Protein Science (1993), *2*, 1482–1489. Cambridge University Press. Printed in the USA.

Interactions and inhibition of blood coagulation factor Va involving residues 311–325 of activated protein C

ROLF M. MESTERS, MARY J. HEEB, AND JOHN H. GRIFFIN

Department of Molecular and Experimental Medicine and the Committee on Vascular Biology, The Scripps Research Institute, La Jolla, California 92037

(RECEIVED February 23, 1993; REVISED MANUSCRIPT RECEIVED May 17, 1993)

Abstract

Activated protein C (APC) exerts its physiologic anticoagulant role by proteolytic inactivation of the blood coagulation cofactors Va and VIIIa. The synthetic peptide-(311–325) (KRNRTFVLNFIKIPV), derived from the heavy chain sequence of APC, potently inhibited APC anticoagulant activity in activated partial thromboplastin time (APTT) and Xa-1-stage coagulation assays in normal and in protein S-depleted plasma with 50% inhibition at 13 μM peptide. In a system using purified clotting factors, peptide-(311–325) inhibited APC-catalyzed inactivation of factor Va in the presence or absence of phospholipids with 50% inhibition at 6 μM peptide. However, peptide-(311–325) had no effect on APC amidolytic activity or on the reaction of APC with the serpin, recombinant [Arg^{358}]α_1-antitrypsin. Peptide-(311–325) surprisingly inhibited factor Xa clotting activity in normal plasma, and in a purified system it inhibited prothrombinase activity in the presence but not in the absence of factor Va with 50% inhibition at 8 μM peptide. The peptide had no significant effect on factor Xa or thrombin amidolytic activity and no effect on the clotting of purified fibrinogen by thrombin, suggesting it does not directly inhibit these enzymes. Factor Va bound in a dose-dependent manner to immobilized peptide-(311–325). Peptide-(311–315) inhibited the binding of factor Va to immobilized APC or factor Xa. These data are consistent with the hypothesis that residues 311–325 in APC bind to factor Va at a site that can bind either APC or factor Xa, and that peptide-(311–325) interferes with both APC inactivation of factor Va and expression of factor Xa activity in the prothrombinase complex by binding to this site.

Keywords: activated protein C; anticoagulant; factor Va; factor Xa; peptide; protein–protein interaction

Protein C, a vitamin K-dependent zymogen of a serine protease (Stenflo, 1976), is activated in blood by the thrombin–thrombomodulin complex (Kisiel, 1979a; Esmon & Owen, 1981; Esmon et al., 1982). Activated protein C, in conjunction with its nonenzymatic cofactor protein S, acts as a natural anticoagulant by proteolytic inactivation of the blood coagulation factors Va and VIIIa (Kisiel et al., 1977; Walker et al., 1979; Walker, 1980; Vehar & Davie, 1980; Marlar et al., 1982). The important role of PC for the hemostatic balance in vivo is demonstrated by the fact that heterozygous PC deficiency in some families is associated with venous thromboembolism (Griffin et al., 1981), whereas homozygous PC deficiency or acquired inhibitors of PC have been associated with severe and generalized thrombotic disease (Branson et al., 1983; Seligsohn et al., 1984; Mitchell et al., 1987). The functional importance of the Gla-containing region (Stenflo & Suttie, 1977; Esmon et al., 1983; Sugo et al., 1985; Zhang & Castellino, 1990, 1991) and the EGF-like regions (Johnson et al., 1983; Hill et al., 1987; Öhlin & Stenflo, 1987; Öhlin et al., 1988a,b, 1990) to the biological activity of APC has been described, and potential sequences of bovine factor Va and human factor VIIIa for the binding to bovine APC have been reported (Walker et al., 1990). Studies of synthetic peptides as inhibitors of APC activities and protein–protein interactions support the suggestion that residues 390–404 of APC provide a site for recognition of factor Va by APC (Mesters et al., 1991). Studies using the synthetic peptide-(311–325) de-

Reprint requests to: John H. Griffin, Department of Molecular and Experimental Medicine, SBR-5, The Scripps Research Institute, 10666 North Torrey Pines Road, La Jolla, California 92037.

