Factor Va is inactivated by APC in the absence of cleavage sites at Arg306, Arg506 and Arg679

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Running title: Inactivation of FVa by APC in the absence of R306, R506 and R679
Summary

Activated protein C (APC) exerts its anticoagulant activity via proteolytic degradation of the heavy chains of activated factor VIII (FVIIIa) and activated factor V (FVa). So far, three APC cleavage sites have been identified in the heavy chain of FVa: R306, R506 and R679. To obtain more insight in the structural and functional implications of each individual cleavage, recombinant factor V mutants were constructed in which two or three of the APC-cleavage sites were mutated. After expression in COS-1 cells, rFV mutants were purified, activated with thrombin and inactivated by APC. During this study we observed that activated rFV-GQA (rFVa-GQA) -in which the arginines at positions 306, 506 and 679 were replaced by glycine, glutamine and alanine, respectively- was still inactivated by APC. Further analysis showed that the inactivation of rFVa-GQA by APC was phospholipid dependent and sensitive to an inhibitory monoclonal antibody against protein C. Inactivation proceeded via a rapid phase \( (k_{x1} = 5.4 \times 10^4 \text{ M}^{-1}\text{s}^{-1}) \) and a slow phase \( (k_{x2} = 3.2 \times 10^3 \text{ M}^{-1}\text{s}^{-1}) \). Analysis of the inactivation curves showed that the rapid phase yielded a reaction intermediate which retained \(~80\%\) of the original FVa activity, whereas the slow cleavage resulted in formation of a completely inactive reaction product. Inactivation of rFVa-GQA was accelerated by protein S, most likely via stimulation of the slow phase. Immunoblot analysis using a monoclonal antibody recognizing an epitope between R306 and R506 indicated that during the rapid phase of inactivation a fragment of 80 kD was generated that resulted from cleavage at a residue very close to R506. The slow phase was associated with the formation of fragments resulting from cleavage at a residue 1.5-2 kD carboxyterminal to R306. Our observations may explain the unexpectedly mild APC resistance associated with mutations at R306 (FV HongKong and FV Cambridge) in the heavy chain of FV.
Introduction

Human blood coagulation factor V (FV)\(^1\) is the inactive precursor of activated factor V (FVa), the non-enzymatic cofactor of the serine protease factor Xa (FXa) (1;2). FVa is formed via limited proteolysis of its single-chain precursor FV (\(M_r \sim 330\) kD) by thrombin. It consists of a heavy chain (\(M_r \sim 105\) kD) and light chain (\(M_r 71-74\) kD), which are held together via a Ca\(^{2+}\) ion. FVa is an essential part of the prothrombinase complex, which comprises FVa, FXa, calcium ions and a negatively charged membrane surface. In this complex FVa acts as a non-enzymatic cofactor that accelerates FXa-catalyzed prothrombin activation \(10^3-10^5\) fold (3-5). Proteolytic inactivation of FVa by activated protein C (APC) is an essential reaction in the anticoagulant protein C pathway (6), which is important in the regulation of thrombin formation and in the inhibition of acute inflammation triggered by coagulation (7). Congenital deficiencies in this pathway are associated with thrombotic disease (8;9). Inactivation of FVa activity by APC is associated with cleavage of three peptide bonds in the heavy chain of FVa, which have been localized at R306, R506 and R679 (10).

Several mutations have been reported in the APC cleavage sites of human FV. The most important one is FV Leiden (FVL), in which arginine 506 has been replaced by glutamine (11-13). This mutation results in the laboratory phenotype of APC resistance and is associated with an increased risk of deep-vein thrombosis (11;14;15). The high allele frequency of FVL (2-7% in Caucasian populations (16)), makes this mutation the most important heritable risk factor for venous thrombosis. Besides FVL, three other mutations have been found in the APC cleavage sites of FV. All three affect the R306 position and are much less frequent than FV Leiden (17-20). There are no indications that the mutations at R306 are associated with an increased risk of venous thrombosis (18;21). Moreover, the effect of these mutations on the
sensitivity to APC is not entirely clear. Studies using recombinant FV have shown that mutations at R306 result in an unexpectedly mild APC resistant phenotype (22-24).

Several studies have been performed to obtain more insight in the complex mechanism of APC-catalyzed FVa inactivation. These investigations demonstrated that cleavage of the heavy chain of FVa by APC is stimulated by negatively charged phospholipids (25-27) and protein S (27-29), which is a non-enzymatic cofactor of APC. In the presence of negatively charged phospholipids the inactivation of FVa by APC proceeds via a rapid and a slow phase, which are associated with cleavages at R506 and R306, respectively (10;30). Cleavage at R506 yields a reaction intermediate with reduced FVa activity, which is completely inactivated by the subsequent cleavage at R306. Inactivation of activated FVL is monophasic and associated with cleavage at R306, which was stimulated ~20-fold by protein S (31). In the presence of negatively charged phospholipids the contribution of the cleavage at R679 to the inactivation of FVa seems to be minor and is difficult to assess (30).

The original objective of the present study was to investigate the functional and structural implications of each individual APC cleavage in more detail. Recombinant factor V mutants were constructed in which a major part of the B-domain (rFV∆B) was removed and two or three of the APC-cleavage sites were mutated. After expression in COS-1 cells, rFV mutants were purified, activated with thrombin and inactivated by APC. Interestingly, rFVa-GQA (rFVa in which the arginines at positions 306, 506 and 679 were replaced by glycine, glutamine and alanine, respectively) was still inactivated by APC. In this manuscript we report on the mechanism of APC-catalyzed inactivation of rFVa -GQA.
Experimental Procedures

Materials

Restriction enzymes were from New England Biolabs, Beverly, MA, USA. Rapid ligation kit and BM Chemiluminescence blotting substrate (POD) were obtained from Boehringer Mannheim, Mannheim, Germany. Plasmid isolation kits were purchased from Qiagen, Chatsworth, CA, USA. DNA restriction fragments were purified from agarose gel using the cleanmix kit (Talent, Trieste, Italy). CNBr-Sepharose and SP-Sepharose were obtained from Pharmacia, Uppsala, Sweden. Bovine serum albumin (BSA), 98% fatty free, was from ICN Biomedicals (Aurora, Ohio, USA). Ovalbumin, benzamidine, phosphatidylcholine (PC), N-hydroxysuccinimidobiotine and Hepes were from Sigma (St. Louis, MO, USA). Dioleoylphosphatidylcholine (DOPC) and dioleoylphosphatidylserine (DOPS) were obtained from Avanti Polar Lipids, Alabaster, AL, USA. EDTA was from Serva, Heidelberg, Germany. NH₄Cl, TRIS and CaCl₂ were purchased from Merck, Whitehouse Station, NJ, USA. Chromogenic substrate S-2238 was obtained from Chromogenix, Uppsala, Sweden. APMSF was from Roche Diagnostics, Mannheim, Germany.

