*, 97(1):233–238, 1997.
- [63] O. Safa, J.H. Morrissey, C.T. Esmon, and N. Esmon. Factor VIIa/tissue factor generates a form of factor V with unchanged specific activity, resistance to activation by thrombin, and increased sensitivity to activated protein C. *Biochemistry*, 38(6):1829–1837, 1999.
- [64] L. Shen and B. Dahlbäck. Factor V and protein S as synergistic cofactors to activated protein C in degradation of factor VIIIa. *Journal of Biological Chemistry*, 269(29):18738, 1994.
- [65] P. Simioni, A. Scudeller, and A. Girolami. Factor V Leiden and thrombophilia. *New England Journal of Medicine*, 332(20):1382, 1995. copy present (with entry 1337).
- [66] 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. *Journal of Biological Chemistry*, 263(29):14884–14890, 1988.
- [67] L. Speroff and A. DeCherney. Evaluation of a new generation of oral contraceptives. the advisory board for the new progestins. *Obstetrics and Gynecology*, 81:1034–1047, 1993.
- [68] K Suzuki, J. Stenflo, B. Dahlbäck, and B. Teodorsson. Inactivation of human coagulation factor V by activated protein C. *Journal of Biological Chemistry*, 258(3):1914–1920, 1983.
- [69] P.J. Svensson, G. Benoni, H. Fredin, O. Bjorgell, P. Nilsson, U. Hedlund, G. Nylander, D. Bergqvist, and B. Dahlbäck. Female gender and resistance to activated protein C (FV Q(506)) as potential risk factors for thrombosis after elective hip arthroplasty. *Thrombosis and Haemostasis*, 78(3):993–996, 1997.
- [70] G. Tans, G.A. Nicolaes, M.C. Thomassen, H.C. Hemker, A.J. van Zonneveld, H. Pannekoek, and J. Rosing. Activation of human factor V by meizothrombin. *Journal of Biological Chemistry*, 269(23):15969–15972, 1994.
- [71] E. Thorelli, R.J. Kaufman, and B. Dahlbäck. Cleavage of factor V at Arg⁵⁰⁶ by activated protein C and the expression of anticoagulant activity of factor V. *Blood*, 93(8):2552–2558, 1999.
- [72] H.H. van Boven, P.H. Reitsma, F.R. Rosendaal, T.A. Bayston, V. Chowdurry, K.A. Bauer, I. Scharer, J. Conard, and D. Lane. Factor V Leiden (FV R506Q) in families with inherited antithrombin deficiency. *Thrombosis and Haemostasis*, 75:417–421, 1996.
- [73] F.J.M. van der Meer, T. Koster, J.P. Vandenbroucke, E. Briët, and F.R. Rosendaal. The Leiden Thrombophilia study (LETS). *Thrombosis and Haemostasis*, 78(1):631–635, 1997.
- [74] J.P. Vandenbroucke, T. Koster, E. Briët, P.H. Reitsma, R.M. Bertina, and F.R. Rosendaal. Increased risk of venous thrombosis in oral-contraceptive users who are carriers of FV Leiden mutation. *Lancet*, 344:1453–1457, 1994.
- [75] K. Váradi, B. Moritz, H. Lang, K. Bauer, E. Preston, I. Peake, G.E. Rivard, B. Keil, and H.P. Schwarz. A chromogenic assay for activated protein C resistance. *British Journal of Haematology*, 90:884–891, 1995.
- [76] K. Váradi, 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 V^{R506Q} mutation. *Thrombosis and Haemostasis*, 76:208–214, 1996.
- [77] K. Váradi, J. Rosing, G. Tans, and H.P. Schwarz. Influence of factor V and factor Va on APC-induced cleavage of human factor VIII. *Thrombosis and Haemostasis*, 73(4):730–731, 1995.
- [78] J. Voorberg, J. Roelse, R. Koopman, H. Büller, 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*, 343(8912):1535–1536, 1994.
- [79] F.J. Walker, P.W. Sexton, and C.T. Esmon. The inhibition of blood coagulation by activated protein C through the selective inactivation of activated factor V. *Biochim-Biophys-Acta*, 571(2):333–342, 1979.
- [80] D. Williamson, K. Brown, R. Luddington, C. Baglin, and T. Baglin. Factor V Cambridge: A New Mutation (Arg³⁰⁶ → Thr) Associated with Resistance to Activated Protein C. *Blood*, 91(4):1140–1144, 1998.
- [81] A. Zivelin, J.H. Griffin, X. Xu, I. Pabinger, M. Samama, J. Conard, B. Brenner, A. Eldor, and U. Seligsohn. A single genetic origin for a common caucasian risk factor for venous thrombosis. *Blood*, 89:397–402, 1997.
- [82] B. Zöller and B. Dahlbäck. Linkage between inherited resistance to activated protein C and factor V gene mutation in venous thrombosis. *Lancet*, 343(8912):1536–1538, 1994.

