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DOWN REGULATION OF PROTHROMBINASE BY ACTIVATED PROTEIN C DURING PROTHROMBIN ACTIVATION*

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SUMMARY

Activated protein C (aPC) proteolytically inactivates factor Va (FVa) and thereby down regulates prothrombinase. Although FVa inactivation by aPC has been studied extensively, the inactivation of prothrombinase during prothrombin activation has not. Therefore, prothrombin activation initiated both without and with aPC (5.0, 7.5 or 10.0nM) was monitored over time by fluorescence. The experiments were performed with 0.075nM FVa and 1.0nM FXa, and with these concentrations reversed. The time courses of the residual prothrombinase activity with aPC, determined from the slopes of fluorescence over time, were pseudo first order with both limiting and excess FVa. With FVa limiting or in excess, the second rate constants for inactivation of prothrombinase were $1.98 \pm 0.09 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ and $2.54 \pm 0.13 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$, respectively. The former value is 101-fold smaller than that for FVa inactivation by aPC alone. Since with limiting FVa the second order rate constants for prothrombinase inactivation and FVa inactivation are equal, FVa is protected 101-fold, presumably by both FXa and prothrombin. In contrast, with excess FVa, the calculated rate constant for FVa inactivation exceeds that for prothrombinase inactivation 17.3-fold, which reflects a loss of protection by FXa. Since the protective effects of the two proteins are theoretically multiplicative, FXa protected 17.3-fold and prothrombin protected 5.8-fold. With 150nM protein S and limiting FVa, prothrombinase inactivation was two-fold faster, yet it was still protected 91-fold. These studies show that FVa is down-regulated by aPC during prothrombin activation, but FXa and prothrombin together are highly protective, both with and without protein S.

INTRODUCTION

Factor Va (FVa) is the non-enzymatic cofactor of prothrombinase, which comprises, along with FVa, the enzyme factor Xa (FXa), a negatively charged phospholipid surface, and calcium ions (1–4). FVa consists of a heavy chain (residues 1–709) and a light chain (residues 1546–2196) that are held together by a calcium-ion dependent interaction (5). FVa increases the rate of prothrombin activation by 10,000-fold (6), and switches the activation pathway from prethrombin-2 to meizothrombin (7).

Activated protein C (aPC) is a serine protease that down-regulates coagulation by proteolytically inactivating cofactors FVa and factor VIIIa (FVIIIa). aPC cleaves FVa at Arg306, Arg506, and Arg679 of the heavy chain (5,8), and the reactions are enhanced by

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phospholipid (9–13). Protein S, a vitamin K-dependent protein, increases the rate of FVa inactivation by aPC in a phospholipid dependent manner (11,13). The cleavage of FVa at Arg506 is less dependent on protein S or the phospholipid composition than the cleavage at Arg306, and is kinetically favoured over cleavage at Arg306 (8). This cleavage results in a 40-fold loss in affinity for FXa, with partial retention of procoagulant activity (8,14). Cleavage at Arg306 is required for complete inactivation of FVa, and results in the dissociation of the A2 domain of the heavy chain (14). Protein S also increases the rate of cleavage at Arg306 (15), possibly by enhancing the binding of aPC to phospholipid (13). Cleavage at Arg679 is thought not to be relevant. aPC cleaves FVIIIa at Arg562 and Arg336 (16), but these cleavages likely are not physiologically irrelevant because inactivation of FVIIIa occurs without aPC due to spontaneous dissociation of its A2 domain (17,18).

FVa binds aPC through both the light (19) and heavy chains (20,21). FXa also binds FVa through both the heavy (22,23) and light chains (23), and FXa protects FVa from aPC mediated proteolysis (24,25). Prothrombin also binds FVa through its Gla-domain, both kringles, and the protease domain (26–29). The prothrombin binding sites on FVa, however, are still unclear, although some studies suggest that they involve the C-terminal portion of the FVa heavy chain (30–32). In addition, prothrombin protects FVa from aPC mediated proteolysis (15,28).

The total loss of FVa activity upon cleavage at Arg306 is consistent with the study by Kojima *et al.* showing that FVa residues 311–325, which overlap the A2 domain (residues 317–656), are involved in its interaction with FXa (22). The same group demonstrated that these residues also are involved in an interaction with aPC (20), which suggests that aPC down-regulates coagulation by not only by proteolytically removing a FXa binding site on FVa but also by competing with FXa binding.