Abbreviations: α_1-AT, α_1-antitrypsin; APC, activated protein C; APTT, activated partial thromboplastin time; BSA, bovine serum albumin; EGF, epidermal growth factor; Gla, γ-carboxyglutamic acid; NHP, normal human plasma; PC, protein C; PSDP, protein S-depleted plasma; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SPDP, *N*-succinimidyl-3-(2-pyridyldithio)propionate; S-2222, *N*-benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine-*p*-nitroanilide hydrochloride; S-2238, H-D-phenylalanyl-L-pipecolyl-L-arginine-*p*-nitroanilide dihydrochloride; S-2366, L-pyroglutamyl-L-prolyl-L-arginine-*p*-nitroanilide hydrochloride; TBS, Tris-buffered saline.

rived from the sequence in the heavy chain of APC are presented here that suggest that the sequence of residues 311–325 in APC provides an important factor Va recognition site, and that the complementary site in factor Va is also a factor Xa binding site.

Results

When a limited number of synthetic pentapeptides comprising APC sequences were screened for their ability to inhibit APC activity (Mesters et al., 1991), we found that the synthetic peptide-(317–331) appeared to be a weak inhibitor of APC anticoagulant activity in APTT and Xa-1-stage coagulation assays with 50% inhibition at ≥500 μM peptide. To define whether this observation was artifactual or whether the 317–331 region of APC was important for APC function, we tested several peptides containing neighboring or overlapping sequences, and peptide-(311–325) was chosen for further detailed study. Peptide-(311–325) potently inhibited APC anticoagulant activity in APTT and Xa-1-stage assays in NHP and in APTT assays in PSDP, with 50% inhibition at 7–20 μM and 90% inhibition at approximately 50 μM (Fig. 1). A control peptide with a randomized sequence of the 311–325 residues had no significant effect on APC anticoagulant activity when tested at various concentrations up to 500 μM peptide (Fig. 1).

Studies were performed to test if the inhibitory peptide inhibited more specific functional activities of APC using purified proteins and chromogenic substrates. In contrast to the observations of APC inhibition in the coagulation assays, when 250 μM peptide-(311–325) was preincubated with 10 nM APC for 30 min at 37 °C prior to the addition of the chromogenic substrate, it had no significant effect on APC amidolytic activity toward the chromogenic substrate S-2366 (data not shown). Because the number of amino acid residues involved in the neutralization of APC by macromolecular plasma protease inhibitors is likely to be higher than the number of residues of APC involved in the cleavage of a small tripeptide chromogenic substrate like S-2366 (Janin & Chothia, 1990), we investigated the effect of peptide-(311–325) on the interaction of APC with the recombinant mutant [Arg^{358}]α_1-AT using previously described methods (Mesters et al., 1991). Substitution of Met^{358} by Arg in the reactive center of recombinant α_1-AT results in an increase of over 4,400-fold in the association rate constant for APC (Heeb et al., 1990). Peptide-(311–325) (250 μM final) had no significant effect on the second-order rate constant, k_2, for inhibition of APC by recombinant [Arg^{358}]α_1-AT (data not shown). The value of k_2 for this reaction in the presence of peptide was 3.3×10^4 M^{-1} s^{-1} versus 3.8×10^4 M^{-1} s^{-1} for the control.

To see if peptide-(311–325) directly affected recognition of factor Va by APC, studies were performed using only purified proteins. Peptide-(311–325) was tested for its ability to inhibit APC-catalyzed inactivation of factor Va in the presence of phospholipid vesicles in a system using 0.25 nM APC, 10.0 nM Va, and 50 μM phosphatidylcholine/phosphatidylserine (80/20%, M/M) vesicles. As shown in Figure 2, peptide-(311–325) inhibited the APC-catalyzed inactivation of factor Va with half-maximal inhibition at 6 μM peptide and with >95% inhibition at 50 μM peptide. Moreover, peptide-(311–325) inhibited the APC-catalyzed inactivation of factor Va in the absence of phospholipids (data not shown). For example, at 5 nM APC and 40 nM Va, the rate of factor Va inactivation under conditions described in the Materials and methods

