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Effects of Factor Xa and Protein S on the Individual Activated Protein C-mediated Cleavages of Coagulation Factor Va*

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Activated protein C inhibits the procoagulant function of activated factor V (FVa) through proteolytic cleavages at Arg-306, Arg-506, and Arg-679. The cleavage at Arg-506 is kinetically favored but protected by factor Xa (FXa). Protein S has been suggested to annihilate the inhibitory effect of FXa, a proposal that has been challenged. To elucidate the effects of FXa and protein S on the individual cleavage sites of FVa, we used recombinant FVa:Q306/Q679 and FVa:Q506/Q679 variants, which can only be cleaved at Arg-506 and Arg-306, respectively. In the presence of active site blocked FXa (FXa-1.5-dansyl-Glu-Gly-Arg), the FVa inactivation was followed over time, and apparent second order rate constants were calculated. Consistent with results on record, we observed that FXa-1.5-dansyl-Glu-Gly-Arg decreased the Arg-506 cleavage by 20-fold, with a half-maximum inhibition of ~2 nM. Interestingly and in contrast to the inhibitory effect of FXa on the 506 cleavage, FXa stimulated the Arg-306 cleavage. Protein S counteracted the inhibition by FXa of the Arg-506 cleavage, whereas protein S and FXa yielded additive stimulatory effect of the cleavage at Arg-306. This suggests that FXa and protein S interact with distinct sites on FVa, which is consistent with the observed lack of inhibitory effect on FXa binding to FVa by protein S. We propose that the apparent annihilation of the FXa protection of the Arg-506 cleavage by protein S is due to an enhanced rate of Arg-506 cleavage of FVa not bound to FXa, resulting in depletion of free FVa and dissociation of FXa-FVa complexes.

Blood coagulation factor Va (FVa)[2] is a nonenzymatic cofactor that together with factor Xa (FXa) forms the prothrombinase (PTase) complex on negatively charged phospholipid surfaces (1–3). The result is a 10^5–10^6-fold increase in the catalytic efficiency of FXa in its conversion of prothrombin to thrombin (4, 5). Factor V circulates in plasma as a procofactor with a domain structure of A1-A2-B-A3-C1-C2 (1–3, 6, 7). Upon activation by thrombin, the B domain is released, and the heavy chain (A1-A2) is linked to the light chain (A3-C1-C2) by a calcium ion. The release of the B domain results in an increased affinity for FXa, which is a prerequisite for its function in the PTase complex (8, 9).

The procoagulant function of FVa is down-regulated by activated protein C (APC) through proteolytic cleavages at three sites in the heavy chain, Arg-306, Arg-506, and Arg-679 (1–3). The importance of the individual APC cleavage sites in FV is demonstrated by several known naturally occurring mutations at the APC cleavage sites in FV. The most prevalent is a substitution of Arg-506 with Gln (FV Leiden), which results in APC resistance and increases the risk for venous thrombosis 5–10-fold (1, 3, 10–12). Two other mutations result in amino acid substitutions at the Arg-306 site, FV Cambridge (R306T) and FV Hong Kong (R306G) (13, 14). These mutations do not yield as severe decrease in APC response in plasma as FV Leiden and are not known to be risk factors for thrombosis (15–17).

The cleavage of FVa by APC results in a loss of binding affinity for FXa (18, 19). Of the three binding sites, the Arg-506 cleavage is the kinetically favored cleavage site and results in an ~40-fold loss of affinity for FXa (19–21). However, this molecule has still some procoagulant activity left, and the cleavage at Arg-306 is required for full loss of procoagulant function (12). The Arg-306 cleavage *per se* also reduces FXa affinity (7-fold) (20), but more importantly, when FVa is cleaved at both Arg-306 and Arg-506, the A2 domain dissociates, which results in a complete loss of procoagulant function of FVa (22). The cleavage at Arg-679 has not been studied in detail, but the cleavage is believed to be of minor importance for the inactivation. Removal of the C-terminal region of the heavy chain of FVa, Asp-683—Arg-709, by a snake venom has been shown to lead to a partial loss of FXa affinity (23). This suggests that the Arg-679 cleavage could also have an effect on the FXa affinity. The FXa-binding site on FVa is not fully localized. Several regions have been reported to be involved in the binding of FXa; amino acids 493–506 and 311–325 as well as 323–331 were based on peptide inhibition proposed to be part of the FXa-binding site (24–26). In addition we have shown that the regions around positions 467, 511, and 652 in the heavy chain of FVa as well as position 1683 in the light chain are important for the FXa binding (27). Recently, a computer-based structural model of the PTase complex has been proposed (28).

FXa is known to inhibit the APC-mediated inactivation of FVa (29, 30). Protein S has been proposed to counteract this

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[2] The abbreviations used are: FVa, factor Va; FXa, factor Xa; APC, activated protein C; DEGR, 1.5-dansyl-Glu-Gly-Arg; FXa-DEGR, FXa inhibited by DEGR; PTase, prothrombinase complex; PE, phosphatidylethanolamine; PS, phosphatidylserine; PC, phosphatidylcholine; BSA, bovine serum albumin.

effect (31). Peptide inhibition experiments have suggested FXa and protein S to have overlapping binding sites on FVa, implying that competitive binding for FVa could be a mechanism behind the counteraction (24). However, a later study claimed that the protein S function and the FXa inhibition are two independent effects and that the FXa only inhibits the cleavage at Arg-506, whereas protein S selectively stimulates the Arg-306 cleavage (21).

Previous studies have been based on kinetic investigation of the inactivation of human purified normal FVa and FVa from individuals being homozygous for the FV Leiden mutation (21, 31). Assuming that the role of Arg-679 for the inactivation is negligible, the FVa Leiden variant made it possible to study the Arg-306 cleavage. However, conclusions regarding the Arg-506 cleavage were made on indirect assumptions based on comparison between normal FVa and FVa Leiden. The aim of the present investigation was to elucidate the effect of FXa on the individual APC cleavage sites in FVa and to clarify if the inhibitory effect of FXa is counteracted by protein S. Recombinant FVa molecules that can only be cleaved at one cleavage site were used, allowing investigation of each APC cleavage site separately. FXa was found only to inhibit the cleavage at the Arg-506 site, whereas the Arg-306 cleavage was stimulated. Protein S counteracted the inhibitory effect of FXa on the Arg-506 cleavage, and a possible mechanism for this effect is proposed.