Results of studies of the stimulation of the kinetics of aPC mediated FVa inactivation by protein S, and suppression of these kinetics by both FXa and prothrombin, imply that the inactivation of FVa is modulated in complex ways. Thus, characterization of the inactivation of prothrombinase by aPC, as opposed to inactivation of isolated FVa, requires measurements of the inactivation of prothrombinase activity during prothrombin activation. Toward this end Rosing and coworkers (33) measured the inactivation of FVa under these conditions. In that study, they showed that protein S specifically stimulates cleavage at Arg306 and that FXa specifically retards cleavage at Arg506. In addition, they showed that during prothrombin activation, FXa protects FVa from inactivation with a concentration dependence profile that is virtually identical to that for stimulation of prothrombin activation. They concluded that FVa is optimally protected when it is incorporated in the prothrombinase complex during prothrombin activation, and that protection by FXa likely involves that same interaction of FXa with FVa that promotes prothrombin activation. They also provided a solid mechanistic explanation, based on protein S and FXa effects, for the observation that the R506Q mutation of FVa is only mildly prothrombotic, even though it profoundly affects the kinetics of inactivation of isolated FVa by aPC. Although Rosing and coworkers (33) determined the kinetics of FVa inactivation by aPC during prothrombin activation by sub-sampling at intervals and measuring residual FVa, they did not directly determine the kinetics of down regulation of prothrombinase activity by aPC. Since these latter kinetics ultimately determine the effects of aPC on prothrombinase activity, the following studies were undertaken to determine the kinetics of inactivation of prothrombinase *in situ* when FVa is engaged in prothrombin activation.

MATERIALS AND METHODS

Materials

Phosphatidyl-L-serine and phosphatidyl-L-choline were obtained from Sigma. Human activated protein C and protein S were from Haematologic Technologies Inc. (Essex Junction, VT). The fluorescent thrombin inhibitor dansylarginine-N-(3-ethyl-1,5-pentanediyl)amide (DAPA) was prepared as described previously (34). Phospholipid vesicles (75% PC and 25% PS (PCPS)) were prepared as described previously (35). Human prothrombin (36), thrombin (37), FXa (36), and FVa (38) were prepared as described.

aPC Mediated Inactivation of Prothrombinase Activity

Reactions were carried out in white 96-well microtitre plates that were pretreated with 0.02M HEPES, 0.15M NaCl, pH 7.4 (HBS), 1% Tween80 and rinsed thoroughly with distilled water and dried. Prothrombin (1.4 μ M) was incubated with 5mM CaCl₂, 20 μ M PCPS, 5 μ M DAPA and 75pM FVa in HBS with 0.01% Tween80, and the fluorescence signal was allowed to stabilize. The reactions were initiated by adding 1nM FXa with aPC at varying concentrations (0, 5nM, 7.5nM and 10nM). The fluorescence of the DAPA-thrombin complex was monitored at 22°C over time by a SpectraMax Gemini XS (Molecular Devices), using excitation at 280nm, emission at 545nm, and a 530nm emission cutoff filter. Similar experiments were performed with the concentrations of FVa and FXa reversed.

Further experiments were carried out with protein S. Prothrombin (1.4 μ M) was incubated with 5mM CaCl₂, 20 μ M PCPS, 5 μ M DAPA, 75pM FVa with protein S at varying levels (0, 15, 30, 50, 75, 100, 150 and 300nM) in HBS with 0.01% Tween80. The reactions were initiated by adding 1nM FXa with 3.4nM aPC. The concentrations of FVa and FXa again were reversed, and the experiments were carried out with 0, 150, and 300nM protein S.

Analysis of the Fluorescence Time Course Data to determine the kinetics of inactivation of prothrombinase

Smoothed lines were fit to the fluorescence time course profiles using TableCurve (SPSS Inc.). Equations provided by the software were chosen empirically to provide the best fit. The smoothed fluorescence units in the presence of aPC and/or protein S then were normalized to the total fluorescence change measured in the control without aPC and protein S. The best fit values were calculated for 1.2 second intervals and these values were exported to Excel 2003. At selected identical extents of reaction (5% increments, starting at about 10–15% of the reaction), 3 point slopes were calculated by the software for the control and the samples with aPC. The time to reach a given extent of reaction (prothrombin activation) was noted and residual prothrombinase activity at that time was determined from the ratio of the slope of DAPA fluorescence with aPC to the control slope without aPC, at the same reaction extent. The prothrombinase level *versus* time data were fit to the single exponential decay function to determine the pseudo first order rate constant for inactivation of prothrombinase activity.

