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EFFECTS OF FACTOR Xa AND PROTEIN S ON THE INDIVIDUAL ACTIVATED PROTEIN C-MEDIATED CLEVAGES OF COAGULATION FACTOR Va*

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Running Title: FXa and the inactivation of FVa

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Activated protein C inhibits the procoagulant function of activated factor V (FVa), through proteolytic cleavages at Arg306, Arg506 and Arg679. The cleavage at Arg506 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 Arg506 and Arg306, respectively. In the presence of active site blocked FXa (FXa-DEGR), the FVa inactivation was followed over time and apparent second order rate constants were calculated. Consistent with results on record, we observed that FXa-DEGR decreased the Arg506 cleavage by 20-fold, with a half-maximum inhibition of approximately 2 nM. Interestingly and in contrast to the inhibitory effect of FXa on the 506 cleavage, FXa stimulated the Arg306 cleavage. Protein S counteracted the inhibition by FXa of the Arg506 cleavage, whereas protein S and FXa yielded additive stimulatory effect of the cleavage at Arg306. This suggests that FXa and protein S interact with distinct sites on FVa, which is consitent 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 Arg506 cleavage by protein S is due to enhanced rate of Arg506 cleavage of FVa not bound to FXa resulting in depletion of free FVa and dissociation of FXa-FVa complexes.

Blood coagulation factor Va (FVa)[1] is a non-enzymatic cofactor, which 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, Arg306, Arg506, and Arg679 (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 Arg506 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 Arg306 site, FV Cambridge (Arg306Thr) and FV Hong Kong (Arg306Gly) (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 Arg506 cleavage is the kinetically favored cleavage site, and results in an around 40-fold loss of affinity for FXa (19-21). However, this molecule has still some procoagulant activity left, and the cleavage at Arg306 is required for full loss of procoagulant function (12). The Arg306 cleavage *per se* also reduces FXa affinity (7-fold) (20), but more importantly when FVa is cleaved at both Arg306 and Arg506 the A2 domain dissociates, which results in a complete loss of procoagulant function of FVa (22). The cleavage at Arg679 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, Asp683-Arg709, by a snake venom, has been shown to lead to a partial loss of FXa affinity (23). This suggests that the Arg679 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 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 Arg506 while protein S selectively stimulates the Arg306 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 Arg679 for the inactivation is negligible, the FVa Leiden variant made it possible to study the Arg306 cleavage. However, conclusions regarding the Arg506 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 Arg506 site, whereas the Arg306 cleavage was stimulated. Protein S counteracted the inhibitory effect of FXa on the Arg506 cleavage and a possible mechanism for this effect is proposed.

EXPERIMENTAL PROCEDURES

Materials – BioTrace PVDF membranes were from Pall Corporation (Ann Arbor, Michigan). 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 Corporation (La Jolla, California). Human FXa and human prothrombin were from Kordia (Leiden, Netherlands). α-thrombin was from Haematologics 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 determined by chromogenic substrate S2366. An active site mutant recombinant APC variant (Ser195Ala) 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 5 molar excess of DEGR for 15 min. followed by dialysis. Monoclonal antibody AHV-5146 against the heavy chain of FV was from Haematologics Inc. (Essex Junction, Vermont). 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 Chemical Co. (St. Louis, Missouri). 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 (Rockford, Ilinois).

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, Germany). The phospholipid mixtures dissolved in buffer were subjected to freeze-thaw circles and subsequently extruded multiple times through a membrane with 100 nm pore size. The extruded phospholipids were used for two 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 (Gibco, Paisley, Scotland) and incubated for 4 hours. The cells were thereafter shocked with 10% DMSO for 2 minutes. The proteins were harvested in serum-free medium (Optimem) 60-70 hours 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 ELISA and PTase assay. ELISA 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. Since the epitope of MK30 is in the B-doman of FV, the activated form of FV is released from the beads while the B domain remains associated with the MK-30 coated beads. The affinity purified FVa yielded similar APC-degradation curves and kinetic konstants 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 U/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 minutes, the prothrombin activation was stopped by 40-fold dilution in ice-cold EDTA-buffer. The lower FXa concentration used for the FVa:306Q/679Q variant was chosen as 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 Arg506 cleavage rate. The EDTA buffer contained 50 mM Tris, 100 mM NaCl, 20 mM EDTA, 1% PEG 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 Arg306 and Arg506, 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 of FV was incubated with thrombin (0.5 U/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 25μM) were added and a subsample was drawn from the mixture and diluted 1/5 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 1/5 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 (5nM) 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 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 aliquoted and FXa-DEGR (0-100 nM) was added. After 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 minutes by the 1/5 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 aliquoted 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 and the reaction was stopped after 10 minutes by a 1/5 dilution in HNBSACa on ice and the FVa activity 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 addition of denaturing sample preparation buffer and run under reducing conditions in a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to PVDF 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 chemilumniscence substrate. The chemilumniscence 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 Arg306 and Arg506 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 following equation (*eq.1*):