EXPERIMENTAL PROCEDURES

Materials—BioTrace polyvinylidene difluoride membranes were from Pall Corp. (Ann Arbor, MI). Chromogenic substrates S2238 and S2366 were kindly provided by Chromogenix (Milano, Italy). 1.5-Dansyl-Glu-Gly-Arg (DEGR) chloromethyl ketone was from Calbiochem-Novabiochem. Human FXa and human prothrombin were from Kordia (Leiden, Netherlands). α-Thrombin was from Hematologics Inc. (Essex Junction, Vermont). Hirudin was from Pentapharm (Basel, Switzerland). Human FV was purified from plasma as described (32) with minor modifications (33). Recombinant human APC was prepared as described (34), and its concentration was determined by chromogenic substrate S2366. An active site mutant recombinant APC variant (S195A) was created essentially as described by Gale *et al.* (35). Human protein S was purified as described (36) with minor modifications (37). FXa-DEGR was prepared by incubating FXa with a 5 molar excess of DEGR for 15 min followed by dialysis. Monoclonal antibody AHV-5146 against the heavy chain of FV was from Hematologics. A biotinylated monoclonal antibody against the B domain of FV (MK30) has previously been described (38). DAKO 0447 polyclonal goat anti-mouse antibody was from DakoCytomation (Glostrup, Denmark). Ovalbumin and bovine serum albumin were obtained from Sigma. Natural phospholipids phosphatidylserine (PS, brain extract), phosphatidylethanolamine (PE, egg extract), phosphatidylcholine (PC, egg extract), and synthetic phospholipids 1-palmitoyl-2-oleoyl-phosphatidylserine, 1-palmitoyl-2-oleoyl-phosphatidylethanolamone, and 1-palmitoyl-2-oleoyl-phosphatidylcholine were from Avanti Polar Lipids (Birmingham, Alabama). Dynabeads M-280 was from Dynal biotech ASA (Oslo, Norway). Supersignal West Dura extended duration substrate was from Pierce.

Phospholipid Vesicle Preparation—The phospholipid stocks were dissolved in 10/90 methanol/chloroform, and the concentrations were determined by phosphate analysis (39). Mixtures of the lipids (weight-based) were prepared in 10/90 methanol/chloroform and kept at −20 °C. Aliquots were drawn from the stocks and dried under N_2 and then resuspended in Hepes buffer at room temperature. Phospholipids for PTase assay were sonicated in a XL 2020 sonicator (Misonix, New York) at amplitude 3 for 10 min. Fresh phospholipid vesicles were prepared every day. For the inactivation assay, extruded phospholipid vesicles were used. The extrusion was performed using LiposoFast basic extruder (Armatis, Mannheim, Germany). The phospholipid mixtures dissolved in buffer were subjected to freeze-thaw circles and subsequently extruded multiple times through a membrane with a 100-nm pore size. The extruded phospholipids were used for 2 days.

Expression and Quantification of Recombinant Factor V Variants—The recombinant FV variants 306Q/679Q and 506Q/679Q were prepared as previously described (16). The recombinant proteins were transiently expressed in Cos-1 cells using the diethyl aminoethyl (DEAE)-dextran transfection method as described (40) with minor modifications. Briefly, FV cDNA in pMT2 vector was mixed with Tris, pH 7.3, 0.1 mM chloroquine and DEAE dextran in Dulbecco's modified essential medium (Invitrogen) and incubated for 4 h. The cells were thereafter shocked with 10% Me_2SO for 2 min. The proteins were harvested in serum-free medium (Opti-MEM) 60–70 h after transfection and concentrated in Vivaspin with a molecular weight cut off of 100,000 (Vivaspin). Aliquots were frozen at −80 °C. The concentrations of the recombinant proteins were determined with both enzyme-linked immunosorbent assay and PTase assay. Enzyme-linked immunosorbent assay was performed as described (16).

Affinity Purification of Recombinant FV Variants—The recombinant FV variants were purified as previously described (38). Briefly, a biotinylated monoclonal antibody against the B domain of FV (MK30) was bound to streptavidin-coated magnetic beads. Thereafter, the recombinant variants were incubated with the beads and subjected to a series of washing steps. To release FV from the beads and to activate FV, the beads were incubated with thrombin. Because the epitope of MK30 is in the B-domain of FV, the activated form of FV is released from the beads, whereas the B domain remains associated with the MK-30 coated beads. The affinity-purified FVa yielded similar APC degradation curves and kinetic constants as the unpurified FV in the condition medium. For the Western blotting experiments, the affinity-purified FVa was used.

Prothrombinase Assay—To determine the procoagulant activity of Factor Va, a PTase-based assay was used as described (16). Briefly, a mixture of 0.5 μM prothrombin and 50 μM phospholipid vesicles (10/90 w/w PS/PC) was prepared in 25 mM Hepes, 150 mM NaCl, 2 mM $CaCl_2$, pH 7.7, containing 0.5 mg/ml ovalbumin (HNO buffer). FV was activated by thrombin (final concentration 0.5 units/ml) at 37 °C for 10 min. FXa (final concentration 5 nM for FVa:506Q/679Q and 0.5 nM for FVa:306Q/679Q) and the FVa samples were added to the PTase mix, and after 2 min the prothrombin activation was stopped by a 40-fold dilution in ice-cold EDTA buffer. The lower FXa con-

centration used for the FVa:306Q/679Q variant was chosen because the remaining FVa activity of position 506-cleaved FVa was much lower at 0.5 nM FXa than at 5 nM FXa, which facilitated evaluation of the Arg-506 cleavage rate. 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.

FVa Inactivation with or without FXa and Protein S—To examine the time course of APC cleavage at Arg-306 and Arg-506, a FVa inactivation assay was performed using recombinant FV variants FV:506Q/679Q and FV:306Q/679Q, respectively. A final concentration of 0.8 nM FV was incubated with thrombin (0.5 units/ml) for 10 min at 37 °C in 25 mM Hepes, 150 mM NaCl, pH 7.7, with 5 mg/ml BSA and 5 mM $CaCl_2$ (HNBSACa). After activation of FV, phospholipid vesicles (PS/PE/PC 10/20/70, final concentration of 25 μM) were added, and a subsample was drawn from the mixture and diluted ⅕ in ice-cold HNBSACa buffer. APC was subsequently added. For the FV:506Q/679Q variant the concentration of APC was 0.8 nM when the degradation was performed in the absence of protein S and 0.2 nM when protein S was present. For the FV variant 306Q/679Q, the concentration of APC was 0.025 nM. At different time points, samples were drawn from the inactivation mixture and diluted ⅕ in ice-cold HNBSACa to stop the reaction. To measure remaining FVa activity, 10 μl of the diluted samples were added to 240 μl of the PTase mix, and the thrombin generation was determined as described above.