The rationale for this approach to determine prothrombinase levels is as follows. In the absence of aPC, the total concentration of prothrombinase was assumed to be stable and constant over the course of the experiment. In addition, at any time during the reaction, the rate was assumed to conform to the Michaelis-Menten equation ($\text{rate} = [\text{Total Prothrombinase}] \times [\text{Prothrombin}] / (K_m + [\text{Prothrombin}])$). Thus, at any given extent of the reaction (*i.e.* any given prothrombin concentration), the term $[\text{Prothrombin}] / (K_m + [\text{Prothrombin}])$ will be the same for the control and the experiment with aPC (and protein S).

Thus, the ratio of the slope of the reaction with aPC (and protein S) to the slope without aPC is equal to the residual prothrombinase concentration, relative to its initial concentration.

The relationships between rates of inactivation of prothrombinase and rates of inactivation of FVa

The rate of inactivation of FVa is given by Equation 1, where $k_{2(Va)}$ is a second order rate constant equal to the k_{cat}/K_m ratio.

$$d[FVa]/dt = -(k_{cat}/K_m)[aPC][FVa] = -k_{2(Va)}[aPC][FVa] \quad (\text{Eq. 1})$$

This equation predicts first order inactivation kinetics of FVa with a pseudo first order rate constant equal to $k_{2(Va)} \cdot [aPC]$. First order inactivation kinetics were assumed for prothrombinase inactivation as well, regardless of whether FVa or FXa were limiting. This assumption was validated experimentally (see below). Thus, the rate of inactivation of prothrombinase is given by Equation 2, where $k_{2(pase)}$ is the second order rate constant for the inactivation of prothrombinase.

$$d[FXa \bullet FVa]/dt = -(k_{cat}/K_m)[aPC][FXa \bullet FVa] = -k_{2(pase)}[aPC][FXa \bullet FVa] \quad (\text{Eq. 2})$$

The relationship between the rate of inactivation of FVa and the rate of inactivation of prothrombinase is found as follows. The concentration of prothrombinase, $[FXa \bullet FVa]$, is determined by the binding equilibrium between FXa and FVa, as in Equation 3.

$$[FXa \bullet FVa] = [FXa][FVa]/K_d \quad (\text{Eq. 3})$$

When FVa is limiting, the concentration of prothrombinase is equal to the concentration of bound FVa, as in Equation 4, where $[FVa]_0$ is the total concentration of FVa.

$$[FXa \bullet FVa] = [FVa]_0[FXa]/(K_d + [FXa]) \quad (\text{Eq. 4})$$

Differentiation of both sides of Equation 4 with respect to time yields the relationship between the rate of inactivation of prothrombinase and the rate of inactivation of total FVa. The result is shown in Equation 5.

$$d[FXa \bullet FVa]/dt = (d[FVa]_0/dt) \times ([FXa]/(K_d + [FXa])) \quad (\text{Eq. 5})$$

Under these conditions $[FXa \bullet FVa]$ and $[FVa]_0$ are directly proportional and the rate of inactivation of total FVa exceeds that of $FXa \bullet FVa$ by a factor of $(K_d + [FXa])/[FXa]$. This factor approaches unity when $[FXa] \gg K_d$ and therefore the concentration of prothrombinase approaches the total concentration of FVa. In the present experiments, with $[FXa] = 1.0 \text{ nM}$ and $K_d = 0.08 \text{ nM}$ (33), this factor evaluates to 1.08 and the rate of inactivation of total FVa is 1.08 times the rate of inactivation of prothrombinase.

