Factor Va Is Inactivated by Activated Protein C in the Absence of Cleavage Sites at Arg-306, Arg-506, and Arg-679*

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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: Arg-306, Arg-506, and Arg-679. To obtain more insight in the structural and functional implications of each individual cleavage, recombinant factor V (rFV) 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 \( k_{1} = 5.4 \times 10^{4} \text{ M}^{-1} \text{s}^{-1} \) and a slow phase \( k_{2} = 3.2 \times 10^{3} \text{ M}^{-1} \text{s}^{-1} \). Analysis of the inactivation curves showed that the rapid phase yielded a reaction intermediate that retained \( \approx 80\% \) of the original FVa activity, whereas the slow cleavage resulted in formation of a completely inactive reaction product. Inactivation of rFV-GQA was accelerated by protein S, most likely via stimulation of the slow phase. Immunoblot analysis using a monoclonal antibody recognizing an epitope between Arg-306 and Arg-506 indicated that during the rapid phase of inactivation a fragment of 80 kDa was generated that resulted from cleavage at a residue very close to Arg-506. The slow phase was associated with the formation of fragments resulting from cleavage at a residue 1.5-2 kDa carboxyl-terminal to Arg-306. Our observations may explain the unexpectedly mild APC resistance associated with mutations at Arg-306 (FV HongKong and FV Cambridge) in the heavy chain of FV.

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 (\(-330 \text{ kDa}\)) by thrombin. It consists of a heavy chain (\(-105 \text{ kDa}\)) and a light chain (71–74 kDa), which are held together via a Ca\(^{2+}\) ion.

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\(^{3}\) The abbreviations used are: FV, factor V; APC, activated protein C; DOPS, dioleoylphosphatidylserine; DOPC, dioleoylphosphatidylcholine; 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; BSA, bovine serum albumin; mAbs, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay; FVL, FV Leiden.

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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 ΔΔ indicates that a major part of the B-domain (Δ326-1499) is lacking. Mutated positions are boldface and underlined.

### Factor V molecules used in this study

| Factor V | Mutation | Comments |
|----------|----------|----------|
| hFVLeiden | R506Q | FV isolated from homozygous FVLeiden carrier |
| rFVΔB-wt | No mutations | "Wild type FV" |
| rFVΔB-RQA | R506Q, R679A | Arg-306 intact |
| rFVΔB-GQA | R306G, R679A | Arg-506 intact |
| rFVΔB-GQR | R306G, R506Q | Arg-679 intact |
| rFVΔB-GQA | R306G, R506Q, R679A | No APC cleavage sites left |

506 yields a reaction intermediate with reduced FVa activity, which is completely inactivated by the subsequent cleavage at Arg-306. Inactivation of activated FV is monophasic and associated with cleavage at Arg-306, which was stimulated 20-fold by protein S (31). In the presence of negatively charged phospholipids, the contribution of the cleavage at Arg-679 to the inactivation of FVAs 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 FVa mutants were constructed in which a major part of the B-domain (rFVΔB) 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, rFVAs-GQA (rFv 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 paper we report on the mechanism of APC-catalyzed inactivation of rFvA-GQA.

### Experimental Procedures

**Materials**—Restriction enzymes were from New England Biolabs, Beverly, MA. Rapid ligation kit and BM chemiluminescence blotting substrate (peroxidase) were obtained from Roche Diagnostics. Plasmid isolation kits were purchased from Qiagen, Chatsworth, CA. DNA restriction fragments were purified from agarose gel using the Cleanmix kit (Talent, Trieste, Italy). CNBr-Sepharose and SP-Sepharose were obtained from Amersham Biosciences. Bovine serum albumin (BSA), 98% fatty acid-free, was from ICN Biomedicals (Aurora, OH). Ovalbumin, benzamidine, phosphatidylcholine (PC), N-hydroxysuccinimidobiotin, and Hepes were from Sigma. Dioleophosphatidylcholine (DOPC) and dioleophosphatidylserine (DOPS) were obtained from Avanti Polar Lipids, Alabaster, AL. EDTA was from Serva, Heidelberg, Germany. NH₄Cl, Tris, and CaCl₂ were purchased from Merck. Chromogenic substrate S-2238 was obtained from Chromogenix, Uppsala, Sweden. 4-amidinophenylmethanesulfonyl fluoride was from Roche Diagnostics.

