Enhanced Rate of Cleavage at Arg-306 and Arg-506 in Coagulation Factor Va by Gla Domain-mutated Human-activated Protein C
*

Yong-Hui Sun‡, Sinh Tran‡, Eva A. Norstrøm, and Björn Dahlbäck§

From the Department of Laboratory Medicine, Division of Clinical Chemistry, Lund University, University Hospital, Malmö, S-20502 Malmö, Sweden

A Gla domain-mutated protein C variant, QGNSEDY, modified at positions 10–12, 23, 32–33, and 44, having enhanced affinity for negatively charged phospholipid and increased anticoagulant potential, was used to elucidate the importance of the interaction between the Gla domain and the phospholipid for the ability of activated protein C (APC) to inactivate factor Va (FVa). FVa degradation by wild type (WT)-APC and QGNSEDY-APC yielded similar fragments on Western blotting; QGNSEDY-APC was, however, considerably more efficient. The kinetic parameters for individual APC-mediated cleavages in FVa, i.e. at Arg-306 and Arg-506, were investigated at high and low phospholipid concentrations in the presence and absence of protein S. FVa variants 306Q679Q and 506Q679Q, which can only be cleaved at Arg-506 and Arg-306, respectively, were used. In the absence of protein S, QGNSEDY-APC was 17.8- and 4-fold more efficient than WT-APC in cleaving at Arg-306 and Arg-506, respectively, at high phospholipid. Similar values were obtained at low phospholipid. In the presence of protein S, QGNSEDY-APC was 6.8- and 3.2-fold more active than WT-APC in cleaving at Arg-306 and Arg-506, respectively, at high phospholipid. At low phospholipid, the corresponding values were 14- and 6.5-fold. In conclusion, the modification of the Gla domain in QGNSEDY-APC yielded increased rates of cleavage at both sites in FVa, the increase being particularly pronounced for the Arg-306 site in the absence of protein S. The results obtained with QGNSEDY-APC provide insights into the importance of the APC-phospholipid interaction for the APC-mediated cleavages at Arg-306 and Arg-506 in FVa.

Blood coagulation comprises a series of enzymatic reactions, many of which take place on negatively charged phospholipid membranes upon which enzymes and cofactors form highly efficient complexes (1–3). Several of the coagulation enzymes, e.g. factor IX (FIX), factor X (FX) and prothrombin, are vitamin K-dependent and thus contain a γ-carboxyl glutamic acid (Gla)-rich domain, which binds to negatively charged phospholipid (4). Activated factor IX and activated factor X (FXa) interact with their respective cofactors, activated factor VIII (FVIIIa) and activated factor V (FVa), on the phospholipid surface, thus forming the tenase and prothrombinase complexes that activate FX and prothrombin, respectively (1, 3, 5). The cofactors FVIIIa and FVa provide several orders of magnitude enhancement to the efficiency of their respective enzyme.

The protein C anticoagulant pathway provides efficient and specific regulation of the coagulation system by inhibiting the activity of the two cofactors, FVIIIa and FVas (3, 6–8). Protein C, the key component of the pathway, is converted to activated protein C (APC) on the surface of endothelial cells by thrombin bound to the membrane protein thrombomodulin. The specific proteolysis of FVIIIa and FVa by APC is stimulated by protein S, which serves as an APC cofactor (9–11). Protein C and protein S are both vitamin K-dependent proteins containing Gla domains that bind to negatively charged phospholipid surfaces upon which the two proteins form an anticoagulant complex (8). This complex is effective in cleaving and inhibiting FVas but is insufficient to down-regulate FVIIIa, which takes part in the tenase complex. In this reaction the APC-protein S complex is supported by yet another APC cofactor, namely the non-activated form of FX (12, 13). Thus, circulating FX has the potential to express both pro- and anticoagulant functions as a procoagulant FXa cofactor after activation by thrombin or FXas and as an anticoagulant APC cofactor (11).

In FVa three peptide bonds at Arg-306, Arg-506, and Arg679 are sensitive to proteolysis by APC (14). The three cleavage reactions have distinct kinetic properties, a dependence on phospholipid, are differently stimulated by protein S, and yield FVa-inactivation intermediates with different FVa activities. The cleavage at Arg-506, which is kinetically favored, is not dependent on protein S and yields a FVa inactivation intermediate with decreased FXa-cofactor activity (14–16). The APC-mediated cleavage at Arg-306 is stimulated by protein S and results in almost complete inactivation of FVa (9, 14, 15, 17, 18). This loss of FVa activity is partly due to reduced affinity for FXas (15, 19, 20), but the Arg-306 cleavage also leads to dissociation of the A2 domain, which is crucial for full loss of function (20, 21). The cleavage at Arg-679 is not characterized in detail but is believed to be of minor importance for the inactivation of FVas (22). The dependence of phospholipid concentration and composition for each cleavage reaction has not been characterized in detail. Results on record, however, indicate that the cleavage at Arg-506 may occur in the absence of phospholipid, whereas the cleavage at Arg-306 is more dependent on the presence of phospholipid (14, 16).