To investigate the effect of FXa, FXa-DEGR (5 nM) was added to the FVa-phospholipid mixture immediately after the activation of FVa. In experiments containing protein S, FXa-DEGR was either preincubated with the FVa-phospholipid mixture for 5 min before the addition of protein S and APC, or protein S was preincubated with the FVa-phospholipid mixture, and FXa-DEGR was subsequently added together with APC.

The concentration of FXa-DEGR in the FVa-phospholipid mixture was varied both in the absence or presence of protein S. In these experiments the FVa-phospholipid mixture was separated into aliquots, and FXa-DEGR (0–100 nM) was added. After a 5-min incubation, APC (final concentration 0.025 nM) with or without protein S (final concentration 100 nM) was added to start the inactivation assay, and the FVa degradation was stopped after 10 min by a ⅕ dilution in ice-cold HNBSACa.

In another experimental setting the protein S concentration was varied in the presence or absence of FXa-DEGR. The FVa-phospholipid mixture was separated into aliquots, and different concentrations of protein S (0–100 nM) were added. APC (final concentration 0.025 nM) with or without FXa-DEGR (final concentration 5 nM) was added to start the FVa inactivation, the reaction was stopped after 10 min by a ⅕ dilution in HNBSACa on ice, and the FVa activity was measured in the PTase assay.

Western Blot Analysis of Recombinant FV Variants—Affinity-purified FVa variants (final concentration 0.8 nM) in HNBSACa were incubated with APC (concentration indicated for each individual experiment) with or without 5 nM FXa-DEGR and 100 nM protein S in the presence of 25 μM phospholipids (PS/PE/PC 10/20/70). At different time points, the inactivation was stopped by the addition of denaturing sample preparation buffer, run under reducing conditions in a 10% SDS-PAGE, and transferred to polyvinylidene difluoride membranes. To detect the FVa fragments a monoclonal antibody against the heavy chain of FVa, AHV5146, was used together with horseradish peroxidase-conjugated goat anti-mouse IgG and the Supersignal West Dura extended duration chemiluminescence substrate. The chemiluminescence was traced with a Fuji LAS 3000IR CCD camera, and the signals were quantified with Image-Gauge program.

Equations Used for Curve Fitting—To calculate pseudo-first order rates for the Arg-306 and Arg-506 cleavages, the APC-mediated inactivation of FVa:506Q/679Q and FVa:306Q/679Q, respectively, was followed over time. The curves were fitted to an equation earlier reported (21). The equation was modified due to the fact that only one cleavage occurs in our FV variants. For calculation of the 506 cleavage, the time curves obtained for FVa:306Q/679Q were fitted to the equation,

$$Va_t = Va_0 \cdot e^{-(k_{506}) \cdot t} + B \cdot Va_0 \cdot (1 - e^{(-(k_{506}) \cdot t)}) \quad \text{(Eq. 1)}$$

in which Va_t is the cofactor activity determined at time t, Va_0 is the cofactor activity determined before APC is added, B is the fraction of remaining procoagulant cofactor activity of FVa cleaved at position 506, and k_{506} is the rate constant of cleavage at position 506.

For calculation of the 306 cleavage, the following equation used

$$Va_t = Va_0 \cdot e^{-(k_{306}) \cdot t} + C \cdot Va_0 \cdot (1 - e^{(-(k_{306}) \cdot t)}) \quad \text{(Eq. 2)}$$

and fitted to the time curve of FVa:506Q/679Q. Here, C is the fraction of remaining procoagulant cofactor activity of FVa cleaved at position 306, and k_{306} is the rate constant of cleavage at position 306. The use of the equations requires that the inactivation curves are independent of FVa concentration (% FVa inactivation *versus* time) and the rates are linear for APC concentration. Control experiments were performed, and the inactivation curves fulfilled these criteria.

Titration of Active Site Mutated APC in the Prothrombinase Assay—To examine the ability of APC to compete with FXa for binding to FVa, active site-mutated APC was utilized. Recombinant wild type FV was activated by incubation with 0.5 units/ml thrombin for 10 min at 37 °C, and the reaction was stopped with the addition of 5 units/ml hirudin. Activated FV (50 pM) and active site-mutated APC (0–40 nM) were incubated with a mixture of FXa (FC 5 nM), 50 μM phospholipids vesicles (10/90 w/w PS/PC) in buffer (HNBSACa) for 20 min in the presence or absence of protein S (100 nM). The PTase assay was started by the addition of prothrombin (0.5 μM), and after 1 min the assay was terminated by an 8-fold dilution in ice-cold EDTA buffer. The amount of thrombin formed was measured kinetically with a chromogenic substrate, S2238.

RESULTS

FXa Inhibits the APC-mediated Cleavage at Arg-506—To elucidate the effect of FXa-DEGR on the APC-mediated cleavage at Arg-506, the inactivation of FVa:306Q/679Q was followed over time in a PTase-based assay (Fig. 1*A*). The inactiva-