### Proteins

Human APC, protein S, prothrombin, and thrombin were purchased from Enzyme Research Laboratories (South Bend, IN). Human APC, protein S, prothrombin, and thrombin were purchased from Amersham Biosciences. Bovine serum albumin (BSA), 98% fatty acid-free, was from ICN Biomedicals (Aurora, OH). Ovalbumin, benzamidine, phosphatidylcholine (PC), N-hydroxysuccinimidobiotin, and Hepes were from Sigma. Dioleophosphatidylcholine (DOPC) and dioleophosphatidylserine (DOPS) were obtained from Avanti Polar Lipids, Alabaster, AL. EDTA was from Serva, Heidelberg, Germany. NH₄Cl, Tris, and CaCl₂ were purchased from Merck. Chromogenic substrate S-2238 was obtained from Chromogenix, Uppsala, Sweden. 4-amidinophenylmethanesulfonyl fluoride was from Roche Diagnostics.

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This model is similar to the inactivation of plasma FVα via cleavages at Arg-506 and Arg-306 (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, $i.e.$, conditions at which the inactivation rate is directly proportional to the factor Vα and APC concentration, the loss of FVα cofactor activity is described by Equation 1 (30).

$$V_a = \frac{V_a}{k_1 + k_2 + k_3} \left(1 - e^{-k_1 t}\right)$$

In this equation $V_a$ is the FVα activity at time $t$; $V_0$ is the FVα activity at time 0; $B$ is the activity of the FVα 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: $k_{2a} = k_1/APC$, $k_{2b} = (k_2 - k_1)/APC$ and $k_{2c} = (k_3 - k_1)/APC$, in $k_1$, $k_2$, and $k_3$ are the first-order rate constants of spontaneous inactivation of the FVα intermediate (FVa-h) and intact FVα, respectively.

**RESULTS**

**Expression and Purification of Recombinant Factor V Mutants**—Recombinant FvΔB mutants were constructed with mutations either in two or in three of the known APC cleavage sites (Arg-306, Arg-506, and Arg-679). In analogy to the naturally occurring FV HongKong (R306G) and FV Leiden (R506Q) mutations, Arg-306 was replaced by glycine and Arg-506 by glutamine, whereas Arg-679 was replaced by alanine (Table I).

After transfection of COS-1 cells, conditioned media containing the rFvΔB molecules were collected, centrifuged, and stored at $-20^\circ$C. Factor V expression levels (activity and antigen) of rFvΔB-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, rFvΔB proteins were purified by ion exchange and immunoaffinity chromatography. The rFvΔB mutants were activated with thrombin and analyzed by SDS-PAGE followed by silver staining or immunoblotting. Both detection methods showed two dominant bands with a molecular mass of 105 and 71 kDa, which corresponded to the expected molecular mass of the heavy and light chain of factor Vα, respectively (data not shown). Apart from these two bands, also variable amounts of 90-kDa fragment were observed, which was most likely the result of thrombin cleavage at Arg-643 (37). The specific activities (activity/antigen) of the purified rFVα mutants were slightly lower than in conditioned medium, being around 0.7.