Although the Gla domains of the different vitamin K-depend-
ent proteins share a high degree of sequence similarity, their affinities for negatively charged phospholipid vary considerably (4, 23). Protein S is one of the proteins with highest affinity for negatively charged phospholipid, whereas protein C belongs to the group having low affinity. It has been proven possible to modulate the affinity of protein C for the phospholipid by mutagenesis of the Gla domain to and obtain protein C variants with increased affinity for the membrane (24, 25). We recently described a protein C variant, QGNSEDY protein C, having increased phospholipid binding ability and enhanced anticoagulant efficiency as a result of multiple mutations in the Gla domain (26). This protein C variant is a useful reagent for the elucidation of the importance of the interaction between the Gla domain of protein C and the phospholipid membrane in the degradation of FVa and FVIIa and the mechanism by which protein S stimulates APC. In this study we have investigated the effect of QGNSEDY-APC on the individual cleavages at Arg-306 and Arg-506 in FVa using recombiant FV variants 306Q679Q and 506Q679Q that can only be cleaved at single APC-cleavage sites.

EXPERIMENTAL PROCEDURES

Materials—BioTrace polyvinylidene difluoride membranes were from Pall Corp. (Ann Arbor, MI). Chromogenic substrates S-2238 and S-2368 were kindly provided by Chromogenix (Milano, Italy). l-α-Phosphatidylserine (PS) from brain and l-α-phosphatidylcholine (PC) from egg were from Avanti Polar Lipids (Alabaster, AL). Hirudin was from Pentapharm Ltd (Basel, Switzerland). Ovalbumin and bovine serum albumin were obtained from Sigma-Aldrich. Human FXa and prothrombin were from Kordia (Leiden, Netherlands). Human protein S and human albumin were obtained from Sigma-Aldrich. Human FXa and prothrombin were from Kordia (Leiden, Netherlands). Human protein S and human albumin were obtained from Sigma-Aldrich. Human FXa and prothrombin were from Kordia (Leiden, Netherlands). Human protein S and human albumin were obtained from Sigma-Aldrich. Human FXa and prothrombin were from Kordia (Leiden, Netherlands). Human protein S and human albumin were obtained from Sigma-Aldrich. Human FXa and prothrombin were from Kordia (Leiden, Netherlands). Human protein S and human albumin were obtained from Sigma-Aldrich. Human FXa and prothrombin were from Kordia (Leiden, Netherlands). Human protein S and human albumin were obtained from Sigma-Aldrich. Human FXa and prothrombin were from Kordia (Leiden, Netherlands). Human protein S and human albumin were obtained from Sigma-Aldrich. Human FXa and prothrombin were from Kordia (Leiden, Netherlands). Human protein S and human albumin were obtained from Sigma-Aldrich. Human FXa and prothrombin were from Kordia (Leiden, Netherlands). Human protein S and human albumin were obtained from Sigma-Aldrich. Human FXa and prothrombin were from Kordia (Leiden, Netherlands). Human protein S and human albumin were obtained from Sigma-Aldrich. Human FXa and prothrombin were from Kordia (Leiden, Netherlands). Human protein S and human albumin were obtained from Sigma-Aldrich. Human FXa and prothrombin were from Kordia (Leiden, Netherlands). Human protein S and human albumin were obtained from Sigma-Aldrich. Human FXa and prothrombin were from Kordia (Leiden, Netherlands). Human protein S and human albumin were obtained from Sigma-Aldrich. Human FXa and prothrombin were from Kordia (Leiden, Netherlands). Human protein S and human albumin were obtained from Sigma-Aldrich. Human FXa and prothrombin were from Kordia (Leiden, Netherlands). Human protein S and human albumin were obtained from Sigma-Aldrich. Human FXa and prothrombin were from Kordia (Leiden, Netherlands). Human protein S and human albumin were obtained from Sigma-Aldrich. Human FXa and prothrombin were from Kordia (Leiden, Netherlands). Human protein S and human albumin were obtained from Sigma-Aldrich. Human FXa and prothrombin were from Kordia (Leiden, Netherlands). Human protein S and human albumin were obtained from Sigma-Aldrich. Human FXa and prothrombin were from Kordia (Leiden, Netherlands). Human protein S and human albumin were obtained from Sigma-Aldrich. Human FXa and prothrombin were from Kordia (Leiden, Netherlands). Human protein S and human albumin were obtained from Sigma-Aldrich. Human FXa and prothrombin were from Kordia (Leiden, Netherlands). Human protein S and human albumin were obtained from Sigma-Aldrich. Human FXa and prothrombin were from Kordia (Leiden, Netherlands). Human protein S and human albumin were obtained from Sigma-Aldrich. Human FXa and prothrombin were from Kordia (Leiden, Netherlands). Human protein S and human albumin were obtained from Sigma-Aldrich. Human FXa and prothrombin were from Kordia (Leiden, Netherlands). Human protein S and human albumin were obtained from Sigma-Aldrich. Human FXa and prothrombin were from Kordia (Leiden, Netherlands). Human protein S and human albumin were obtained from Sigma-Aldrich. Human FXa and prothrombin were from Kordia (Leiden, Netherlands). Human protein S and human albumin were obtained from Sigma-Aldrich. Human FXa and prothrombin were from Kordia (Leiden, Netherlands). Human protein S and human albumin were obtained from Sigma-Aldrich. Human FXa and prothrombin were from Kordia (Leiden, Netherlands). Human protein S and human albumin were obtained from Sigma-Aldrich. Human FXa and prothrombin were from Kordia (Leiden, Netherlands). Human protein S and human albumin were obtained from Sigma-Aldrich. Human FXa and prothrombin were from Kordia (Leiden, Netherlands). Human protein S and human albumin were obtained from Sigma-Aldrich. Human FXa and prothrombin were from Kordia (Leiden, Netherlands). Human protein S and human albumin were obtained from Sigma-Aldrich. Human FXa and prothrombin were from Kordia (Leiden, Netherlands). Human protein S and human albumin were obtained from Sigma-Aldrich. Human FXa and prothrombin were from Kordia (Leiden, Netherlands). Human protein S and human albumin were obtained from Sigma-Aldrich. Human FXa and prothrombin were from Kordia (Leiden, Netherlands). Human protein S and human albumin were obtained from Sigma-Aldrich. Human FXa and prothrombin were from Kordia (Leiden, Netherlands). Human protein S and human albumin were obtained from Sigma-Aldrich. Human FXa and prothrombin were from Kordia (Leiden, Netherlands). Human protein S and human albumin were obtained from Sigma-Aldrich. Human FXa and prothrombin were from Kordia (Leiden, Netherlands). Human protein S and human albumin were obtained from Sigma-Aldrich. Human FXa and prothrombin were from Kordia (Leiden, Netherlands). Human protein S and human albumin were obtained from Sigma-Aldrich. Human FXa and prothrombin were from Kordia (Leiden, Netherlands). Human protein S and human albumin were obtained from Sigma-Aldrich. Human FXa and prothrombin were from Kordia (Leiden, Netherlands). Human protein S and human albumin were obtained from Sigma-Aldrich. Human FXa and prothrombin were from Kordia (Leiden, Netherlands). Human protein S and human albumin were obtained from Sigma-Aldrich. Human FXa and prothrombin were from Kordia (Leiden, Netherlands). Human protein S and human albumin were obtained from Sigma-Aldrich. Human FXa and prothrombin were from Kordia (Leiden, Netherlands). Human protein S and human albumin were obtained from Sigma-Aldrich.
Increased FVa Degradation by Gla-mutated APC