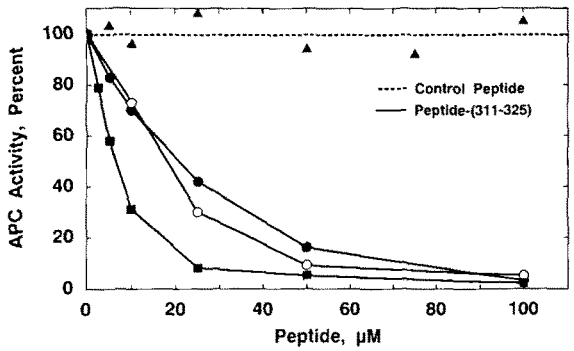

Fig. 1. Inhibition of APC anticoagulant activity by peptide-(311–325). The APTT and Xa-1-stage coagulation assays were performed as described in the Materials and methods. Solid lines with solid circles represent inhibition of anticoagulant activity of 5.0 nM APC by peptide-(311–325) in an APTT assay in NHP, solid lines with open circles represent inhibition of anticoagulant activity of 5.0 nM APC by peptide-(311–325) in an APTT assay in PSDP, and solid lines with solid squares represent inhibition of anticoagulant activity of 30.0 nM APC by peptide-(311–325) in a Xa-1-stage assay in NHP, and dashed lines with solid triangles represent APC activity in APTT assays in the presence of the control peptide.

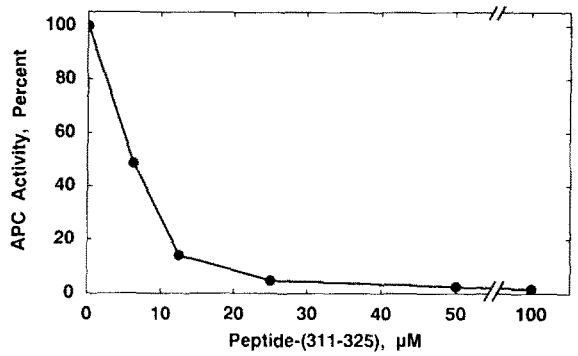

Fig. 2. APC-catalyzed inactivation of factor Va in the presence of peptide-(311–325) and phospholipids. The assay was carried out in TBS-BSA, 2.5 mM $CaCl_2$, pH 7.4, using 0.25 nM APC, 10.0 nM Va, 50 μM phospholipid vesicles, and 0–100 μM peptide-(311–325) that had been preincubated for 20 min at 22 °C with factor Va. Factor Va inactivation by APC was monitored using the cofactor activity of factor Va in a prothrombinase assay. Residual APC activity in the presence of the peptide was calculated from a standard curve constructed using varying APC concentrations in the absence of peptide.

was −4.0% factor Va/min in the absence of peptide and −0.5% factor Va/min in the presence of 50 μM peptide-(311-325), corresponding to 87% inhibition of APC activity in this assay. Thus, peptide-(311-325) potently inhibited the activity of APC on purified factor Va in the presence or absence of phospholipids.

In the course of coagulation assay studies performed in the absence of exogenously added APC, peptide-(311-325) was unexpectedly anticoagulant (Fig. 3). For example, peptide-(311-325) inhibited factor Xa clotting activity in NHP with 50% inhibition at 7 μM peptide, whereas the control peptide with the randomized sequence only slightly affected factor Xa activity (Fig. 3). In order to test whether peptide-(311-325) interfered with the interaction of factor Xa or prothrombin with the cofactor factor Va, studies using purified proteins were performed. Peptide-(311-325) potently inhibited the generation of thrombin in the prothrombinase assay in the presence of factor Va (in the absence or presence of phospholipid vesicles) with 50% inhibition of prothrombinase activity at 8 μM peptide (Fig. 4). However, in the absence of factor Va even at concentrations up to 200 μM of peptide-(311-325), very little if any significant inhibition of prothrombinase activity occurred (Fig. 4). A small amount of inhibition of prothrombinase activity was also apparently observed for the control peptide with a random sequence in the absence as well as in the presence of factor Va (Fig. 4). Thus, the presence of factor Va was essential for the inhibitory effect of the peptide on the prothrombinase complex.