**Stability of Activated rFVαB Mutants**—Recombinant FvΔB 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 rFvΔB mutants was assessed by following the loss of FVα 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

![Fig. 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 (10, 30), APC-mediated cleavage should generate fragments with molecular weights as indicated.](http://www.jbc.org/)

![Fig. 2. Effect of phospholipids on the inactivation of activated rFvΔB-GQA by APC.](http://www.jbc.org/)

and the activated rFvΔB mutants were approximately similar, being around $3.0 \times 10^{-7}$ s$^{-1}$, which corresponds to a spontaneous loss of FVα 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 rFvα-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 rFvα-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 4-aminophenylmethanesulfonyl fluoride (a nonspecific inhibitor of serine proteases) inhibited the activation of rFvα-GQA by APC, whereas preincubation with a monoclonal antibody against protein S (S18) did not affect the rate of inactivation (Table III). This indicates that the loss of FVα 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 rFvα-GQA by APC results from one or more proteolytic cleavages at still unidentified cleavage sites. To
FIG. 3. Inactivation of activated rFVΔB-GQA with different APC concentrations. Activated rFVΔB-GQA (1 nM) was incubated with 0 nM (∗), 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 under...
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. Because 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 (k2) for the second reaction and the activity of the FVa intermediate (FVaB) from semi-logarithmic plots (Fig. 3b). The slope of the lines yields k2 at different APC concentrations, and the intercept at the y-axis yields an estimate for the FVa activity remaining in the intermediate (FVaB), which was found to be ~80%. Fig. 3c shows that the pseudo first-order rate constant k2 obtained for the second slow reaction is a linear function of the APC concentration, which enabled calculation of the second-order rate constant (k2x2 = 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^{-6} \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 FVaB (represented as k’2). This rate constant is close to that determined for native FVa (k2 = -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 1 under “Experimental Procedures”), in which rFVa-GQA is rapidly cleaved by APC resulting in an intermediate (FVaB) with slightly lower FVa activity, whereas a much slower cleavage in FVA and FVaB results in the formation of a FVa derivative 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 Arg-506 (fast reaction) and Arg-306 (slow reaction). By using the pseudo-first-order rate constants (k2B and B) calculated from Fig. 3, b and c, the pseudo first-order rate constants k1 and k2 were calculated by fitting the experimental data (Fig. 3a) to Equation 1. Like k2B and k1, k2 also increased with higher APC concentrations (Fig. 3, d and e), 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 (k1x1) and Rx2 (k2x2 and k’2x2). From these plots, the second-order rate constants k2x2 were calculated (see “Experimental Procedures”). The second-order rate constant of the first reaction (k2x1 = 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 k2x2 and k’2x2 in Table IV). These rate constants are much lower than those reported for APC-catalyzed cleavage at Arg-506 (k206 = 2.3 \times 10^6 \text{ M}^{-1} \text{s}^{-1}) and Arg-506 (k’206 = 4.3 \times 10^7 \text{ M}^{-1} \text{s}^{-1}) (30).

Effect of Protein S on the APC-catalyzed Inactivation of Activated rFVAB-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 Arg-306 (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 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 (FVaB) still has a high FVa activity. Assuming that k’1x1 (5.4 \times 10^4 \text{ M}^{-1} \text{s}^{-1}) and B (0.82) were not affected by the addition of protein S, k2 and k3 values were determined by fitting the data to Equation 1 (Table IV). The second-order rate constant k’2x2 (calculated from k2x1) was -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 rFVAB-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 Arg-306 and Arg-506. Experiments with synthetic peptides identified the epitope for AHV-5146 between residues 427 and 440.2 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 kDa, whereas the second slow phase was associated with the generation of a doublet of 55/60 kDa.

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"Experimental Procedures." Data are presented as means ± S.D. The solid lines in a represent exponential curves obtained by fitting the data to Equation 1 (using k2 and B obtained from b) with nonlinear least squares regression of the data. The pseudo first-order rate constants of the second slow phase (k2) and the activity of the reaction intermediate B were estimated from the plots in b. The slope of the lines from 90 to 180 min represents k2, whereas the intercept of these lines with the y-axis represents the activity of the reaction intermediate B. c, the pseudo first-order rate constants of k2 (s−1) have been plotted versus the APC concentration. The slope of this line corresponds to the second-order rate constant k2x2. The pseudo first-order rate constants for k2 (s−1) and k’2x2 were obtained by fitting the experimental data to Equation 1, in which k2x2 (s−1) and B were fixed, d, the pseudo first-order rate constants of k1 (s−1) have been plotted versus the APC concentration. From the slope of this line, the second-order rate constant k1x1 was determined. e, the pseudo first-order rate constants of k3 (s−1) have been plotted versus the APC concentration. The slope of this line represents the second-order rate constant k’2x2.

