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Binding sites for blood coagulation Factor Xa and protein S involving residues 493–506 in Factor Va

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Abstract

Inactivation due to cleavage of Factor Va (FVa) at Arg 506 by activated protein C (APC) helps to downregulate blood coagulation. To identify potential functional roles of amino acids near Arg 506, synthetic overlapping pentadecapeptides comprising FVa heavy chain residues 481–525 were tested for their ability to inhibit prothrombin activation by prothrombinase complexes [Factor Xa (FXa):FVa:phospholipids:Ca^{2+}]. The most potent inhibition was observed for peptide VP493 (residues 493–506), with 50% inhibition at 2.5 μM. VP493 also inhibited FXa in plasma in FXa-1-stage clotting assays by 50% at 3 μM. When the C-terminal carboxamide group of VP493 was replaced by a carboxyl group, most prothrombinase inhibitory activity was lost. VP493 preincubated with FXa inhibited prothrombinase with a pattern of mixed inhibition. Homologous peptides from Factor VIII sequences did not inhibit prothrombinase. Affinity-purified antibodies to VP493 inhibited prothrombinase activity and prolonged FXa-1-stage clotting times. VP493 also blocked the ability of protein S to inhibit prothrombinase independently of APC. Immobilized VP493 bound specifically with similar affinity to both FXa and protein S ($K_d \sim 40$ nM), but did not measurably bind prothrombin or APC. These studies suggest that FVa residues 493–506 contribute to binding sites for both FXa and protein S, providing a rationale for the ability of protein S to negate the protective effect of FXa toward APC cleavage of FVa. Possible loss of this FVa binding site for FXa due to cleavage at Arg 506 by APC may help explain why this cleavage causes 40% decrease in FVa activity and facilitates inactivation of FVa.

Keywords: anticoagulant; blood coagulation; Factor Va; Factor Xa; protein–protein interaction; protein S

Blood coagulation Factor Va (FVa) is a cofactor for Factor Xa (FXa) in the prothrombinase complex, which also consists of negatively charged phospholipids and calcium ions (Mann et al., 1981; Tracy & Mann, 1983; Krishnaswamy et al., 1987). FVa increases by ~500-fold the k_{cat} for conversion of prothrombin to thrombin by FXa (Rosing et al., 1980). The precise location of binding sites for FXa on FVa are unclear, but may consist of regions in both the heavy and light chains of FVa (Tracy & Mann, 1983; Tucker et al., 1983; Kalafatis et al., 1994b). Proteolytic cleavages of the heavy chain of human FVa by activated protein C (APC) at arginines 506, 306, and 679 lead to inactivation of FVa. Cleavage at Arg 506 in FVa takes place first, possibly facilitating cleavage at Arg 306, and leading to loss of over 80% of FVa activity (Suzuki et al., 1983; Odegaard & Mann, 1987; Kalafatis et al., 1994a).

The importance of the Arg 506 site is underscored by the fact that a mutation present in about 5% of the Caucasian population that leads to Arg 506 Gln FV (Bertina et al., 1994; Greengard et al., 1994; Voorberg et al., 1994) is associated with APC resistance that causes an increased risk of venous thrombosis (Dahlbäck et al., 1993; Griffin et al., 1993; Koster et al., 1993; Svensson & Dahlbäck, 1994; Dahlbäck, 1995). The mutant Arg 506 Gln FVa is inactivated about ten times more slowly than normal FVa (Bertina et al., 1994; Sun et al., 1994; Heeb et al., 1995; Kalafatis et al., 1995; Nicolaes et al., 1995). The homologous region in FVIIIa (residues 558–565) that contains the APC cleavage site Arg 562 also contains a Factor IXa binding site (Fay et al., 1994; O'Brien et al., 1995). This investigation examines whether loss of FVa activity due to cleavage at Arg 506 might be associated, at least in part, with the loss of a FXa binding site. We also questioned whether this putative FXa binding site might overlap a binding site for protein S, because protein S is known to ablate the ability of FXa to protect FVa from proteolytic inactivation by APC (Solymoss et al., 1988; Jane et al., 1991).