On the other hand, when FVa is in excess, and FXa is limiting, the concentration of prothrombinase is equal to the concentration of bound FXa, as in Equation 6, where $[FXa]_0$ is the total concentration of FXa.

$$[FVa \bullet FXa] = [FXa]_0 \cdot [FVa] / (K_d + [FVa]) \quad (\text{Eq. 6})$$

As expected, when $[FVa]$ is in excess and is much greater than K_d , the concentration of prothrombinase approaches the total concentration of FXa. In addition, the relationship between the concentrations of prothrombinase and FVa is not linear. The time rate of change of prothrombinase, expressed in terms of the time rate of change of FVa, is found by differentiating Equation 6 on both sides with respect to time. The result is given by Equation 7.

$$d[FVa \bullet FXa]/dt = (d[FVa]/dt) \times ([FXa]_0 \cdot K_d / (K_d + [FVa])^2) \quad (\text{Eq. 7})$$

Thus, when FVa is in excess, the rates of inactivation of prothrombinase and FVa are not linearly related and can be quite different. The former differs from the latter by a factor of $[FXa]_0 \cdot K_d / (K_d + [FVa])^2$, which in this case evaluates to 0.00566 at the initial concentration of FVa.

The relationships between the second order rate *constants* for inactivation of FVa and prothrombinase in both cases are found as follows. When FVa is limiting the term $[FXa]/K_d$ of Equation 3 can be considered constant. Consequently, $d[FXa \bullet FVa] = (d[FVa]/dt)[FXa]/K_d$. Division of the left and right hand sides of this equation by the left and right hand sides of Equation 3 yields Equation 8. Since this equation shows that the *fractional* change ratios of prothrombinase and FVa are equal, their pseudo first order and second rate constants for inactivation are equal.

$$d[FXa \bullet FVa]/[FXa \bullet FVa] = d[FVa]/[FVa] \quad (\text{Eq. 8})$$

The situation is different when FVa is in excess. Dividing the left and right hand sides of Equation 7 by the left and right hand sides of Equation 6 yields Equation 9.

$$(d[FXa \bullet FVa]/[FXa \bullet FVa]) = (d[FVa]/[FVa]) \times K_d / (K_d + [FVa]) \quad (\text{Eq. 9})$$

The terms in parentheses on the left and right sides of Equation 9 are equal to $-k_{2(\text{pase})} [aPC]/dt$ and $-k_{2(FVa)} [aPC]/dt$, respectively, where $k_{2(\text{pase})}$ is the second order rate constant for prothrombinase inactivation and $k_{2(FVa)}$ is the second order rate constant for FVa inactivation. Thus, with excess FVa, the second order rate constants are not equal, but instead are related according to Equation 10.

$$k_{2(FVa)} = k_{2(\text{pase})} \times (K_d + [FVa]) / K_d \quad (\text{Eq. 10})$$

With excess FVa (1.0nM) and $K_d = 0.08\text{nM}$ (33), the second order rate constant for FVa inactivation exceeds that for prothrombinase inactivation by a factor of 13.5.

Analysis of Prothrombin Activation with and without aPC by SDS-PAGE and Densitometry

Prothrombin (1.4 μ M) was incubated with 20 μ M PCPS, 5mM CaCl₂, 5 μ M DAPA, and FVa (75pM when limiting or 1nM when in excess) in HBS with 0.01% Tween80 at 25°C, in the presence or absence of 10nM aPC. The reactions were initiated by the addition of FXa (1nM when FVa is limiting or 75pM when FVa is in excess). Samples were removed at various times (0, 1, 2, 3, 4, 5, 7.5, 10 and 15 min) and analyzed by SDS-PAGE and densitometry as described previously (39,40).

RESULTS

Time courses of prothrombin, intermediates and products during prothrombin activation in the absence and presence of aPC

Because the analyses described below are based on measuring DAPA fluorescence, the validity of using time rates of change of fluorescence to calculate prothrombinase activity requires that aPC does not alter the pathway from prothrombin to thrombin. If, for example, aPC were to direct conversion to prothrombin-2 but restrict subsequent conversion to thrombin, rapid consumption of prothrombin would not be detected because DAPA detects only thrombin and meizothrombin. Thus, the time courses of prothrombin and the intermediate and end products of prothrombin activation were assessed by SDS-PAGE and densitometry during prothrombin activation (Figure 1). These data indicate that very little prothrombin-2 accumulates in either the absence or presence of aPC and that both the consumption of prothrombin and the appearance of the B-chain, which is proportional to the sum of meizothrombin and thrombin, are delayed by aPC. Thus, the DAPA fluorescence can be used reliably to infer prothrombinase activity.