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

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 was used (*eq.2*):

$$Va_t = Va_0 \cdot e^{-(k_{306})\cdot t} + C \cdot Va_0 \cdot (1 - e^{(-(k_{306})\cdot t)})$$

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 that 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 WT-FV was activated by incubation with 0.5 U/ml thrombin for 10 minutes at 37°C and the reaction was stopped with addition of 5 U/ml hirudin. Activated FV (50 pM) and active site mutated APC (0-40 nM) were incubated with a mixture of FXa (FC 5nM), 50 μM phospholipids vesicles (10/90 w/w PS/PC) in buffer (HNBSACa) for 20 minutes in the presence or absence of protein S (100 nM). The PTase assay was started by 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 Arg506 – To elucidate the effect of FXa-DEGR

on the APC-mediated cleavage at Arg506, the inactivation of FVa:306Q/679Q was followed over time in a PTase based assay (Fig 1A). The inactivation was followed both in 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 Arg506. After approximately 5 minutes, a 40% plateau-level of activity was reached, which represent the remaining procoagulant function of Arg506-cleaved FVa. FXa-DEGR severely hampered the FVa inactivation, and after 20 minutes 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 Arg506. 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 Arg506 occurred almost instantly in the absence of FXa-DEGR, and after only a few minutes the plateau level of 40% was reached (Fig 1A, 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 minutes the remaining activity had reached 40%, indicating that all FVa had been cleaved at Arg506. Based on second-order rate constants, protein S stimulated the APC-mediated cleavage at Arg506 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 Arg506 by a 9-fold decrease in rate constant in the presence of protein S, as compared to 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 minutes before addition of the APC/protein S mixture. Second, protein S was preincubated with FVa for 5 minutes before FXa-DEGR was added and thereafter APC. The magnitude of inhibition was independent on which approach 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 Arg306 and Arg506. 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 chemiluminesence (Fig. 1B). In the absence of FXa-DEGR and protein S, the Arg506 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 Arg506 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.

Protein S counteracts the FXa-mediated inhibition of Arg506 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 (Arg506-cleavage studied) was incubated in the presence and absence of FXa-DEGR with APC and increasing concentrations of protein S for 10 minutes where-after 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 approximately 10 nM protein S.

To evaluate how much FXa-DEGR was required to inhibit the APC-mediated cleavage at Arg506 in the presence and absence of protein S, the degradation of FVa:306Q/679Q by APC was followed for 10 minutes 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 Arg506 cleavage, with half-maximum effect being observed at approximately 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 Arg306 – To investigate

the influence of FXa on the cleavage at Arg306, 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 Arg306 cleavage rate was unexpectedly observed when FXa-DEGR was added to the inactivation mixture (Table 2). The stimulatory effect of FXa-DEGR on the Arg306 cleavage was also observed in the presence of protein S with a 6-fold increase of rate constants for cleavage at Arg306 (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 figure 1 was used and the loss of heavy chain and appearance of fragments were quantified using chemiluminesence (Fig. 4B). The Arg306 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 Arg306.

A possible mechanism for the stimulatory effect of FXa-DEGR on cleavage at Arg306 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 mono-saturated synthetic phopholipids, 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 Arg306 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 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 lead 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. 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 (31). Conversely, Rosing et al. 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 Arg506 cleavage, whereas protein S stimulates the Arg306 cleavage (21).

