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Prothrombin amino terminal region helps protect coagulation factor Va from proteolytic inactivation by activated protein C

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Summary

The hypothesis that prothrombin (FII) protects coagulation factor Va (FVa) from proteolytic inactivation by activated protein C (APC) was tested using purified proteins. FII dose-dependently protected FVa from APC proteolysis under conditions where competition of proteins for binding to negatively-charged phospholipid surface was not relevant (i.e. either at high phospholipid vesicle concentrations or using soluble dicaproylphosphatidylserine at levels below its critical micellar concentration). Cleavages in FVa at both Arg⁵⁰⁶ and Arg³⁰⁶ by APC were inhibited by FII. FII did not alter the amidolytic activity of APC towards chromogenic oligopeptide substrates or inhibit FVIIIa inactivation by APC, implying that the FII-mediated protection of FVa from APC proteolysis was due to the ability of FII to inhibit protein-protein interactions between FVa and APC. FII also protected FVa from inactivation by Gla-domainless APC, ruling out a role for the APC Gla domain for these observations. To identify domains of FII responsible for the observed phenomenon, various forms or fragments of FII were employed. Biotin-Phe-ProArg-CMK-inhibited meizothrombin and fII-fragment 1•2 protected FVa from proteolysis by APC. In contrast, no significant protection of FVa from APC cleavage was observed for Gla-domainless-FII, prethrombin-1, prethrombin-2, FII fragment 1 or active site inhibited-thrombin (DEGR-thrombin). Overall, these data demonstrate that the Gla domain of FII linked to kringle 1 and 2 is necessary for the ability of FII to protect FVa from APC cleavage and support the general concept that assembly of the FII activation complex (FXa•FVa•FII•lipid surface) protects FVa from APC inactivation so that the procoagulant, thrombin generating pathway can act unhindered by APC. Only following FII activation and dissociation of the FII Gla domain fragments from the FII-ase complex, can APC inactivate FVa and down-regulate thrombin generation.

Keywords

Prothrombin; factor Va; activated protein C; inhibition; inactivation

Introduction

In the penultimate step of blood coagulation, prothrombin (FII) is cleaved by the so-called prothrombinase (FII-ase) complex (factor Xa•factor Va•phospholipids) to generate procoagulant alpha thrombin (FIIa) (1). Factor Va (FVa) serves as a cofactor in this reaction and accelerates the production of FIIa by the FII-ase complex by five orders of magnitude (2,

3). Thus, FVa is absolutely necessary for achieving physiologically relevant rates of FIIa generation.

FII is cleaved at Arg²⁷¹ and at Arg³²⁰ to generate FIIa. FII activation can proceed either via the prethrombin-2 intermediate (initial cleavage at Arg²⁷¹) or via a meizo-thrombin intermediate (initial cleavage at Arg³²⁰) (4,5). In the presence of FVa, FII activation proceeds primarily via the meizo-thrombin intermediate (6–8). The mechanism(s) by which FII is activated by the FII-ase complex has been a topic of discussion and is thought to involve both “channeling”, whereby FII is directly converted to FIIa without the release of intermediates (9,10), and “ratcheting”, whereby the reaction intermediate meizothrombin is proposed to undergo a spontaneous conformational change thereby making the second cleavage feasible at Arg²⁷¹ (11).

Activated protein C (APC) inactivates FVa by cleaving at Arg⁵⁰⁶, Arg³⁰⁶, and Arg⁶⁷⁹, thus regulating FIIa generation (12). The cleavages occur sequentially with Arg⁵⁰⁶ cleavage being kinetically favoured over the Arg³⁰⁶ cleavage (13,14). Cleavage at Arg⁵⁰⁶ results in partial loss of FVa activity, whereas proteolysis at Arg³⁰⁶ results in complete loss of FVa activity. The cleavage at Arg⁶⁷⁹ is believed to be of minor importance for the inactivation of FVa. Inactivation of FVa results primarily from the loss of ability of FVa to bind factor Xa (FXa) (15).

