Importance of Protein S and Phospholipid for Activated Protein C-mediated Cleavages in Factor Va*

Eva A. Norström, Mårten Steen, Sinh Tran, and Björn Dahlbäck‡

From the Department of Laboratory Medicine, Division of Clinical Chemistry, Lund University, and the Wallenberg Laboratory, University Hospital Malmö, SE-205 02 Malmö, Sweden

Received for publication, April 11, 2003
Published, JBC Papers in Press, April 21, 2003, DOI 10.1074/jbc.M303829200

Activated factor V (FVa) is a procoagulant cofactor to activated factor X (FXa) in the conversion of prothrombin to thrombin (1–3). The procoagulant function of FXa is down-regulated by the anticoagulant serine protease activated protein C (APC) (2, 4). APC cleaves FVa at three sites, Arg306, Arg506, and Arg679 (5). The Arg306 site is kinetically most favored, and cleavage results in decreased affinity for FXa and partial loss of procoagulant activity (6). Cleavage at Arg306 impairs the procoagulant function more severely, and the combined cleavage at Arg506 and Arg506 results in the dissociation of the A2 fragment and complete loss of FVa activity (7). The role of the Arg579 cleavage in the regulation of FVa activity is not fully elucidated, but appears to be of minor importance. Recently, we found that, in the presence of protein S, the Arg579 cleavage might play a role in the degradation of naturally occurring FVa variants that are mutated at Arg579, such as FV Cambridge and FV Hong Kong (8).

Efficient inactivation of FVa by APC requires the presence of negatively charged phospholipids, with phosphatidylserine (PS) being the most potent stimulator of the APC cleavage of FVa (9). In addition, incorporation of phosphatidylethanolamine (PE) into the phospholipid membrane has been found to enhance the inactivation of FVa by APC (10). This was proposed to be a selective effect on the FVa inactivation because the effect of PE on the APC cleavage of FVa was more pronounced than what had been reported for other coagulation complexes (11, 12). Other membrane components that have been reported to increase the efficiency of APC cleavage of FVa include cardiolipin and glycolipids (13, 14). The phospholipid composition affects reorientation of the active site of APC closer to the phospholipid membrane. To investigate the importance of protein S and phospholipid in the APC-mediated cleavages of individual sites, recombinant FV variants FV(R306Q/R679Q) and FV(R506Q/R679Q) (can be cleaved only at Arg306 and Arg506, respectively) were created. The cleavage rate was determined for each cleavage site in the presence of varied protein S concentrations and phospholipid compositions. In contrast to results on record, we found that protein S stimulated both APC cleavages in a phospholipid composition-dependent manner. Thus, on vesicles containing both phosphatidylserine and phosphatidylethanolamine, protein S increased the rate of Arg306 cleavage 27-fold and that of Arg506 cleavage 5-fold. Half-maximal stimulation was obtained at ~30 nM protein S for both cleavages. In conclusion, we demonstrate that APC-mediated cleavages at both Arg306 and Arg506 in FVa are stimulated by protein S in a phospholipid composition-dependent manner. These results provide new insights into the mechanism of APC cofactor activity of protein S and the importance of phospholipid composition.
based assay, and apparent second-order rate constants for the Arg⁵⁰⁶ and Arg⁵⁰⁶ cleavages were calculated. Surprisingly, we found that protein S enhanced cleavage not only at Arg⁵⁰⁶, but also at Arg⁵⁰⁶. The protein S effect was dependent on the presence of PS and stimulated by the inclusion of PE in the membrane. These results provide new insights into the importance of membrane composition for APC-mediated degradation of FVa and the mechanism of protein S activity.

EXPERIMENTAL PROCEDURES

Materials—BioTrace polyvinylidene difluoride membrane was from Pall Corp. (Ann Arbor, MI). Chromogenic substrates S2238 and S2366 were kindly provided by Chromogenix (Milano, Italy). Human FXa and human prothrombin were from Kordia (Leiden, The Netherlands). α-Thrombin and monoclonal antibody AH-5146 (against the heavy chain of FVa) were from Hematologics Inc. (Essen Junction, VT). Human FVa was purified from plasma as described (22) with minor modifications (23). Recombinant human APC was prepared as described (24), and its concentration was determined with S2368. Human protein S was purified as described (25) with minor modifications (26). PD-10 columns were purchased from Amersham Biosciences AB (Uppsala, Sweden). Triton X-100, brijun, ovalbumin, and bovine serum albumin were from Sigma. Benzamidine (Geel, Belgium). FS (brain extract), PE (egg extract), phosphatidylcholine (PC; egg extract), 1-palmitoyl-2-oleoylphosphatidylserine, 1-palmitoyl-2-oleoylphosphatidylethanolamine, and 1-palmitoyl-2-oleoylphosphatidylycholine were from Avanti Polar Lipids (Alabaster, AL).

Phospholipid Vesicle Preparation—The phospholipid stocks were dissolved in 1:90 methanol/chloroform solution, and the concentrations were determined by phosphatase analysis (27). Mixtures of the lipids (weight/weight) were prepared in 10:90 methanol/chloroform and kept at −20 °C. Aliquots were taken from the stocks, dried under N₂, and then resuspended in Hepes at room temperature. Phospholipids for the prothrombinase assay were sonicated in a Misonix XL 2020 sonicator at 100 W for 10 min. Fresh phospholipid vesicles were prepared every day. For the inactivation assay, extruded phospholipid vesicles were used to avoid heterogeneity in the size of the vesicles (10). The extrusion was performed using a LiposFast basic extruder (Armatis, Mannheim, Germany) as described (28). The phospholipid mixtures dissolved in buffer were subjected to freeze-thaw circles and subsequently extruded 19 times through a membrane with a 100-nm pore size. The extruded phospholipid vesicles were used for 2 days.