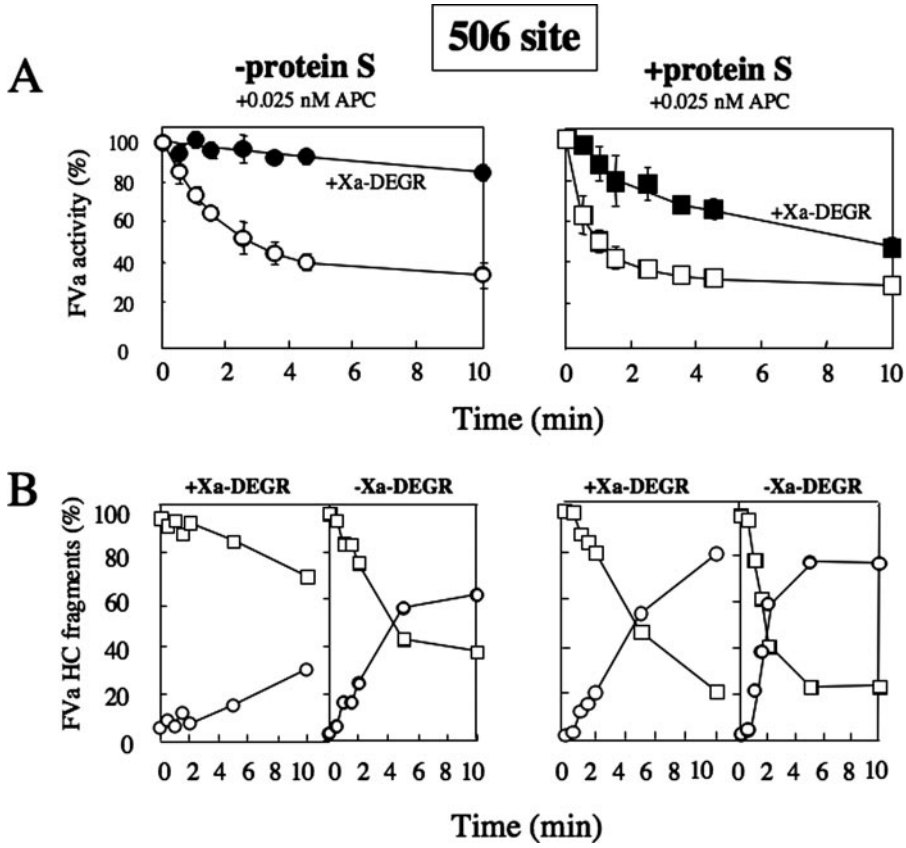

FIGURE 1. **Effects of FXa and protein S on APC-mediated inactivation of FVa:306Q/679Q.** *A*, the affinity-purified FVa:306Q/679Q variant (0.8 nM final concentration) was incubated with phospholipid vesicles (PS/PE/PC 10/20/70) in the presence (*closed symbols*) or absence (*open symbols*) of 5 nM FXa-DEGR with (*right*) or without (*left*) protein S. APC was added to start the inactivation assay and at intervals, samples were drawn, and the FVa degradation was stopped by 1/5 dilution in ice-cold HNBSACa. The FVa activity was measured with the PTase assay using 0.5 nM FXa. The FVa activity was related to the activity observed before the addition of APC. The plotted values in *A* represent the mean of three individual experiments; *error bars* represent SD. The *lines* indicate the curve fit as described under "Experimental Procedures." *B*, samples drawn from inactivation reactions using affinity-purified FVa 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 situated in the 307–506 fragment. FV-heavy chain (*HC*) fragments were visualized with SuperSignal West chemiluminescent substrate using a chemiluminescence reader (FUJIFILM LAS-3000 IR), and the bands were quantified by using the ImageGauge program (Fujifilm); *open squares* indicate the intact heavy chain, and *open circles* denote the 1–506 band.

TABLE 1

Effects of FXa-DEGR and protein S on the cleavage rate at Arg-506

The data used to calculate the rate constants were those presented in Fig. 1*A*.

	+FXa-DEGR, k_{506}	−FXa-DEGR, k_{506}	Times inhibition by FXa-DEGR
	$M^{-1}\,s^{-1}$	$M^{-1}\,s^{-1}$	
−Protein S	$1.7 \pm 0.02 \times 10^7$	$2.7 \pm 0.6 \times 10^8$	20
+Protein S:	$1.0 \pm 0.25 \times 10^8$	$9.0 \pm 3.2 \times 10^8$	9
Times stimulation by protein S	6	3	

tion was followed both in the absence (*left*) and presence (*right*) of protein S. In the absence of protein S and FXa-DEGR, incubation with APC yielded a rapid but partial loss of activity, which is consistent with cleavage at Arg-506. After ~5 min, a 40% plateau level of activity was reached, which represented the remaining procoagulant function of Arg-506-cleaved FVa. FXa-DEGR severely hampered the FVa inactivation, and after 20 min as much as 80% of the original FVa activity still remained. Based on the FVa inactivation time curves, apparent second order rate constants were calculated (Table 1). In the absence of protein S, the addition of FXa-DEGR yielded a 20-fold inhibition of the rate of APC cleavage at Arg-506. The effect of FXa-DEGR was also studied in the presence of protein S. With protein S, the initial loss of activity caused by cleavage at Arg-506 occurred almost instantly in the absence of FXa-DEGR, and after only a few minutes the plateau level of 40% was reached (Fig. 1*A*, *right*). FXa-DEGR hampered the inactivation also in the presence of protein S but not to the same extent as in the absence of protein S. After 20 min the remaining activity had reached 40%, indicating that all FVa had been cleaved at Arg-506. Based on second-order rate constants, protein S stimulated the APC-mediated cleavage at Arg-506 3-fold in the absence of FXa-DEGR and 6-fold in the presence of FXa-DEGR (Table 1). FXa-DEGR inhibited the cleavage at Arg-506 by a 9-fold decrease in rate constant in the presence of protein S as compared with the 20-fold decrease in the absence of protein S. This experiment was performed using two approaches. First, FVa was preincubated with FXa-DEGR for 5 min before the addition of the APC/protein S mixture. Second, protein S was preincubated with FVa for 5 min before FXa-DEGR was added and, thereafter, APC. The magnitude of inhibition was independent on which approach was used (data not shown).

To analyze the appearance of proteolytic products, aliquots were drawn from the inactivation mixture and subjected to Western blotting. The monoclonal antibody recognized an epitope located between Arg-306 and Arg-506. Incubation of the FVa:306Q/679Q variant with APC resulted in a fragment of about 75 kDa, corresponding to the 1–506 fragment. The appearance of this fragment and the loss of heavy chain were quantified using chemiluminescence (Fig. 1*B*). In the absence of FXa-DEGR and protein S, the Arg-506 site was rapidly cleaved, and the 75 kDa fragment appeared early and increased in intensity during the incubation with APC. The addition of FXa-DEGR delayed the appearance of the 75-kDa fragment, as the Arg-506 cleavage was inhibited. In the presence of protein S, the 75-kDa fragment appeared very early when FXa-DEGR was not added, and the appearance was only slightly delayed in the presence of both protein S and FXa-DEGR.