Since FXa, APC, FII and its fragments all bind to FVa, competition among these proteins for FVa appears likely. Consistent with this notion, FVa is protected from APC-mediated inactivation by the presence of FXa (16–19). This is likely because both FXa and APC share at least one or more binding site(s) on FVa, e.g. residues 311–325 in the heavy chain of FVa (20,21). Thus, the assembly of FXa•FVa complex could sterically hinder the formation of the FVa•APC complex. The effect of FII in APC mediated inactivation of FVa is less well characterized. Earlier reports showed that FII levels could modulate the anticoagulant activity of APC and that in purified system assays, FII inhibited the ability of APC to inactivate FVa (22–24). Here we have characterized FII-mediated inhibition of APC activity using various mutants of FVa and different fragments of FII and find that FII blocks APC cleavage at both Arg⁵⁰⁶ and Arg³⁰⁶ and that an intact N-terminal region of FII is responsible for blocking these cleavages.

Materials and methods

Proteins and reagents

FVa, FXa, FX, prethrombin-1, prethrombin-2, fragment 1, fragment 1•2 and APC were obtained from Haematologic Technologies Inc., Essex Junction, VT, USA. FII, Gla-domainless FII and Gla-domainless APC were obtained from Enzyme Research Laboratories, South Bend, IN, USA. Recombinant A2 and A3 domains disulfide crosslinked FVa (A2-S-S-A3 FVa) and its variants (RR506/679QQ and RR306/679QQ) were constructed on a B-domain deleted S2183A platform expressed and purified as described (15). Full length factor VIII (FVIII) was obtained as a gift from Bayer Healthcare (Berkley, CA, USA). Chromogenic substrates PefaChrome TH and PCa were purchased from Pentapharm Ltd., Basel, Switzerland. Substrate S2765 was purchased from Chromogenix (Lexington, MA, USA). Synthetic 1,2-dioleoyl-*sn*-glycero-3 phosphatidylcholine and 1,2-dioleoyl-*sn*-glycero-3-phosphatidylserine and 1,2-dihexanoyl-*sn*-glycero-3 phosphatidylserine (C6PS) were obtained from Avanti Polar Lipids, Alabaster AL, USA. Phosphatidylethanolamine (PE) was obtained from Sigma-Aldrich company. L-3-Phosphatidylcholine-1,2-di [1-¹⁴C]oleoyl PC was purchased from Amersham Biosciences. Phospholipid vesicles containing phosphatidylcholine and phosphatidylserine in the ratio 4:1 (PC/PS) were essentially prepared by sonication and ultracentrifugation as

described earlier (25). PC/PS/PE (2:1:2 mole ratio) vesicles were prepared as described before (26).

Amidolytic assays

APC (20 nM final) in buffer containing 25 μ M PC/PS in 50 mM Hepes (pH 7.4), 150 mM NaCl, 2 mM CaCl_2 was added to Pefachrome PCa (0–1.6 mM) in the presence or absence of 3.75 μ M FII (final). The initial rate of substrate cleavage was immediately recorded using a OPTImax plate reader (Molecular Devices, Sunnyvale, CA, USA) at 405 nm.

APC-dependent inactivation of FVa in the presence or absence of FII and its intermediates

A three-step reaction was used to evaluate the APC-mediated inactivation of FVa. The first step involved the inactivation of FVa by APC in the presence of phospholipids vesicles with or without FII (15). The second step involved the generation of FIIa by the FXa•FVa•FII•PL complex. The final step involved the quantification of FIIa generated by the FII-ase complex with the aid of a chromogenic substrate. One notes that both meizo-thrombin and α -FIIa exhibited similar amidolytic activity and we herein describe FII activation without distinguishing meizo-thrombin from alpha FIIa.