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Results

Screening of peptides representing FVa sequences near Arg 506

In order to elucidate the role of the FVa heavy-chain region near the Arg 506 cleavage site, a series of overlapping 15-mer synthetic

Table 1. *Inhibition of prothrombinase activity by peptides related to FV*

Residues	Factor V peptide sequence	% Inhibition[a]
481–495	VDIMRDIASGLIGLL	0
491–505	LIGLLLICKSRSLDR	99
501–515	RSLDR**R**GIQRAADIE	83
507–520	GIQRAADIEQQAVFC	50
511–525	AADIEQQAVFAVFDE	22

[a] Prothrombinase assays were performed as described in Materials and methods, in the presence and absence of 200 μM of the respective peptides. The rate of thrombin formation was determined in each case, with % inhibition calculated relative to the rate of thrombin generation observed in the absence of peptide.

peptides representing FVa sequences from residues 481 to 525 was tested for their ability to inhibit prothrombinase activity. All peptides were in the C-terminal carboxamide form unless otherwise stated. The most inhibitory peptide in this series represented residues 491–505, near the Arg 506 cleavage site for APC. Inhibitory activity of peptides gradually decreased as residues in the C-terminal direction became more distant from this region (Table 1).

In testing variations of peptides in the region of the Arg 506 cleavage site, the most potent peptide for prothrombinase inhibition was designated VP493, analogous to FVa residues 493–506, terminating at Arg 506. This peptide inhibited prothrombinase by 50% at 2.5 μM when preincubated with FVa, FXa, and phospholipids prior to addition of prothrombin (Table 2). The potency of peptide VP493 was retained after HPLC purification and a second synthesis of VP493 yielded peptide of nearly identical potency. A peptide overlapping the Arg 506 site (VP501) was much less potent in inhibition of prothrombinase, whereas analogous peptides from the corresponding APC cleavage site at Arg 562 in FVIIIa and from the APC cleavage site at Arg 306 in FVa did not inhibit prothrombinase at doses up to 400 μM (Table 2). Note that the control peptide VIIIP554 contains the sequence 558–565 from FVIIIa that was recently shown to inhibit Factor Xase activity and to contain a binding site for FIXa on FVIIIa (Fay et al., 1994; O'Brien et al., 1995).

Because VP493 contains three Arg residues, two occurring sequentially, two control peptides containing the same net charge and the same Arg content were tested: VRGFHAIGRRSAY and VCIRGYHLVGRRTFL. These peptides did not inhibit prothrombinase activity at concentrations of 200 μM. The data in Table 2 also show this type of specificity from the standpoint that peptide VP501, which contains the same three Arg residues as VP493, is 44-fold less inhibitory in prothrombinase assays.

Anticoagulant activity of peptide VP493 in prothrombinase and clotting assays

Figure 1 shows the dose dependency of inhibition of prothrombinase by peptide VP493 and two control peptides. VP493 was a poor inhibitor of prothrombinase when prepared with a C-terminal carboxyl group, as exists following cleavage at Arg 506 by APC, rather than with a C-terminal carboxamide group. Peptide VP493 in either the amide form or the acid C-terminal form had the correct mass for the monomeric form and contained an internal disulfide bond between the naturally occurring cysteine at residue 498 and the non-natural cysteine at the N terminus. The conformational constraints imposed by the disulfide bond contributed to part of the potency of VP493 amide, because 2.5–10-fold higher concentrations of the reduced and alkylated peptide were required for 50% inhibition of prothrombinase (data not shown). The homologous FVIII peptide did not inhibit prothrombinase.

Figure 2 shows Lineweaver–Burk plots for prothrombinase activity in the presence of various concentrations of VP493 at varying prothrombin concentrations. In this case, the peptide was preincubated with FXa prior to addition of other prothrombinase

Table 2. *Inhibition of prothrombinase by selected peptides*

Peptide[a]	Residues	Sequence[b] APC site ↓	Conc. for 50% inhibition (μM)
VP493	493–506	CGLLLICKSRSLDR**R**	2.5
VP501	501–515	RSLDR**R**GIQRAADIE	110
VIIIP549	549–562	CGPLLICYKESVDQ**R**	>400
VIIIP554	554–567	CYKESVDQ**R**GNQIMS	>400
VP292	292–306	MQAYIDIKNCPKKT**R**	>400
VP301	301–315	CPKKT**R**NLKKITREQ	>400

[a] VP designates a peptide related to FV sequences, whereas VIIIP designates a peptide related to FVIII sequences. Each peptide was preincubated 15 min with FXa, FVa, and phospholipid prior to addition of prothrombin and measurement of the rate of thrombin generation relative to a control without peptide.