FIG. 1. Inactivation of wild type FVAs by APC variants in the presence and absence of protein S. Affinity-purified recombinant WT-FVAs (0.8 nM final concentration) was incubated with 10/90 PS/PC phospholipid vesicles (25 μM final concentration) with (right) or without (left) protein S (100 nM final concentration) in HNSBSACA-buffer. A, the FVa inactivation was initiated by the addition of WT APC (□) or QGNSEDY APC (○), with final APC concentrations of 0.2 nM in the absence of protein S and 0.05 nM in the presence of protein S. At intervals aliquots drawn and diluted 1/5 in ice-cold HNSBSACA buffer, and the FVa activity was subsequently measured in the PTase assay using 5 nM FXa. B, separate aliquots drawn at 0.5, 1, 3, 5, 10, and 15 min were mixed with SDS-containing sample preparation buffer (reducing conditions) and analyzed by Western blotting (10% SDS-PAGE) using the monoclonal antibody AHV5146, the epitope of which is located in the 307–506 fragment. FV-HC fragments were visualized with Supersignal West chemiluminescent substrate (Pierce) using a chemiluminescence reader (FUJIFILM LAS-3000 IR), and the quantification of the bands was performed using the ImageGauge program (Fujifilm). The three fragments 1–506, 307–709, and 307–506 are indicated. The weak band (marked with an asterisk) in the starting material of B, left, having a slightly higher molecular weight than the 1–506 fragment, represents a heavy chain which is cleaved somewhere before position 506. The disappearance of this band is correlated with the appearance of a weak band (marked with a star) located just below the 307–709 fragment. C, the quantification of the different immune-reactive bands: □, the intact HC; ⌧, the 1–506 fragment; ○, the 307–506 fragment; ●, the 307–709 fragment. The quantification of the weak bands in B, left, that are marked with asterisks and stars are not shown but were found to represent around 20% of the total HC signal.

activity of position 506-cleaved FVAs was much lower at 0.1 nM FXa than that at 5 nM FXa, which facilitated evaluation of the Arg-506 cleavage rate (16). The EDTA buffer contained 50 mM Tris, 100 mM NaCl, 20 mM EDTA, 1% polyethylene glycol 6000, pH 7.9. The amount of thrombin formed was measured kinetically with a chromogenic substrate, S2238.

Immunoprecipitation with HV-1 to Quantify FvAs Light-Heavy Chain Dissociation—To quantify the dissociation between the FVAs heavy and light chains in FVAs, occurring before and during the APC degradation, a biotinylated light chain antibody, HV-1, was used together with streptavidin-coated magnetic beads. The beads were coated with 80 μg/ml HV-1 before the immunoprecipitation experiment. The affinity-purified 306Q679Q-FVa (0.8 nM final concentration) was incubated with 25 μM phospholipids (PS/PC w/w 10/90) in HNSBSACA with buffer WT- or QGNSEDY-APC (0.05 nM final concentrations) for 15 min at 37 °C in a final volume of 200 μl. Aliquots (20 μl) were drawn before and 15 min after the addition of the APC, and the FVa activity was measured with the PTase assay. Aliquots (20 μl) were drawn for Western blotting, and separate aliquots (50 μl) were drawn and subjected to immunoprecipitation with the HV-1-coated magnetic beads (a 60-μl bead solution was used for each precipitation; the beads buffer was removed before the addition of the 50-μl aliquot from the FVa degradation). After 30 min of incubation at 4 °C, the beads were removed, and the supernatant was withdrawn for Western blotting and PTase assay. The beads were washed with 3 × 60 μl of HNSBSACA, and the proteins were then eluted with 100 μl of 95 °C sample preparation buffer and subjected to Western blotting using the heavy chain antibody AHV5146, as described above.