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 wt 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 Arg506 cleavage thus supporting the conclusion of Solymoss et al. (31). The experimental design of the study of Rosing et al. may provide an explanation for their conclusions that the effects of protein S and FXa were independent (21). 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 Arg306 cleavage, assuming the cleavage at Arg679 to be of minor importance. However, all conclusions regarding the rate of cleavage at Arg506 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 Arg506. 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 Arg306. 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 Arg506 cleavage and assumed that the increased rate observed when protein S was added to FXa, was caused by the stimulatory effect of protein S at the Arg306 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 Arg506 cleavage

The APC-mediated inactivation of the FVa:506Q/679Q variant, which can only be cleaved at Arg306, 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 the finding that FXa stimulated the cleavage even more when synthetic phospholipid vesicles, which are sub-optimal 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. Since protein S can stimulate the Arg306 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 Arg306 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 Arg506 may be provided by the recently identified ability of protein S to stimulate the cleavage at Arg506 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). As the Kd 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 Arg506 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 Arg506), the clinical consequences of the mutation are not as serious as would be expected. Rosing et al. suggested that the inhibition by FXa of the APC-mediated cleavage of FVa could partly explain this discrepancy (21). If FXa inhibits the Arg506 cleavage and both FXa and protein S stimulate the Arg306 cleavage, the consequence would be that in the presence of both protein S and FXa, the Arg306 cleavage would be equally fast or faster than the the Arg506 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 Arg306 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 Arg306 site, such as FV Hong Kong (Arg306Gly) and FV Cambridge (Arg306Thr), are not severe risk factors for venous thrombosis and are not associated with APC resistance (13,14,16,17). This argues that the Arg506 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, like for instance protein S deficiency, 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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FOOTNOTES

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[1]The abbreviations used are: APC, activated protein C; DEGR, 1.5-Dansyl-Glu-Gly-Arg; FVa, factor Va; FXa, factor Xa; FXa-DEGR, FXa inhibited by DEGR; HNBSACa, 25 mM Hepes, 150 mM NaCl, pH 7.7 with 5 mg/ml BSA and 5 mM $CaCl_2$; HNO, 25 mM Hepes, 150 mM NaCl, 2 mM $CaCl_2$, pH 7.7 containing 0.5 mg/ml ovalbumin; PTase, prothrombinase complex; PE, phosphatidylethanolamine; PS, phosphatidylserine; PC, phosphatidylcholine; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

FIGURES LEGENDS

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 5nM 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 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 section A represent the mean of 3 individual experiments; error bars represent SD. The lines between the symbols indicate the curve fit as described in materials and methods. 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-HC fragments were visualized with SuperSignal West chemilumniscent 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.

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 25μM, were subsequently added. The FVa-phospholipid mixture was aliquoted 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 minutes 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 and (closed symbols) in the presence of FXa-DEGR. The dashed line marks the level of procoagulant activity of the Arg506-cleaved FVa. The plotted values represent the mean of 3 individual experiments; error bars represent SD.

Figure 3. Concentration dependence of FXa-inhibition of Arg506 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 25μM, were subsequently added. The FVa-phospholipid mixture was aliquoted 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 minutes 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 and (closed symbols) in the presence of protein S. The plotted values represent the mean of 3 individual experiments; error bars represent SD.

Figure 4. Effects of FXa and protein S on APC-mediated inactivation of FVa:506Q/679Q.
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) 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 stopped by 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 (section A) represent the mean of 3 individual experiments; error bars represent SD. The lines between the symbols indicate the curve fits calculated as described in materials and methods. B) aliquots from inactivation mixtures were analyzed by western blotting (10% SDS-PAGE) using the monoclonal antibody AHV5146. FV-HC fragments were visualized with SuperSignal West chemilumniscent 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 the 307-709 band.

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 figure 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 data obtained in the presence of FXa-DEGR.

Figure 6. FXa-titration in the presence and absence of protein S
Recombinant FV-wt was activated with 0.5 U/ml thrombin for 10 minutes at 37°C and 5 U/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. PT (0.5 μM, final concentration) was added and after 15 seconds, thrombin generation was started by the addition of FXa (5-50000 pM). After 1 minute, 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 ± SD

Figure 7. Active site mutated APC-mediated inhibition of prothrombinase in presence and absence of protein S.
Recombinant FV-wt was activation with 0.5 U/ml thrombin for 10 minutes at 37°C and then 5 U/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 minute, 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.

Figure 8. Scheme summarizing the effects of protein S and FXa on APC-mediated cleavages at Arg306 and Arg506. In free FVa, protein S stimulates the cleavage at both Arg506 and Arg306, whereas only the Arg306 site is stimulated by protein S when FVa is

bound to FXa. Cleavage of free FVa at Arg506 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 Arg506 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 Arg306 site where both protein S and FXa stimulates the cleavage by APC. Vi(506) and Vi(306) denote FVa cleaved at Arg506 and Arg306, respectively.

TABLES

Table 1. Effects of FXa-DEGR and protein S on the cleavage rate at Arg506. The data used to calculate the rate constants were those presented in Fig. 1 A.