[b] C designates a Cys residue not in the sequence of the native protein.

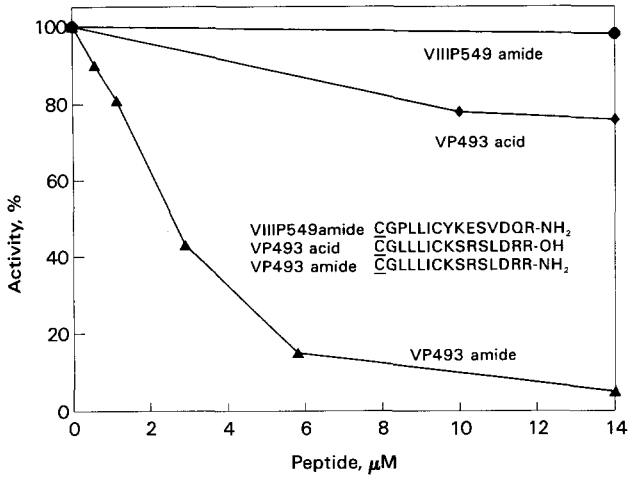

Fig. 1. Dose dependence of inhibition of prothrombinase by various peptides. Peptide dilutions were preincubated for 15 min with FXa, FVa, and phospholipid vesicles in duplicate. Prothrombin was added and the rate of thrombin formation (activity) was determined as a percentage of the rate in the absence of peptide.

components. The pattern is that of mixed (or noncompetitive parabolic) inhibition. Replot of the slopes in Figure 2 versus [VP493] yielded a parabolic-shaped curve.

Peptide VP493 had similar potency in a FXa-1-stage clotting assay. When preincubated with FXa, clot time was increased. By comparison with a standard curve for various concentrations of FXa, it was determined that 50% inhibition of FXa activity was achieved at about 3 μM peptide (Fig. 3). The amide form of VP493 was much more inhibitory than the acid form. The homologous FVIII peptide VIIIP549 did not inhibit FXa activity significantly (data not shown).

Peptide VP493Q, containing the substitution R506Q as in the mutant FV that causes APC resistance, was prepared and tested. This peptide required 2.7-fold the dose of VP493 to inhibit prothrombinase activity and prolonged the FXa-1-stage times to a lesser extent than did peptide VP493 (data not shown). It is unknown whether this moderate difference in the behavior of peptides VP493Q versus VP493 reflects on any differences in behavior of native FVa versus R506Q-FVa toward FXa.

Peptide VP493 inhibited the amidolytic activity of FXa toward the chromogenic substrate S-2765 only at high concentrations during a 50-min preincubation. Inhibition of FXa amidolytic activity was ≤10% at 5 μM peptide, 21% at 200 μM peptide, and 25% at 400 μM peptide. Other experiments showed that 400 μM VP493 did not significantly alter thrombin amidolytic activity or thrombin time in a clotting assay (data not shown).

Anticoagulant effects of antibody to VP493

Immunoaffinity-purified antibodies to VP493 were preincubated with FVa and tested for ability to inhibit prothrombinase activity. Prothrombinase was inhibited by 50% at 170 nM IgG (Fig. 4), whereas nonimmune IgG was not inhibitory even at much higher concentrations. Similarly, these antibodies were inhibitory in a FXa-1-stage clotting assay when preincubated with FVa in FV-deficient plasma (data not shown).