Proteins

Human activated protein C (APC), protein S, prothrombin and thrombin were purchased from Enzyme Research Laboratories (South Bend, IN, USA). The APC concentration was determined from APC antigen and activity measurements. APC antigen was measured by ELISA using IgG fractions of rabbit anti–human protein C (DakoCytomation Denmark A/S, Glostrup, Denmark), whereas APC activity was measured with S2366 using the kinetic parameters reported by Sala et al. (32). Human factor Xa, monoclonal antibodies AHV-5146 and AHV-5112 were from Haematologic Technologies Inc., Essex, VT, USA. Monoclonal antibody 3B1 directed against the heavy chain of human FV was a kind gift from Prof. B.N.
Bouma. Goat-anti-mouse IgG conjugated with horse-radish peroxidase (GAMPO) was from Bio-Rad Laboratories Inc., Hercules, CA, USA. Neutralite™ Avidin-HRP conjugate was from Southern Biotechnology Associated, Inc., Birmingham, AL, USA. Human FVLeiden (FVL) was isolated from human plasma according to Nicolaes et al. (30).

**FV antigen assay**

FV light chain antigen was measured by ELISA using two different monoclonal antibodies directed against the light chain of FV (33). In this ELISA, mAb V-6 was used as coating antibody and biotinylated mAb V-9 as tagging antibody.

**Mutagenesis**

In this study expression vectors (pMT2) were used containing the cDNA of FV in which two or three APC-cleavage sites were mutated (23;34). In these mutants the arginines at position 306, 506 or 679 were replaced by glycine, glutamine and alanine, respectively (see Table 1). The vectors were used to construct FV expression vectors lacking a major part of the B-domain (deletion from amino acid 827 to 1499) as described before (35). B-domainless constructs, labelled pMT2FV∆B, were checked by direct DNA sequencing using the CEQ2000™ Dye Terminator Cycle Sequencing Kit from Beckman Coulter Inc., Fullerton, CA, USA.

**Transient expression and purification of rFV∆B mutants**

B-domainless recombinant FV (rFV∆B) mutants were transiently expressed in COS-1 cells (175 cm² culture flasks) using Fugene 6 Transfection Reagent from Roche Molecular Biochemicals, Hague Road, IN, USA. Twenty-four hours after transfection cells were washed with phosphate-buffered saline (PBS) and incubated with serum-free medium (Optimem
Glutamax, Life Technologies Ltd., Paisley, Scotland). Conditioned medium was harvested after 72h, centrifuged for 20 min at 3000 rpm (4°C) and frozen at -20°C. FV expression was measured by functional FV assay and ELISA.

Recombinant FVΔB mutants were isolated in a two step procedure essentially as previously described (35). Briefly, conditioned medium was thawed, supplemented with 10 mM benzamidine and loaded on an ion exchange column (SP-Sepharose fast flow), which was subsequently washed with 25 mM Heps, 100 mM NH₄Cl, 5 mM CaCl₂, 10 mM benzamidine (pH 7.5), until the fall-through was protein free. Recombinant FVΔB was eluted from the column with 25 mM Heps, 1.5 M NH₄Cl, 5 mM CaCl₂, 10 mM benzamidine, pH 7.5. Fractions containing FVa activity were supplemented with 2 mg/ml ovalbumine, dialysed against a buffer containing 25 mM Heps, 50 mM NaCl, 5 mM CaCl₂, 10 mM benzamidine (pH 7.3), pooled and applied at a speed of 3 ml/h to an affinity column, consisting of 1 mg mAb-3B1/ml Sepharose. The column was washed with 25 mM Heps, 50 mM NaCl, 5 mM CaCl₂ (pH 7.3) and eluted with 25 mM Heps, 1.8 M NaCl, 5 mM CaCl₂, pH 7.3. Eluted fractions were screened for FV (activity/antigen) and analysed by SDS-PAGE and immunoblotting. FV containing fractions were supplemented with 2 mg/ml ovalbumin, dialysed against 25 mM Heps, 50 mM NaCl (pH 7.3), and stored at –80°C.

*Factor Va activity assay*

FVa activity was measured in a two step procedure as previously described (35). This assay was calibrated using dilutions of pooled normal plasma corresponding with 0-3 pM of FV.

*Inactivation of FVa by APC*

FVa (~1nM) was incubated with APC (see legends) in 25 mM Heps (pH 7.5), 175 mM NaCl, 3 mM CaCl₂, 5 mg/ml BSA, 24.5 μM phospholipids (DOPS:DOPC 10:90). At different
time intervals 10 µl samples were transferred to 240 µl of prothrombinase mix (containing 25 mM Hapes (pH 7.5), 175 mM NaCl, 3 mM CaCl₂, 0.5 mg/ml ovalbumin, FXa, prothrombin and DOPS/DOPC 10:90 molar ratio) yielding final concentrations of 5 nM FXa, 540 nM prothrombin and 50 µM DOPS/DOPC vesicles. After 3 min incubation at 37°C prothrombin activation was stopped by subsampling 40 µl in 460 µl TN-EDTA (50 mM TRIS, 175 mM NaCl, 20 mM EDTA, 0.5 mg/ml ovalbumin, pH 7.9). Subsequently, 200 µl of this mix were transferred to a 96-wells plate and 25 µl of chromogenic substrate S-2238 (2.35 mM) was added, after which the amount of thrombin formed was measured kinetically on a Spectra III Thermo microtiter plate reader from Tecan (Salzburg, Austria). Under these conditions the formation of thrombin was linearly dependent on the FVa concentration present in the prothrombinase mixture. The FVa activity measured before the addition of APC (t=0) was set at 100%.

Curve fitting of time courses of FVa inactivation

Time courses of APC-catalyzed FVa inactivation were obtained by measuring loss of FVa activity as a function of time. Data reported in this paper indicate that inactivation of the activated rFVΔB-GQA mutant by APC proceeds via two pathways:

\[
Va \xrightarrow{k_1} Va_B \xrightarrow{k_2} Vi \quad (1)
\]

\[
Va \xrightarrow{k_3} Vi \quad (2)
\]

This model is similar to the inactivation of plasma FVa via cleavages at R506 and R306 (30). \(k_1, k_2\) and \(k_3\) are pseudo first-order rate constants for cleavage at the as yet unidentified cleavage sites Rx1 and Rx2.