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and fragments of 45 and 30 kDa (Fig. 5). Note that besides the heavy chain fragment of 105 kDa (intact heavy chain from position 1 to 709), a fragment of ~90 kDa was also detected, which was already present before the addition of APC. Most likely, this fragment was formed during the activation with thrombin, which can cleave the heavy chain of FVa at Arg-643 (37).

In the presence of protein S, the loss of FVa activity was essentially associated with the generation of a 60-kDa fragment (Fig. 5c). After 15 min 45- and faint 30-kDa fragments were also 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-kDa fragment (Fig. 5, a and 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 amino or the carboxyl terminus of the heavy chain. Because the amino-terminal region (residue 150–200) 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 Gln-506. Cleavage in this region will result in the formation of an 80-kDa fragment from both the 105- and 90-kDa heavy chains and is also compatible with the high activity of the intermediate (FVα ~80%) after cleavage at Rx1. Consequently, the slow cleavage (Rx2), which results in complete inactivation, should be positioned in the region of Gly-306. This would explain the generation of the observed 60-kDa fragment (cleavage in the intact heavy chain of 105 kDa) and the 30-kDa fragment (cleavage in the intermediate fragment of 80 kDa). A cleavage close to position 306 can also explain the generation of a 45-kDa fragment (cleavage in heavy chain fragment of 90 kDa), which has been described before (37). Furthermore, a location of Rx2 close to Arg-306 is compatible with the stimulation of k’x2 by protein S (formation of 60-kDa fragment in Fig. 5c), because cleavage at Arg-306 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 rFVΔB-GRA (Arg-506 present) and rFVΔB-RQA (Arg-306 present) were incubated with APC. Inactivation of activated rFVΔB-GRA with APC (20 nM) generated an expected fragment of 75 kDa (amino acids 1–506), which was gradually cleaved into a 30-kDa fragment (data not shown), suggesting that in the region of Gly-306 indeed another APC cleavage site (Rx2) is present. The generation of this 30-kDa fragment was strongly accelerated in the presence of protein S (Fig. 6a). Inactivation of activated rFVΔB-RQA with 20 nM APC generated fragments with the expected sizes of 60 (fragment 307–709) and 45 kDa (fragment 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 Gln-506 region, which may be due to dissociation of the 307–643 and the 307–709 fragments after cleavage at Arg-306 (39).

Finally, the position of Rx2 could be estimated more precisely by comparing the mobility of the 30-kDa fragments, which are the final products formed after inactivation of activated rFVΔB-wt, rFVΔB-GRA, and rFVΔB-GQA by APC (Fig. 7). The immunoblot analysis. Fragments from the heavy chain were visualized with mAb AHV-5146 (epitope between Arg-306 and Arg-506). 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 (c).
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Theoretical, cleavage of Rx1 in Activated FVL—Theoretically, cleavage at Rx1 (located in the region of residue 506) may serve as an alternative for a cleavage at Arg-506. To check this, purified plasma FVL 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 and 90 kDa. Initially a 50/60-kDa doublet was generated, which suggests the result of cleavage at Arg-306 in the 1–679 and 1–709 fragments. Also a fragment of 45 kDa was generated, which was probably produced by cleavage of the 90-kDa fragment resulting in a fragment from residues 307–643. The fragments were not further cleaved at Rx1, analogous to what was observed during inactivation of activated rFVΔB-RQA. This might be the result of dissociation of the domain carboxyl-terminal to Arg-306 (A2-domain).