For the first step, FVa (0.55 nM) was incubated at room temperature with PC/PS (4:1, ~28 μ M) in 40 μ l of 50 mM Hepes (pH 7.4), 150 mM NaCl, 5 mM CaCl_2 , 0.1 mM MnCl_2 , 0.5% BSA (FII-ase buffer) containing or in the absence of varying concentrations of FII or its fragments. At time (t) = 0, two aliquots (1.8 μ l each) of this mixture were withdrawn and tested in FII-ase assay. Controls lacking APC were performed. At t = 0, human APC (0.5 nM) was added into the reaction mixture and subsequently 2 μ l of reaction mixture was withdrawn at t = 1, 2, 3, 4, 6, 8, 12, 18, 24, and 30 min and added to II-ase mixture of 38 μ l of FXa/PCPS (1.25 nM FXa/31 μ M PC/PS) and 10 μ l of II (1.5 μ M). Finally, at 2.67 min IIa production by the II-ase mixture was stopped by the addition of 15 μ l of this reaction mixture into 55 μ l of 50 mM Tris (pH 7.4), 100 mM NaCl, 10 mM EDTA, 0.5% BSA, and 0.02% NaN_3 buffer. IIa produced was quantified by the addition of 35 μ l of Pefa-Th (0.6 mM) to the mixture and colour generation was monitored at 405 nm on an OPTImax tunable plate reader (Molecular Devices, Sunnyvale, CA, USA). Inactivation assays of recombinant-RR506/679QQ FVa (2.2 nM) and recombinant-RR306/679QQ FVa (2.2 nM) were performed essentially as previously described except that the concentration of APC was 6 nM and 1.25 nM, respectively. Inactivation curves were fit according to equations described earlier (13).

The protective effects of FII and its fragments were assessed by adding suitable concentrations of FII (or its analogs) into the first step of FVa inactivation. Each experiment was compared with appropriate controls containing equimolar concentrations of FII and its fragments in the second step.

APC-dependent inactivation of factor VIIIa in the presence or absence of FII

Factor VIIIa inactivation by APC was performed essentially as described before (26). Briefly, FVIII (5 nM) was incubated with 25 μ M PC/PS/PE (2:1:2 molar ratio) vesicles in buffer containing 50 mM Hepes (pH 7.4), 150 mM NaCl, 5 mM CaCl_2 , 0.1 mM MnCl_2 , 5 mg/ml BSA prior to activation by FIIa (0.8 U/ml) for 1 min at room temperature. Subsequently, thrombin activation was stopped by the addition of 4.8 U/ml of hirudin. An aliquot of this sample was removed to determine FVIIIa cofactor activity in a FX-ase assay and designated as the activity of FVIIIa at time (t) = 0. Next, APC (20 nM) was added in the presence or absence of FII and aliquots were withdrawn at regular intervals for a time period of 45 minutes to determine the residual FVIIIa cofactor activity in a FX-ase assay. FX-ase activity was assessed as follows; FVIIIa (150 pM final) aliquots were added to FIXa (2.5 nM) in buffer containing 25 μ M PC/PS/PE before initiating the FX-ase reaction with 0.85 μ M FX. Finally, the reaction was stopped

by the addition of EDTA (5 mM) and FXa generated measured with substrate S2765 (Chromogenix, Lexington, MA, USA).

FVa proteolysis by APC in the presence or absence of FII monitored by Western blotting

A2-S-S-A3 wt FVa, A2-S-S-A3 RR506/679QQ FVa, or A2-S-S-A3 RR306/679QQ FVa (20 nM) in buffer containing 25 μ M PC/PS vesicles were incubated with APC (1 nM) in the presence or absence of FII (20 μ M) at room temperature for 60 minutes. APC proteolysis of FVa was stopped by the addition of 20 mM EDTA immediately followed by hot SDS. Aliquots were withdrawn and analyzed by gel electrophoresis. Subsequently, the protein bands were transferred to a PVDF membrane using a semi-dry transfer apparatus and stained for the FVa heavy chain using an anti-FVa heavy-chain primary antibody (AHV-5146, HaemTech) followed by biotinylated rat anti-mouse secondary antibody (Pierce, Rockford, IL, USA). Bands were finally visualized by using streptavidin-alkaline phosphatase colorimetric substrate 1-step™ NBT/BCIP (Pierce).