Under first order conditions, \textit{i.e.} conditions at which the inactivation rate is directly proportional to the factor Va and APC concentration, the loss of FVa cofactor activity is described by the following equation (30):

\[
\text{FVa activity} = \text{FVa activity at } t=0 \times e^{-k \times t}
\]
\[ V_a(t) = V_{a_0} \cdot e^{-(k_1+k_3)t} + B \cdot V_{a_0} \cdot \frac{k_1 \cdot e^{-k_2 t}}{k_1 + k_3 - k_2} \cdot (1 - e^{-(k_1+k_3-k_2)t}) \]  \hspace{1cm} (3)

In this equation \( V_a(t) \) is the FVa activity at time \( t \), \( V_{a_0} \) is the FVa activity at time zero, \( B \) is the activity of the FVa intermediate generated after cleavage at Rx1. \( k_1, k_2 \) and \( k_3 \) are the observed pseudo first-order rate constants, from which the second-order rate constants were calculated as follows: \( k_{x1} = k_1/[APC] \), \( k_{x2} = (k_2-k')/[APC] \) and \( k_{x3} = (k_3-k_s)/[APC] \), in which \( k' \) and \( k_s \) are the first-order rate constants of spontaneous inactivation of the FVa intermediate (FVa\(_b\)) and intact FVa, respectively.

**SDS-PAGE and immunoblot analysis**

Polyacrylamide gel electrophoresis was performed on SDS/PAGE (TRIS/HCl prefab gels, Gradipore, French Forest, Australia) under reducing conditions according to Laemmli (36). Protein transfer to PVDF membranes (Millipore Corporation, Bedford, MA, USA) was carried out semi-dry on a blot system from Pharmacia (Uppsala, Sweden). To detect FV, murine monoclonal antibodies against the heavy and light chain (AHV-5146 and AHV-5112, respectively) were used. GAMPO in combination with the blotting substrate POD was used to visualise the immobilised antibodies. Both antibodies were checked for any cross-reaction with BSA present in the FV fractions and were found negative. Visualisation with AHV5146 was optimized to detect low molecular degradation fragments from the heavy chain. Due to the high sensitivity of this procedure small amounts of intact heavy chains still show significant staining (see also reference (41)).
Results

Expression and purification of recombinant factor V mutants

Recombinant FV∆B mutants were constructed with mutations either in two or in three of the known APC cleavage sites (R306, R506, R679). In analogy to the naturally occurring FV HongKong (R306G) and FV Leiden (R506Q) mutations R306 was replaced by glycine and R506 by glutamine, whereas R679 was replaced by alanine (Table 1).

After transfection of COS-1 cells, conditioned media containing the rFV∆B molecules were collected, centrifuged and stored at -20°C. Factor V expression levels (activity and antigen) of rFV∆B-wt and its mutants were similar and ranged (depending on the transfection efficiency) between 0.2 and 4 nM. After thawing and pooling of conditioned media, rFV∆B proteins were purified by ion-exchange- and immunoaffinity-chromatography. The rFV∆B mutants were activated with thrombin and analyzed by SDS-PAGE followed by silverstaining or immunoblotting. Both detection methods showed two dominant bands with a MW of 105 kD and 71 kD, which corresponded to the expected MW of the heavy and light chain of factor Va, respectively (data not shown). Apart from these two bands also variable amounts of 90 kD fragment was observed, which was most likely the result of thrombin cleavage at R643 (37). The specific activities (activity/antigen) of the purified rFV mutants were slightly lower than in conditioned medium, being around 0.7.

Stability of activated rFV∆B mutants

Recombinant FV∆B proteins were activated with 9 nM thrombin for 20 min at 37°C and incubated in the absence or presence of phospholipids. The stability of the activated rFV∆B mutants was assessed by following the loss of FVa activity as a function of time in a reaction mixture that did not contain APC. The first-order rate constants for spontaneous inactivation
of activated rFVΔB-wt and the activated rFVΔB mutants were approximately similar, being around ~3.0 x 10^{-5} \text{s}^{-1}, which corresponds to a spontaneous loss of FVa activity of ~0.2% per min. The presence of phospholipids did not influence the stability of the activated rFV molecules.

**APC-catalyzed inactivation of activated rFVΔB-GQA**

Preliminary experiments showed that apart from the activated rFVΔB double mutants (rFVΔB-RQA, rFVΔB-GRA, rFVΔB-GQR) also the activated triple mutant rFVa-GQA was inactivated by APC and that this inactivation was strongly dependent on the presence of negatively charged phospholipids (Fig. 2). In the absence of phospholipids inactivation of rFVa-GQA by 20 nM APC was only slightly faster than the spontaneous loss of activity.

Preincubation of APC with a monoclonal antibody against protein C (C12), which blocks the active site of APC, or with APMSF (a non-specific inhibitor of serine proteases), inhibited the activation of rFVa-GQA by APC, whereas preincubation with a monoclonal antibody against protein S (S18) did not affect the rate of inactivation (Table 3). This indicates that the loss of FVa activity observed in the presence of APC and phospholipids (Fig. 2) was indeed mediated by APC and not a contaminating protease.

**Inactivation of activated rFVΔB-GQA with increasing APC concentrations**

The results presented above indicate that the inactivation of rFVa-GQA by APC results from one or more proteolytic cleavages at as yet unidentified cleavage sites. To learn more about the mechanism of inactivation, rFVa-GQA was incubated with increasing concentrations of APC under pseudo-first order conditions. Increasing the APC concentration resulted in an acceleration of FVa inactivation (Fig. 3a). The FVa inactivation curves could not be fitted with a single exponential (data not shown), indicating that inactivation involves at least two
different reactions, a rapid reaction resulting in a partially active FVa molecule that is subsequently completely inactivated via a slow reaction. Since the rate constant for the second reaction was much lower than that for the first reaction, it was possible to calculate the pseudo-first order rate constant \((k_2)\) for the second reaction and the activity of the FVa intermediate \((\text{FVa}_B)\) from semi-logarithmic plots (Fig. 3b). The slope of the lines yields \(k_2\) at different APC concentrations and the intercept at the y-axis yields an estimate for the FVa activity remaining in the intermediate \((\text{FVa}_B)\), which was found to be ~80%. Fig. 3c shows that the pseudo-first order rate constant \(k_2\) obtained for the second, slow reaction is a linear function of the APC concentration, which enabled calculation of the second-order rate constant \((k_{s2} = 3.2 \times 10^3 \text{ M}^{-1}\text{s}^{-1})\) for the second cleavage reaction. The line extrapolated to a rate constant of \(5.6 \times 10^{-5} \text{ s}^{-1}\) at [APC] = 0 (intercept of the line with the y-axis), which most likely reflects the spontaneous loss of activity of the reaction intermediate \(\text{FVa}_B\) (represented as \(k_{s}^{'}\)). This rate constant is close to that determined for native rFVa \((k_s \sim 3.0 \times 10^{-5} \text{ s}^{-1})\).