Expression and Quantification of Recombinant FVa Variants—The recombinant FVa(R306Q/R679Q) and FVa(R306Q/R679Q) variants were constructed as previously described (8). The recombinant proteins were transiently expressed in COS-1 cells using the DEAE-dextran transfection method (29). Briefly, FV cDNA, derived from Acros Genetix (Geel, Belgium). FS (brain extract), PE (egg extract), phosphatidylcholine (PC; egg extract), 1-palmitoyl-2-oleoylphosphatidylserine, 1-palmitoyl-2-oleoylphosphatidylethanolamine, and 1-palmitoyl-2-oleoylphosphatidylycholine were from Avanti Polar Lipids (Alabaster, AL).

Prothrombinase Assay—To determine the activity of FVa, a prothrombinase-based assay was used as described (8). Briefly, a mixture

of 0.5 μm prothrombin and 50 μm phospholipid vesicles (10:90 (w/v) FS/PC) was prepared in 25 mM Hepes, 150 mM NaCl, and 2 mM CaCl2 (pH 7.7) containing 0.5 mg/ml ovalbumin. FV was activated by thrombin (final concentration of 0.5 units/ml) at 37 °C for 10 min. FXa (final concentrations of 5 nM for FVa(R506Q/R679Q) and 0.05 nM for FVa(R306Q/R679Q) and the FVa samples were added to the prothrombinase mixture; and after 2 min, the prothrombin activation was stopped by 40-fold dilution in ice-cold EDTA buffer (50 mM Tris, 100 mM NaCl, 20 mM EDTA, and 1% polyethylene glycol 6000 (pH 7.9)). The amount of thrombin formed was measured kinetically with S2238.

FVa Inactivation Assay—FVa was incubated with thrombin (0.5 units/ml) for 10 min at 37 °C in buffer B. After activation of FVa, phospholipid vesicles (final concentration of 25 μM) and protein S were added, and a subsample was taken from the mixture and diluted 1.5 in ice-cold buffer B. APC was subsequently added. The amounts of APC and protein S added varied in the different experiments as indicated in the description of the individual experiments. At different time points, samples were taken from the inactivation mixture and diluted 1.5 in ice-cold buffer B to stop the reaction. The FVa activities in the diluted samples were then measured with the prothrombinase assay for the remaining FVa activity.

Equations Used for Curve Fitting—Inactivation of recombinant FVa(R506Q/R679Q) and FVa(R306Q/R679Q) was followed in time to calculate pseudo first-order rates for Arg⁵⁰⁶ and Arg⁵⁰⁶ cleavages by APC, respectively. The inactivation curves obtained were fitted to an exponential function already reported (18). The equation was modified due to the fact that only one cleavage occurs in our FV variants. For calculation of cleavage at position 506, the time curves obtained for FVa(R306Q/R679Q) were fitted to Equation 1,

\[
V_{306} = V_{306e} - k_{306} e^{-k_{306}t} + B \cdot V_{306c} (1 - e^{-k_{306}t})
\]

(1) in which \(V_{306}\) is the cofactor activity determined at time \(t\), \(V_{306e}\) is the cofactor activity determined before APC is added, \(B\) is the remaining procoagulant cofactor activity of FVa cleaved at position 506, and \(k_{306}\) is the rate constant of cleavage at position 506. The residual activity \(B\) was determined from time curves where complete cleavage at Arg⁵⁰⁶ had been accomplished, i.e. in the presence of 10:20:70 PS/PE/PC and protein S. The complete inactivation was obvious from the fact that prolonged incubation with APC did not lead to more loss of activity, i.e. the time curve leveled off at the residual FVa activity. Additionally, the validity of the \(B\) value was investigated in experiments using increasing concentrations of APC.

For calculation of cleavage at position 506, Equation 2 was used,

\[
V_{306} = V_{306e} - k_{306} e^{-k_{306}t} + B \cdot V_{306c} (1 - e^{-k_{306}t})
\]

(2) and fitted to the time curve of FVa(R506Q/R679Q). Here, \(C\) is the remaining procoagulant cofactor activity of FVa cleaved at position 506, and \(k_{306}\) is the rate constant of cleavage at position 506. The residual activity \(B\) was determined in a manner similar to Equation 1, i.e.