Down-Regulation of Prothrombinase Activity by aPC During Prothrombin Activation

The progress of prothrombin activation was measured over time by the fluorescence of the DAPA-thrombin complex, in the absence or presence of aPC at varying concentrations. The data with limiting FVa are shown in Figure 2, panel A, while those with limiting FXa are shown in Figure 2, panel B. In the absence of aPC, the thrombin concentration increased over time and reached a plateau when prothrombin was exhausted. Similar time courses were observed with aPC, but reactions terminated prior to prothrombin exhaustion. The magnitude of this effect increased with increasing levels of aPC. Similar results were obtained when FVa was in excess, but the total reaction extents were smaller than with limiting FVa.

The fluorescence data were smoothed and normalized to the total change observed by the control in the absence of aPC. An example of the smoothed data is provided in Supplemental Materials (Supp. Fig. S1). From these data the time courses of the residual prothrombinase with aPC were determined. Figure 3 panel A shows the decay of prothrombinase activity when FVa was limiting at three concentrations of aPC, and Figure 3 panel B shows the decay of prothrombinase activity when FVa was in excess.

These prothrombinase time course data were fit individually to the equation for exponential decay, and the pseudo first order rate constants for inactivation were determined (Table 1). The pseudo first order rate constants for inactivation of prothrombinase with limiting FVa were similar to those with excess FVa, and in both cases they increased linearly with the aPC concentration. The second order rate constants for prothrombinase inactivation were calculated by dividing the pseudo first order rate constants by the aPC concentrations. The values for the three concentrations of aPC with limiting FVa were very similar and the average value was $(1.98 \pm 0.15) \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$. With excess FVa the values for the three

concentrations of aPC again were very similar to one another and the average value was $(2.54 \pm 0.23) \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$.

In addition, second order rate constants for FVa inactivation were calculated. When FVa was limiting the second order rate constants for inactivation of prothrombinase and FVa are expected to be equal (see Experimental Procedures). The situation is more complex when FVa is in excess (see Experimental Procedures). In this case, the second order rate constant for FVa inactivation is equal to the second order rate constant for prothrombinase inactivation multiplied by $(K_d + [\text{Va}])/K_d$. The average second order rate constant for FVa inactivation was calculated to be $(3.36 \pm 0.30) \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$. This value is 17.3-fold greater than the value obtained with limiting FVa. This increase undoubtedly reflects loss of protection by FXa when FVa is in excess, because most of the FVa population cannot be bound to FXa under these conditions. This magnitude of the apparent FXa protective effect is very similar to that measured directly by others (25,33).

Prothrombinase is inactivated by aPC during prothrombin activation much more slowly than Factor Va is inactivated in the absence of Factor Xa and prothrombin

When FVa is the limiting component of prothrombinase, the extent to which FVa is protected from inactivation by aPC during prothrombin activation can be found by comparing the second order rate constant for prothrombinase inactivation to that for inactivation of FVa by aPC in the absence of both prothrombin and FXa. Solymoss *et al.* determined the kinetics of FVa inactivation by aPC under these conditions (41). The k_{cat} and K_m values were 0.395 s^{-1} and 19.7 nM , respectively. Thus, the second order rate constant (k_{cat}/K_m) under these conditions is $2.0 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$. Although the data are not shown here, the k_{cat} and K_m values reported by Solymoss *et al.* were confirmed by us in similar experiments. This second order rate constant is 101-fold greater than the value obtained here for limiting FVa in prothrombinase (Table 1). Thus, under otherwise identical circumstances, FVa is inactivated 101 times faster in the absence of both FXa and prothrombin than it is during prothrombin activation.

Tran *et al.* reported time courses of FVa inactivation by aPC plus protein S in both the presence or absence of prothrombin, but without FXa (15). In the absence of prothrombin, the time course of FVa inactivation with 0.1 nM aPC (Fig. 1, reference(15)), was very similar to the time course of prothrombinase decay obtained with 10 nM aPC here (Fig. 3, panel A). This comparison implies a very extensive protection of FVa from aPC during prothrombin activation, because similar rates of FVa inactivation were obtained with aPC concentrations that differed by 100-fold. The rate constants for FVa inactivation can be calculated from the data of Tran *et al.* (Fig. 1, reference(15)) by estimating the initial rates of FVa inactivation. The values estimated in this way are $9.7 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ and $1.2 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ in the absence or presence of prothrombin, respectively. In this instance, the rate constant obtained in the absence of prothrombin is 422-fold greater from that found here for prothrombinase (and FVa) inactivation during prothrombin activation with limiting FVa. This ratio, however, likely over estimates the degree of protection because of the protein S in the experiments of Tran *et al.* Their data show, though, that FVa is protected by prothrombin about 7.6-fold in the absence of FXa.