Equations Used for Curve Fitting—The APC-mediated inactivation of 506Q679Q-FVa and 306Q679Q-FVa was followed over time, and the results were used to calculate pseudo first order rates for the Arg-306 and Arg-506 cleavages, respectively. The curves were fitted to an equation earlier reported (9, 15). The equation was modified because only one cleavage occurs in our FVa variants. For calculation of the Arg-506 cleavage, the time curves obtained for 306Q679Q-FVa were fitted to the following equation,

\[ V_{A_t} = V_{A0}e^{-kt \cdot t} + BV_a(1 - e^{-kt \cdot t}) \]  

in which \( V_{A0} \) is the cofactor activity determined at time \( t \), \( Vo \) is the cofactor activity determined before APC is added, \( B \) is the remaining procoagulant cofactor activity of FVa cleaved at Arg-506, and \( k_{app} \) is the rate constant of cleavage at Arg-506. For calculation of the Arg-306 cleavage, the time curves were used to fit the time curve of 506Q679Q-FVa.

\[ V_{A_t} = V_{A0}e^{-kt \cdot t} + CV_a(1 - e^{-kt \cdot t}) \]  

Here \( C \) is the remaining procoagulant cofactor activity of FXa cleaved at Arg-306, and \( k_{app} \) is the rate constant of cleavage at Arg-306. The use of the equations requires that the inactivation curves are independent of FXa concentration (% FXa inactivation versus time) and that the rates are linear for APC concentration. Control experiments were performed, and the inactivation curves fulfilled these criteria.

RESULTS

Degradation of Recombinant Fvas by Wild Type- and Gla-mutated APC—Purified recombinant WT-FVa was incubated...
with recombinant WT-APC or the Gla domain-mutant QGNSEDY-APC in the presence of negatively charged phospholipid vesicles (Fig. 1). At intervals, aliquots were drawn, and the remaining FVa activity was measured with a PTase-based FVa assay. The APC-mediated fragmentation of the FVa heavy chain was visualized by Western blotting using the monoclonal antibody AHV5146 that recognized the 307–506 fragment. A chemiluminescence-based technique was used that allowed quantification of the immune-reactive bands. The inactivation was performed in the absence (Fig. 1, left panels) and presence of protein S (Fig. 1, right panels). Protein S stimulated the rate of FVa degradation, and lower APC concentrations were, therefore, used in incubations containing protein S. WT-APC alone yielded an initial rapid loss of FVa activity, which is consistent with selective cleavage at Arg-506. At the high concentration of FXa (5 nM) used in the PTase assay, the remaining FVa activity after Arg-506 cleavage was around 60%. According to the Western blot (Fig. 1B, left panel), the heavy chain was cleaved within the first 5 min, and the 1–506 fragment appeared. This was slowly cleaved at Arg-306 to generate the 307–506 fragment. The 10% remaining uncleaved heavy chain (quantification of bands in Fig. 1C) represented heavy chain that had dissociated from the light chain already, before the APC degradation experiment (see the immunoprecipitation experiment below). Based on the results presented in Fig. 1, it is valid to conclude that QGNSEDY-APC was considerably more efficient than WT-APC in cleaving FVas at both Arg-506 and Arg-306.

In the presence of protein S, both WT-APC and QGNSEDY-APC were more efficient in cleaving FVas than in the absence of protein S (Fig. 1, right). 4-Fold lower APC concentrations were used in the presence of protein S to be able to follow the
degradation over time. WT-APC cleaved both the Arg-506 and Arg-306 sites, resulting in the sequential appearance of the 1–506 and 307–506 fragments, which is consistent with the contribution of the APC-cofactor activity of protein S in the Arg-306 cleavage. In addition, a weak band representing the 307–709 fragment, generated when the Arg-306 site was cleaved before the Arg-506 site, was observed. The extra band that is marked with an asterisk in the left (see above) was not observed in this Western blot. The QGNSEDY-APC was found to be more efficient than WT-APC in cleaving at both Arg-506 and Arg-306 also in the presence of protein S. The results of the quantification of the immuno-reactive bands are shown in Fig. 1C.

Individual Cleavages at Arg-506 and Arg-306 by WT-APC and QGNSEDY-APC—To quantify the difference in catalytic efficiency between the two recombinant APC variants at the Arg-506 and Arg-306 sites, individually 306Q679Q-FVa and 506Q679Q-FVa were used. The results obtained with the 306Q679Q-FVa are illustrated in Fig. 2. In the absence of protein S (Fig. 2, A–C, left), the loss of FVa activity was faster in the presence of QGNSEDY-APC than in the presence of WT-APC, and on Western blotting, the 1–506 fragment appeared earlier. The presence of protein S (Fig. 2, right) enhanced the rates of FVa degradation for both APC variants, but there was still a difference between them, QGNSEDY-APC being more efficient of the two. The quantification of the 1–506 fragment in the experiment containing QGNSEDY-APC and protein S demonstrated a peak at 3 min and then a decline, which may be due to slow cleavage at an unknown site. The existence of one or more such sites in FVa was recently proposed (31).