Western Blot Analysis of Recombinant FVa Variants—Recombinant and plasma-derived human FVa were incubated with 0.3 units/ml thrombin for 10 min at 37 °C to activate FVa to FVα. FVa (final concentration of 0.8 nM) was subsequently incubated with APC (at the concentration indicated for each individual experiment) with or without phospholipid S in the presence of 25 μM phospholipids (10:20:70 PS/PE/PC). At different time points, the inactivation was stopped by addition of denaturing solution. The samples were run under reducing conditions on a 10% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride membranes. A monoclonal antibody against the heavy chain of FVa (HAV-5146) was used to detect the FVa fragments, and the Western blots were developed using a Vectastain Elite ABC kit (Vector Labs, Inc., Burlingame, CA) according to the manufacturer's instructions.

\[\text{FXa Titration of APC-cleaved FVa(R306Q/R679Q) and FVa(R506Q/R679Q)} \]
Protein S in FVa degradation

RESULTS

Residual FVa activity after APC cleavage of FVAs at Arg^306 and Arg^506—The FVa(R506Q/R679Q) and FVa(R306Q/R679Q) variants were cleaved with APC, and their residual FVa activities were determined at increasing concentrations of FXa (Fig. 1). The FXa titration was performed using wild-type FVa. The Arg^506 cleavage resulted in a molecule expressing decreased maximal FVa activity (~70% at 20 nM FXa) as well as decreased FXa affinity (~10-fold). After cleavage at Arg^306, the FVa activity was severely impaired; and even at the highest FXa concentration, the activity was only 10–20% of the activity of uncleaved FVa.

Protein S Stimulates APC-mediated Cleavages at Both Arg^306 and Arg^506—The time courses of APC-mediated inactivation of the recombinant FVAs were determined using the prothrombinase-based assay to measure the remaining FVa activity (Figs. 2 and 3). As the residual FVa activities after APC cleavage of the FVAs (R506Q/R679Q) and FVa(R306Q/R679Q) variants were cleaved with APC, and their residual FVa activities were determined at increasing concentrations of FXa (Fig. 1). In comparison, a FXa titration was performed using wild-type FVa. The Arg^506 cleavage resulted in a molecule expressing decreased maximal FVa activity (~70% at 20 nM FXa) as well as decreased FXa affinity (~10-fold). After cleavage at Arg^306, the FVa activity was severely impaired; and even at the highest FXa concentration, the activity was only 10–20% of the activity of uncleaved FVa.

Protein S Stimulates APC-mediated Cleavages at Both Arg^306 and Arg^506—The time courses of APC-mediated inactivation of the recombinant FVAs were determined using the prothrombinase-based assay to measure the remaining FVa activity (Figs. 2 and 3). As the residual FVa activities after APC cleavage of the FVAs (R506Q/R679Q) and FVa(R306Q/R679Q) variants were cleaved with APC, and their residual FVa activities were determined at increasing concentrations of FXa (Fig. 1). In comparison, a FXa titration was performed using wild-type FVa. The Arg^506 cleavage resulted in a molecule expressing decreased maximal FVa activity (~70% at 20 nM FXa) as well as decreased FXa affinity (~10-fold). After cleavage at Arg^306, the FVa activity was severely impaired; and even at the highest FXa concentration, the activity was only 10–20% of the activity of uncleaved FVa.

Protein S Stimulates APC-mediated Cleavages at Both Arg^306 and Arg^506—The time courses of APC-mediated inactivation of the recombinant FVAs were determined using the prothrombinase-based assay to measure the remaining FVa activity (Figs. 2 and 3). As the residual FVa activities after APC cleavage of the FVAs (R506Q/R679Q) and FVa(R306Q/R679Q) variants were cleaved with APC, and their residual FVa activities were determined at increasing concentrations of FXa (Fig. 1). In comparison, a FXa titration was performed using wild-type FVa. The Arg^506 cleavage resulted in a molecule expressing decreased maximal FVa activity (~70% at 20 nM FXa) as well as decreased FXa affinity (~10-fold). After cleavage at Arg^306, the FVa activity was severely impaired; and even at the highest FXa concentration, the activity was only 10–20% of the activity of uncleaved FVa.

Protein S Stimulates APC-mediated Cleavages at Both Arg^306 and Arg^506—The time courses of APC-mediated inactivation of the recombinant FVAs were determined using the prothrombinase-based assay to measure the remaining FVa activity (Figs. 2 and 3). As the residual FVa activities after APC cleavage of the FVAs (R506Q/R679Q) and FVa(R306Q/R679Q) variants were cleaved with APC, and their residual FVa activities were determined at increasing concentrations of FXa (Fig. 1). In comparison, a FXa titration was performed using wild-type FVa. The Arg^506 cleavage resulted in a molecule expressing decreased maximal FVa activity (~70% at 20 nM FXa) as well as decreased FXa affinity (~10-fold). After cleavage at Arg^306, the FVa activity was severely impaired; and even at the highest FXa concentration, the activity was only 10–20% of the activity of uncleaved FVa.

Protein S Stimulates APC-mediated Cleavages at Both Arg^306 and Arg^506—The time courses of APC-mediated inactivation of the recombinant FVAs were determined using the prothrombinase-based assay to measure the remaining FVa activity (Figs. 2 and 3). As the residual FVa activities after APC cleavage of the FVAs (R506Q/R679Q) and FVa(R306Q/R679Q) variants were cleaved with APC, and their residual FVa activities were determined at increasing concentrations of FXa (Fig. 1). In comparison, a FXa titration was performed using wild-type FVa. The Arg^506 cleavage resulted in a molecule expressing decreased maximal FVa activity (~70% at 20 nM FXa) as well as decreased FXa affinity (~10-fold). After cleavage at Arg^306, the FVa activity was severely impaired; and even at the highest FXa concentration, the activity was only 10–20% of the activity of uncleaved FVa.
To study the effect of protein S on the APC-mediated cleavage at Arg506, the same set of phospholipid vesicles was used as for cleavage at position 306, but the APC concentration was lowered to 0.05 nM because the Arg506 cleavage is much faster than the Arg306 cleavage (Fig. 3). In contrast to results on record, protein S was found to enhance the Arg506 cleavage by APC. The protein S-dependent stimulation of the APC-mediated Arg506 cleavage was ∼5-fold at all three phospholipid compositions (Table II), which was lower than the stimulation observed for the Arg306 cleavage. As described for cleavage at position 306, the residual FVa activity of APC-cleaved FVa can be determined from the experiment using 10:20:70 PS/PE/PC in the presence of protein S, and this was used as the B value in Equation 1.