Blockage of protein S anticoagulant activity by VP493

Protein S inhibits prothrombinase in the absence of APC due to its binding to FVa and FXa (Heeb et al., 1993b, 1994b; Hackeng et al., 1994). When both VP493 and protein S were included in a prothrombinase assay, combined inhibition was much less than the sum of the inhibition due to protein S alone plus VP493 alone (Fig. 5). For example, under the conditions in this study, protein S alone at 16 nM inhibited prothrombinase by 44%, and peptide VP493 alone at 4.5 μM inhibited prothrombinase by 28%, but inhibition for the combination of these two was only 35%. Relative to peptide VP493 alone taken as 100% activity and 0% inhibition, protein S inhibition in the presence of 4.5 μM peptide was calculated as only 15%. This suggested that VP493 might bind to protein S and block its ability to inhibit prothrombinase, and/or vice versa.

Binding of FXa and protein S to peptide VP493

FXa, DIP-FXa, and protein S bound to immobilized VP493 with similar affinity (Fig. 6 and data not shown). Apparent K_ds calcu-

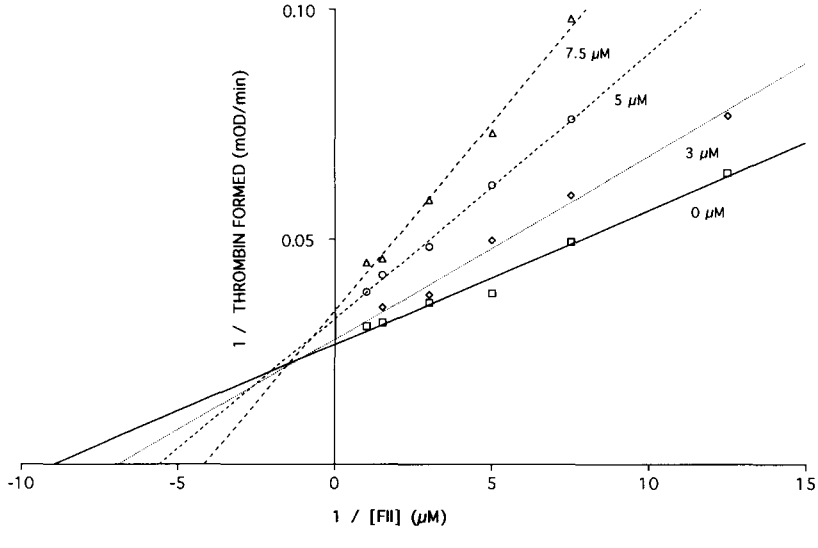

Fig. 2. Lineweaver–Burk plot of prothrombinase activity in the presence of peptide VP493. VP493 at the indicated final concentrations was preincubated for 15 min with FXa prior to addition of other prothrombinase components, including variable prothrombin concentrations. The rate of thrombin formation was measured in duplicate and averaged.

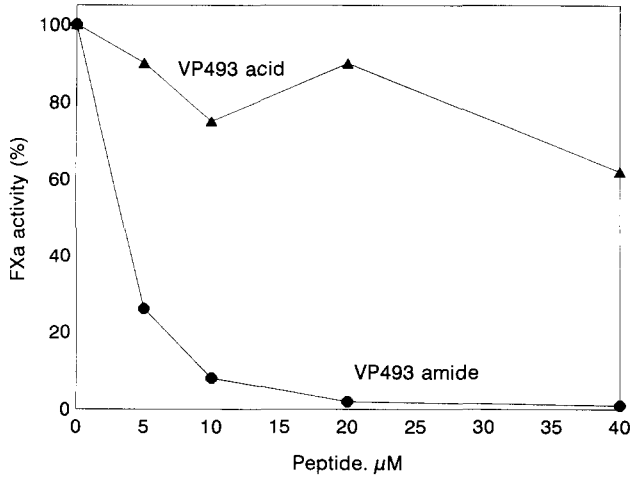

Fig. 3. Inhibition of FXa activity in a clotting assay. An FXa-1-stage clotting assay was performed in the presence and absence of various concentrations of peptides in duplicate. Percent FXa activity in each case was determined by comparison of clot times in the presence of peptide to a standard curve for clot times in the absence of peptide for various concentrations of FXa.