The Effect of Protein S on Prothrombinase Inactivation by aPC During Prothrombin Activation

Protein S was titrated in the presence or absence of aPC at 3.4 nM , a concentration chosen because studies *in vivo* in baboons showed a peak aPC blood level ranging from 2.5 to 3.5 nM upon thrombin infusion (42). The second order rate constant of prothrombinase inactivation with FVa limiting increased linearly from $1.98 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ to 7.04×10^5

$\text{M}^{-1}\text{s}^{-1}$, as the level of protein S increased from 0 to 300nM (Supp. Fig. S2). With limiting FXa, at protein S concentrations of 0, 150, and 300nM, the second order rate constants increased linearly from $3.25 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ to $8.21 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$. In the absence of aPC, protein S at concentrations ranging from 0 to 300nM had negligible effects on the time courses of thrombin generation (Supp. Fig. S3), which indicates that under the present conditions the protein S effect is aPC-dependent.

Whether, and to what extent protein S might modify the protection of FVa from aPC during prothrombin activation can be answered by the following considerations. Protein S at 150nM increases the rate constant for free FVa inactivation by 2-fold (17,43). Based on this, and the second order rate constants of Solymoss *et al.*, the expected rate constant for free FVa inactivation with protein S is $4.0 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$. The value found here with 150 nM protein S for prothrombinase inactivation during prothrombin activation was $(4.40 \pm 0.02) \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ at limiting FVa. This value is 91-fold smaller than that expected for free FVa. Thus, when protein S is present and FVa is limiting, it is still highly protected even though protein S stimulates inactivation during prothrombin activation.

DISCUSSION

This work shows that aPC down regulates prothrombinase activity during the process of prothrombin activation. Under these conditions, however, when FVa is limiting with respect to the concentrations of both FXa and prothrombin, it is protected to an extent not previously appreciated. Compared to the rate of inactivation of free FVa, the rate of inactivation of prothrombinase is down-regulated at least 100-fold. The phenomenon of protection during prothrombin activation has been reported by Rosing and coworkers (33). The magnitude of the effect that can be attributed to FXa, as inferred from the decrease in rate constant for FVa inactivation, was about 20-fold, which is very similar to the 17-fold effect found here. The extent to which prothrombin may have protected was not determined in those studies because the reference experiment without prothrombin in that experimental series was not performed. The absolute values of the second order rate constants found by Rosing *et al.* (33) and those found here cannot be compared because the former experiments were carried out at 37°C whereas the current ones were carried out at 22°C.

The present studies and those of others (9,15,20–22,24,25,33,41,44) show that both FXa (X) and prothrombin (P) protect FVa (V) from inactivation by aPC (A). The current studies suggest that the effects of the two proteins are multiplicative. This is expected if both prothrombin and FXa bind to FVa and each competes with the binding of aPC. In that case complexes comprising V·A, V·P, V·X, and V·X·P would exist along with free FVa (Supp. Fig. S4). If the dissociation constants for the binding of the proteins to FVa are K_A , K_P , and K_X , respectively, and no binding linkage between prothrombin and FXa exists so that the binding of one does not affect the affinity of the binding of the other, and the concentration of aPC is much smaller than K_A , then the relationship between free and total FVa (V_0) is given by Equation 11.

$$[V]_0 \cong [V] \cdot \left(1 + \frac{[P]}{K_P}\right) \cdot \left(1 + \frac{[X]}{K_X}\right) \quad (\text{Eq. 11})$$

In the absence of P and X the terms in parentheses are in unity and no protection exists. If the rate of inactivation of FVa by aPC is proportional to the level of free FVa, dividing the equation having $[P] =$ and $[X] = 0$ by the above equation gives the ratio of rates of inactivation in the absence and presence of prothrombin, FXa, or both. This ratio can be

defined as the “protection factor” (pf), which is a unit-less number equal to the product of the terms in parentheses in Equation 11.

$$pf = (1 + [P]/K_p) \times (1 + [FXa]/K_x) \quad (\text{Eq. 12})$$

The individual terms in parentheses in Equation 12 quantify the protection afforded by prothrombin and FXa, respectively. Clearly, the effects of the two are proteins are multiplicative in this case. For example, if the terms with P and X each evaluate at 10, each alone would protect 10-fold. Together, however, they would protect 100-fold.