**DISCUSSION**

To obtain more insight in the functional and structural implications of the individual cleavages at Arg-306, Arg-506, or Arg-679 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 re-
combimnant proteins showed that rFVa-GQA (in which the known APC cleavage sites Arg-306, Arg-506, and Arg-679 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 II). Also the presence of a heavy chain fragment of 90 kDa (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 III), 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 FVas 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, an FVa intermediate (FVa<sub>int</sub>), 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<sub>x1</sub> = 5.4 × 10<sup>4</sup> M<sup>-1</sup> s<sup>-1</sup>) was 20-fold higher than that of the slow reaction (k<sub>x2</sub> = 3.2 × 10<sup>3</sup> M<sup>-1</sup> s<sup>-1</sup> and k<sup>'</sup>x2 = 2.6 × 10<sup>3</sup> M<sup>-1</sup> s<sup>-1</sup>). The rate constant of the rapid reaction is 50- and 1000-fold lower than those reported for cleavage at Arg-306 and Arg-506, respectively (30), but is somewhat higher than the second-order rate constant for cleavage at Arg-679, which has been estimated at 7.6 × 10<sup>3</sup> M<sup>-1</sup> s<sup>-1</sup> (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 Arg-306, Arg-506, or Arg-679 and need to be taken into account (especially in the case of analysis of cleavage at Arg-679). Protein S strongly stimulated the inactivation of rFVa-GQA by APC (Fig. 4). Kinetic analysis revealed that the slow cleavage at Rx2 (k<sub>x2</sub>) that fully inactivates rFVa-GQA was stimulated about 50-fold by protein S (Table IV). 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-kDa fragment (cleavage at Rx2) was generated by APC instead of the 80-kDa 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 residues 306 and 506 on the heavy chain of FVa. At time 0 of the inactivation curves, some 90-kDa fragment was also present in the activated rFVΔB mutants (Figs. 5 and 6) and activated FVL (Fig. 8) apart from the expected 105-kDa heavy chain fragment. SDS-PAGE analysis of the time course of thrombin activation revealed that this fragment was the result of cleavage by thrombin (data not shown), most likely at Arg-643 (37). Due to the high sensitivity of the detection method, it was not possible to quantify the relative amounts of 105- and 90-kDa fragments. However, under the conditions used, no effect of the cleavage at Arg-643 on the FVa 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 kDa during the initial phase of the inactivation of rFVa-GQA by APC (Fig. 5c). It also would explain the relatively high FVa activity of the intermediate formed after cleavage at Rx1, because cleavage of factor Vra at Arg-506 also results in the formation of an intermediate with relatively high factor Vra activity (30, 41). Location of Rx2 near residue 306 would explain the formation of peptides of 60 and 45 kDa (from the heavy chains fragments of 105 and 90 kDa, respectively) and 30 kDa (from the 80-kDa 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 IV), because protein S exerts its stimulatory effect on the inactivation of normal FVas by selec-

### Table II

| Activated factor V | k<sub>x1</sub> (s<sup>-1</sup>) | k<sub>x2</sub> (s<sup>-1</sup>) |
|-------------------|-----------------|-----------------|
| rFVΔB-wt         | 3.3 × 10<sup>-5</sup> | 3.7 × 10<sup>-5</sup> |
| rFVΔB-RQA        | 5.4 × 10<sup>-5</sup> | 6.4 × 10<sup>-5</sup> |
| rFVΔB-GQA        | 2.9 × 10<sup>-5</sup> | 3.3 × 10<sup>-5</sup> |
| rFVΔB-GQR        | 3.1 × 10<sup>-5</sup> | 2.8 × 10<sup>-5</sup> |
| rFVΔB-GQA        | 3.8 × 10<sup>-5</sup> | 4.0 × 10<sup>-5</sup> |

### Table III

| Condition | APC<sup>c</sup> + APC | APC + APC/C12 | + APC/S18 | + APC/AMPSF |
|-----------|----------------------|----------------|----------|-------------|
| FVa activity | 100                  | 75             | 34        | 81          |

<sup>c</sup> Residual FVa activity determined after 180 min in the absence of APC was set at 100%.