The time courses of inactivation of rFVa-GQA by APC (Fig. 3a) were further analyzed in a random ordered two-step model (Equation 3 in Experimental Procedures), in which rFVa-GQA is rapidly cleaved by APC resulting in an intermediate \((\text{FVa}_B)\) with slightly lower FVa activity, while a much slower cleavage in FVa and \(\text{FVa}_B\) results in the formation of a FVa derivate that is completely inactive. This model is similar to that proposed by Nicolaes et al. (30) for the inactivation of plasma FVa via cleavages at R506 (fast reaction) and R306 (slow reaction). Using the pseudo-first order rate constants \((k_2)\) and \(B\) calculated from Fig. 3b and 3c, the pseudo-first order rate constants \(k_1\) and \(k_3\) were calculated by fitting the experimental data (Fig. 3a) to Equation 3. Like \(k_2\), also \(k_1\) and \(k_3\) increased with higher APC concentrations (Fig. 3d and 3e), which indicates that the rapid and slow inactivation phases both are associated with APC-mediated cleavages at two as yet unidentified cleavage sites, designated Rx1 \((k_{s1})\) and Rx2 \((k_{s2}, k_{s2}^{'})\). From these plots the second-order rate constants were calculated

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(see Experimental Methods). The second-order rate constant of the first reaction \( (k_{x1} = 5.4 \times 10^4 \text{ M}^{-1}\text{s}^{-1}) \) appeared to be ~20-fold higher than that of the second reaction (compare with \( k_{x2} \) and \( k'_{x2} \) in Table 4). These rate constants are much lower than those reported for APC-catalyzed cleavage at R306 (\( k_{306} = 2.3 \times 10^6 \text{ M}^{-1}\text{s}^{-1} \)) and R506 (\( k_{506} = 4.3 \times 10^7 \text{ M}^{-1}\text{s}^{-1} \)) (30).

Effect of protein S on the APC-catalyzed inactivation of activated rFV\(\Delta\)B-GQA

Previous studies have shown that in the presence of saturating concentrations of protein S (490 nM) the inactivation of FVa by APC is accelerated by selective stimulation of the cleavage at R306 (31). The inactivation of rFVa-GQA by APC was also strongly stimulated in the presence of 490 nM protein S (Fig. 4). The time course of inactivation of rFVa-GQA by APC in the presence of protein S suggests that the acceleration of FVa inactivation was mainly due to an increased rate of the slow cleavage reaction (cleavage at Rx2). Acceleration of the rapid reaction (cleavage at Rx1) will hardly contribute to the final inactivation curve, because the intermediate formed after cleavage at Rx1 (FVa\(\beta\)) still has a high FVa activity. Assuming that \( k_{x1} \) (\( 5.4 \times 10^4 \text{ M}^{-1}\text{s}^{-1} \)) and B (0.82) were not affected by the addition of protein S, \( k_2 \) and \( k_3 \) were determined by fitting the data to equation 3 (Table 4). The second-order rate constant \( k'_{x2} \) (calculated from \( k_3 \)) was \( \sim 1.5 \times 10^5 \text{ M}^{-1}\text{s}^{-1} \), which indicates that protein S stimulates the APC-catalyzed cleavage at Rx2 about 50-fold.

SDS-PAGE analysis of APC-catalyzed inactivation of activated rFV\(\Delta\)B-GQA

APC-mediated proteolysis of the heavy and light chain of rFVa-GQA was monitored by SDS-PAGE and immunoblotting. In this procedure, heavy chain fragments were detected by monoclonal antibody AHV-5146, which recognizes an epitope between R306 and R506. Experiments with synthetic peptides identified the epitope for AHV-5146 between residues 427 and 440 (unpublished results). FVa light chains were detected by mAb AHV-5112.
During the inactivation of rFVa-GQA with 20 nM APC no degradation of the light chain was observed (data not shown). Analysis of the heavy chain, however, revealed the generation of several fragments, which could be associated with the loss of FVa activity (Fig. 5). The initial phase of inactivation (0-30 min) was associated with generation of a fragment of ~80 kD, whereas the second slow phase was associated with the generation of a doublet of 55/60 kD and fragments of 45 kD and 30 kD (Fig 5). Note that besides the heavy chain fragment of 105 kD (intact heavy chain from position 1 to 709) also a fragment of ~90 kD was detected, which was already present before addition of APC. Most likely, this fragment was formed during the activation with thrombin, which can cleave the heavy chain of FVa at R643 (37).

In the presence of protein S the loss of FVa activity was essentially associated with the generation of a 60 kD fragment (Fig. 5c). After 15 min also 45 kD and faint 30 kD fragments were generated.

Localization of potential APC cleavage sites in rFVΔB-GQA

The experimental data and the amino acid sequence of FV were used to localize potential APC-cleavage sites in the heavy chain of rFVa-GQA. The rapid cleavage at Rx1 in the absence of protein S was associated with formation of a 80 kD fragment (Fig. 5a/b), which still contained ~80% FVa activity. Such a fragment may result from a cleavage in a region 150-200 amino acids from either the N- or the C-terminus of the heavy chain. Since the N-terminal region (residue 150 to 200) contains no arginines and only a few lysines, whereas the region between residues 499 and 513 contains a surface-exposed loop with several arginines and lysines (38), it is likely that Rx1 is positioned in the region surrounding Q506. Cleavage in this region will result in the formation of a 80 kD fragment from both the 105 kD and 90 kD heavy chains and is also compatible with the high activity of the intermediate (FVaB~80%) after cleavage at Rx1. Consequently, the slow cleavage (Rx2), which results in
complete inactivation, should be positioned in the region of G306. This would explain the generation of the observed 60 kD fragment (cleavage in the intact heavy chain of 105 kD) and the 30 kD fragment (cleavage in the intermediate fragment of 80 kD). A cleavage near position 306 can also explain the generation of a 45 kD fragment (cleavage in heavy chain fragment of 90 kD), which has been described before (37). Furthermore, a location of Rx2 close to R306 is compatible with the stimulation of \( k'_{x2} \) by protein S (formation of 60 kD fragment in Fig. 5c), because cleavage at R306 is also stimulated by protein S (31).