The APC-mediated degradation of 506Q679Q-FVa monitoring the cleavage at the Arg-306 site is shown in Fig. 3. In the absence of protein S (left), the 306Q679Q-FVa cleavage was slow in the incubation mixture containing WT-APC even though the APC was 10-fold higher than in the experiment illustrated in Fig. 2. In contrast, QGNSEDY-APC efficiently cleaved at Arg-306 also in the absence of protein S. Protein S is an efficient cofactor to WT-APC in the cleavage at Arg-306, and in the presence of this cofactor, the APC concentrations used were, therefore, 10-fold lower than in the experiment performed in the absence of protein S. The QGNSEDY-APC was found to be more effective than WT-APC also in the presence of protein S, as illustrated by the experiment shown in Fig. 3, right.
Increased FVa Degradation by Gla-mutated APC

Phospholipid Dependence of APC-mediated Cleavages at Arg-506 and Arg-306—The Arg-506 site can be cleaved by APC also in the absence of phospholipid, albeit at low efficiency. This allowed us to investigate whether QGNSEDY-APC had the same catalytic efficiency as WT-APC in the cleavage at Arg-506 and Arg-306—i.e., between the isolation of the FVa and the start of the experiment.

Phospholipid dependence of FVa inactivation. A. phospholipid-free purified 306Q679Q-FVa (0.8 nM) was incubated with WT-APC (■), QGNSEDY-APC (2 nM final concentration) (○), or buffer (▲) in the absence of phospholipid. At intervals aliquots were drawn and diluted ½, and the FVa activity was measured in the PTase assay using 0.1 nM FXa. The phospholipid dependence of the APC-mediated degradation of the two mutated FVa variants 306Q679Q and 506Q679Q are illustrated in B and C, respectively. In B, increasing concentrations of phospholipid vesicles (10/90 PS/PC) were incubated with 0.8 nM 506Q679Q-FVa, 1.0 nM WT-APC (■) or QGNSEDY-APC (○) for 10 min at 37 °C. The reactions were stopped by ½ dilutions in HNBSAc buffer, and the remaining FVa activity was measured in a prothrombinase assay using 0.1 nM FXa. In C, increasing concentrations of phospholipid vesicles (10/90 PS/PC) were incubated with 0.8 nM 506Q679Q-FVa, 1.0 nM WT-APC (■), or 1.0 nM QGNSEDY-APC (○) for 10 min at 37 °C. The reaction was stopped by ½ dilutions in HNBSAc buffer, and the remaining FVa activity was measured in a prothrombinase assay using 5 nM FXa.

The Arg-506 site can be cleaved by APC also in the absence of phospholipid, albeit at low efficiency. This allowed us to investigate whether QGNSEDY-APC had the same catalytic efficiency as WT-APC in the cleavage at Arg-506, and therefore, the degradation of 306Q679Q-FVa was performed in the absence of negatively charged phospholipid vesicles (Fig. 5A). Relatively high concentrations (2 nM) of WT-

Fig. 4. Quantification of the spontaneous dissociation of heavy and light chains of FVa. Affinity-purified 306Q679Q-FVa (0.8 nM) was incubated with 10/90 PS/PC phospholipid vesicles (25 μM final concentration) in the presence of WT-APC or QGNSEDY-APC (final concentrations 0.05 nM) or with buffer. A, at time 0 and after 15 min of incubation, aliquots were drawn and diluted ½ in ice-cold HNBSAc buffer, and the FVa activity was measured in the PTase assay using 0.1 nM FXa. (black bars) Heavy and light chains of FVa from the precipitated control were visualized and quantified as described in legend to Fig. 1. In the Western blot, the heavy (Ig-HC) and light (Ig-LC) chains of the HV-1 monoclonal antibody are seen as well as the heavy chain of FVa and the 1–506 fragment. In the lanes marked S, the FVa heavy chain and traces of the 1–506 fragment present in the supernatants are seen. In C, the results of the quantification of the FVa heavy chain (black bars) and the 1–506 fragment (open bars) are shown. The amounts of fragments are related to the total signal of the heavy chain of FVa in the non-digested and non-precipitated control.

The amount of dissociated heavy chain was visualized by Western blotting using the monoclonal antibody AHV5146. The FVa activity remaining in the supernatant after immunoprecipitation was measured (open bars). B, samples from both non-precipitated FVa (N) and from the precipitated FVa (P) and the supernatant after immunoprecipitation (S) were analyzed by Western blotting using the monoclonal antibody AHV5146. The Fv-HC fragments were visualized and quantified as described in legend to Fig. 1. In the lanes marked P in the Western blot, the heavy (Ig-HC) and light (Ig-LC) chains of the HV-1 monoclonal antibody are seen as well as the heavy chain of FVa and the 1–506 fragment. In the lanes marked S, the FVa heavy chain and traces of the 1–506 fragment present in the supernatants are seen. In C, the results of the quantification of the FVa heavy chain (black bars) and the 1–506 fragment (open bars) are shown. The amounts of fragments are related to the total signal of the heavy chain of FVa in the non-digested and non-precipitated control.