The plausibility of this relationship can be assessed by taking into account the protection factors inferred from the experimental data and using them to calculate the apparent dissociation constants for the binding of FXa and prothrombin to FVa. In our experiments, the overall protection factor was 101 and that inferred for FXa was 17.3. From these two values, a value of 5.8 can be attributed to prothrombin, which compares well to the value of 7.6 reported by Tran *et al.* (15). From these and the concentrations of prothrombin and FXa (with limiting FVa) used here, dissociation constants K_x and K_p for the binding of FXa and prothrombin to FVa are calculated to be $K_x = 0.061\text{nM}$ and $K_p = 0.29\mu\text{M}$. The former value agrees very well with the value of 0.08nM reported by Rosing *et al.* (33) for this interaction. The latter value cannot be supported by independent data, but it is reasonable if it is related to the K_m for prothrombin activation, which in turn might be related to an exosite dependent interaction of prothrombin with prothrombinase.

Other investigators have used SDS-PAGE with or without immunoblotting, along with assays of FVa over time, to correlate cleavage of FVa with inactivation (9,11,12,14,15,25,28,33). This approach was used to work out the sequences and consequences of the cleavages. This approach could be used here under conditions with FVa in excess, but not with limiting FVa, because in the latter case the level would be below the limit of detection. With excess FVa the expected result is that FVa cleavage would be more rapid than prothrombinase inactivation because FVa is in excess and, although protected by prothrombin, would not be protected by FXa. In addition, the rate constant for cleavage would be similar to that inferred from kinetics (Table 1); that is, about 14-fold greater than the rate constant for of inactivation of prothrombinase. In the case of limiting FVa, the expected result would be that not only would the rate of inactivation of prothrombinase be highly reduced compared to that of free FVa, but also would the rate of cleavage. Alternatively, cleavage might occur rapidly, but for as yet unknown reasons, prothrombinase might be stabilized in spite of cleavage. Whether one or the other of these circumstances actually occurs will require more sensitive methods for detecting cleavage.

Others have observed that the protein S concentration dependence on the rates of either individual cleavages or overall FVa inactivation approaches saturation around 100–150nM (12,15,25). Our data indicate during prothrombin activation, the second order rate constants for prothrombinase inactivation increased linearly with the concentration of protein S between 0 and 300nM. This suggests that the inclusion of both FXa and prothrombin creates competition for binding of protein S to FVa (22,45), thereby increasing the apparent K_d for protein S binding and a failure to achieve saturation. The protein S mediated relative stimulation of prothrombinase (and FVa) inactivation, however, was comparable to that reported previously, showing about a two-fold increase in FVa inactivation in the presence of 150nM protein S (12,17,43).

In summary, our results show that FVa can be down regulated by aPC during prothrombin activation. It is highly protected, however, and the effect is multiplicative with respect to FXa and prothrombin. Protein S stimulates inactivation but does not eliminate protection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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The abbreviations used are

FVa	factor Va
FXa	factor Xa
aPC	activated protein C
DAPA	dansylarginine-N-(3-ethyl-1,5-pentanediy)amide
PCPS	phospholipid vesicles (75% phosphatidyl-L-choline (PC) and 25% Phosphatidyl-L-serine (PS))
HBS	HEPES-buffered saline

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1. What is known on this topic

- Activated protein C (aPC) inactivates factor Va (FVa) with rate constant $2.0 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$
- Prothrombin protects FVa from aPC mediated inactivation by ~10-fold
- Factor Xa (FXa) protects FVa from aPC mediated inactivation by 3 to 20-fold
- When FVa is complexed in prothrombinase, less FXa is required to achieve comparable level of protection than when either prothrombin or FXa is present alone