More information about the position of Rx1 and Rx2 was obtained from experiments in which the activated double mutants rFV\( \Delta B \)-GRA (R506 present) and rFV\( \Delta B \)-RQA (R306 present) were incubated with APC. Inactivation of activated rFV\( \Delta B \)-GRA with APC (20 nM) generated an expected fragment of 75 kD (1-506), which was gradually cleaved into a 30 kD fragment (data not shown), suggesting that in the region of G306 indeed another APC cleavage site (Rx2) is present. The generation of this 30 kD fragment was strongly accelerated in the presence of protein S (Fig. 6a). Inactivation of activated rFV\( \Delta B \)-RQA with 20 nM APC generated fragments with the expected sizes of 60 kD (307-709) and 45 kD (307-643), which were both accelerated in the presence of protein S (Fig. 6b). These fragments were no longer susceptible to APC cleavage in the Q506 region, which may be due to dissociation of the 307-643 and the 307-709 fragments after cleavage at R306 (39).

Finally, the position of Rx2 could be estimated more precisely by comparing the mobility of the 30 kD fragments, which are the final products formed after inactivation of activated rFV\( \Delta B \)-wt, rFV\( \Delta B \)-GRA and rFV\( \Delta B \)-GQA by APC (Fig. 7). The final fragment generated during the inactivation of rFV\( \Delta B \)-wt has an expected MW of 30 kD, corresponding to fragment 307-506. The fragment generated from the inactivation of activated rFV\( \Delta B \)-GRA, most likely corresponding to position Rx2-506, was about 28 kD suggesting that the position of Rx2 is about 10-20 amino acids carboxy-terminal to residue 306. Consequently, the
position of Rx1 should be very close to residue 506, because the final product in the
inactivation of rFVΔB-GQA (Rx2-Rx1) also has a MW of 28 kD.

Cleavage of Rx1 in activated FVLeiden

Theoretically, cleavage at Rx1 (located in the region of residue 506) may serve as an
alternative for a cleavage at R506. To check this, purified plasma FVLeiden was activated and
inactivated using the same conditions as for the rFVΔB mutants and subsequently subjected to
analysis by SDS-PAGE and immunoblotting (Fig. 8). The immunoblot showed a very similar
cleavage pattern as observed for the activated rFVΔB-RQA mutant starting with a heavy chain
doublet of 105 kD and 90 kD. Initially a 50/60 kD doublet was generated, which was most
likely the result of cleavage at R306 in the 1-679 and 1-709 fragments. Also a fragment of 45
kD was generated, which was probably produced by cleavage of the 90 kD fragment resulting
in a fragment from residue 307 to 643. The fragments were not further cleaved at Rx1,
alogous to what was observed during inactivation of activated rFVΔB-RQA. This might be
the result of dissociation of the domain carboxy-terminal to R306 (A2 domain).
Discussion

To obtain more insight in the functional and structural implications of the individual cleavages at R306, R506 or R679 in the heavy chain of FVa by APC, recombinant FV mutants were generated in which two or three of these APC-cleavage sites had been mutated. Because the B-domain of FV has no crucial role in the APC-catalyzed inactivation of FVa, we used mutants lacking a major part of the B-domain in order to increase the yield of rFV mutants. The expression of these B-domainless mutants was about 10 times higher than that of the full-length constructs, which is in line with previous studies (35;40). Initial inactivation experiments with the purified recombinant proteins showed that rFVa-GQA (in which the known APC cleavage sites R306, R506 and R679 have been mutated) was still inactivated by APC, although at a much lower rate than normal FVa. The main objective of this study was to learn more about the mechanism underlying this unexpected observation.

All activated B-domainless rFV mutants used in this investigation were stable under the conditions chosen to study FVa inactivation by APC (Table 2). Also the presence of a heavy chain fragment of 90 kD (most likely representing fragment 1-643 (37) generated during the activation with thrombin) did not affect the stability of the activated rFV molecules. The inactivation of rFVa-GQA by APC is dependent on negatively charged phospholipids (Fig. 2) and is inhibited by a monoclonal antibody against protein C (Table 3), indicating that the observed inactivation is mediated by APC.

Kinetic analysis of the inactivation of rFVa-GQA by APC revealed that under pseudo-first order conditions (FVa concentration ranging from 1 to 5 nM (30)) rFVa-GQA was inactivated in a biphasic reaction, indicating that analogous to the inactivation of normal FVa also the inactivation of rFVa-GQA was associated with at least two cleavages (designated Rx1 and Rx2). This conclusion is supported by SDS-PAGE analysis of the reaction products (Fig. 5). During inactivation of rFVa-GQA a FVa intermediate (FVaB) with ~80% of the original FVa
activity was formed via rapid cleavage at Rx1. This intermediate was completely inactivated via a second slow cleavage at Rx2. The calculated second-order rate constant of the rapid reaction ($k_{x1} = 5.4 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$) was approximately 20-fold higher than that of the slow reaction ($k_{x2} = 3.2 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$ and $k'_{x2} = 2.6 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$). The rate constant of the rapid reaction is 50 and 1000-fold lower than those reported for cleavage at R306 and R506, respectively (30), but is somewhat higher than the second-order rate constant for cleavage at R679, which has been estimated at $7.6 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$ (30). Therefore, the knowledge of the existence of additional cleavage sites for APC, one close to residue 306 and one close to residue 506, is important for the determination of the kinetic parameters for cleavage at the individual sites R306, R506 or R679 and need to be taken into account (especially in the case of analysis of cleavage at R679).

Protein S strongly stimulated the inactivation of rFVa-GQA by APC (Fig. 4). Kinetic analysis revealed that the slow cleavage at Rx2 ($k'_{x2}$) that fully inactivates rFVa-GQA was stimulated about 50-fold by protein S (Table 4). In this analysis we assumed that cleavage at Rx1 was not affected by protein S. This is supported by the observation that in the presence of protein S (Fig. 5c) mainly a 60 kD fragment (cleavage at Rx2) was generated by APC instead of the 80 kD fragment observed in the absence of protein S. Moreover, under the experimental conditions used acceleration of the cleavage at Rx1 by protein S would hardly contribute to the final inactivation curve due to the high FVa activity associated with the intermediate formed after cleavage at Rx1.