The Arg-506 site can be cleaved by APC also in the absence of phospholipid, albeit at low efficiency. This allowed us to investigate whether QGNSEDY-APC had the same catalytic efficiency as WT-APC in the cleavage at Arg-506, and therefore, the degradation of 306Q679Q-FVa was performed in the absence of negatively charged phospholipid vesicles (Fig. 5A). Relatively high concentrations (2 nM) of WT-

Fig. 5. Phospholipid dependence of FVa inactivation. A, phospholipid-free purified 306Q679Q-FVa (0.8 nM) was incubated with WT-APC (■), QGNSEDY-APC (2 nM final concentration) (○), or buffer (▲) in the absence of phospholipid. At intervals aliquots were drawn and diluted ½, and the FVa activity was measured in the PTase assay using 0.1 nM FXa. The phospholipid dependence of the APC-mediated degradation of the two mutated FVa variants 306Q679Q and 506Q679Q are illustrated in B and C, respectively. In B, increasing concentrations of phospholipid vesicles (10/90 PS/PC) were incubated with 0.8 nM 506Q679Q-FVa, 1.0 nM WT-APC (■) or QGNSEDY-APC (○) for 10 min at 37 °C. The reactions were stopped by ½ dilutions in HNBSAc buffer, and the remaining FVa activity was measured in a prothrombinase assay using 0.1 nM FXa. In C, increasing concentrations of phospholipid vesicles (10/90 PS/PC) were incubated with 0.8 nM 506Q679Q-FVa, 1.0 nM WT-APC (■), or 1.0 nM QGNSEDY-APC (○) for 10 min at 37 °C. The reaction was stopped by ½ dilutions in HNBSAc buffer, and the remaining FVa activity was measured in a prothrombinase assay using 5 nM FXa.
Increased FVa Degradation by Gla-mutated APC

APC and QGNSEDY-APC were required when phospholipid was absent to obtain measurable rates of FVa inactivation. There was no difference in the rates of FVa inactivation obtained with WT-APC and QGNSEDY-APC. In the control without added APC, the FVa activity remained stable throughout the experiment. From these results it can be concluded that the difference between WT-APC and QGNSEDY-APC was only observed in the presence of phospholipid vesicles. The phospholipid concentration dependence of the APC-mediated cleavages at Arg-506 and Arg-306 was investigated using 306Q679Q-FVa and 506Q679Q-FVa, respectively (Fig. 5, B and C). Because the cleavage at Arg-506 is highly sensitive to APC, a low concentration of APC (0.02 nM) was chosen to highlight the difference in efficiency between the two APC variants. Under the conditions used, WT-APC yielded limited inhibition of 306Q679Q-FVa even at the highest concentration of phospholipid used, whereas QGNSEDY-APC was efficient even at low concentrations of phospholipid. These results further illustrated the dependence of phospholipid for the activity of QGNSEDY-APC.

To investigate the phospholipid concentration dependence of the cleavage at Arg-306 by WT-APC and QGNSEDY-APC (1 nM used for both variants), 506Q679Q-FVa was used (Fig. 5C). This experiment, which was performed in the absence of protein S, demonstrated QGNSEDY-APC to be much more efficient than WT-APC and illustrated the dependence of phospholipid for the reaction. At the highest phospholipid concentration used, the 1 nM WT-APC only yielded a small degree of FVa inactivation (80% remaining activity), whereas QGNSEDY-APC at the same concentration was highly efficient, and less than 20% activity remained after 10 min.

**Kinetics of Cleavages at Arg-306 and Arg-506 by WT-APC and QGNSEDY-APC**—To quantify the differences in efficiency of WT-APC and QGNSEDY-APC to cleave at Arg-306 and Arg-506, time-courses of degradation of 506Q679Q-FVa and 306Q679Q-FVa, respectively, were performed at high and low phospholipid concentrations both in the presence and absence of protein S. Each experiment involved a full time-course of FVa degradation, and the rate constants for each experiment were calculated. The original data used to calculate the rate constants were similar to those presented in Figs. 2 and 3. The derived kinetic constants are presented in Table I. The small amount of dissociation of the two FVa chains did not affect the calculated rate constants for the reactions, as the rate of FVa degradation was independent of the exact FVa concentration under the conditions used. QGNSEDY-APC was found to yield higher rate constants than WT-APC for both cleavages in the presence as well as in the absence of protein S and, in addition, at both high and low phospholipid concentrations. However, in the absence of phospholipid, the two APC variants yielded similar rate constants for the Arg-506 cleavage, demonstrating the requirement of phospholipid for expression of higher efficiency of QGNSEDY-APC. The biggest differences between the two APC variants were observed for the Arg-306 cleavage (up to an 17.8-fold increased rate of cleavage). However, at high phospholipid and in the presence of protein S, the difference between the two APCs in rate of cleavage at Arg-306 was smaller than that seen at low phospholipid. It was noteworthy that at both high and low phospholipid, the rate constants for the Arg-306 site obtained with QGNSEDY-APC in the absence of protein S were similar to those obtained for the same cleavage by WT-APC in the presence of protein S. The addition of protein S to the incubations containing QGNSEY-APC further enhanced the rate constants for the cleavage at Arg-306. The differences in rate constants between the two APC variants for the Arg-506 cleavage were lower than those observed for the Arg-306 cleavage site, in particular when protein S was present. Thus, the mutations in QGNSEDY-APC preferentially enhance the rate of cleavage at the Arg-306 site.