In the experiments described, we used well defined synthetic phospholipids with a 1-palmitoyl-2-oleoyl composition. The phospholipids that have been used to investigate FVa inactivation vary a lot between the different studies. Because no other group has reported any stimulation of the Arg506 cleavage, the same set of phospholipid compositions was used (Table II), which was lower than the stimulation observed for the Arg306 cleavage. As described for cleavage at position 306, the residual FVa activity of APC-cleaved FVa can be determined from the experiment using 10:20:70 PS/PE/PC in the presence of protein S, and this was used as the B value in Equation 1.

Western Blot Analysis of APC-mediated FVa Cleavage—To correlate the loss of activity according to the FVa inactivation assay with the formation of proteolytic products, aliquots were taken from the FVa inactivation mixtures and analyzed by Western blotting (Figs. 5 and 6). A monoclonal antibody against the heavy chain of FVa (epitope located between positions 307 and 506) was used in combination with a highly sensitive detection system. This allowed detection of the low amount of APC-generated fragments directly from the FVa degradation incubation mixture, even though it contained only 0.8 nM FVa. The high sensitivity of the Western blot technique also allowed detection of the small amount of uncleaved heavy chain that remained after the APC digestion. According to titration experiments in which decreasing amounts of FVa were applied to the Western blot, ∼5% of the uncleaved heavy chain (corresponding to 0.04 nM FVa) could be detected. Because the remaining uncleaved heavy chain did not express FVa activity (as determined by FXa titration as in Fig. 1), it probably had dissociated from its light chain, which would explain its resistance to APC. As the calculation of rate constants is insensitive to the concentration of FVa, the presence of this FVa species did not affect the calculated constants. In the FVa(R306Q/R679Q)-containing incubation mixtures, APC-mediated proteolysis resulted in the generation of a 60-kDa band, which corresponds to the Arg306–Arg709 fragment generated after cleavage at Arg506 (Fig. 5). In the presence of protein S, this band was clearly detected already after 0.5 min of incubation. In contrast, in the absence of protein S, this band was significantly weaker on the Western blot and occurred only at later time points.

### Table I

Comparison of rates of cleavage at Arg306 on different phospholipid vesicles in the absence or presence of protein S

| Phospholipid composition (synthetic unless indicated) | k<sub>306</sub> | + Protein S | − Protein S |
|-----------------------------------------------------|--------------|-------------|-------------|
| 10:90 PS/PC                                          | (1.5 ± 0.3) × 10<sup>6</sup> | (1.5 ± 0.2) × 10<sup>6</sup> | |
| 5:20:75 PS/PE/PC                                     | (2 ± 0.3) × 10<sup>6</sup> | (3.7 ± 0.2) × 10<sup>6</sup> | |
| 10:20:70 PS/PE/PC                                    | (1.2 ± 0.2) × 10<sup>7</sup> | (4.5 ± 0.8) × 10<sup>6</sup> | |
| 10:20:70 PS/PE/PC (natural)                          | 1 × 10<sup>6</sup> | 3.9 × 10<sup>6</sup> | |

### Table II

Comparison of rates of cleavage at Arg506 on different phospholipid vesicles in the absence or presence of protein S

| Phospholipid composition (synthetic unless indicated) | k<sub>506</sub> | + Protein S | − Protein S |
|-----------------------------------------------------|--------------|-------------|-------------|
| PL<sup>a</sup>                                       | (6.8 ± 1.4) × 10<sup>5</sup> | (5 ± 2) × 10<sup>5</sup> | |
| 100% PC                                              | (6.8 ± 0.8) × 10<sup>10</sup> | (6.7 ± 1.6) × 10<sup>10</sup> | |
| 20:80 PE/PC                                          | (8.8 ± 0.4) × 10<sup>10</sup> | (7.7 ± 1.2) × 10<sup>10</sup> | |
| 10:90 PS/PC                                          | (3.3 ± 0.2) × 10<sup>10</sup> | (1.1 ± 0.1) × 10<sup>10</sup> | |
| 5:20:75 PS/PE/PC                                     | (2.9 ± 0.5) × 10<sup>9</sup> | (0.8 ± 0.2) × 10<sup>9</sup> | |
| 10:20:70 PS/PE/PC                                    | (1.7 ± 0.3) × 10<sup>9</sup> | (3.7 ± 0.8) × 10<sup>9</sup> | |
| 10:90 PS/PE/PC (natural)                             | (4.6 ± 2.6) × 10<sup>9</sup> | (1.9 ± 1.3) × 10<sup>9</sup> | |
| 10:20:70 PS/PE/PC (natural)                          | 9 × 10<sup>9</sup> | 2.7 × 10<sup>9</sup> | |