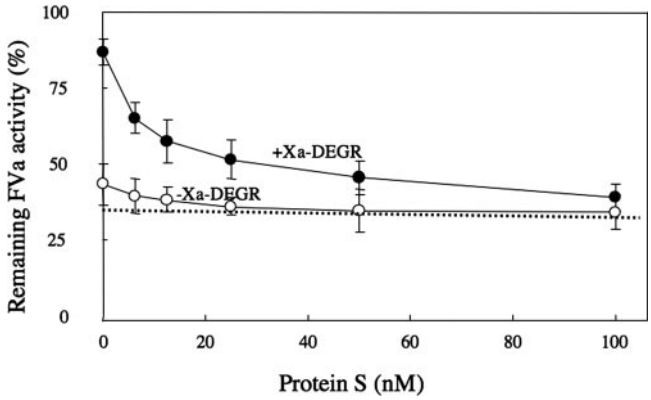

FIGURE 2. **Protein S titration in the presence or absence of FXa-DEGR.** FV:306Q/679Q (0.8 nM FV) was incubated with thrombin for 10 min at 37 °C to activate the FV, and phospholipid vesicles (10/20/70 PS/PE/PC, natural extracts), final concentration 25 μM, were subsequently added. The FVa-phospholipid mixture was separated into aliquots, and increasing concentrations of protein S (0–100 nM) were added to the aliquots. APC (final concentration 0.025 nM) with or without FXa-DEGR (final concentration 5 nM) was added to start the inactivation assay, and the assay was stopped after 10 min by a 1/5 dilution in HNBSACa on ice. FVa activity was measured with the PTase assay. The FVa activity was related to the activity observed before the addition of APC. *Open symbols*, in the absence of FXa-DEGR; *closed symbols*, in the presence of FXa-DEGR. The *dashed line* marks the level of procoagulant activity of the Arg-506-cleaved FVa. The plotted values represent the mean of three individual experiments; *error bars* represent S.D.

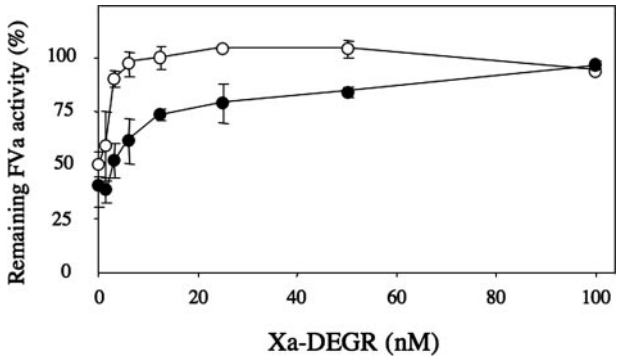

FIGURE 3. **Concentration dependence of FXa inhibition of Arg-506 cleavage by APC in the presence and absence of protein S.** FV:306Q/679Q (0.8 nM FV) was incubated with thrombin for 10 min at 37 °C to activate the FV, and phospholipid vesicles (10/20/70 PS/PE/PC, natural extracts), final concentration 25 μM, were subsequently added. The FVa-phospholipid mixture was separated into aliquots, and FXa-DEGR (0–100 nM) at different concentrations was added to the aliquots. APC (final concentration 0.025 nM) with or without protein S (final concentration 100 nM) was added to start the inactivation assay, and the assay was stopped after 10 min by a 1/5 dilution in HNBSACa on ice. FVa activity was measured with the PTase assay. The FVa activity was related to the activity observed before the addition of APC. *Open symbols*, in the absence of protein S; *closed symbols*, in the presence of protein S. The plotted values represent the mean of three individual experiments; *error bars* represent S.D.

Protein S Counteracts the FXa-mediated Inhibition of Arg-506 Cleavage—To estimate the concentration of protein S needed to counteract the inhibitory effect of FXa, the FVa inactivation was performed at increasing concentrations of protein S (Fig. 2). The FVa:306Q/679Q variant (Arg-506 cleavage studied) was incubated in the presence and absence of FXa-DEGR with APC and increasing concentrations of protein S for 10 min, after which the remaining FVa activity was measured. The addition of FXa-DEGR almost completely blocked the FVa inactivation in the absence of protein S. Increasing concentrations of protein S counteracted the inhibitory effect of FXa-DEGR, resulting in loss of FVa activity. At 100 nM protein S, the APC-mediated FVa inactivation was as efficient as in the absence of FXa-DEGR, *i.e.* protein S completely counteracted the effect of FXa-DEGR. Half-maximum inhibition of the FXa-DEGR effect was observed at ~10 nM protein S.

To evaluate how much FXa-DEGR was required to inhibit the APC-mediated cleavage at Arg-506 in the presence and absence of protein S, the degradation of FVa:306Q/679Q by APC was followed for 10 min in the presence (100 nM) or absence of protein S at increasing concentrations of FXa-DEGR (Fig. 3). In the absence of protein S, the addition of low concentrations of FXa-DEGR resulted in a strong inhibition of APC-mediated Arg-506 cleavage, with a half-maximum effect observed at ~2 nM FXa-DEGR. In the presence of protein S, higher concentrations of FXa-DEGR were required to obtain inhibition of APC-mediated FVa cleavage, and half-maximum inhibition was seen at about 14 nM FXa-DEGR.

Effects of FXa and Protein S on the APC-mediated Cleavage at Arg-306—To investigate the influence of FXa on the cleavage at Arg-306, the inactivation was performed using FVa:506Q/679Q in the presence of 5 nM FXa-DEGR (Fig. 4 and Table 2). In the absence of protein S, a 5-fold increase in the Arg-306 cleavage rate was unexpectedly observed when FXa-DEGR was added to the inactivation mixture (Table 2). The stimulatory effect of FXa-DEGR on the Arg-306 cleavage was also observed in the presence of protein S with a 6-fold increase of rate constants for cleavage at Arg-306 (Table 2). Aliquots from the inactivation reaction were subjected to Western blot analysis to correlate the loss of activity with the appearance of proteolytic cleavage products. The same antibody as in the experiment of Fig. 1 was used, and the loss of heavy chain and appearance of fragments were quantified using chemiluminescence (Fig. 4*B*). The Arg-306-cleaved product (positions 307–709) of around 60 kDa was observed on the gels. The band appeared earlier and with greater intensity when FXa-DEGR was present, confirming that FXa-DEGR stimulated the cleavage at Arg-306.

A possible mechanism for the stimulatory effect of FXa-DEGR on cleavage at Arg-306 may be that FXa-DEGR enhanced the binding of FVa to the phospholipid membrane. Theoretically this effect would be even more evident in the presence of monosaturated synthetic phospholipids, which are less favorable than more unsaturated natural phospholipids for the FVa degradation reaction. We, therefore, investigated the effect of FXa-DEGR on the inactivation of FVa:506Q/679Q in the presence of synthetic monounsaturated (1-palmitoyl-2-oleoyl) PS/PE/PC (10/20/70) vesicles. To focus on the effect of FXa-DEGR, protein S was not added in this experiment. Under these conditions the APC-mediated Arg-306 cleavage was strongly stimulated by the presence of FXa-DEGR (Fig. 5).