2. What this paper adds

- When FVa is complexed in prothrombinase, its protection from aPC-mediated inactivation increases dramatically
- FVa protection increases 101-fold when FVa is the limiting component of prothrombinase
- At physiologic concentrations of protein S, the inactivation rate constants increase by ~2-fold, however, the protection is not ameliorated
- FVa protection in the presence of both prothrombin and FXa is multiplicative

Figure 1. The time courses of prothrombin, fragment 1.2:A-chain, fragment 1.2, prethrombin-2 and the B-chain during prothrombin activation by prothrombinase in the A) absence or B) presence of aPC

Prothrombin (1.4μM) was incubated with CaCl₂ (5mM), PCPS (20μM), DAPA (5μM) and FVa (1nM) in HBS with 0.01% Tween80, and its activation was initiated by the addition of FXa (75pM). Samples were withdrawn at various times and subjected to 4–15% SDS-PAGE. The gels were fixed in 50% methanol, 20% ethanol, and 6% trichloroacetic acid for at least 2 hours and were stained with Coomassie Blue and destained. The time courses were determined by quantitative densitometry. *PT*, prothrombin; *F1.2-A*, fragment 1.2:A-chain; *F1.2*, fragment 1.2; *Pre2*, prethrombin-2; *B*, B-chain.

Figure 2. DAPA fluorescence time course profiles observed upon prothrombin activation in the presence or absence of aPC when A) FVa or B) FXa is limiting

Prothrombin ($1.4\mu\text{M}$) was incubated with CaCl_2 (5mM), PCPS ($20\mu\text{M}$), DAPA ($5\mu\text{M}$) and FVa (75pM when FVa is limiting, or 1nM when FXa is limiting) in HBS with 0.01% Tween80, and the fluorescence signal was allowed to stabilize. The reactions were initiated by adding FXa (75pM when FXa is limiting or 1nM when FVa is limiting) with aPC at varying concentrations (0 , 5nM , 7.5nM and 10nM). Fluorescence of the DAPA-thrombin complex was monitored over time, using excitation at 280nm and emission at 545nm , with the cutoff filter at 530nm .

Figure 3. Time courses of prothrombinase activity during prothrombin activation in the presence of aPC at varying levels

Relative residual prothrombinase levels were calculated as described in Materials and Methods are plotted against the time required to reach a given extent of the reaction when A) FVa was limiting or B) FVa was in excess. The data were individually fit to the single exponential decay equation, and the respective pseudo first order rate constants for inactivation were determined. The lines are regression lines obtained from the fit.

Table 1

Kinetics of inactivation of prothrombinase and factor Va by aPC during prothrombin activation.

APC (nM)	0.075nM FVa			1.0nM FVa		
	$a k_1$ (s^{-1})	$b k_{2(\text{pase})}$ ($M^{-1}s^{-1}$)	$c k_{2(\text{FVa})}$ ($M^{-1}s^{-1}$)	$a k_1$ (s^{-1})	$b k_{2(\text{pase})}$ ($M^{-1}s^{-1}$)	$c k_{2(\text{FVa})}$ ($M^{-1}s^{-1}$)
5.0	0.00107	2.14×10^5	2.14×10^5	0.00140	2.80×10^5	3.78×10^6
7.5	0.00147	1.96×10^5	1.96×10^5	0.00179	2.39×10^5	3.23×10^6
10.0	0.00183	1.83×10^5	1.83×10^5	0.00243	2.43×10^5	3.28×10^6
Average		$(1.98 \pm 0.15) \times 10^5$	$(1.98 \pm 0.15) \times 10^5$		$(2.54 \pm 0.23) \times 10^5$	$(3.43 \pm 0.3) \times 10^6$

^aThe pseudo first order rate constants for prothrombinase decay were found by fitting the data of Figure 3 to the exponential decay function.

^bThe second order rate constants for prothrombinase inactivation were calculated by dividing the pseudo first order rate constants for prothrombinase inactivation by the concentration of aPC.

^cThe second order rate constants for FVa inactivation, with limiting FVa (0.075nM), are equal to those for prothrombinase inactivation. With excess FVa (1.0nM), the values are equal to the second order rate constant for prothrombinase inactivation, multiplied by 13.5; *i.e.*, by $(K_d + [FVa])/K_d$ (see Experimental Procedures). The value of K_d was set at 0.08nM (33).