<sup>a</sup> Phospholipid.
Protein S in FVa degradation

The APC cleavage of the FVa(R306Q/R679Q) variant yielded a 75-kDa band (Fig. 6, upper panel). This is consistent with cleavage at Arg\(^{306}\) and the generation of a fragment comprising residues 1–506. When protein S was present in the incubation mixture, the 75-kDa band appeared at earlier time points and with greater intensity. The same band was observed when normal plasma-derived FVa was used (Fig. 6, middle panel), but not when FVa purified from an individual homozygous for the FV Leiden mutation (human FVa(R506Q)) was used (lower panel), confirming that the fragment is indeed the result of cleavage at Arg\(^{306}\). When normal plasma-derived FVa was degraded in the presence of protein S, a barely detectable 30-kDa band was also observed, corresponding to the fragment after cleavage both at Arg\(^{306}\) and Arg\(^{506}\) (Fig. 6, middle panel). Also a faint band was observed in the presence of protein S with a size of ~60 kDa, corresponding to the fragment after isolated cleavage at Arg\(^{506}\). This band could also be observed faintly in human FVa(R506Q) (Fig. 6, lower panel).

Protein S Concentration Dependence of the APC-mediated Cleavage at Arg\(^{306}\) and Arg\(^{506}\)—To further elucidate the effect of protein S on the individual APC cleavage sites in FVa, the initial rate of FVa inactivation (first 20% loss of activity) was determined at different concentrations of protein S. Pseudo first-order rates were calculated, and the protein S concentration was plotted as a function of the rate constants obtained (Fig. 7). At saturating levels of protein S, the Arg\(^{306}\) cleavage proceeded ~25-fold faster in the presence of protein S, whereas the corresponding number for the Arg\(^{506}\) cleavage was only 4-fold. The concentration of protein S giving half-maximal stimulation of APC cleavage was similar for the two cleavages (~30 nM).

Phospholipid Composition Dependence of Arg\(^{306}\) and Arg\(^{506}\) Cleavages in the Absence and Presence of Protein S—Time courses of FVa inactivation were performed in the presence of numerous different phospholipid compositions, and the results are summarized in Fig. 8 and Tables I and II. Even though the rates of Arg\(^{506}\) cleavage under all conditions were at least 10-fold higher than the those of Arg\(^{306}\) cleavage, the two cleavages demonstrated similar responses to changes in phospholipid composition. In the absence of protein S, the rate of

![Western blot analysis of the APC-mediated cleavage at Arg\(^{306}\).](image)

![Western blot analysis of the APC-mediated cleavage at Arg\(^{506}\).](image)
The regulation of FVα activity by APC is a complicated phospholipid membrane-bound process involving the three cleavages at Arg<sup>306</sup>, Arg<sup>506</sup>, and Arg<sup>679</sup> (5). These cleavages have been reported to proceed at different kinetics, to have different protein S and phospholipid composition requirements, to yield products with different levels of FVα activity, and to be differently influenced by other components of the prothrombinase complex (18, 31). Despite a wealth of investigations, our knowledge is limited because it has not been possible to investigate each individual cleavage site separately. To study the influence of phospholipid composition and protein S on individ-

![Protein S titration in the inactivation of FVa(R306Q/R506Q) and FVa(R306Q/R506Q)](image)

**Fig. 7.** Protein S titration in the inactivation of FVa(R306Q/R506Q) and FVa(R306Q/R506Q). Inactivation of FVa(R506Q/R679Q) and FVa(R306Q/R679Q) was followed in time as described in the legends to Figs. 1 and 3 (APC concentration of 0.1–0.8 and 0.01–0.05 nM, respectively) at varying concentrations of protein S. The rate constants $k_{306}$ and $k_{506}$ were calculated for each protein S concentration. Upper panel, $k_{306}$ as a function of protein S concentration; lower panel, $k_{506}$ as a function of protein S concentration.

Inactivation of FVa(R506Q/R679Q) was also performed in the presence of 20:80 PE/PC. No significant differences in the rates of APC-mediated cleavage could be observed in the presence and absence of such vesicles, regardless of whether protein S was present or not. The APC-mediated cleavage of FVa(R506Q/R679Q) was also followed in the absence of phospholipid vesicles. Because very little cleavage was expected under these conditions, an APC titration was performed instead of a time course. FVa(R506Q/R679Q) was incubated with increasing concentration of APC for 10 min, and the FVα activity was measured with the prothrombinase assay. No inactivation of FVa(R506Q/R679Q) could be observed even at 6.4 nM APC, which is 8-fold higher than the FVα concentration (data not shown).

**Fig. 8.** Influence of membrane composition on Arg<sup>306</sup> and Arg<sup>506</sup> cleavages. Inactivation of FVa(R506Q/R679Q) (upper panel) and FVa(R306Q/R679Q) (lower panel) was followed in time as described in the legends to Figs. 1 and 3, and the rate constants $k_{306}$ and $k_{506}$ were calculated. Values represent the mean of three individual experiments; error bars represent S.D.

phospholipids, the rate of cleavage at Arg<sup>506</sup> was $-7 \times 10^5$ m<sup>-1</sup> s<sup>-1</sup>, with no significant influence of protein S. The APC-mediated inactivation of FVa(R306Q/R679Q) was also performed in the presence of vesicles composed of 100% PC. No stimulation of the Arg<sup>506</sup> cleavage could be observed upon addition of these vesicles. Moreover, protein S did not stimulate the cleavage rate in the presence of 100% PC vesicles. Inactivation assays were also performed in the presence of 20:80 PE/PC. No significant differences in the rates of APC-mediated cleavage could be observed in the presence and absence of such vesicles, regardless of whether protein S was present or not. The APC-mediated cleavage of FVa(R506Q/R679Q) was also followed in the absence of phospholipid vesicles. Because very little cleavage was expected under these conditions, an APC titration was performed instead of a time course. FVa(R506Q/R679Q) was incubated with increasing concentration of APC for 10 min, and the FVα activity was measured with the prothrombinase assay. No inactivation of FVa(R506Q/R679Q) could be observed even at 6.4 nM APC, which is 8-fold higher than the FVα concentration (data not shown).