Results

FII-mediated protection of FVa from inactivation by APC

The hypothesis that FII protects FVa from APC inactivation was tested. Figure 1 shows that FII protects FVa from APC inactivation in a dose-dependent fashion. Complete FII-mediated protection was achieved by the addition of $\sim 2.4 \mu$ M FII in the first step of FVa inactivation assay. The inset in Figure 1 is a replot of the FII-dependence of residual FVa activity at 8 minutes time point in the FVa inactivation assay. From this plot the half maximal concentration of FII at which protection was observed is 0.5 μ M.

Effect of FII on APC amidolytic activity

The hypothesis that the inhibition of APC inactivation of FVa was due to an effect of FII on APC activity was tested. The activity of PC/PS-bound APC towards small oligopeptidic substrate PefaChrome PCa was tested in the presence and absence of FII. The k_{cat} and K_M values observed for APC cleaving the chromogenic substrate in the absence and presence of FII were not significantly different ($K_M = 0.9$ and 0.8 mM, respectively, and $k_{cat} = 37$ and 45 sec^{-1} , respectively) suggesting that FII does not affect the amidolytic activity of APC (data not shown).

Effect of FII on APC-mediated inactivation of FVIIIa

To examine if the FII-mediated protection was limited only to the inactivation of FVa by APC, the effect of FII on APC-mediated inactivation of FVIIIa was tested. 7.5μ M FII had no effect on the inactivation of FVIIIa by APC (data not shown). These data suggest that the FII-mediated inhibition of APC activity is limited to the substrate FVa, suggesting that protein-protein interactions between FVa and FII are likely to be responsible for the expression of FII-mediated inhibition of APC inactivation of FVa.

Phospholipid surface dependence of FII-mediated protection of FVa inactivation

To assess if the FII-mediated protection of FVa from APC is due to competition for phospholipids surfaces between FVa, APC and FII, inactivation assays were performed at various concentrations of PC/PS vesicles. FVa was incubated with APC in the presence or absence of FII at 14, 25 and 56 μ M PC/PS before the residual FVa activity was assessed in the coupled FII-ase assay. Figure 2A shows that FII-mediated protection was still evident at all three concentrations of PC/PS, suggesting that this phenomenon is not solely due to competition for PC/PS vesicles. Alternatively, FVa inactivation assays were also performed in the presence of 2 mM of the soluble phosphatidylserine, C6PS. Using a variety of biophysical techniques,

Koppaka and coworkers (27) have shown that the critical micellar concentration (*cmc*) of C6PS is above 3 mM under conditions similar to our assay conditions. Thus, at a concentration of 2 mM, C6PS should exist as monomers. Figure 2B shows that FII inhibits APC-dependent inactivation of FVa even when the reaction lacks a “phospholipids carpet”, suggesting that the mechanism of inhibition is something other than competition between FII, APC and FVa for phospholipids surfaces. Our results concur with the observations by Smirnov and coworkers who showed that FII inhibited APC inactivation of FVa even in the absence of phospholipids (22).