**DISCUSSION**

The availability of recombinant protein C variants with enhanced affinity for phospholipid provides an opportunity to investigate the functional importance of the phospholipid binding for the individual APC-mediated cleavage reactions in FVa. We have previously demonstrated QGNSEDY-APC to have increased affinity for negatively charged phospholipid and to be more potent as anticoagulant than WT-APC (26). To quantify the effects of QGNSEDY-APC on the individual APC-mediated cleavages in FVa, we now used recombinant FVa-variants 306Q679Q and 506Q679Q. Cleavages at both Arg-506 and Arg-506 sites by the QGNSEDY-APC variant were more efficient than by WT-APC at both high and low phospholipid concentrations. The differences between the two APC variants was particularly pronounced for the Arg-306 cleavage when performed in the absence of protein S. This shows that Gla domain mutagenesis of APC can result in enhanced efficiency of cleavage at the Arg-306 site, a reaction that for WT-APC is dependent on the presence of the cofactor protein S. The results are consistent with the hypothesis that the enhanced affinity for negatively charged phospholipid of QGNSEDY-APC is an important mechanism for its increased functional activity. However, the increased equilibrium binding affinity of QGNSEDY-APC for the phospholipid may not be the sole determinant for its increased efficiency. In surface plasmon resonance (Biacore) experiments, it was found that the association and dissociation rates of the phospholipid binding of QGNSEDY-APC were much higher than those observed for WT-APC (26). It is not unlikely that the kinetics of the phospholipid interaction of the vitamin K-dependent protein is of importance for their functions and not only the equilibrium binding constants.

**Table I**

| Protein S          | WT-APC | QGNSEDY-APC | ×-fold |
|--------------------|--------|-------------|--------|
| High phospholipid (25 μM) |        |             |        |
| Arg-306 site       | 1.8 ± 0.5 x10^6 | 3.2 ± 0.83 x10^7 | 17.8   |
| +                  | 3.7 ± 0.8 x10^7 | 2.5 ± 0.35 x10^8 | 6.8    |
| ×-fold             | 20.5   | 7.8         |        |
| Arg-506 site       | 1.1 ± 0.14 x10^8 | 4.4 ± 0.35 x10^8 | 4      |
| +                  | 1.3 ± 0.24 x10^8 | 4.1 ± 0.66 x10^8 | 3.2    |
| ×-fold             | 1.2    | 0.9         |        |
| Low phospholipid (2.5 μM) |        |             |        |
| Arg-306 site       | 2.7 ± 0.08 x10^6 | 4.6 ± 1.32 x10^6 | 17.0   |
| +                  | 4.7 ± 1.2 x10^6 | 6.6 ± 0.21 x10^7 | 14.0   |
| ×-fold             | 17.0   | 14.3        |        |
| Arg-506 site       | 3.5 ± 0.12 x10^7 | 1.5 ± 0.1 x10^8 | 4.3    |
| +                  | 3.2 ± 0.91 x10^7 | 2.1 ± 0.4 x10^8 | 6.5    |
| ×-fold             | 0.9    | 1.4         |        |
| Zero phospholipid  | 3.00 ± 0.32 x10^5 | 3.10 ± 0.41 x10^5 | 1.0    |
APC were stimulated by protein S, whereas the Arg-506 cleavages by the two APC variants were not affected by protein S. One of the mechanisms by which protein S is believed to stimulate the activity of APC is by enhancing the affinity of APC for the phospholipid (38). However, it has also been suggested that other mechanisms are involved in the protein S effect, e.g. relocation of the active site by APC closer to the membrane by protein S (39, 32). In this context it is of interest to note that protein S provides potentiation to both WT-APC and QGNSEDY-APC in the cleavage of Arg-306 in FVa. The present results are compatible with the existence of dual mechanisms being important for the cofactor function of protein S, i.e. the enhanced phospholipid binding and the effect on the active site location of APC. The enhanced affinity for phospholipid of QGNSEDY-APC is one mechanism to explain the increased efficiency in the Arg-306 cleavage. It remains to be determined if the distance to the phospholipid of the active site of QGNSEDY-APC is affected by protein S or if it is directly affected by the Gla mutations, as was reported for an APC variant carrying a Gla domain being a hybrid between protein C and prothrombin (32).

The two chains in FVa are non-covalently associated, but the exact affinity for the interaction between the two chains in human FVa is unknown; in bovine FVa the Kd is 3.7–5.9 nm (33, 34). Presumably the FVa concentration used in these experiments (0.8 nm) is not too different from the Kd of the heavy-light chain interaction in human FVa, which would explain the observed 20% dissociation of the chains. Because the dissociated heavy chain was unable to associate with the phospholipid membrane (light chain binds to the phospholipid), it had no activity and was not cleaved by APC. Accordingly, it remained uncleaved in the supernatant after the incubation with APC. However, the small amount of chain dissociation did not affect the cleavage rate of the kinetic parameters because the relative rate of FVa degradation was independent of the exact FVa concentration under the conditions used, i.e. when the FVa concentration was much lower than the Kc of the APC-cleavage reactions, discussed in detail by Nicolaes et al. (15), who also outlined the theoretical background for the calculation of the rate constants for APC-mediated FVa degradation performed under experimental conditions similar to those now used.

APC has been proven to be useful in the treatment of severe septic shock, a condition associated with disseminated intravascular coagulation (35, 36). The exact mechanisms by which APC exerts its beneficial effects are not understood in detail but are believed not only to inhibit initiation of coagulation but also to be due to expression of anti-inflammatory properties of APC (37). It is possible that the increased phospholipid binding affinity and the enhanced anticoagulant potential of the now characterized recombinant APC variants will prove useful in severe septic shock.

Acknowledgments—This work was supported by grants from the Network for Cardiovascular Research funded by the Swedish Foundation for Strategic Research, Swedish Council Grant 07143, a Senior Investigator’s Award from the Foundation for Strategic Research, the Albert Påhlsson trust, and by research funds from the University Hospital Malmö.