**DISCUSSION**

The regulation of FVα activity by APC is a complicated phospholipid membrane-bound process involving the three cleavages at Arg<sup>306</sup>, Arg<sup>506</sup>, and Arg<sup>679</sup> (5). These cleavages have been reported to proceed at different kinetics, to have different protein S and phospholipid composition requirements, to yield products with different levels of FVα activity, and to be differently influenced by other components of the prothrombinase complex (18, 31). Despite a wealth of investigations, our knowledge is limited because it has not been possible to investigate each individual cleavage site separately. To study the influence of phospholipid composition and protein S on individ-

![Protein S in FVα degradation](image)
Arg306, whereas Arg506 is unaffected by protein S. In contrast to protein S selectively enhances the APC-mediated cleavage at ever, the conclusions on the Arg 506 cleavage were indirect, of FVa activity corresponds to cleavage at Arg506, whereas the of protein S occurs in a biphasic manner. The initial rapid loss of APC-mediated inactivation of normal FVa in the absence being based on comparisons of normal FVa and FVa Leiden.

The APC-mediated inactivation of purified recombinant FVAs. Partially purified or non-purified FV(R306Q/R679Q) or FV(R506Q/R679Q) (final concentration of 0.8 nM) was incubated with 0.5 units/ml thrombin for 5 min at 37 °C, and hirudin (final concentration of 5 units/ml) was subsequently added. APC was added to the reaction mixture, which also contained 25 μM phospholipids (10:20:70 PS/PE/PC). At intervals, samples were taken, and the FVa degradation was stopped by 1.5 dilution in ice-cold buffer B. FVa activity was measured with the protrombinase assay at a FXa concentration of 0.05 nM. The FVa activity was related to the activity observed before the addition of APC. Upper panel, inactivation of purified (■) or non-purified (○) FVa(R506Q/R679Q) in the presence of 0.8 nM APC and 100 nM protein S; lower panel, inactivation of purified (●) or non-purified (○) FVa(R306Q/R679Q) in the presence of 0.05 nM APC. The experiment was performed twice, and the data represent the mean.

Fig. 9. APC-mediated inactivation of purified recombinant FVAs. Partially purified or non-purified FV(R306Q/R679Q) or FV(R506Q/R679Q) (final concentration of 0.8 nM) was incubated with 0.5 units/ml thrombin for 5 min at 37 °C, and hirudin (final concentration of 5 units/ml) was subsequently added. APC was added to the reaction mixture, which also contained 25 μM phospholipids (10:20:70 PS/PE/PC). At intervals, samples were taken, and the FVa degradation was stopped by 1.5 dilution in ice-cold buffer B. FVa activity was measured with the protrombinase assay at a FXa concentration of 0.05 nM. The FVa activity was related to the activity observed before the addition of APC. Upper panel, inactivation of purified (■) or non-purified (○) FVa(R506Q/R679Q) in the presence of 0.8 nM APC and 100 nM protein S; lower panel, inactivation of purified (●) or non-purified (○) FVa(R306Q/R679Q) in the presence of 0.05 nM APC. The experiment was performed twice, and the data represent the mean.

usual APC cleavage sites, we constructed the recombinant FV(R306Q/R679Q) and FV(R506Q/R679Q) variants, which can be cleaved only at Arg506 and Arg206, respectively (8). These FV variants have similar procoagulant activity/antigen ratios as wild-type FV and yield the expected cleavage patterns upon Western blotting after incubation with thrombin and APC (8). In the present investigation, we used these FV variants for detailed studies on the influence of protein S and the phospholipid composition on the rate of APC-mediated cleavages.

It has previously been reported by Rosing et al. (18) that protein S selectively enhances the APC-mediated cleavage at Arg206, whereas Arg506 is unaffected by protein S. In contrast to this, we now demonstrate a protein S-dependent acceleration of the APC-mediated cleavage at Arg206. Several experimental differences between our study and that of Rosing et al. explain the difference in the results. Rosing et al. based their conclusions on studies of plasma-derived FVAs from individuals homozygous for normal FV or for FV(R506Q) (FV Leiden) (18). As the Arg679 cleavage was negligible under the conditions used, they could selectively investigate the Arg206 cleavage. However, the conclusions on the Arg206 cleavage were indirect, being based on comparisons of normal FVAs and FV Leiden. The APC-mediated inactivation of normal FVAs in the absence of protein S occurs in a biphasic manner. The initial rapid loss of FVa activity corresponds to cleavage at Arg206, whereas the slower phase is caused by cleavage at Arg506. When protein S was added to the inactivation, Rosing et al. observed that the slow phase was accelerated and that full loss of activity was achieved in a much shorter period of time. Therefore, they concluded that protein S preferentially enhances cleavage at Arg506. This is consistent with our results because we also observed that the APC-mediated cleavage at Arg506 is enhanced to a much greater extent than that at Arg206. However, their results did not exclude that Arg206 could also be stimulated by protein S. Because the protein S effect on the Arg206 cleavage is much larger than that on the Arg506 cleavage, the protein S-dependent stimulation is hard to detect by simply comparing the inactivation of purified human FVAs and FVas(R506Q) in the presence and absence of protein S. The use of recombinant FV variants that can be cleaved only at one of the APC cleavage sites enabled us to specifically investigate the protein S effects on each cleavage site under optimized conditions for the particular cleavage site.