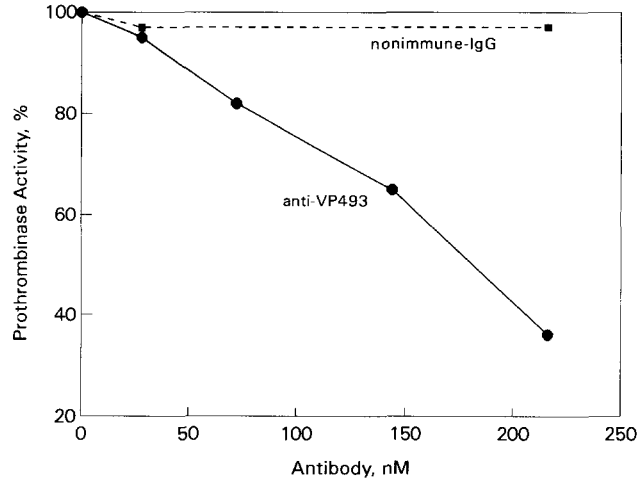

Fig. 4. Inhibition of prothrombinase activity by immunoaffinity purified antibody to peptide VP493. FVa was preincubated for 30 min with various concentrations of IgG fraction of rabbit antibodies to VP493, IgG fraction of nonimmunized rabbits, or binding buffer alone. Other prothrombinase components were added and the rate of thrombin formation was measured. Prothrombinase activity in the absence of IgG was defined as 100%.

lated by Enzfitter and Scatchard analysis averaged 32 nM for FXa (n = 3 separate experiments), 48 nM for DIP-FXa (n = 7), and 42 nM for protein S (n = 7). Prothrombin and APC did not bind to immobilized VP493 with comparable or measurable affinity (apparent K_d > 400 nM). Protein S did not bind with measurable affinity to several other peptides related to FVa heavy chain or to several FVIII-related peptides from the region homologous to FV residues 493–506, including those shown in Table 2 (data not shown). In three experiments, the apparent K_d for binding of FXa to VP493Q (with the substitution R506Q) was 2.8-fold higher than for binding to VP493, and the apparent K_d for binding of protein S to VP493Q was 1.8-fold higher than for binding to VP493 (data not shown). It would be speculative to extrapolate these moderate differences in peptide behavior to the behavior of native FVa versus R506Q-FVa upon binding of FXa or protein S.

Binding data obtained from solid-phase ligand binding experiments yield only *apparent* K_ds that are useful to compare the specificity of binding of different proteins and different peptides. Experiments were performed to estimate the K_ds in fluid phase as described in Materials and methods. Binding constants for VP493 in fluid phase were 1.3, 1.8, and 5.2 μM for binding to FXa, and 0.3 and 1.0 μM for binding to DIP-FXa in several experiments (data not shown). These values are similar to the concentration of peptide needed for 50% inhibition of prothrombinase following preincubation with FXa/FVa/phospholipid (2.5 μM).

Discussion

Potent inhibition of prothrombinase activity and FXa clotting activity by peptide VP493 (50% inhibition at 2.5–6 μM) suggested