### Table IV

| Rate constants | Protein S | Protein S |
|----------------|-----------|-----------|
| k<sub>x1</sub> (m<sup>-1</sup> s<sup>-1</sup>) | 5.4 × 10<sup>4</sup> | 5.4 × 10<sup>4</sup> |
| k<sub>x2</sub> (m<sup>-1</sup> s<sup>-1</sup>) | 3.2 × 10<sup>3</sup> | 8.1 × 10<sup>4</sup> |
| k<sup>'</sup>x2 (m<sup>-1</sup> s<sup>-1</sup>) | 2.6 × 10<sup>3</sup> | 1.5 × 10<sup>5</sup> |

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tively stimulating the cleavage at Arg-306 (31). A more detailed localization of the position of Rx2 was obtained by comparing the 30-kDa 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 Arg-506 is followed by cleavage at Rx2 (Fig. 7). The fragment of Rx2-506 was about 1.5 kDa smaller than the fragment of 307–506, indicating that the position of Rx2 is localized 10–20 residues carboxyl-terminal to residue 306. This confines the candidates for the position of Rx2 to the residues Arg-316, Arg-317, Lys-320, and Arg-321. Consequently, the position of Rx1 should be very close to residue 506, because the Rx2-Rx1 fragment produced during the inactivation of rFVα-GQA has the same molecular weight as the Rx2-506 fragment (Fig. 7). This would make residues Arg-505 and Arg-510 strong candidates for the location of Rx1. However, the localization of Rx1 at Arg-505 seems less likely, because this residue is positioned in a putative Xa-binding site (amino acids 493–506) (42). Hence, cleavage at Arg-505 would likely result in an 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 FV mutants (Fig. 6) to confirm some of the results for the location of Rx1. However, the localization of Rx1 should be very close to residue 506, because the Rx2-Rx1 fragment produced during the inactivation of rFVα-GQA can still be detected by amino-terminal sequencing.

Interestingly, the degradation patterns of the activated rFVΔB-RQA and rFVΔB-GRA mutants (Fig. 6) confirmed some of the structural implications of cleavage at Arg-306 or Arg-506. The final fragments in the APC-mediated degradation of activated rFVΔB-RQA were 60 and 45 kDa (Fig. 6b), which indicated that after cleavage at Arg-306, Rx1 was no longer susceptible to APC cleavage. This might be explained by the dissociation of the 60- and 45-kDa fragments from the FVa heterotrimer, a mechanism that has been proposed as explanation for the low FVa activity after cleavage at Arg-306 (39). In contrast, the final fragment in the degradation of activated rFVΔB-GRA was 30 kDa, which indicates that Rx2 can still be cleaved by APC after cleavage at Arg-506.

This is the first time that APC cleavage sites are reported in the heavy chain of FVa different from the sites at Arg-306, Arg-506, and Arg-679. 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 Arg-506 and Arg-306, 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 Arg-306 and Arg-506 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 Arg-506 during the inactivation of activated FV (Fig. 7), most likely because of dissociation of the A2-domain after cleavage at Arg-306. Inactivation of activated rFVΔB-GRA on the other hand clearly demonstrated that cleavage at Rx2 may occur as an alternative for cleavage at Arg-306. Therefore, cleavage at Rx2 may be relevant in the FV-catalyzed inactivation of activated FV-Hongkong or FV-Cambridge and may explain the unexpected mild APC-resistant phenotype associated with the carriage of these FV mutants.
Factor Va Is Inactivated by Activated Protein C in the Absence of Cleavage Sites at Arg-306, Arg-506, and Arg-679

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