7.6 References

- [83] B. Zöller, 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. *Journal of Clinical Investigation*, 94:2521–2524, dec 1994.

Summary

Chapter 1 is the introduction to this thesis and gives an overview of the regulation of blood coagulation and the role of factor V therein.

In **chapter 2**, the roles of protein S and factor Xa in the APC-catalyzed down-regulation of factor Va activity are analyzed. In the absence of these modulators, inactivation of factor Va activity by APC occurs via limited proteolysis, primarily at Arg³⁰⁶ and at Arg⁵⁰⁶. Since cleavage at Arg⁵⁰⁶ is approximately 20-fold faster than at Arg³⁰⁶, Arg⁵⁰⁶ is a very important cleavage site for APC. Inactivation of factor Va by APC in the presence of protein S occurs at a higher rate than in the absence of protein S. Analysis of the factor Va inactivation curves and assignment of rate constants to cleavages at Arg⁵⁰⁶ and Arg³⁰⁶ showed that protein S specifically enhances the rate of cleavage at Arg³⁰⁶ approximately 20-fold. Half-maximum stimulation is obtained at 490 nM protein S, a value close to the plasma concentration of protein S. The effect of factor Xa on APC-catalyzed factor Va inactivation is also restricted to one cleavage site in factor Va. In contrast to protein S, factor Xa diminishes factor Va inactivation and prevents specifically cleavage at Arg⁵⁰⁶. Interestingly, when the APC-catalyzed inactivation of factor Va and factor Va from a homozygous carrier of the factor V_{Leiden} mutation are studied in the presence of protein S and factor Xa, the difference in rate of inactivation of the 2 forms is annihilated. This observation may explain why, in the absence other risk factors, factor V_{Leiden} is only a mild risk factor for thrombotic complications.

Chapter 3 describes the properties of the two factor V isoforms (factor V₁ and factor V₂) in the pro- and anticoagulant pathways. In plasma, 2 forms of factor V circulate which are differently glycosylated in the C-terminal part of the C2 domain. As a consequence the 2 forms of factor V have different affinities for negatively charged phospholipid vesicles (factor V₁ has lower affinity), have different properties in prothrombin activation and differ in their susceptibility for APC. At phospholipid vesicles with a negatively charged phospholipid content of 10 mole percent and lower, factor Va₁ is increasingly less well inactivated by APC than factor Va₂. However, the expression of cofactor activity in prothrombin activation requires much less phospholipids. This predicts that conditions can be found where factor Va₁ and factor Va₂ have identical cofactor activities in prothrombin activation, but factor Va₁ is less efficiently down-regulated by APC than factor Va₂ and thus results in more thrombin generation. Indeed it was shown in an experiment which mimics the physiological situation, i.e. in the presence of membranes at a physiological concentration and composition at which factor V is activated, expresses its cofactor activity in prothrombin activation and is down-regulated by APC and

protein S, that in the presence of factor V_1 7 fold higher levels of thrombin are formed than in the presence of factor V_2 . This raised the hypothesis that altered amounts of factor V_1 and factor V_2 , in favor of the more thrombogenic form, factor V_1 , contribute to APC-resistance in the absence of the FV_{Leiden} mutation.