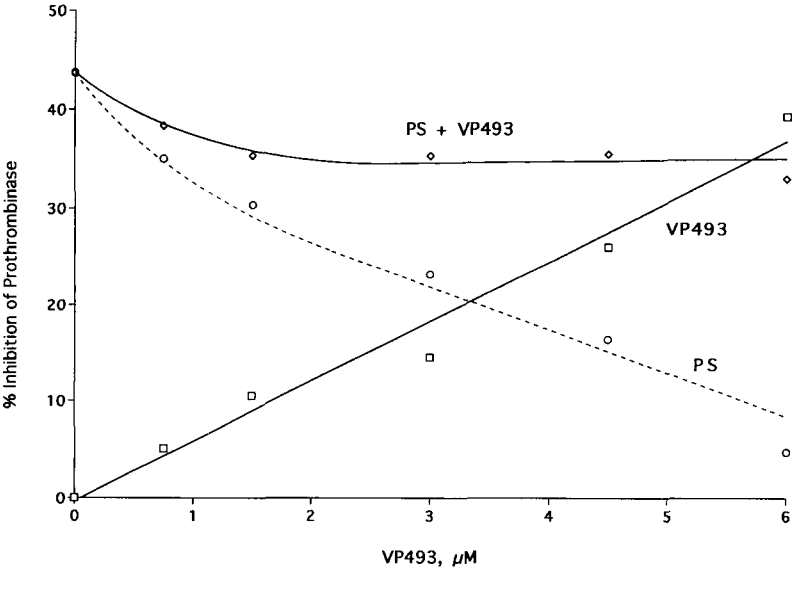

Fig. 5. Inhibition of prothrombinase activity by protein S as a function of VP493 concentration. Protein S (16 nM final concentration) was preincubated with variable concentrations of VP493 in 30 μL of binding buffer for 20 min. FVa, FXa, and phospholipids were added in a volume of 60 μL and preincubation was continued for 10 min. Prothrombin was added in 10 μL and the rate of thrombin formation was followed in each sample. ◇, inhibition by protein S and peptide in combination, relative to a control without peptide or protein S taken as 100% activity, i.e., 0% inhibition; □, inhibition of prothrombinase by peptide without protein S, also relative to a control without peptide or protein S; ○, calculated inhibition by protein S in the presence of peptide, using prothrombinase activity in the presence of peptide alone defined as 100% activity, i.e., 0% inhibition (----).

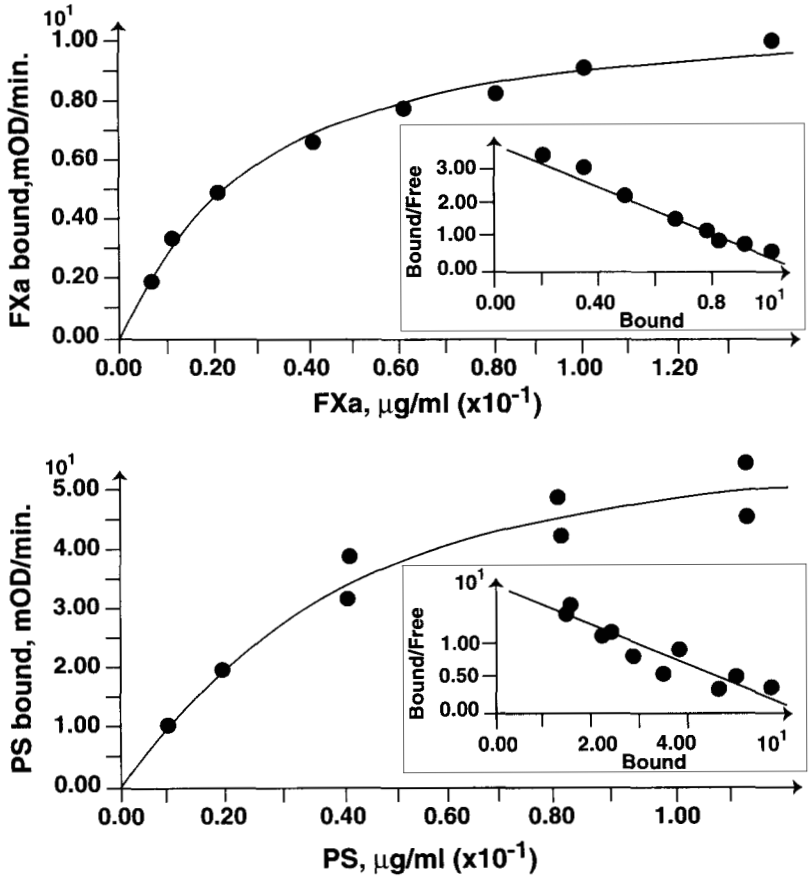

Fig. 6. Enzfitter analysis of binding of FXa and protein S to immobilized peptide VP493. Binding assays were performed as described in Materials and methods. Insets show Scatchard analysis of data used to calculate K_d = 2.8 μg/mL (45 nM) for FXa and K_d = 3.1 μg/mL (41 nM) for protein S.

that the region of residues 493–506 of FVa contains a Factor FXa binding site. The apparent blockage of protein S inhibition of prothrombinase by VP493 suggested that this region might also contribute to a protein S binding site. Indeed, both proteins bound specifically to this immobilized peptide with similar apparent K_ds of ~40 nM. Possible competition between protein S and FXa for this binding site on FVa may help explain why protein S negates the protective effect of FXa toward FVa inactivation by APC (Solymoss et al., 1988; Jane et al., 1991).