To localize the position of the cleavage sites Rx1 and Rx2, the fragments generated during the APC-catalyzed degradation of rFVa-GQA were analyzed by SDS-PAGE and immunoblotting using an antibody with an epitope between residue 306 and 506 on the heavy chain of FVa. At time zero of the inactivation curves also some 90 kD fragment was present in the activated rFVΔB mutants (Fig. 5, 6) and activated FVLeiden (Fig. 8) apart from the expected 105 kD
heavy chain fragment. SDS-PAGE analysis of the time courses of thrombin activation revealed that this fragment was the result of cleavage by thrombin (data not shown), most likely at R643 (37). Due to the high sensitivity of the detection method it was not possible to quantify the relative amounts of 105 kD and 90 kD fragments. However, under the conditions used no effect of the cleavage at R643 on the FVα activity was observed (data not shown).

Positioning of Rx1 and Rx2 close to residue 506 and 306, respectively, would best fit the experimental data obtained via immunoblot and kinetic analysis. A position of Rx1 near residue 506 would explain the generation of a fragment of ~80 kD during the initial phase of the inactivation of rFVα-GQA by APC (Fig 5a). It also would explain the relatively high FVα activity of the intermediate formed after cleavage at Rx1, because cleavage of factor Vα at R506 also results in the formation of an intermediate with relatively high factor Vα activity (30;41). Location of Rx2 near residue 306 would explain the formation of peptides of 60 kD and 45 kD (from the heavy chains fragments of 105 kD and 90 kD, respectively) and 30kD (from the 80 kD intermediate) during the second slow inactivating phase. A position of Rx2 close to residue 306 is also compatible with the observation that the cleavage at Rx2 is stimulated by protein S (Table 4), because protein S exerts its stimulatory effect on the inactivation of normal FVα by selectively stimulating the cleavage at R306 (31). A more detailed localization of the position of Rx2 was obtained by comparing the 30 kD fragment (residues 307-506) generated in the inactivation of rFVΔB-wt with the fragment (Rx2-506) generated during the inactivation of activated rFVΔB-GRA by APC, when cleavage at R506 is followed by cleavage at Rx2 (Fig. 7). The fragment of Rx2-506 was about 1.5 kD smaller than the fragment of 307-506 indicating that the position of Rx2 is localized 10-20 residues carboxy-terminal to residue 306. This confines the candidates for the position of Rx2 to the residues R316, R317, K320 and R321. Consequently, the position of Rx1 should be very close to residue 506, because the Rx2-Rx1 fragment produced during the inactivation of
rFVa-GQA has the same molecular weight as the Rx2-506 fragment (Fig. 7). This would make residues R505 and R510 strong candidates for the location of Rx1. However, the localization of Rx1 at R505 seems less likely, because this residue is positioned in a putative Xa binding site (493-506) (42). Hence, cleavage at R505 would likely result in a FVa intermediate with a lower FVa activity than the 80% activity calculated for the intermediate after cleavage at Rx1. Currently, attempts are made to produce sufficient amounts of rFV∆B-GQA to identify the precise location of Rx1 and Rx2 by N-terminal sequencing.

Interestingly, the degradation patterns of the activated rFV∆B-RQA and rFV∆B-GRA mutants (Fig 6) confirmed some of the structural implications of cleavage at R306 or R506. The final fragments in the APC-mediated degradation of activated rFV∆B-RQA were 60 kD and 45 kD (Fig. 6b), which indicated that after cleavage at R306, Rx1 was no longer susceptible to APC cleavage. This might be explained by the dissociation of the 60 kD and 45 kD fragments from the FVa heterotrimer, a mechanism that has been proposed as explanation for the low FVa activity after cleavage at R306 (39). In contrast, the final fragment in the degradation of activated rFV∆B-GRA was 30 kD, which indicates that Rx2 can still be cleaved by APC after cleavage at R506.

This is the first time that APC cleavage sites are reported in the heavy chain of FVa different from the sites at R306, R506 and R679. During the inactivation of plasma FVa by APC, cleavage at Rx1 or Rx2 will hardly occur, because the second-order rate constants are much lower than those reported for cleavage at R506 and R306, even in the presence of protein S. This might explain why the sites of Rx1 and Rx2 have not been identified before in studies using normal FVas (10;30). Also, no novel APC-cleavage sites have been observed in studies using rFV molecules in which R306 and R506 had been mutated (22;24;41;43). However, the conditions used in these studies were such (much lower APC concentrations and shorter incubation time) that cleavages at Rx1 and Rx2 could not be detected.
During the present study we observed that Rx1 is not cleaved by APC as an alternative for R506 during the inactivation of activated FVLeiden (Fig. 7), most likely because of dissociation of the A2 domain after cleavage at R306. Inactivation of activated rFVΔB-GRA on the other hand clearly demonstrated that cleavage at Rx2 may occur as an alternative for cleavage at R306. Therefore, cleavage at Rx2 may be relevant in the APC-catalyzed inactivation of activated FV-Hongkong or FV-Cambridge and may explain the unexpected mild APC resistant phenotype associated with the carriernship of these FV mutants.
References


Abbreviations

The abbreviations used are: APC, activated protein C; DOPS, dioleoylphosphatidylserine; DOPC, dioleoylphosphatidylcholine; FV, factor V; FVa, activated factor V; rFV, recombinant FV, rFV\(\Delta B\)-GQA, recombinant factor V with a partial deletion of the B-domain containing the following mutations, R306G, R506Q, R679A; rFVa-GQA, activated rFV\(\Delta B\)-GQA; FXa, activated factor X; TN-EDTA, buffer containing 50 mM TRIS, 175 mM NaCl, 20 mM EDTA, 0.5 mg/ml ovalbumin, pH 7.9.

Acknowledgements

We would like to thank Dr. R.J. Kaufman for providing the expression vector pMT2FV. Furthermore, we thank Stella Thomassen for determining the APC concentration.
Table 1. Factor V molecules used in this study. Recombinant proteins were named after the amino acids present at the APC cleavage sites at positions 306, 506 and 679 using the one letter code for amino acids. The symbol $\Delta B$ indicates that a major part of the B-domain ($\Delta$ 827-1499) is lacking. Mutated positions are bold and underlined.