REFERENCES

1. Furie, B., and Furie, B. C. (1988) Cell 53, 505–518
2. Mann, K. G., Nesheim, M. E., Church, W. R., Haley, P., and Krishnaswamy, S. (1996) Blood 76, 1–16
3. Dahlback, B. (2000) Lancet 355, 1627–1632
4. Nelsestuen, G. L. (1999) Trends Cardiovas. Med. 9, 162–167
5. Mann, K. G., Jenny, R. J., and Krishnaswamy, S. (1988) Annu. Rev. Biochem. 57, 915–956
6. Esmon, C. T. (1993) Thromb. Haemostasis 70, 29–35
7. Esmon, C. T., Gu, J. M., Xu, J., Qa, D., Stearns-Kurosawa, D. J., and Kurosawa, S. (1999) Haematologica 84, 363–368
8. Dahlback, B., and Stenflo, J. (2001) in The Molecular Basis of Blood Disease (Stamatoyannopoulos, G., Majerus, P. W., Perlmutter, R. M., and Varmus, H., eds) 3rd Ed., pp. 614–656, W. B. Saunders Co., Philadelphia, PA
9. Rosing, J., Hoekema, L., Nicolaes, G. A., Thomassen, M. C., Hemker, H. C., Varadi, K., Schwarz, H. P., and Tans, G. (1995) J. Biol. Chem. 270, 27852–27858
10. Dahlback, B. (1997) Semin. Hematol. 34, 217–234
11. Nicolaes, G. A., and Dahlback, B. (2002) Arterioscler. Thromb. Vasc. Biol. 22, 530–538
12. Shen, L., and Dahlback, B. (1994) J. Biol. Chem. 269, 18735–18738
13. Varadi, K. R. J., Tans, G., Pabinger, I., Keil, B., and Schwarz, H. P. (1996) Thromb. Haemostasis 76, 208–214
14. Kalafatis, M., Rand, M. D., and Mann, K. G. (1994) J. Biol. Chem. 269, 31869–31880
15. Nicolaes, G. A., Tans, G., Thomassen, M. C., Hemker, H. C., Pabinger, I., Varadi, K., Schwarz, H. P., and Rosing, J. (1995) J. Biol. Chem. 270, 21108–21116
16. Norstrom, E., Steen, M., Tran, S., and Dahlback, B. (2003) J. Biol. Chem. 278, 24804–24811
17. Rosing, J., Kojima, Y., Greengard, J. S., and Griffin, J. H. (1995) Blood 85, 3405–3411
18. Kalafatis, M., Bertina, R. M., Rand, M. D., and Mann, K. G. (1995) J. Biol. Chem. 270, 4053–4057
19. Kojima, Y., Heeb, M. J., Gale, A. J., Hackeng, T. M., and Griffin, J. H. (1998) J. Biol. Chem. 273, 14900–14905
20. Gale, A. J., Xu, X., Pellequer, J. L., Getzoff, E. D., and Griffin, J. H. (2002) J. Biol. Chem. 277, 5120–5127
21. Mann, K. G., Kockin, M. F., Begin, K. J., and Kalafatis, M. (1997) J. Biol. Chem. 272, 20678–20683
22. Norstrom, E., Thorsbl, E., and Dahlback, B. (2002) Blood 100, 524–530
23. McDonald, J. F., Shah, A. M., Schwable, R. A., Kiel, W., Dahlback, B., and Nelsestuen, G. L. (1997) Biochemistry 36, 5120–5127
24. Shen, L., Shah, A. M., Dahlback, B., and Nelsestuen, G. L. (1997) Biochemistry 36, 16025–16031
25. Shen, L., Shah, A., Dahlback, B., and Nelsestuen, G. (1998) J. Biol. Chem. 273, 31086–31091
26. Sun, Y. H., Shen, L., and Dahlback, B. (2003) Blood 101, 2277–2284
27. Dahlback, B. (1983) Biochem. J. 209, 837–846
28. Dahlback, B., Wiedmer, T., and Sims, P. (1992) Biochemistry 31, 12769–12777
29. Dahlback, B., and Hildebrand, B. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 1396–1400
30. Kaufman, R. J. (1990) Methods Enzymol. 185, 487–511
31. van der Neut Kolfschoten, M., Dirven, R. J., Vos, H. L., Tans, G., Rosing, J., and Bertina, R. M. (2004) J. Biol. Chem. 279, 6567–6575
32. Yegneswaran, S., Smirnov, M. D., Safa, O., Emon, N. L., Eson, C. T., and Johnson, A. E. (1999) J. Biol. Chem. 274, 5462–5468
33. Nelsestuen, G. L., and Dahlback, B. (1997) Biochemistry 36, 16025–16031
34. Krishnaswamy, S., Russell, G. D., and Mann, K. G. (1989) J. Biol. Chem. 264, 3160–3168
35. Bernard, G. R., Vincent, J. L., Laterre, P. P., LaRosa, S. P., Dhainaut, J. F., Lopez-Rodriguez, A., Steinigub, J. S., Garber, G. E., Helterbrand, D. J., Ely, E. W., and Fisher, C. J., Jr. (2001) N. Engl. J. Med. 344, 699–709
36. Esmon, C. T. (2001) Crit. Care Med. 29, 51–52
37. Joyce, D. E., Gelbert, L., Ciaccia, A., DeHoff, B., and Grinnell, B. W. (2001) J. Biol. Chem. 276, 11199–11203
38. Walker, F. J. (1981) J. Biol. Chem. 256, 11128–11131
39. Yegneswaran, S., Wood, G. M., Eson, C. T., and Johnson, A. E. (1997) J. Biol. Chem. 272, 25013–25021