To test if peptide-(311-325) directly inhibited the enzymatic activity of factor Xa or thrombin, further studies were made. Peptide-(311-325) (250 μM final) as well as the control peptide did not significantly inhibit the amidolytic activity of 4 nM factor Xa or 2 nM thrombin toward the chromogenic substrates, S-2222 and S-2238, respectively, in the presence or absence of calcium ions (data not shown). Furthermore, peptide-(311-325) and the control peptide had no significant effect on the clotting of purified fibrinogen by thrombin when tested at six different peptide concentrations (0-500 μM final) (data not shown).

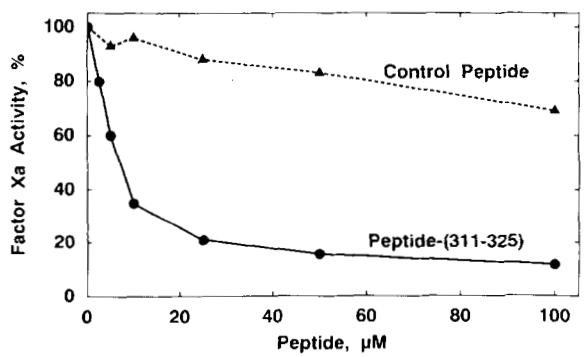

Fig. 3. Inhibition of factor Xa clotting activity by peptide-(311-325). The assay was performed as described in the Materials and methods. Solid circles with solid lines indicate factor Xa activity of 0.15 nM factor Xa in the presence of peptide-(311-325), and solid triangles with dashed lines indicate factor Xa activity in the presence of the control peptide.

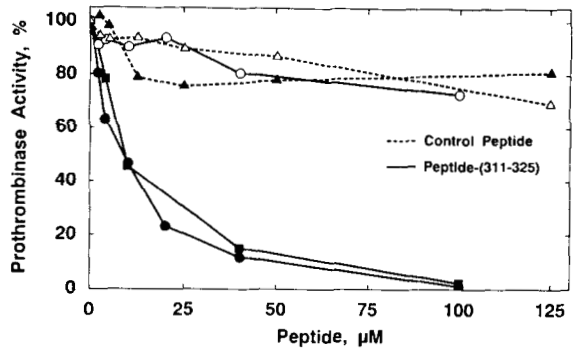

Fig. 4. Inhibition of prothrombinase activity by peptide-(311-325). The assay was performed using 1 nM factor Xa, 0.4 nM factor Va, 1.2 μM prothrombin in TBS-BSA, 2.5 mM $CaCl_2$, pH 7.4. In the absence of factor Va, 2.5 nM factor Xa was used. Unless otherwise specified, all prothrombinase assays were carried out in the presence of 50 μM phospholipid vesicles. In all cases solid symbols indicate prothrombinase activity in the presence of factor Va, whereas open symbols indicate prothrombinase activity in the absence of factor Va. Solid circles with solid lines indicate prothrombinase activity (with factor Va) in the presence of peptide-(311-325), solid squares with solid lines indicate prothrombinase activity (with factor Va) in the presence of peptide-(311-325) but in the absence of phospholipid vesicles, and solid triangles with dashed lines indicate prothrombinase activity (with factor Va) in the presence of the control peptide. Open circles with solid lines denote prothrombinase activity (without factor Va) in the presence of peptide-(311-325), and open triangles with dashed lines represent prothrombinase activity (without factor Va) in the presence of the control peptide.

To test whether factor Va bound to peptide-(311-325), the peptide and a control peptide with random sequence were coated to the wells of microtiter plates at concentrations ranging from 12.5 to 225 μM. Factor Va at 3 nM bound to the immobilized peptide-(311-325), but not to the control peptide, in a manner dependent on the concentration of peptide coated (Fig. 5). These data demonstrate that the sequence 311-325 in APC contains a binding site for factor Va.