<table>
<thead>
<tr>
<th>Factor V</th>
<th>Mutation</th>
<th>Comments</th>
</tr>
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<tbody>
<tr>
<td>hFVLeiden</td>
<td>R506Q</td>
<td>FV isolated from homozygous FVLeiden carrier</td>
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<tr>
<td>rFV$\Delta B$-wt</td>
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<td>“wild type FV”</td>
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<tr>
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<td>R506Q, R679A</td>
<td>R306 intact</td>
</tr>
<tr>
<td>rFV$\Delta B$-GRA</td>
<td>R306G, R679A</td>
<td>R506 intact</td>
</tr>
<tr>
<td>rFV$\Delta B$-GQR</td>
<td>R306G, R506Q</td>
<td>R679 intact</td>
</tr>
<tr>
<td>rFV$\Delta B$-GQA</td>
<td>R306G, R506Q, R679A</td>
<td>No APC cleavage sites left</td>
</tr>
</tbody>
</table>
Table 2. Stability of activated rFVΔB mutants in the absence or presence of phospholipids. Spontaneous inactivation rates ($k_s$) were obtained by fitting time courses (30 hours) of rFVa inactivation with a single exponential. Incubation conditions were similar to those for FVa inactivation in the presence of APC (~1 nM FVa in 25 mM Hepes (pH 7.5), 175 mM NaCl, 3 mM CaCl$_2$, 5 mg/ml BSA +/- 24.5 µM phospholipids (10:90 DOPS:DOPC) at 37°C).

<table>
<thead>
<tr>
<th>Activated Factor V</th>
<th>$k_s$ (s$^{-1}$) + phospholipids</th>
<th>$k_s$ (s$^{-1}$) - phospholipids</th>
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<td>rFVΔB-wt</td>
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<td>3.7 x 10$^{-5}$</td>
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<td>6.4 x 10$^{-5}$</td>
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<td>rFVΔB-GQA</td>
<td>3.8 x 10$^{-5}$</td>
<td>4.6 x 10$^{-5}$</td>
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Table 3. Effect of APC inhibitor C12 on APC-catalyzed loss of FVa activity of activated rFV∆B-GQA. Activated rFV∆B-GQA was incubated for 180 min at 37°C in 25 mM Hepes (pH 7.5), 175 mM NaCl, 3 mM CaCl₂, 5 mg/ml BSA with 20 nM APC, which had been preincubated with anti-protein C antibody C12 (~60 µg/ml), anti-protein S antibody S18 (~60 µg/ml) or APMSF (80µM). The remaining FVa activity is shown.

<table>
<thead>
<tr>
<th>Condition</th>
<th>-APC</th>
<th>+ APC</th>
<th>+ APC/ C12</th>
<th>+ APC/ S18</th>
<th>+ APC/ APMSF</th>
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<tr>
<td>FVa activity (%)</td>
<td>100</td>
<td>32</td>
<td>75</td>
<td>34</td>
<td>81</td>
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</table>

1Residual FVa activity determined after 180 min in the absence of APC was set at 100%
Table 4. Second-order rate constants for inactivation of activated rFVΔB-GQA by APC via peptide bond cleavages at unidentified cleavage sites (Rx1 and Rx2). The second-order rate constants (M⁻¹s⁻¹) in the absence of protein S were calculated from the slopes of the plots of the pseudo first-order rate constants versus the APC concentrations (Fig. 3c,d,e). The second-order rate constants in the presence of protein S were measured at a single APC concentration of 20 nM. The pseudo-first order rate constants $k_2$ and $k_3$ were determined by fitting the data to equation 3 (Fig. 4), assuming that $k_{x1} (5.4 \times 10^4$ M⁻¹s⁻¹) and B (0.82) were not affected by the presence of protein S (see text). The second-order rate constants $k_{x2}$ and $k'_{x2}$ were calculated from the pseudo first-order rate constants as described under Experimental Procedures).

<table>
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<tr>
<th>Rate constants</th>
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<th>+ protein S</th>
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<tr>
<td>$k_{x1}$ (M⁻¹s⁻¹)</td>
<td>5.4 x 10⁴</td>
<td>5.4 x 10⁴</td>
</tr>
<tr>
<td>$k_{x2}$ (M⁻¹s⁻¹)</td>
<td>3.2 x 10³</td>
<td>8.1 x 10⁴</td>
</tr>
<tr>
<td>$k'_{x2}$ (M⁻¹s⁻¹)</td>
<td>2.6 x 10³</td>
<td>1.5 x 10⁵</td>
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</tbody>
</table>
Figure 1. Schematic representation of fragments generated during APC-mediated proteolysis of the heavy chain of activated rFVΔB mutants with a single APC cleavage site. Three rFVΔB double mutants were prepared for this study in each of which only one APC-cleavage site was left. Based on previous reports APC-mediated cleavage should generate fragments with molecular weights as indicated (10;30).

Figure 2. Effect of phospholipids on the inactivation of activated rFVΔB-GQA by APC. 1 nM of activated rFVΔB-GQA was incubated with 20 nM APC in the presence (●) or in the absence (■) of 24.5 µM phospholipid vesicles (DOPS/DOPC, 10/90) in 25 mM Hepes (pH 7.5), 175 mM NaCl, 3 mM CaCl₂ and 5 mg/ml BSA at 37°C. At the indicated time points FVa activity was measured as described in “Experimental Procedures”. In the presence of phospholipids vesicles the activated rFVΔB-GQA was also incubated without APC (▲) to determine spontaneous inactivation under these conditions.

Figure 3. Inactivation of activated rFVΔB-GQA with different APC concentrations. Activated rFVΔB-GQA (1 nM) was incubated with 0 nM (x), 20 nM (■), 40 nM (●), 60 nM (▲) or 80 nM (●) APC in the presence of 24.5 µM phospholipid vesicles (DOPS/DOPC, 10/90) in 25 mM Hepes (pH 7.5), 175 mM NaCl, 3 mM CaCl₂ and 5 mg/ml BSA at 37°C. At the indicated time intervals FVa activity was measured as described in “Experimental Procedures”. Data are presented as means +/- SD. The solid lines in Fig 3a represent exponential curves obtained by fitting the data to equation 3 (using $k_2$ and B obtained from Fig. 3b) with nonlinear least-squares regression of the data. The pseudo first-order rate constants of the second slow phase ($k_2$) and the activity of the reaction intermediate (B) were estimated from the plots in Fig 3b. The slope of the lines from 90 to 180 min represents $k_2$. 
while the intercept of these lines with the Y-axis represents the activity of the reaction intermediate (B). In Fig. 3c the pseudo first-order rate constants of \( k_2 \) (s\(^{-1}\)) have been plotted versus the APC concentration. The slope of this line corresponds to the second-order rate constant \( k_{x2} \). The pseudo first-order rate constants for \( k_1 \) (s\(^{-1}\)) and \( k_3 \) (s\(^{-1}\)) were obtained by fitting the experimental data to equation 3, in which \( k_2 \) (s\(^{-1}\)) and \( B \) were fixed. In Fig. 3d the pseudo first-order rate constants of \( k_1 \) (s\(^{-1}\)) have been plotted versus the APC concentration. From the slope of this line the second-order rate constant \( k_{x1} \) was determined. In Fig. 3e the pseudo first-order rate constants of \( k_3 \) (s\(^{-1}\)) have been plotted versus the APC concentration. The slope of this line represents the second-order rate constant \( k'_{x2} \).