To test this hypothesis, an assay was developed that quantifies factor V_1 and factor V_2 in plasma (**chapter 4**). This assay is based on the differences in prothrombin activation between factor V_{a1} and factor V_{a2} at phospholipid vesicles with a low content of negatively charged phospholipid vesicles. With this assay it was shown that normal plasma contains twice as much factor V_2 as factor V_1 (67% factor V_2), and by determining the amounts of factor V_1 and factor V_2 in plasma from 40 individuals it was shown that the relative amounts of factor V_1 and factor V_2 in plasma are under strict control (range 50-80% factor V_2).

Chapter 5 describes the results obtained in collaboration with the group of Prof. Bernardi from Italy. This group discovered the so-called R2-polymorphism, which encodes for several amino acid changes in factor V and is associated with mild APC-resistance. In this chapter it is shown that carriers of the R2-polymorphism have an increased risk for coronary artery disease. One of the amino acid changes in R2-factor V results in increased relative amounts of the more thrombogenic form of factor V (factor V_1) in plasma of carriers of this polymorphism. This may well explain the increased risk for thrombotic complications observed for carriers of the R2-polymorphism.

In **chapter 6** a detailed kinetic analysis of the functional properties of the R2-factor V molecule is performed, in order to elucidate the biochemical mechanism by which the R2-polymorphism is associated with an increased risk for arterial and venous thrombosis. Compared to normal factor V, R2-factor V is equally well activated by thrombin and has subsequently the same sensitivity to inactivation by APC. Moreover, R2-factor V_a and normal factor V_a have the same properties in the prothrombinase complex. However, when plasmas from homozygous carriers of the R2-polymorphism are compared with plasmas from age- and sex-matched controls, it appears that R2-plasma contains higher amounts of factor VIII, lower amounts of factor V and is more resistant to APC in a diluted aPTT-based APC-resistance test. Plasma from homozygous R2-carriers is more resistant to APC in an assay that quantifies the cofactor activity of factor V in the APC-catalyzed inactivation of factor VIII.

Together with the increased relative amount of the more thrombogenic form of factor V (factor V_1) in plasma of R2-carriers this may well explain the increased risk for thrombotic complications associated with carriership of the R2-gene. This chapter describes new mechanisms underlying thrombotic disease. Further research is required to answer the question whether these mechanisms, independent of the R2-polymorphism, contribute to the development of thrombosis.

Samenvatting

Het stollen van bloed is essentieel voor het menselijk lichaam om bloedverlies tegen te gaan. Bloedstolling is daarom een bijzonder efficiënt en snel proces dat te vergelijken valt met een lawine. Tengevolge van een kleine stimulus worden grote hoeveelheden stollingseiwitten geactiveerd die ervoor zorgen dat de bloeding stopt. Het stollen van bloed moet beperkt blijven tot de plaats van de verwonding en mag bovendien niet langer doorgaan dan strikt noodzakelijk is voor het stoppen van de bloeding aangezien anders trombose kan optreden. Eén van de mechanismen die beschikbaar zijn om bloedstolling onder controle te houden is het zogenaamde protein C systeem: door inactivering van stollingseiwitten (o.a. factor V) wordt het stollingsproces onder controle gehouden. Het protein C systeem heeft de afgelopen jaren veel belangstelling getrokken van wetenschappers omdat er een erfelijke afwijking in factor V is gevonden (factor V_{Leiden}) dat geassocieerd is met een verhoogd risico op trombose. Deze afwijking leidt ertoe dat het factor V molecuul slechter door het protein C systeem geïnactiveerd kan worden.