Another important difference between our study and that of Rosing et al. (18) is related to the phospholipids. In this study, we used synthetic phospholipids with monounsaturated fatty acid chains, whereas Rosing et al. used synthetic phospholipids with a higher degree of polyunsaturated chains. It is known that the inactivation of FVAs by APC is enhanced on vesicles composed of polyunsaturated chains compared with unsaturated chains (15). One of the proposed effects of protein S is that it enhances the binding of APC to the phospholipid membrane (19). Therefore, it is conceivable that the specific stimulation of the Arg206 cleavage by protein S has remained undetected because of the use of phospholipid vesicles with optimal binding properties. For this reason, the inactivation of FVas(R306Q/R679Q) was also performed on vesicles containing phospholipids from normal extracts, which have a high degree of polyunsaturation. Even though the Arg206 cleavage was faster on these vesicles than on those prepared from synthetic phospholipids, it was still possible to detect a specific protein S-dependent stimulatory effect, which was, however, smaller than that observed using the synthetic vesicles.

Even though the rate of cleavage at Arg206 was enhanced to a greater extent than that at Arg506 by protein S, the concentration giving half-maximal stimulation was the same for the two cleavages. This indicates that the mechanism of protein S stimulation is similar for the two cleavage sites. Taken together with the previously observed enhancement of the Arg679 cleavage by protein S (8), the data suggest that protein S gives a general enhancement of the efficiency of APC on the membrane surface. The mechanism by which protein S enhances the APC-mediated inactivation of FVAs is not fully elucidated. One proposed mechanism is that protein S enhances the binding of APC to the membrane surface (19), with the two proteins forming a membrane-bound complex, which is consistent with a general increase in cleavage rates. Protein S has also been shown to decrease the distance between the active site of APC and the membrane surface (20). It has been hypothesized that this change would bring the active site of APC closer to Arg206 than to Arg506. However, the lack of inhibition of the Arg206 cleavage by protein S argues against this hypothesis; in fact, in this study, we saw the opposite, i.e. stimulation by protein S. Thus, our results do not argue against protein S having a specific effect on the orientation of the active site of APC, but do not support the idea that this would result in a specific enhancement of the Arg206 cleavage.

In the study, we have also analyzed the effect of membrane composition on the individual APC cleavage sites in FVAs. It has been suggested that the Arg206 cleavage site is more dependent on the phospholipid membrane compared with the other cleavage sites (32). We observed that the Arg206 and Arg506 cleavages were affected to the same extent by changes in the membrane composition. The effect of protein S and changes in phospholipid composition were synergetic, which is reasonable
because a membrane with a high content of negatively charged phospholipids binds protein S with greater affinity, thereby increasing the effective pool of cofactor. Appreciably high rates of Arg306 cleavage could also be observed in the absence of phospholipids, whereas no Arg506 cleavage was observed under these conditions. Thus, even though both cleavages are dependent on the presence of phospholipids, cleavage at position 506 can occur in their absence.

The effect of PE on the APC cleavage rates that we observed was smaller than that reported by Smirnov et al. (15). Moreover, they reported that the PE effect on the FVa inactivation could not be overcome by increasing the PS content, which stands in contrast to what has been observed for the FVIIIa-tissue factor complex, the prothrombinase complex, and FVIIIa binding. Based on this, Smirnov et al. proposed that PE selectively enhances the FVa inactivation. Our present results do not support this because we observed the same cleavage rates on 5:20:75 PS/PE/PC as on 10:90 PS/PE. This indicates that the effect of PE is more general, increasing the affinity of the phospholipid membrane for many of the coagulation proteins. Probably, the differences in the observed effects of PE are explained by the different phospholipids used in the two studies. Whereas we used PE containing the same monounsaturated side chain as PS and PC, Smirnov et al. used a polyunsaturated phospholipid. Also, their vesicles contained more PE than ours (50% compared with 20%).

In conclusion, our results indicate that the two APC-mediated cleavages at Arg306 and Arg506 in FVa are more similar in their dependence on phospholipid composition and the presence of protein S than previously reported. Thus, both cleavages are enhanced by the presence of protein S, even though the effect is more pronounced for the Arg506 cleavage site, and changes in membrane composition similarly influence both cleavages. The differences in kinetics between the Arg306 and Arg506 cleavages are presumably due to differences in specific interactions between exosites in APC and the FVa surfaces surrounding the cleavage sites. In agreement with this hypothesis is the demonstration of a positively charged exosite in APC, which is important for cleavage at Arg306, but not for cleavage at Arg506 (33, 34). Thus, elimination of the positive cluster in APC by site-directed mutagenesis resulted in specific loss of Arg506 cleavage potential, but did not affect the ability of APC to cleave Arg306. Therefore, the efficiency of each APC-mediated cleavage in FVa is determined by multiple specific molecular interactions between APC, FVa, protein S, and the phospholipid membrane.

Acknowledgments—We gratefully acknowledge the expert technical assistance of Ann-Louise Tholander and Ing-Marie Persson. We thank Dr. G. A. F. Nicolaes for helpful discussion.