**Figure 4. Effect of protein S on the APC-catalyzed inactivation of activated rFVΔB-GQA.** 1 nM of activated rFVΔB-GQA was incubated at 37°C with 20 nM APC in the absence (■) or in the presence (●) of 490 nM protein S (in 25 mM Hepes (pH 7.5), 175 mM NaCl, 3 mM CaCl\(_2\), 5 mg/ml BSA and 24.5 µM phospholipid vesicles, DOPS/DOPC, 10/90). At indicated time points FVa activity was measured as described in “Experimental Procedures”. Data are presented as means +/- SD. The fitted curves (solid lines) were obtained after fitting the data with equation 1 using non-linear least-squares regression.

**Figure 5. Immunoblot analysis of APC-catalyzed inactivation of activated rFVΔB-GQA.** 1 nM of activated rFVΔB-GQA was incubated at 37°C in an inactivation mixture containing 20 nM APC and 24.5 µM phospholipid vesicles (DOPS/DOPC, 10/90) in 25 mM Hepes (pH 7.5), 175 mM NaCl, 3 mM CaCl\(_2\) and 5 mg/ml BSA. At indicated time points samples were drawn from the inactivation mixture and subjected to 8% (Fig. 5a) and 4-15% (Fig. 5b) SDS-PAGE under reducing conditions followed by immunoblot analysis. Fragments from the heavy chain were visualized with mAb AHV-5146 (epitope between R306 and R506). The
same procedure was also performed in the presence of 490 nM protein S, after which the samples were subjected to 8% SDS/PAGE and immunoblot analysis (Fig. 5c).

**Figure 6. Immunoblot analysis of APC-catalyzed inactivation of activated rFVΔB-GRA and rFVΔB-RQA in the presence of protein S.** 1 nM of activated rFVΔB-GRA (Fig. 6a) was incubated at 37°C in an inactivation mixture containing 20 nM APC, 490 nM protein S and 24.5 µM phospholipid vesicles (DOPS/DOPC, 10/90) in 25 mM Hepes (pH 7.5), 175 mM NaCl, 3 mM CaCl₂ and 5 mg/ml BSA. 1 nM of rFVΔB-RQA (Fig. 6b) was incubated using the same conditions with 6 nM APC. At indicated time points samples were drawn from the inactivation mixture and subjected to 4-15% SDS-PAGE under reducing conditions followed by immunoblot analysis. Fragments from the heavy chain were visualized with mAb AHV-5146 (epitope between R306 and R506). In the blot of rFVΔB-GRA (Fig. 6a) a small band with a MW around 50 kD was observed, which appeared to be present in all time samples. Most likely, purified samples of this FV mutant were enriched with some IgG during the purification procedure, which could also be stained.

**Figure 7. Comparison of 30 kD fragments generated during the APC-catalyzed inactivation of activated rFVΔB-GQA, rFVΔB-wt and rFVΔB-GRA.** The sample containing rFVa-GQA was the same as used in Fig. 5b (T = 90 min). The sample containing activated rFVΔB-wt was drawn after 30 min from an inactivation mixture containing 1 nM rFVΔB-wt, 0.15 nM APC, 490 nM protein S and 24.5 µM phospholipid vesicles (DOPS/DOPC, 10/90) in 25 mM Hepes (pH 7.5), 175 mM NaCl, 3 mM CaCl₂ and 5 mg/ml BSA at 37°C. The sample containing rFVΔB-GRA was the same as used in Fig. 6a (T = 30 min). Samples were subjected to 12-20% SDS-PAGE under reducing conditions followed by immunoblot analysis. Fragments from the heavy chain were visualized with mAb AHV-5146
(epitope between R306 and R506). The 30 kD fragments represent the following cleavage products: rFV∆β-GQA, Rx2-Rx1; rFV∆β-wt, 307-506 and rFV∆β-GRA, Rx2-506.

**Figure 8. Immunoblot analysis of APC-catalyzed inactivation of activated plasma FVLeiden in the presence of protein S.** 1 nM of activated FVLeiden was incubated at 37°C in an inactivation mixture containing 6 nM APC, 490 nM protein S and 24.5 µM phospholipid vesicles (DOPS/DOPC, 10/90) in 25 mM Hepes (pH 7.5), 175 mM NaCl, 3 mM CaCl₂ and 5 mg/ml BSA. At indicated time points samples were drawn from the inactivation mixture and subjected to 4-15% SDS-PAGE under reducing conditions followed by immunoblot analysis. Fragments from the heavy chain were visualized with mAb AHV-5146 (epitope between R306 and R506).
Fig. 1

<table>
<thead>
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<th>Protein</th>
<th>Mass</th>
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<td>rFVΔB-RQA</td>
<td>45 kDa</td>
</tr>
<tr>
<td></td>
<td>60/62 kDa</td>
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<tr>
<td>rFVΔB-GRA</td>
<td>75 kDa</td>
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<td>26/28 kDa</td>
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<tr>
<td>rFVΔB-GQR</td>
<td>100 kDa</td>
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<tr>
<td></td>
<td>6 kDa</td>
</tr>
</tbody>
</table>
Fig 2.
Fig 3a.

![Graph showing the activity of FVa over time.](image)

Fig 3b.

![Graph showing the natural logarithm of the activity of FVa over time.](image)
Fig. 3c

![Graph showing the relationship between APC (nM) and $k_2$ (10^{-4} s^{-1}).](image)

Fig. 3d

![Graph showing the relationship between APC (nM) and $k_1$ (10^{-4} s^{-1}).](image)
Fig. 3e

![Graph showing the relationship between APC concentration (nM) and $k_3$ ($10^{-4}$ s$^{-1}$).]
Fig. 4

![Graph showing FVa activity (%)](image-url)
Fig. 5a

Fig. 5b
Fig. 5c
Factor Va is inactivated by APC in the absence of cleavage sites at Arg306, Arg506 and Arg679
M. van der Neut Kolfschoten, Richard J. Dirven, Hans L. Vos, Guido Tans, Jan Rosing and Rogier M. Bertina

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