To test the hypothesis that the peptide-(311-325) binding site on factor Va was a site common for binding APC or factor Xa, the ability of the peptide to inhibit factor Va binding to immobilized APC or factor Xa was studied. The results in Figure 6 show that peptide-(311-325) did inhibit binding of factor Va to each of these proteins with 50% inhibition at ~10 μM peptide.

Discussion

Synthetic peptides offer useful probes of protein-protein interaction, and these have been used to identify sites for recognition of factor Va by the antithrombotic enzyme, APC. Peptide-(311-325), containing the APC sequence,

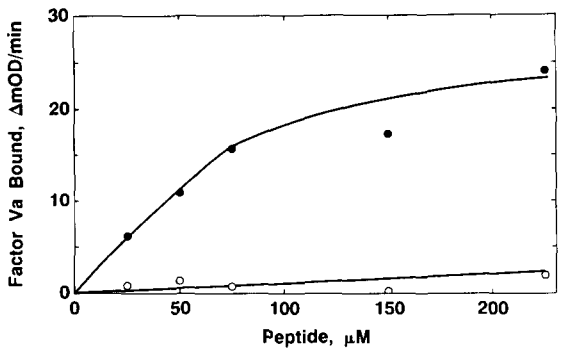

Fig. 5. Binding of factor Va to immobilized peptide-(311-325). Peptides at the concentrations shown on the abscissa were coated to the wells of microtiter plates as described in the Materials and methods. Factor Va was incubated in the wells, the wells were washed, and bound factor Va was detected as described. Closed circles indicate binding of factor Va to wells containing peptide-(311-325), and open circles indicate binding to wells containing a control peptide with random sequence.

KRNRTFVLNFIKIPV, potently inhibits APC anticoagulant activity in various coagulation assays. The inhibitory effect of peptide-(311-325) is specific because a control peptide with a randomized sequence of residues 311-325 has no significant effect on APC anticoagulant activity. The observed dose-response of peptide-(311-325) is identical using APTT assays and either NHP or PSDP, suggesting that the inhibitory effect of this peptide is not dependent on the presence of protein S and therefore that these residues are not involved in APC interactions with protein S.

Peptide-(311-325) did not significantly affect the amidolytic activity of APC toward the chromogenic substrate S-2366 or the inhibition of APC by the serpin, recombinant [Arg^{358}]α_1-AT. These data indicate that peptide-(311-325) does not affect the reactivity of the active-site residues and does not exert its inhibition of APC anticoagulant activity by blocking primary substrate binding sites close to the active-site serine that are involved in cleavage of a small tripeptide substrate like S-2366 or in recognition of the recombinant 55,000 M_r [Arg^{358}]α_1-AT.

The observation that peptide-(311-325) inhibits APC-catalyzed inactivation of purified factor Va in the presence as well as in the absence of phospholipids with 50% inhibition at 6 μM suggests that the region of residues 311-325 represents an exosite on APC essential for its anticoagulant activity and for the recognition of its macromolecular substrate, factor Va. It furthermore excludes the possibility that peptide-(311-325) exerts its action by inhibiting binding of APC or factor Va to phospholipids.

The heavy chain of APC is homologous to other serine proteases (Beckmann et al., 1985; Plutzky et al., 1986; Greer, 1990) and this class of enzymes has a common three-dimensional structure, allowing some inferences about structure-function relationships of APC using models based on structurally conserved regions of homologous enzymes. However, any inferences about the detailed structure of the hypothetical exosite based on homology to the X-ray crystallographic structure of chymotrypsin or trypsin are limited because human PC has an insertion of four amino acids preceding residue 307. Although the sequence of residues 311-325 in human PC is highly conserved compared to the bovine sequence (Table 1), bovine PC does not have this four-residue insertion. The hypothesized exosite in APC can be located in homology to residues 148-162 of chymotrypsin in the three-dimensional X-ray crystallographic model. Residues 134-143 in trypsin or 148-157 in chymotrypsin, homologous to residues 311-320 in PC (Table 1), form an exposed loop on the surface of the catalytic domain, whereas trypsin residues 144-148 or chymotrypsin 159-162, homologous to 321-325 in PC, are rather buried in the structure.