REFERENCES
1. Butenas, S., and Mann, K. G. (2002) Biochemistry (Mosc.) 67, 3–12
2. Dahlback, B. (2000) Lancet 355, 1627–1632
3. Mann, K. G. (1999) Thromb. Haemostasis 82, 165–174
4. Esmon, C. T., Ding, W., Yashuhiko, K., Gu, J. M., Ferrell, G., Regan, L. M., Stearns-Kurosawa, D. J., Kurosawa, S., Matier, T., Laskzik, Z., and Esmon, N. L. (1997) Thromb. Haemostasis 78, 70–74
5. Kalafatias, M., Rand, M. D., and Mann, K. G. (1994) J. Biol. Chem. 269, 31869–31880
6. Nicolaes, G. A., Tans, G., Thomassen, M. C., Hemker, H. C., Pabinger, I., Varadi, K., Schwartz, H. P., and Rosing, J. (1995) J. Biol. Chem. 270, 21158–21166
7. Mann, K. G., Heckin, M. F., Begin, K. J., and Kalafatias, M. (1997) J. Biol. Chem. 272, 20678–20683
8. Norstrom, E., Thorelli, E., and Dahlback, B. (2002) Blood 100, 524–530
9. Bakker, H. M., Tans, G., Janssen-Claessen, T., Thomassen, M. C., Hemker, H. C., Griffin, J. H., and Rosing, J. (1992) Eur. J. Biochem. 208, 171–178
10. Smirnov, M. D., and Esmon, C. T. (1994) J. Biol. Chem. 269, 816–819
11. Smeets, E. F., Comfurius, P., Bevers, E. M., and Zwaal, R. F. (1996) Thromb. Res. 81, 419–426
12. Neunesserwander, P. F., Bianco-Fisher, E., Rezaie, A. R., and Morrissey, J. H. (1995) Biochemistry 34, 13988–13993
13. Fernandez, J. A., Kajima, K., Petaja, J., Hackeng, T. M., and Griffin, J. H. (2000) Blood Cells Mol. Dis. 26, 115–123
14. Deguchi, H., Fernandez, J. A., and Griffin, J. H. (2002) J. Biol. Chem. 277, 8861–8865
15. Smirnov, M. D., Ford, D. A., Esmon, C. T., and Esmon, N. L. (1999) Biochemistry 38, 3591–3598
16. Saha, O., Hensley, K., Smirnov, M. D., Esmon, C. T., and Esmon, N. L. (2001) J. Biol. Chem. 276, 1829–1836
17. Walker, F. J. (1980) J. Biol. Chem. 255, 5521–5524
18. Rosing, J., Hoekema, L., Nicolaes, G. A., Thomassen, M. C., Hemker, H. C., Varadi, K., Schwartz, H. P., and Tans, G. (1995) J. Biol. Chem. 270, 27852–27858
19. Walker, F. J. (1981) J. Biol. Chem. 256, 11128–11131
20. Yegneswaran, S., Smirnov, M. D., Saha, O., Esmon, N. L., Esmon, C. T., and Johnson, A. E. (1999) J. Biol. Chem. 274, 5462–5468
21. Solymos, S., Tucker, M. M., and Tracy, P. B. (1988) J. Biol. Chem. 263, 14884–14890
22. Dahlback, B. (1980) J. Clin. Invest. 66, 583–591
23. Tans, G., Rosing, J., Thomassen, M. C., Heeh, M. J., Zwaal, R. F., and Griffin, J. H. (1991) Blood 77, 2641–2648
24. Chen, L., Shah, A. M., Dahlback, B., and Nelsestuen, G. L. (1997) Biochemistry 36, 16025–16031
25. Dahlback, B. (1983) Biochem. J. 209, 837–846
26. Dahlback, B., Wiedmer, T., and Sims, P. J. (1992) Biochemistry 31, 12769–12777
27. Fiske, C. H., and Subbarow, Y. (1925) J. Biol. Chem. 66, 374–389
28. MacDonalld, R. C., Macdonald, R. I., Menzo, B. P., Takeshita, K., Subbarao, N. K., and Hu, L. R. (1991) Biochem. Biophys. Acta 1061, 297–303
29. Kaufman, R. J. (1990) Methods Enzymol. 185, 487–511
30. Steen, M., and Dahlback, B. (2002) J. Biol. Chem. 277, 38424–38430
31. Smirnov, M. D., Saha, O., Esmon, N. L., and Esmon, C. T. (1999) Blood 94, 3839–3846
32. Kalafatias, M., and Mann, K. G. (1993) J. Biol. Chem. 268, 27246–27257
33. Friedrich, U., Nicolaes, G. A., Villelurex, B. O., and Dahlback, B. (2001) J. Biol. Chem. 276, 23105–23108
34. Gale, A. J., Travaled, A., and Griffin, J. H. (2002) J. Biol. Chem. 277, 26836–26840
Importance of Protein S and Phospholipid for Activated Protein C-mediated Cleavages in Factor Va

Eva A. Norstrøm, Mårten Steen, Sinh Tran and Björn Dahlbäck

J. Biol. Chem. 2003, 278:24904-24911.
doi: 10.1074/jbc.M303829200 originally published online April 21, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M303829200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 34 references, 18 of which can be accessed free at
http://www.jbc.org/content/278/27/24904.full.html#ref-list-1