Effect of F II on APC-mediated cleavages at Arg⁵⁰⁶ and Arg³⁰⁶ of FVa

APC mediates the cleavage of Arg⁵⁰⁶ and Arg³⁰⁶ in FVa which subsequently results in the release of the A2 domain and inactivation of FVa. Earlier work by Gale and coworkers using an A2-S-S-A3 inter-domain disulfide crosslinked FVa showed that the critical events that effectively inactivate FVa are the cleavages at Arg⁵⁰⁶ and Arg³⁰⁶ and that A2 domain dissociation has a modest contribution towards the inactivation of FVa (15). Importantly, the A2-S-S-A3 based FVa mutants used by Gale and coworkers are useful tools to assess the contribution of Arg³⁰⁶ and Arg⁵⁰⁶ bond cleavage without the contribution of A2 domain dissociation. Figure 3A shows that A2-S-S-A3 FVa was protected from APC inactivation by FII similar to the plasma-derived FVa used in Figure 1 and Figure 2. FVa disulfide linked mutants were used to define protection for cleavage of individual Arg residues. FII also protected A2-S-S-A3 RR306/679QQ FVa (Fig. 3B), in which only Arg⁵⁰⁶ was cleaved and partially protected RR506/679QQ FVa (Fig. 3C), where only Arg³⁰⁶ was cleaved, demonstrating that both Arg⁵⁰⁶ and Arg³⁰⁶ bond cleavages are protected by FII.

Effect of fragments of FII on APC inactivation of FVa

The effect of various fragments of FII (residues 1–579) on the APC mediated inactivation of FVa was tested. PC/PS-bound FVa was incubated in the presence and absence of various fragments of FII before the addition of APC. Residual FVa activity was then tested using a coupled FII-ase assay as described. Biotin-Phe-ProArg-inhibited meizothrombin (bFPR-mIIa) completely protected FVa from APC inactivation similar to intact FII (Fig. 4A). To test if the Gla domain and Kringle 1 and 2 of FII were sufficient to protect FVa from APC, FVa inactivation assays were performed in the presence and absence of FII fragment 1•2 (residues 1–271, Gla domain plus kringle 1 and kringle 2). FII fragment 1•2 protected FVa from APC cleavage, albeit weakly (Fig. 4B). Half maximal protection of FVa was observed at ~10 μM fragment 1•2 concentration. Panels 4C–F show that desGla FII (residues 40–579), prethrombin-1 (residues 156–579), pre-thrombin-2 (residues 272–579) and dansyl-GluGlyArg-inhibited FIIa (DEGR-FIIa) did not protect FVa from APC inactivation. Figure 4G shows that FII fragment 1 (residues 1–155, Gla and kringle 1) did not detectably protect FVa from APC inactivation upto 20 μM. These results show that once FII loses the Gla domain, it is not able to protect FVa from APC inactivation. These data suggest that the Gla domain in combination with kringle 1 and 2 of FII is sufficient/necessary to protect FVa from APC.

Effect of FII on DesGla APC-mediated cleavage of FVa

To test if the Gla domain of APC is necessary for the FVa-protective effect of FII, the effect of FII on desGla APC mediated inactivation of FVa was tested. Figure 5 shows that FII protected FVa from desGla APC-mediated cleavage, suggesting that APC Gla domain does not play a role in the protein-protein interactions that mediate this protection of FVa.

Discussion

In this study, we have extended the previously reported findings that FII inhibits the APC-dependent inactivation of FVa using disulfide stabilized mutants of FVa and using different

fragments of FII. Using a multistage APC-mediated FVa inactivation assay, we have confirmed previous work showing that FII inhibits APC proteolysis of FVa in a dose-dependent fashion. FII-mediated protection of APC inactivation of FVa was also confirmed in Western blot analyses using antibodies against FVa heavy chain (data not shown). These data confirm observations by Tran and coworkers that no new cleavages in FVa were observed in the presence of FII other than Arg⁵⁰⁶ and Arg³⁰⁶. Half maximal inhibition of APC activity was obtained at ~0.5 μ M FII comparable to the 0.7 μ M reported by Tran et al. (24). Since the K_m for the FII-ase reaction is on this same order of magnitude, our results concur with the suggestion that the FII-mediated inhibitory effect could be a physiologically relevant regulatory mechanism (22).

FII-mediated inhibition of APC activity could be caused because FII inhibits the amidolytic activity of APC. To investigate this, we tested the ability of PC/PS-bound APC to hydrolyze small oligopeptidic substrate at various concentrations of FII. No detectable alteration in APC activity was observed at different FII concentrations, suggesting that FII does not inhibit APC in-activation of FVa by altering APC activity.