The specificity of the FV peptide VP493 is demonstrated by the fact that control peptides containing the homologous FVIII sequences or a similar distribution of Arg residues did not inhibit prothrombinase. Further evidence of specificity lies in the poor activity of the C-terminal carboxyl form of VP493 compared with the C-terminal carboxamide form, and the anticoagulant activity of affinity-purified anti-VP493 antibodies that are against this region of FVa. However, the possibility remains that nonspecific steric hindrance due to antibody binding rather than specific blockage of residues 493–506 might explain the antibodies' anticoagulant activity.

Protection of FVa from APC by FXa is apparently exhibited mostly for the Arg 506 cleavage by APC in FVa, whereas the potent 20-fold protein S cofactor effect for APC is exhibited only for the Arg 306 cleavage in FVa (Rosing et al., 1995). To explain the direct protection by FXa of Arg 506 from APC and the ablation of the FXa protection by protein S, one must hypothesize that the geometry of binding of FXa differs from that of protein S. One possible explanation of the Rosing group's data is that protein S binding at the 493–506 site in FVa places protein S in an optimal orientation for acting as a cofactor for APC cleavage at Arg 306.

Additional binding elements for FXa other than those of residues 493–506 on FVa probably exist, including the possible nonoverlapping region of residues 507–520 (Table 1). FXa has binding sites in both the heavy and light chains of FVa (Tracy & Mann, 1983; Tucker et al., 1983; Dharmawardana & Johnson, 1993; Kalafatis et al., 1994b). APC apparently has a binding site in the light chain of FVa (Krishnaswamy et al., 1986; Walker et al., 1990); however, there are likely to be others because several binding sites in the heavy and light chains of APC have been identified as potential binding sites for FVa (Mesters et al., 1991, 1993a, 1993b). Moreover, because APC cleaves the FVa heavy chain at Arg 306, 506, and 679, it seems likely that the protease domain of APC has exosites that bind FVa heavy chain.

A proposed structure for the A domains of FVIII was made by homology modeling (Pan et al., 1995). In that model, the APC cleavage site that is homologous to Arg 506 in FV was found to be solvent exposed and located between two β-barrels in the A2 domain. This suggests that such a site in FVa would also be favorable for a FXa and protein S binding site.

We have identified a binding site for FVa heavy chain on protein S at its C terminal residues 621–635 (Heeb et al., 1993a). It is not likely that the protein S C terminus is the complementary site for FV residues 493–506 because both sites have a substantial net positive charge. Thus, protein S very likely has at least two distinct sites for binding to FVa.

FVa loses affinity for FXa after inactivation by APC (Guinto &

Esmon, 1984). The binding site suggested here for FXa on FVa is adjacent to an important APC cleavage site (Arg 506) and is homologous to a recently elucidated FIXa binding site on FVIIIa (Fay et al., 1994; O'Brien et al., 1995). This suggests that homologous regions in the middle of the A2 domains of FVa and FVIIIa similarly bind the respective homologous enzymes FXa and FIXa, enhancing speculation that other structurally homologous regions in FVa and FVIIIa are responsible for functionally similar protein–protein interactions involving FVa and FVIIIa.

The poor anticoagulant activity of the C-terminal acid form of VP493 suggests that FVa cleaved by APC, with a new carboxyl group on residue 506, might lose some activity, at least in part because of the loss of a FXa binding site. It is also possible that cleavage at Arg 506 by APC leads to a conformational change in FVa, making the Arg 306 cleavage site more accessible to APC. The anticoagulant potency of the C-terminal carboxamide form of VP493 underscores the importance of the region of residues 493–506 in FVa procoagulant function involving FXa and in its regulation by protein S and APC.

Materials and methods

Peptide synthesis

Peptides were prepared under the supervision of Dr. Richard Houghten of Torrey Pines Institute using the simultaneous multiple synthesis method (Houghten, 1985), were in the C-terminal carboxamide form, and were analyzed by reverse-phase HPLC and electrospray mass spectral analysis to verify purity and composition (Mesters et al., 1991). Unless otherwise specified, peptide solutions were made at least one day prior to use and were pretreated with excess iodoacetamide before use in order to block any free sulfhydryl groups that might be present. Controls showed that iodoacetamide did not affect the various assays.