In **hoofdstuk 2** wordt de inactivering van geactiveerd factor V (factor V_a) door geactiveerd proteïne C (APC) in de aanwezigheid van proteïne S en factor Xa beschreven. Via een nauwkeurige kinetische analyse van de inactivering van factor V_a door APC werd gevonden dat proteïne S en factor Xa elk een specifiek effect hebben op een knipplaats voor APC in het factor V_a molecuul. Proteïne S versnelt de inactivering van factor V_a op positie 306, terwijl factor Xa de inactivering op positie 506 remt. Wanneer in de inactivering van normaal factor V_a en factor $V_{a\text{Leiden}}$ vergeleken worden in de aanwezigheid van proteïne S en factor Xa dan verdwijnt het verschil in inactiveringssnelheid volledig. Dit kan mogelijk verklaren waarom de factor V_{Leiden} mutatie een relatief milde risicofactor voor trombose is.

In feite is het niet correct om van *het* factor V molecuul te spreken aangezien er 2 vormen van factor V (factor V_1 en factor V_2) in plasma circuleren. Als gevolg van een verschil in ver-suikering hebben ze verschillende affiniteiten voor, voor de bloedstolling belangrijke, negatief geladen membranen en daarom hebben de 2 vormen verschillende functionele eigenschappen. Deze functionele eigenschappen worden in **hoofdstuk 3** geanalyseerd. De vorm die het slechtst bindt aan membranen, factor V_1 , is een slechtere cofactor in de vorming van trombine dan factor V_2 , maar wordt ook slechter door APC geïnactiveerd dan factor V_2 , hetgeen weer aanleiding zou kunnen geven tot méér trombinevorming. Het netto resultaat van het verschil in functionele eigenschappen tussen factor V_1 en factor V_2 op de uiteindelijke hoeveelheid trombine die gevormd wordt, laat zich niet gemakkelijk voorspellen. Maar in een experiment dat de

fysiologische situatie benadert, werd aangetoond dat factor V_1 aanleiding geeft tot 7 maal meer trombinevorming dan factor V_2 . Dit leidde tot de hypothese dat de verhouding van factor V_1 en factor V_2 in plasma belangrijk is voor de regulatie van de trombinevorming en dat een verandering in het voordeel van de meer trombogene vorm (factor V_1) mogelijk APC-resistentie in de afwezigheid van factor V_{Leiden} kan verklaren.

Om deze hypothese te testen werd een assay ontwikkeld (**hoofdstuk 4**) die beide vormen in plasma kan kwantificeren. Deze assay is gebaseerd op het verschil in cofactor activiteit in de vorming van trombine tussen factor V_{a1} en factor V_{a2} en maakt gebruik van het feit dat onder 'optimale' condities geen verschil is in cofactor activiteit, terwijl onder niet-optimale condities factor V_{a2} ongeveer een 8-maal betere cofactor is dan factor V_{a1} . De meting onder optimale condities levert de totale hoeveelheid factor V (factor V_1 + factor V_2) op. Door dit te combineren met de meting onder niet-optimale condities kunnen de hoeveelheden factor V_1 en factor V_2 in een plasma monster berekend worden. Analyse van plasma monsters van gezonde individuen geeft aan dat de relatieve hoeveelheden factor V_1 en factor V_2 strikt gereguleerd worden; de waardes liggen tussen de 50% en 80% factor V_2 , met een gemiddelde van 67% factor V_2 .

Hoofdstuk 5 beschrijft de resultaten die verkregen zijn in samenwerking met de groep van prof. Bernardi uit Italië. Door deze groep is voor het eerst het zogenaamde R2-polymorfisme beschreven dat codeert voor een aantal aminozuur veranderingen in factor V en geassocieerd is met milde APC-resistentie. In dit hoofdstuk wordt aangetoond dat dragers van dit polymorfisme een verhoogd risico hebben op trombotische problemen in de coronair vaten van het hart. Tengevolge van één van de aminozuur veranderingen in het R2-factor V molecuul is plasma van dragers van dit polymorfisme verrijkt in de meer trombogene factor V_1 vorm van factor V. Dit is een mogelijke verklaring voor het waargenomen verhoogde trombose risico voor dragers van het R2-polymorfisme.