	+FXa-DEGR k_{506} $M^{-1}s^{-1}$	-FXa-DEGR k_{506} $M^{-1}s^{-1}$	Times inhibition by FXa-DEGR
- 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	

Table 2. Effects of FXa-DEGR and protein S on the cleavage rate at Arg306. 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} $M^{-1}s^{-1}$	-FXa-DEGR k_{306} $M^{-1}s^{-1}$	Times stimulation by FXa-DEGR
- 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	

Figure 1

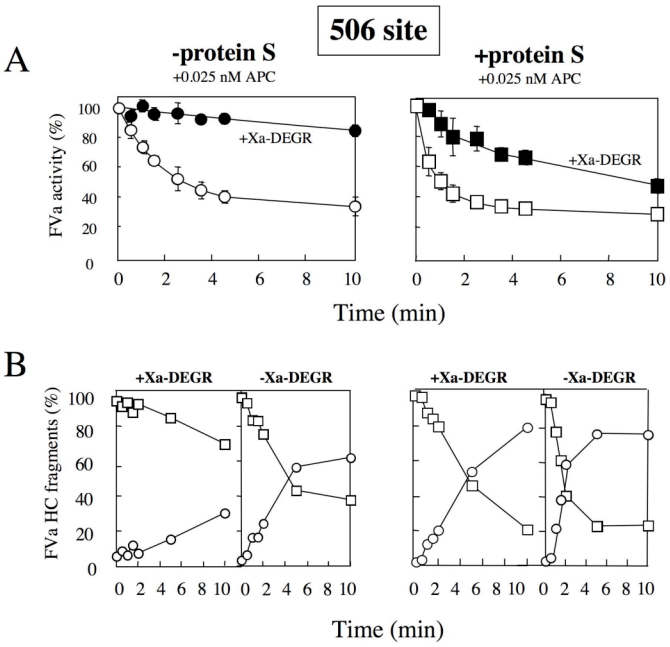
506 site
A
-protein S
+0.025 nM APC
+protein S
+0.025 nM APC
FVa activity (%)
+Xa-DEGR
+Xa-DEGR
Time (min)
B
+Xa-DEGR
-Xa-DEGR
+Xa-DEGR
-Xa-DEGR
FVa HC fragments (%)
Time (min)

Figure 2

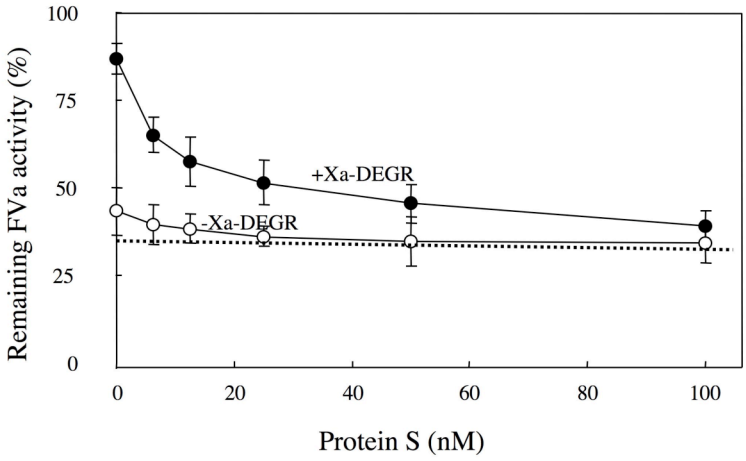
100
75
50
25
0
Remaining FVa activity (%)
+Xa-DEGR
-Xa-DEGR
0
20
40
60
80
100
Protein S (nM)

Figure 3

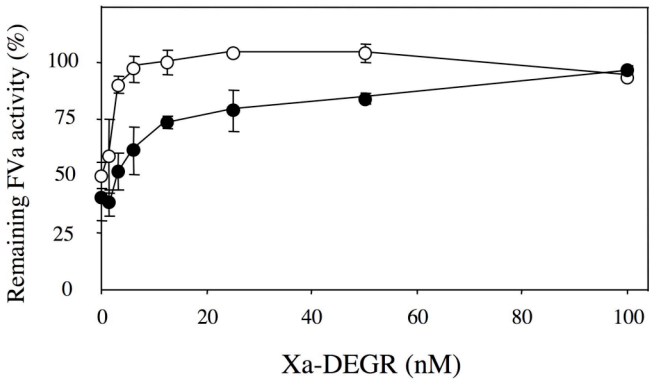
Remaining FVa activity (%)
100
75
50
25
0
0
20
40
60
80
100
Xa-DEGR (nM)