Proteins and other reagents

Human protein S, FVa, prothrombin, monoclonal antibody S7 to protein S, and phospholipid vesicles (80% phosphatidylcholine, 20% phosphatidylserine) were prepared as described (Heeb et al., 1993b, 1994b; Mesters et al., 1991). Antibodies to peptide VP493 were prepared in rabbits and immunoaffinity purified using Sepharose to which FV had been attached covalently (Mesters et al., 1991). FXa was obtained from Enzyme Research Laboratories (South Bend, Indiana) and DIP-FXa was prepared by preincubation of FXa with 2 mM diisopropyl fluorophosphate until less than 1% amidolytic activity remained, followed by dialysis against Hepes-buffered saline.

Binding assays

Binding assays were described in detail elsewhere (Heeb et al., 1993b). Peptides at 20 μM were coated to Xenobind plates (Xenopore, Saddle Brook, New Jersey) according to manufacturer's instructions and blocked with 3% fish skin gelatin (Sigma, St. Louis, Missouri). Detection of bound protein S was with monoclonal antibody S7, and detection of bound DIP-FXa or FXa was with monoclonal antibody (purified IgG from Biodesign, Kennebunkport, Maine) or with FXa substrate S-2765 (Chromogenix, Franklin, Ohio). Bound antibodies were detected with biotin-secondary antibody, steptavidin-alkaline phosphatase, and phosphatase substrate as previously described (Heeb et al., 1993b). Noncoated wells served as nonspecific controls, the values of which were subtracted from corresponding coated wells. Nonspecific binding ranged from 10–30% of total binding in various experiments. Binding studies of protein S or FXa were performed at 37 °C in binding buffer consisting of 0.05 M Tris, 0.2 M NaCl, 5 mM $CaCl_2$, 0.1 mM $MnCl_2$, 0.02% NaN_3, and 0.5% porcine skin gelatin type A (Sigma).

Fluid-phase binding experiments were also performed as previously described (Heeb et al., 1993b). Briefly, FXa or DIP-FXa at 12 nM and at 24 nM was equilibrated with various concentrations of VP493 for 2 h at 37 °C. Standards of various concentrations of FXa or DIP-FXa were incubated in parallel. The mixtures were transferred for 20 min to a microtiter plate coated with 20 μM VP493. Bound FXa on this plate corresponded to free FXa in the FXa-peptide mixtures, and was detected as described in the previous paragraph and quantitated by comparison to a calibration curve for the known concentrations of FXa or DIP-FXa in the standard dilutions.

Functional assays

All experiments were performed in duplicate and repeated on separate days. Prothrombinase assays were described in detail elsewhere (Mesters et al., 1991; Heeb et al., 1993b) and were performed in the same binding buffer as described above except that BSA was substituted for gelatin and [NaCl] was 0.10 M. Unless otherwise stated, FXa was 1 nM, FVa was 20 pM, phospholipid vesicles were 25 μM, and prothrombin was 0.3 μM final concentration, and the temperature was 23 °C. Thrombin substrate S-2238 (Chromogenix) was used at 0.2 mM final in Tris-buffered saline, 0.1% BSA, 10 mM EDTA, pH 8.2, and the rate of thrombin formation was monitored with a Biotek EL312 plate reader with Kineticalc software (Biotek, Winooski, Vermont).

FXa-1-stage clotting assays were performed as follows. FV-deficient plasma (60 μL) was mixed with 20 μL 1% BSA in Hepes-buffered saline ± peptide, 50 μL of 200 μg/mL cephalin, and 10 μL of 4 nM FXa, and preincubated for 2 min at 37 °C. FVa (10 μL of 30 pM FVa) and 50 μL of prewarmed 33 mM $CaCl_2$ in Hepes-buffered saline were added, and clot time was recorded using an ST4 coagulometer (Diagnostica Stago, Asnieres, France). For some experiments, 10 μL of 18 nM FV was substituted for FVa, with similar results.

Acknowledgments

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