In **hoofdstuk 6** wordt een uitgebreide kinetische analyse voor het R2-factor V molecuul beschreven die als doel heeft de biochemische verklaring te vinden van het verhoogde risico op arteriële en veneuze trombose, dat dragers van dit polymorfisme hebben. In vergelijking met normaal factor V wordt R2-factor V net zo snel geactiveerd door trombine en R2-factor V_a is vervolgens even gevoelig voor inactivering door APC als normaal factor V_a . Bovendien hebben R2-factor V_a en normaal factor V_a identieke kinetische eigenschappen als cofactor in de trombine-vormende protrombinase complex. Wanneer echter plasmas van R2-dragers worden vergeleken dat van niet-dragers dan komen een aantal verschillen aan het licht. Zo hebben R2-dragers hogere factor VIII en lagere factor V spiegels in hun plasma en is hun plasma ongevoeliger voor APC in een verdunde APC-resistentietest. Ook verloopt de inactivering van factor VIII in R2-plasma slechter dan in normale plasma's, hetgeen veroorzaakt wordt door een verminderde cofactor activiteit van R2-factor V in de door APC gekatalyseerde inactivering van factor VIII. Samen met de verrijking in de meer trombogene vorm van factor V (factor V_1) in plasma van R2-dragers, vormt dit een goede verklaring voor het waargenomen verhoogde risico op trombose van dragers van het R2-polymorfisme. In dit hoofdstuk zijn nieuwe mechanismen beschreven die ten grondslag liggen aan trombotische klachten. Verder onderzoek is nodig om uit te maken of deze mechanismen, onafhankelijk van het R2-polymorfisme, van belang zijn in de ontwikkeling van trombose.

List of publications

1. Rosing, J., Hoekema, L., Nicolaes, G.A.F., Thomassen, M.C.L.G.D., Hemker, H.C., Váradi, K., Schwarz, H.P. & Tans, G. (1995) Effects of protein S and factor Xa on peptide bond cleavages during inactivation of factor Va and factor Va^{R506Q} by activated protein C. *Journal of Biological Chemistry* **270**, 27852–27858.
2. Hoekema, L., Nicolaes, G.A.F., Hemker, H.C., Tans, G. & Rosing, J. (1997) Human Factor Va₁ and Factor Va₂: Properties in the Procoagulant and Anticoagulant Pathways. *Biochemistry* **36** 3331–3335.
3. Castoldi, E., Rosing, J., Girelli, D., Hoekema, L., Lunghi, B., Mingozzi, F., Ferraresi, P., Friso, S., Corrocher, R., Tans, G. & Bernardi, F. Mutations in the R2 FV gene affect the ratio between the FV isoforms in plasma, *Thrombosis and Haemostasis*, (accepted for publication)
4. Hoekema, L., Castoldi, E., Tans, G., Girelli, D., Gemmati, D., Bernardi, F. & Rosing, J. Functional properties of factor V and factor Va encoded by the R2-gene. (submitted for publication)
5. Hoekema, L., Rosing, J. & Tans, G. An assay to quantify the two plasma isoforms of factor V. (submitted for publication)

Abstracts

1. Rosing, J., Nicolaes, G.A.F., Hoekema, L., Thomassen, M.L.G.C.D., Hemker, H.C. & Tans, G. (1996) Regulation of factor Va cofactor activity in normal and APC-resistant plasma. XIVth International Congress on Thrombosis, Montpellier, France, 1996. *Haemostasis* **26** S3, 443.
2. Nicolaes, G.A.F., Hoekema, L., Tans, G. & Rosing, J. (1997) Human factor Va₁ and Va₂: Different sensitivities for activated protein C result in different procoagulant activities. XVIth Congress on Thrombosis and Haemostasis, Florence, Italy, 1997. *Thrombosis and Haemostasis (suppl.)* 598.
3. Hoekema, L., Nicolaes, G.A.F., Tans, G. & Rosing, J. (1997) APC-mediated regulation of factor Va activity in plasma. XVIth Congress on Thrombosis and Haemostasis, Florence, Italy, 1997. *Thrombosis and Haemostasis (suppl.)* 614.
4. Hoekema, L., Castoldi, E., Tans, G., Manzato, F., Bernardi, F. & Rosing, J. Characterization of blood coagulation factor V(a) encoded by the R2-gene. (1999) XVIIth Congress on Thrombosis and Haemostasis, Washington, USA, 1999. *Thrombosis and Haemostasis, (suppl.)*, 1999, 684.