To test if the FII-mediated inhibition of APC activity was limited to FVa as substrate, inactivation of FVIIIa by APC was performed in the presence and absence of FII. APC-dependent FVIIIa inactivation profiles in the presence and absence of FII were essentially the same, suggesting that FII inhibition of APC activity is limited to FVa as substrate. This suggests that FII may interfere with the interaction of APC with FVa.

Alternatively, FII could inhibit APC action on FVa by competing with these proteins for phospholipids (PC/PS) surfaces. FII inhibited APC inactivation of FVa even at saturating high concentrations of PC/PS, suggesting that the FII-mediated effect was not primarily due to competition for lipids. This conclusion was also reinforced by the fact that FII inhibited APC-dependent FVa inactivation using soluble short-chain phosphatidylserine.

APC proteolyses the heavy chain of FVa at Arg⁵⁰⁶ and at Arg³⁰⁶ to inactivate FVa. Cleavages at Arg⁵⁰⁶ and at Arg³⁰⁶ are also accompanied by a cleavage at Arg⁶⁷⁹, which is of minor importance, and A2 domain dissociation, all of which can contribute to FVa inactivation (15). We have used mutants of FVa which have an A2–A3 inter-domain disulfide bond to get an accurate estimation of the effect of FII on individual Arg bond hydrolysis. Our data shows that FII inhibits APC-mediated proteolysis at Arg⁵⁰⁶ completely at 7.5 μ M FII, whereas it inhibits the Arg³⁰⁶ only partially at these concentrations (only ~2-fold inhibition can be observed at 7.5 μ M FII). Our data agree with very recent observations by Tran and coworkers who showed that FII protects both Arg⁵⁰⁶ and Arg³⁰⁶ of FVa from APC cleavage (24). It is as yet unclear as to the effect of FII on the APC-mediated cleavage of FVa at Arg⁶⁷⁹ because the cleavage of Arg⁶⁷⁹ does not result in any major reduction FVa activity. These observations for FII protection at Arg⁵⁰⁶ and Arg³⁰⁶ are significant because FXa protects FVa from APC cleavage at only Arg⁵⁰⁶ whereas APC-mediated cleavage at Arg³⁰⁶ has been reported to be stimulated by the presence of FXa (19). Based on our data and in conjunction with the previous report, we suggest that in order for FVa to be completely protected from APC-mediated cleavage at Arg³⁰⁶, FVa must complex to FII. Thus, FVa can be completely protected from APC by forming the complete FII-ase complex.

FII activation to FIIa using ping-pong-like mechanism involves the release of multiple intermediates (10). Conversion to products may involve both “channeling” and “ratcheting” mechanisms. Much of the FII is converted to FIIa via the mIIa pathway (28), although evidence of the prethrombin-2 pathway exists and is quantifiable by gel electrophoresis (29). We have tested various fragments of FII for their ability to protect FVa from APC inactivation. While biotin-PheProArg-inhibited mIIa still protects FVa from APC inactivation, Gla-domainless

FII, prethrombin-1, prethrombin-2, and dansyl-GluGlyArg-inhibited FIIa did not exhibit such protection. These data unequivocally demonstrate that the Gla domain of FII is necessary for the FII-mediated inhibition for APC activity.

Since the FII-mediated protection of FVa from APC is not due to competition for lipids, it is likely that protein-protein interaction between FVa and FII play an important role in influencing protection. The binding of FII to FVa has been reported to involve several domains of FII. FVa binding sites have been localized on or near the pro-exosite 1 of serine protease domain (30–32), kringle 2 (33,34), kringle 1 (35) and the Gla domain (36). Since the Gla domain of FII was necessary to mediate FVa protection, N-terminal fragments of FII were also tested for inhibiting FVa inactivation by APC. Fragment 1 did not inhibit APC-mediated FVa inactivation, whereas fragment 1•2 did. These data suggest that the Gla domain of FII by itself is not sufficient for inhibiting FVa inactivation by APC. Rather, it is likely that the gain in free energy obtained from the interactions of each kringle domain plus that from the Gla domain is necessary to achieve sufficient affinity of FII for FVa and to account for this phenomenon.