Protein S Does Not Inhibit FXa Binding to FVa—To investigate if protein S and FXa compete for the binding of FVa, a PTase-based assay was performed with increasing concentrations of FXa in the presence and absence of protein S (Fig. 6). The thrombin generation was not affected by preincubation of protein S, indicating that protein S does not interfere the interaction between FXa and FVa.

Active site-mutated APC is known to inhibit the binding of FXa to FVa (35), and we performed an experiment to elucidate

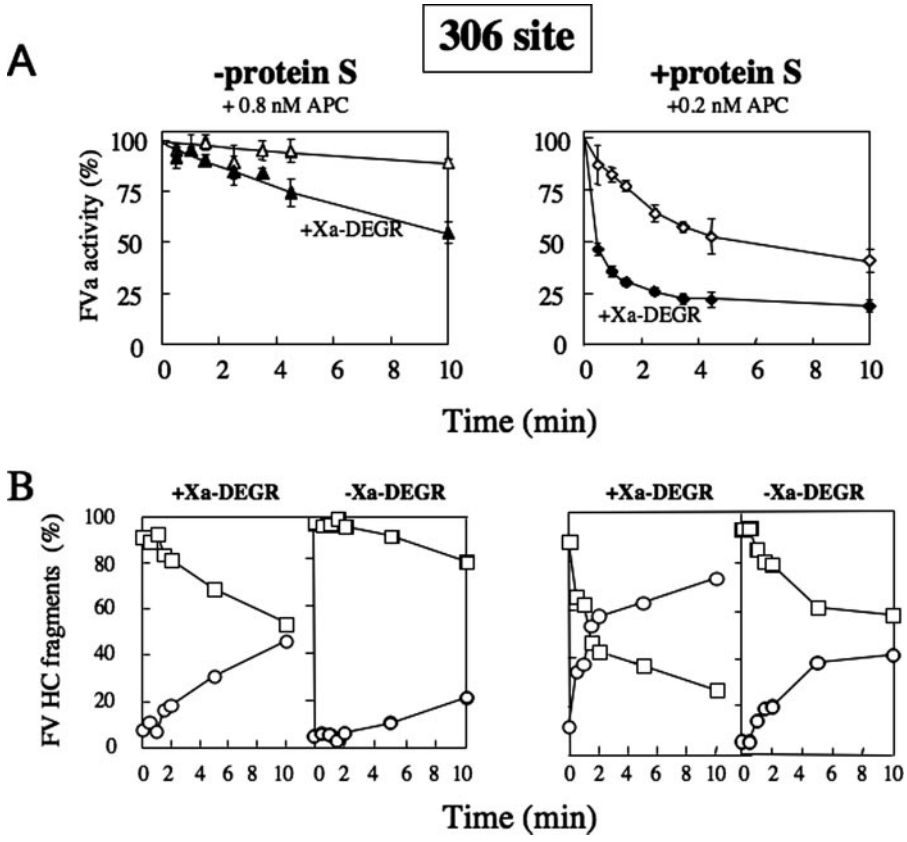

FIGURE 4. **Effects of FXa and protein S on APC-mediated inactivation of FVa:506Q/679Q.** The affinity-purified FVa:506Q/679Q variant (0.8 nM final concentration) was incubated with phospholipid vesicles (PS/PE/PC 10/20/70) in the presence (*closed symbols*) or absence (*open symbols*) of 5 nM FXa-DEGR with (*right*) or without (*left*) 100 nM protein S. APC was added to start the inactivation assay. Please note that the final APC concentration was 0.8 nM in the absence of protein S and 0.2 nM in the presence of protein S. At intervals samples were drawn, and the FVa degradation was stopped by a 1/5 dilution in ice-cold HNBSACa. FVa activity was measured with the PTase assay using 5 nM FXa. The FVa activity was related to the activity observed before the addition of APC. The plotted values (*A*) represent the mean of three individual experiments; *error bars* represent S.D. The *lines* indicate the curve fits calculated as described under "Experimental Procedures." *B*, aliquots from inactivation mixtures were analyzed by Western blotting (10% SDS-PAGE) using the monoclonal antibody AHV5146. FV-heavy chain (*HC*) fragments were visualized with SuperSignal West chemiluminescent substrate using a chemiluminescence reader (FUJIFILM LAS-3000 IR), and the bands were quantified by using the ImageGauge program (Fujifilm). The *open squares* represent the intact heavy chain, and the *open circles* represent the 307–709 band.

TABLE 2

Effects of FXa-DEGR and protein S on the cleavage rate at Arg-306

In this experiment natural phospholipids were used. The data used to calculate the rate constants were those presented in Fig. 4*A*.

	+FXa-DEGR, k_{306}	−FXa-DEGR, k_{306}	Times stimulation by FXa-DEGR
	$M^{-1}\,s^{-1}$	$M^{-1}\,s^{-1}$	
−Protein S	$1.8 \pm 0.38 \times 10^6$	$3.3 \pm 0.72 \times 10^5$	5
+Protein S:	$1.6 \pm 0.07 \times 10^8$	$2.5 \pm 0.28 \times 10^7$	6
Times stimulation by protein S	89	76	

if this inhibition was more pronounced in the presence of protein S (Fig. 7). Increasing concentrations of active site-mutated APC was added in a PTase-based assay, and thrombin generation was measured. Increasing concentrations of active site-mutated APC led to a decrease in thrombin generation, indicating that FXa and APC compete for the binding of FVa. However, the presence of protein S barely enhanced this inhibition.

DISCUSSION

It has been known and well accepted for many years that FXa inhibits the FVa inactivation by APC (29, 30, 41). However, whether protein S annihilates the FXa-mediated inhibition has been a matter of debate. Solymoss *et al.* (31) reported that protein S abrogates the inhibition by FXa, suggesting that protein S enables APC to inactivate FVa even when it is bound to FXa in the PTase complex. Conversely, Rosing *et al.* (21) suggested that the effects of protein S and FXa were independent of each other, since the two proteins influence separate cleavage sites, *i.e.* FXa inhibits the Arg-506 cleavage, whereas protein S stimulates the Arg-306 cleavage.