List of publications

5. Hoekema, L., Rosing, J. & Tans, G. Quantification of factor V isoforms in plasma. Altered factor V₁/factor V₂ ratios in patients with coronary artery disease and venous thrombosis. (1999) XVIIth Congress on Thrombosis and Haemostasis, Washington, USA, 1999. *Thrombosis and Haemostasis (suppl)*, 1999, 217.
6. Castoldi, E., Rosing, J., Lunghi, B., Hoekema, L., Girelli, D., Mingozzi, F., Ferraresi, P., Friso, S., Corrocher, R., Tans, G. & Bernardi, F. Factor V gene mutations (R2 gene) are associated with coronary artery disease in elderly people. (1999) XVIIth Congress on Thrombosis and Haemostasis, Washington, USA, 1999. *Thrombosis and Haemostasis (suppl)*, 1999, 509.

Hobbs, J. R., Kottke, J. & Tans, G. Quantitation of factor V deficiency in plasma. *Journal of Vascular Medicine and Biology* 1997; 9: 115-117.

Curriculum Vitae

Lico (Lijkle) Hoekema werd geboren op 24 december 1967 te Leeuwarden. Na het behalen van het VWO diploma aan het Lienward College (1980-1986) ging hij Scheikunde studeren aan de Rijksuniversiteit Groningen. Onder begeleiding van prof. W.G.J. Hol en prof. B.W. Dijkstra deed hij hoofdvakonderzoek naar de 3-dimensionale structuur van eiwitten met behulp van röntgendiffractietechnieken. Als afgestudeerd biofysisch chemicus begon hij in november 1994 als onderzoeker in opleiding (OIO) bij de vakgroep Biochemie aan de Universiteit Maastricht. In de periode tot september 1999 deed hij onderzoek naar de rol van factor V in de regulatie van trombinevorming in plasma, hetgeen tot dit proefschrift heeft geleid.

In augustus 1999, tijdens het XVIIth Congress of the International Society on Thrombosis and Haemostasis in Washington, kreeg hij de 'President's Plenary Award' uitgereikt voor het abstract 'Characterization of blood coagulation factor V(a) encoded by the R2-gene'.

Hoekema L., Kottke J. & Tans, G. (1997) Quantitation of factor V deficiency in plasma. *Journal of Vascular Medicine and Biology* 9: 115-117.

Abstracts

1. Hoekema L., Kottke J. & Tans, G. (1997) Quantitation of factor V deficiency in plasma. *Journal of Vascular Medicine and Biology* 9: 115-117.
2. Hoekema L., Kottke J. & Tans, G. (1997) Quantitation of factor V deficiency in plasma. *Journal of Vascular Medicine and Biology* 9: 115-117.
3. Hoekema L., Kottke J. & Tans, G. (1997) Quantitation of factor V deficiency in plasma. *Journal of Vascular Medicine and Biology* 9: 115-117.
4. Hoekema L., Kottke J. & Tans, G. (1997) Quantitation of factor V deficiency in plasma. *Journal of Vascular Medicine and Biology* 9: 115-117.

Dankwoord

Zoals alle dankwoorden begint ook dit met de opmerking dat dit proefschrift nooit tot stand was gekomen als ik het helemaal alleen had moeten doen. Er zijn dus een heleboel mensen die in dit dankwoord genoemd gaan worden.

Om te beginnen natuurlijk alle mensen van de vakgroep Biochemie die met raad, daad en reservevoorraden chemicaliën e.d. hebben bijgestaan. Door de aangename sfeer in de groep heb ik de periode van mijn oio-schap als ontzettend prettig ervaren.