In summary, our data suggests that FII concentrations play a very significant role in protecting FVa from APC. The FVa protective effects of FII may be augmented in subjects carrying the G20210A polymorphism in the 3' untranslated region of FII gene which causes elevated FII levels and is associated with an increased risk for venous thrombosis (37,38). Furthermore, our experiments provide further evidence for the paradigm that FVa is completely protected from APC when the FII-ase complex containing FXa:FVa:FII is intact. mIIa has been shown to bind FVa and the FXa:FVa complex (39,40). Here we show that mIIa also protects FVa from APC cleavage, prior to the generation of FIIa. Hence, APC can inactivate FVa once FIIa generation is complete and the FII fragments and FXa dissociate from the FII-ase complex.

What is known about this topic?

- Factor (F)II levels could modulate the anticoagulant activity of activated protein C (APC).
- In purified system assays, FII inhibits the ability of APC to inactivate FVa.
- FII inhibits APC-mediated proteolysis at both Arg⁵⁰⁶ and Arg³⁰⁶ in FVa.

What does this paper add?

- FII does not inhibit the ability of APC to inactivate factor VIIIa, suggesting that the FII inhibition of FVa inactivation by APC is substrate-specific.
- In purified system assays, FII intermediate meizo-thrombin protected FVa from APC inactivation.
- FII activation intermediates prethrombin 1, prethrombin 2 and active site blocked thrombin do not inhibit FVa inactivation by APC.
- FII fragment 1•2 inhibits APC inactivation of FVa, whereas fragment 1 does not.
- Collectively, these data suggest that the Gla domain of FII linked to kringle 1 and 2 is necessary for the ability of FII to protect FVa from APC cleavage.
- Our experiments provide further evidence for the paradigm that FVa is completely protected from APC when the FII-ase complex containing FXa•FVa•FII is intact or when FII is activated to mIIa prior to the generation of FIIa. APC can inactivate FVa once FIIa generation is complete and the FII fragments and FXa dissociate from the FII-ase complex.

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Abbreviations

FVa, factor Va
 FXa, factor Xa
 FII, prothrombin, APC, activated protein C
 FII-ase, prothrombinase
 FVIII, full-length factor VIII
 FVIIIa, thrombin-activated factor VIII
 PC/PS, phosphatidylcholine/phosphatidylserine (4:1 molar ratio) containing vesicles
 PC/PS/PE, phosphatidylcholine/phosphatidylserine/phosphatidylethanolamine (2:1:2 molar ratio) containing vesicles
 A2-S-S-A3 wt FVa, recombinant His609Cys/Glu1691Cys B-domain deleted factor Va with a disulfide bond between the Cys⁶⁰⁹ in the A2 domain and Glu¹⁶⁹ in the A3 domain
 A2-S-S-A3 RR506/679QQ fVa, recombinant B-domain deleted factor Va with a disulfide bond between A2 and A3 domains and with Arg⁵⁰⁶ and Arg⁶⁷⁹ mutated to Gln
 A2-S-S-A3 RR306/679QQ FVa, recombinant B-domain deleted factor Va with a disulfide bond between A2 and A3 domains and with Arg³⁰⁶ and Arg⁶⁷⁹ mutated to Gln
 FIIa, thrombin
 C6PS, dicaproyl phosphatidylserine
 Gla, γ -carboxyglutamic acid
 EDTA, ethylene diamine tetra acetic acid
 BSA, bovine serum albumin

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Figure 1. Effect of FII on APC-mediated inactivation of FVa

The effect of varying concentrations of FII (0–7.5 μM) was tested in APC-mediated FVa inactivation assays as described in *Materials and methods*. FII concentrations (in step 1) were 0 (filled squares), 0.25 (open triangles), 0.5 (open inverted triangles), 1.5 (open squares), 2.4 (open circles) and 7.5 μM (star), respectively. Inset: Depicts the FII-dependence of inhibition of APC mediated FVa inactivation at eight minutes of the reaction.