To study the effects of FXa and protein S on the individual cleavage sites in FVa, we used the recombinant variants FV:306Q/679Q and FV:506Q/679Q (16). These FV variants have similar procoagulant activity/antigen ratios as wild type FV and yield the expected cleavage pattern on Western blotting after incubation with thrombin and APC. Our results demonstrate that protein S counteracts the FXa-mediated inhibition of the Arg-506 cleavage, thus supporting the conclusion of Solymoss *et al.* (31). The experimental design of the study of Rosing *et al.* (21) may provide an explanation for their conclusions that the effects of protein S and FXa were independent. They performed a detailed kinetic analysis using purified human normal FVa and FVa Leiden in the presence of protein S, FXa, or both. The use of FVa Leiden enabled them to investigate the Arg-306 cleavage, assuming the cleavage at Arg-679 to be of minor importance. However, all conclusions regarding the rate of cleavage at Arg-506 were indirect based on the comparison between normal FVa and FVa Leiden (FVa:506Q). They found the inactivation of FVa:506Q not to be influenced by the presence of FXa, indicating that FXa selectively inhibited the cleavage at Arg-506. The rate of inactivation of FVa:506Q was greatly enhanced by protein S, which was the basis for their conclusion that protein S stimulated the APC-mediated cleavage at Arg-306. When normal FVa was subjected to APC-mediated inactivation in the presence of both protein S and FXa, the rate of inactivation did not reach that obtained in the presence of protein S alone. They, therefore, concluded that FXa still inhibited the Arg-506 cleavage and assumed that the increased rate observed when protein S was added to FXa was

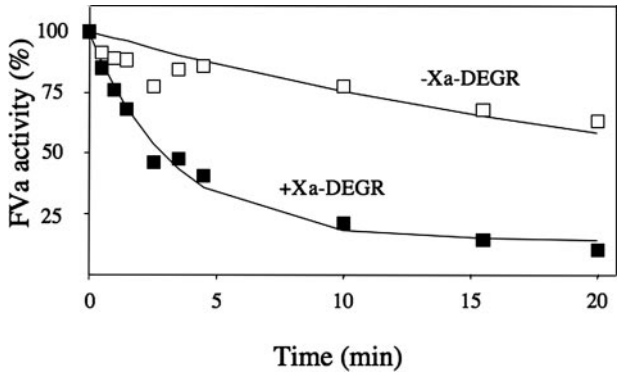

FIGURE 5. **Effects of FXa on APC-mediated inactivation of FVa:506Q/679Q using phospholipid vesicles composed of synthetic phospholipids.** Inactivation assay using FV variant FV:506Q/679Q was performed in the absence of protein S, essentially as described in the legend to Fig. 2. The phospholipid vesicles used were synthetic phospholipids (PS/PE/PC 10/20/70) where the fatty acid chains were composed of 1-palmitoyl-2-oleoyl. *Open symbols* denote data obtained in the absence of FXa-DEGR, and *closed symbols* denote data obtained in the presence of FXa-DEGR.

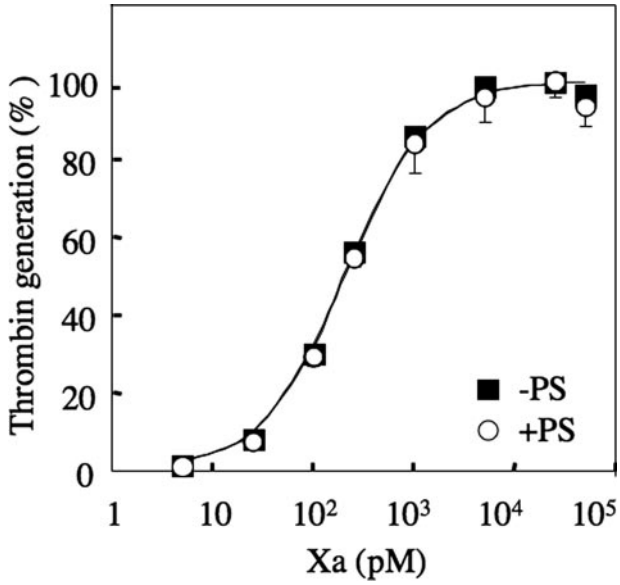

FIGURE 6. **FXa titration in the presence and absence of protein S.** Recombinant wild type FV was activated with 0.5 units/ml thrombin for 10 min at 37 °C, and 5 units/ml hirudin was subsequently added to stop the reaction. After dilution to 50 pM final concentration, the FVa was incubated with 50 μM natural phospholipids (10/90 PS/PC) in the absence (*open circles*) or presence (*closed squares*) of 100 nM protein S. Prothrombin (*PT*; 0.5 μM, final concentration) was added, and after 15 s, thrombin generation was started by the addition of FXa (5–50000 pM). After 1 min, the reaction was stopped by dilution in ice-cold EDTA buffer. The generated thrombin was determined with chromogenic substrate S-2238. The activity was expressed as percentage of maximum activity generated in the absence of protein S. Each data point represents the mean of three independent experiments performed in duplicate. *Error bars* represent ± S.D.

caused by the stimulatory effect of protein S at the Arg-306 site. Yet, their results did not exclude that some of the effect of protein S could be to diminish the FXa-mediated inhibition of the Arg-506 cleavage.

The APC-mediated inactivation of the FVa:506Q/679Q variant, which can only be cleaved at Arg-306, gave surprising results. FXa did not inhibit this cleavage but in fact stimulated the rate of cleavage by APC. This enhancement might be due to a cooperative binding where FXa stimulates FVa binding to the surface in a dose-dependent manner. This was underscored by

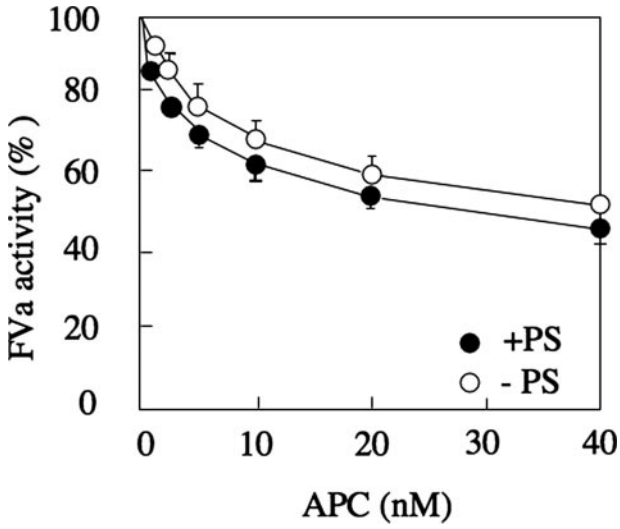

FIGURE 7. **Active site-mutated APC-mediated inhibition of prothrombinase in presence and absence of protein S.** Recombinant wild type FV was activation with 0.5 units/ml thrombin for 10 min at 37 °C, and then 5 units/ml hirudin was added. After dilution (50 pM final concentration) the FV was incubated with 50 μM natural phospholipids (10/90 PS/PC), 2 mM Ca^{2+}, 5 nM FXa, 0–40 nM active site-mutated APC in the presence (*closed circles*) or absence (*opened circles*) of 100 nM protein S for 20 min at 37 °C. Thrombin generation was started by the addition of 0.5 μM PT for 1 min, and the reactions were stopped by dilution with ice-cold EDTA buffer. The generated thrombin was determined with chromogenic substrate S-2238. The FVa activity was expressed as percentage of generated thrombin by FVa without APC.