Figure 4

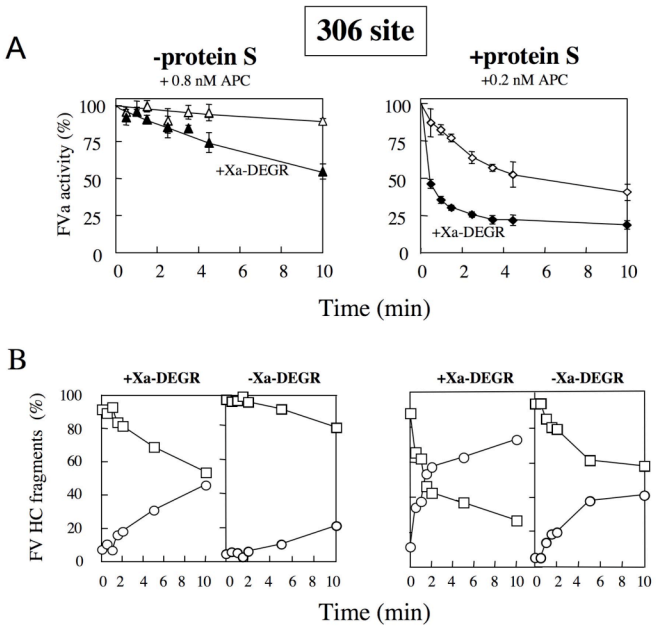
A
306 site
-protein S
+ 0.8 nM APC
+protein S
+0.2 nM APC
FVa activity (%)
+Xa-DEGR
+Xa-DEGR
Time (min)
B
+Xa-DEGR
-Xa-DEGR
+Xa-DEGR
-Xa-DEGR
FV HC fragments (%)
Time (min)

Figure 5

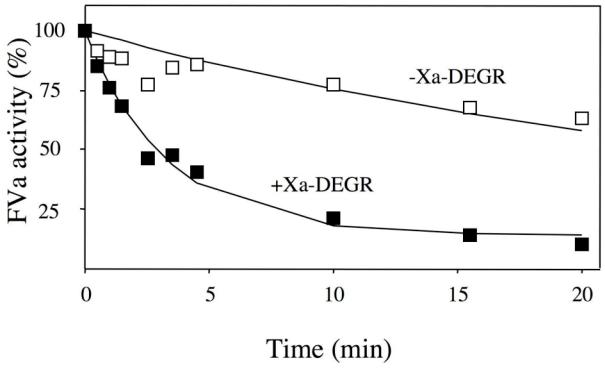

Figure 6

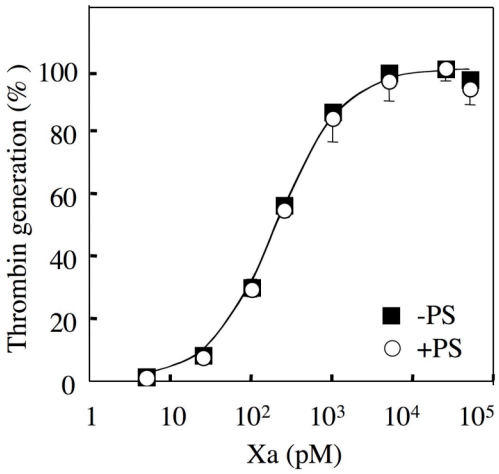
Thrombin generation (%)
100
80
60
40
20
0
1
10
10²
10³
10⁴
10⁵
Xa (pM)
-PS
+PS

Figure 7

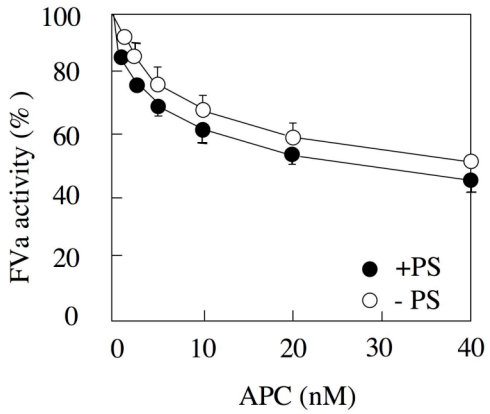

Figure 8

Effects of protein S and FXa on cleavages at Arg506 and Arg306 by APC