Speciale dank gaat uit naar mijn promotoren en co-promotor, Prof. Jan Rosing, Prof. Coen Hemker en Dr. Guido Tans. *Jan*, jouw deur stond altijd open en je maakte altijd tijd voor kleine en grote vragen. Als jonge onderzoeker wilde ik nog wel eens vergeten dat experimenten niet alleen gedaan worden vanwege het experimenten doen. Jij hebt me altijd weer op het rechte spoor gebracht met vragen als 'Wat wil je weten?' en 'Wat is het doel van de proef?' Van jouw systematiek in het benaderen van problemen heb ik veel geleerd. *Prof. Coen Hemker*, Uw betrokkenheid was wat meer op de achtergrond maar ook bij U kon ik altijd aankloppen om iets te bespreken. En natuurlijk *Guido*, niet alleen voor de vele discussies over wetenschap, statistiek en over elk ander mogelijk onderwerp, het samen pipetteren maar vooral ook voor het feit dat je er (indirect) voor gezorgd hebt dat we Pumba, een vertegenwoordiger van het allerliefste hondenras, de Basset Fauve de Bretagne, als huisgenoot hebben. Misschien moet ik voor dat laatste *Geja* wel bedanken, maar ik moet haar in ieder geval bedanken dat ze mijn paranimf wil zijn.

Elisabetta, I would like to thank you for coming to Maastricht with the right samples at right time. It was a pleasure working with you, and learning a little about the molecular biology of factor V. When you were back in Italy I enjoyed reading your e-mails. *Prof. Bernardi*, I would like to thank you for the fruitful collaboration and for lending us Elisabetta several times, it must have been quiet in your lab without her.

Stella, jij bent de vraagbaak voor het hele lab. Jij weet altijd alles te vinden op het lab, soms wist je zelfs mijn spullen beter te vinden dan ik zelf. *Joyce*, met een stralend humeur en veel optimisme prominent aanwezig op het lab, al was het alleen maar door het aantal malen dat er telefoon voor je was. *Gerry*, hoewel je toen nog nooit noordelijker was geweest dan Utrecht (een fout die je nu ruimschoots hebt goedgeemaakt) heb ik met plezier vele uren achter het waterbad met je doorgebracht. *Wilbert*, jammer dat je wegging want in de tijd dat je bij ons werkte heb je ervoor gezorgd dat we je nooit vergeten. *Tilman*, jouw inzichten in wat belangrijke randvoorwaarden zijn voor goed wetenschappelijk onderzoek zijn, heb je duidelijk laten zien toen

jij meteen de biervoorziening voor ons lab professioneel aanpakte. *José*, jouw oog voor detail en jarenlange ervaring op het lab maken je tot een onuitputtelijke 'databank'. Bij jou vond ik altijd een gewillig oor als ik weer eens problemen had met fosfolipiden. *Rory*, nog maar net begonnen dus veel succes kun je wel gebruiken. *Kristin*, je hebt er voor gezorgd dat Stella niet helemaal meer alleen staat als 'Belgische', maar dat maakt het vertellen van Belgenmoppen des te leuker. *Rob*, je hebt Stella bijna overtroffen in het draaien van 'gekmakende' CD's, die waarschijnlijk je teleurstelling moeten wegwerken als Roda weer eens verloren heeft. Ik kijk met spanning uit naar de wedstrijd Roda-Heerenveen.

Mijn ouders, die zich waarschijnlijk vaak hebben afgevraagd waarom ik nou helemaal naar Maastricht moest, omdat jullie me altijd hebben gesteund.

De 'Wolfen' met speciaal Gerard, omdat ik zo vaak van je computer gebruik mocht maken als we een weekend in Epe waren, en Ernst omdat je mij wilt bijstaan als paranimf.

Als laatste wil ik het thuisfront bedanken, Pumba, Lisa en Judy. *Pumba*, je was al genoemd, door je (avond-)wandelingen kon ik tenminste fijn 'afschakelen'. *Lisa*, jouw glimlach en blijdschap als ik thuiskwam deden meteen alle beslommeringen vergeten. *Judy*, ik wil je bedanken voor al je steun en dat je genoeg hebt genomen met minder tijd voor ons samen. Er is niemand liever dan jij.