the finding that FXa stimulated the cleavage even more when synthetic phospholipid vesicles, which are suboptimal for binding of coagulation factors, were used instead of the natural phospholipids.

Results on record have suggested that FXa and protein S have overlapping binding sites (24). A feasible explanation for the annihilation of FXa inhibition by protein S could, therefore, be competition between FXa and protein S for binding to FVa. However, our results indicate that this is not the mechanism involved. We evaluated the ability of protein S to inhibit the FXa binding to FVa by using a PTase-based FXa-titration assay, and the binding of FXa was not affected by protein S preincubation. Because protein S can stimulate the Arg-306 site even in the presence of FXa, it seems reasonable that FXa and protein S can simultaneously be bound to FVa. Under our experimental conditions we even saw a stimulatory effect of FXa on the Arg-306 cleavage. This effect was additive to the effect of protein S, further supporting that protein S does not compete with FXa for binding to FVa.

Another possible mechanism by which protein S hampers the FXa inhibition could involve enhancing the ability of APC to compete with FXa for FVa binding. We evaluated this hypothesis by using active site-mutated APC that binds to FVa without cleaving it. Titration of this APC in the PTase assay inhibited the thrombin generation, indicating a decrease in FVa-FXa complex formation. However, the presence of protein S did not increase the inhibition, indicating that protein S does not to any major extent augment the ability of APC to compete for FVa binding.

The solution to how protein S abrogates the FXa-mediated inhibition of APC cleavage at Arg-506 may be provided by the recently identified ability of protein S to stimulate the cleavage

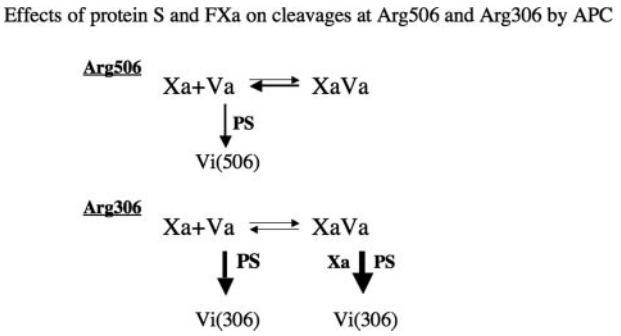

FIGURE 8. **Scheme summarizing the effects of protein S and FXa on APC-mediated cleavages at Arg-306 and Arg-506.** In free FVa protein S stimulates the cleavage at both Arg-506 and Arg-306, whereas only the Arg-306 site is stimulated by protein S when FVa is bound to FXa. Cleavage of free FVa at Arg-506 depletes free FVa and drives the equilibrium between FVa and FXa to the left. The apparent effect of protein S is, thus, an abrogation of the protection of FXa, whereas in fact the Arg-506 site in the FXa-FVa complex is protected from cleavage by APC, and only the free FVa is cleaved. The situation is different for the Arg-306 site where both protein S and FXa stimulates the cleavage by APC. Vi(506) and Vi(306) denote FVa cleaved at Arg-506 and Arg-306, respectively.

at Arg-506 in free FVa, *i.e.* not bound to FXa (42). The FVa-FXa complex is in steady state equilibrium with the free forms of the proteins, and the free form of FVa is available for APC inactivation (Fig. 8). Because the K_d for the phospholipids bound FXa-FVa interaction is around 1 nM, a certain fraction of FVa is free in the presence of 5 nM DEGR-FXa. This FVa serves as a substrate for APC, and the catalytic efficiency of APC is stimulated by the presence of protein S. FVa that has been cleaved by APC at Arg-506 has lower affinity for FXa. The consequence is depletion of procoagulant FVa and decreased concentration of functional FVa-FXa complexes.

Even if the kinetically favored APC cleavage site is mutated in FV Leiden (at Arg-506), the clinical consequences of the mutation are not as serious as would be expected. Rosing *et al.* (21) suggested that the inhibition by FXa of the APC-mediated cleavage of FVa could partly explain this discrepancy. If FXa inhibits the Arg-506 cleavage and both FXa and protein S stimulate the Arg-306 cleavage, the consequence would be that in the presence of both protein S and FXa, the Arg-306 cleavage would be equally fast or faster than the Arg-506 cleavage. As a consequence, the difference between the APC cleavages of FVa Leiden and normal FVa would decrease. However, according to this hypothesis FVa mutated at position Arg-306 would be completely resistant to inactivation by APC in the presence of high concentrations of FXa and, therefore, be associated with a serious risk for venous thrombosis. Nature has proven that this hypothesis is not accurate as naturally occurring mutations in the Arg-306 site, such as FV Hong Kong (R306G) and FV Cambridge (R306T), are not severe risk factors for venous thrombosis and are not associated with APC resistance (13, 14, 16, 17). This argues that the Arg-506 cleavage is of physiological importance and may occur even in the presence of FXa due to the effect of protein S. The balance between the inactivation of FVa by APC and protection from inactivation by FXa is probably dependent on the local concentration of all components and any pathological situation, as for instance protein S deficiency, which may disrupt this intricate balance.

In the present investigation we have focused on the effects of protein S and FXa but not taken into account that *in vivo* there are other factors that may influence the APC-mediated degradation of FVa. One such factor is prothrombin, which has been shown to inhibit the degradation of FVa by APC (43). We are currently investigating the effects of prothrombin on the individual APC-mediated FVa cleavage sites in an effort to gain further understanding of the complex reactions governing the regulation of FVa activity under physiological conditions.

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Enzyme Catalysis and Regulation:

Effects of Factor Xa and Protein S on the Individual Activated Protein C-mediated Cleavages of Coagulation Factor Va

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