Figure 2. Phospholipid surface dependence of fFII-mediated protection of F Va inactivation

A) FVa inactivation assays were performed at varying PC/PS concentrations to assess if the FII-mediated protection is due to competition for PC/PS vesicles. In the first step of the FVa inactivation assay, FVa (0.55 nM) was incubated with APC (0.5 nM) in the presence (open symbols) or absence (closed symbols) of FII (7.5 μM) at varying PC/PS concentrations. Subsequently, residual activity of FVa was measured in a FII-ase assay as described in *Materials and methods*. FVa inactivation profiles at three different PC/PS concentrations are shown; 25 μM (squares), 14 μM (triangles), 56 μM (circles). B) FVa inactivation assays were performed in the presence (open squares) or in the absence (closed squares) of FII as previously described using C6PS (2 mM) in the first step instead of PC/PS vesicles. Subsequently, residual FVa activity was estimated as described before.

Figure 3. Effect of FII on APC-mediated cleavages at Arg⁵⁰⁶ and Arg³⁰⁶ of FVa

A) APC-mediated (0.25 nM) inactivation of recombinant A2-S-S-A3 FVa (0.25 nM) is shown in the presence (open squares) and absence (closed squares) of FII (7.5 μ M) in the first step. B) APC-mediated (1.25 nM) inactivation of recombinant A2-S-S-A3. RR306/679QQ FVa (2.2 nM) is shown in the presence (open squares) and absence (closed squares) of FII (7.5 μ M). C) Inactivation of recombinant A2-S-S-A3 RR506/679QQ FVa (2.2 nM) by APC (6 nM) in the presence (open squares) and absence (closed squares) of FII (7.5 μ M) is shown.

Figure 4. Effect of fragments of FII on APC inactivation of FVa

A) Effect of the presence (open squares) or absence (filled squares) of biotin-PheProArg-mIIa (7.5 μ M) in the first step of FVa (0.55 nM) inactivation by APC (0.5 nM) is shown. B) Inactivation of FVa (0.55 nM) by APC (0.55 nM) in the presence of 7.5 μ M (open triangles), 20 μ M (open inverted triangles), 30 μ M (open diamonds), 40 μ M (open circles) and absence (filled squares) of FII fragment 1•2 in the first step is shown. C) Inactivation of FVa (0.91 nM) by APC (0.91 nM) in the presence (open squares) and absence (filled squares) of gd FII (15.7 μ M) in the first step is shown. D) Effect of the presence (open squares) or absence (filled squares) of prethrombin-1 (7.5 μ M) in the first step of FVa (0.55 nM) inactivation by APC (0.5 nM) is shown. E) Effect of the presence (open squares) or absence (filled squares) of

prethrombin-2 (7.5 μ M) in the first step of FVa (0.55 nM) inactivation by APC (0.5 nM) is shown. F) Effect of the presence (open squares) or absence (filled squares) of DEGR-FIIa (7.5 μ M) in the first step of FVa (0.55 nM) inactivation by APC (0.5 nM) is shown. G) APC-mediated (0.5 nM) inactivation of FVa (0.55 nM) is shown in the presence (open symbols) and absence (closed symbols) of FII fragment 1 in the first step. Inhibition of FVa inactivation was not observed at both concentrations of fragment 1 tested [7.5 μ M (open triangles) and 20 μ M (open squares)].

Figure 5. Effect of FII on DesGla APC mediated cleavage of FVa
Gd-APC-mediated (18 nM) inactivation of FVa (0.55 nM) is shown in the presence (open symbols) and absence (closed symbols) of FII in the first step.