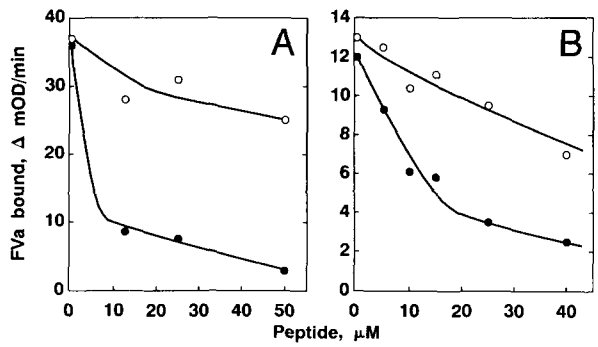

Fig. 6. Inhibition of binding of factor Va to immobilized APC or factor Xa by peptide-(311-325). **A:** Binding of factor Va to immobilized APC in the absence or presence of various concentrations of peptide-(311-325) (closed circles) or a control peptide with random sequence (open circles). **B:** A similar experiment using immobilized factor Xa in place of APC.

Table 1. *Alignment of residues 311-325 of human protein C with homologous sequences in bovine protein C, human factor IX, factor X, prothrombin, factor VII, bovine chymotrypsinogen, and bovine trypsinogen*[a]

Protein	Residue	Sequence[b]
Human PC	311-325	KRNRTFVLNFIKIPV
Bovine PC	309-323	KRNRTFVLSFIKVPV
Factor IX	316-330	KGRSALVLQYLRVPL
Factor X	330-344	KGRQSTRLKMLEVPY
Prothrombin	474-488	KGQ.PSVLQVVNLPI
Factor VII	290-304	RGATALELMVLNVPR
Chymotrypsinogen	148-162	NANTPDRLQQASLPL
Trypsinogen	134-148	GTSYPDVLKCLKAPI

[a] Amino acids in PC are numbered according to Plutzky et al. (1986). Sequence alignment was performed as described (Mesters et al., 1991) with the additional alignment of the sequences of bovine PC (Fernlund & Stenflo, 1982; Stenflo & Fernlund, 1982) and bovine trypsinogen (Mikes et al., 1966).

[b] Underlined letters indicate amino acids that are identical among all listed proteins.

Thus, based on the trypsin and chymotrypsin models, the APC 320–325 sequence is unlikely to be involved in APC interactions with substrates, whereas residues 311–319 of PC (KRNRTFVLN) are probably surface exposed and available for recognition by factor Va.

An unexpected finding was that peptide-(311–325) alone potently inhibits factor Xa clotting activity both in clotting assays in plasma with 50% inhibition at 7 μM peptide and in prothrombinase assays with 50% inhibition at 8 μM peptide. No significant inhibition of prothrombinase activity occurs in the absence of factor Va. Thus, the presence of factor Va is a requirement for the inhibitory effect of the peptide, suggesting that the peptide interacts with factor Va. Peptide-(311–325) inhibits prothrombinase activity in the absence or presence of phospholipids. Peptide-(311–325) has no significant effect on factor Xa or thrombin amidolytic activity toward chromogenic substrates and no effect on the clotting of purified fibrinogen by thrombin. These observations exclude the possibilities that the peptide has anticoagulant properties due to inhibition of the binding of the components of the prothrombinase complex to the phospholipid vesicles, or due to direct inhibition of factor Xa or thrombin or of fibrin polymerization. These data, in combination with the inhibitory effect of peptide-(311–325) on APC inactivation of purified factor Va, suggest that the sequence of residues 311–319 in APC provides a factor Va binding site and that peptide-(311–325) binds factor Va, thereby interfering with both inactivation of factor Va and expression of factor Xa activity in the prothrombinase complex. This suggestion was tested and it was shown that factor Va binds directly to peptide-(311–325).

Previously, we suggested that the region of APC comprising residues 390–404 represents an exosite for the binding of APC to its macromolecular substrate, factor Va (Mesters et al., 1991). Thus, it seems that APC may have at least two spatially distinct exosites for recognition of factor Va, namely the region of residues 390–404 and the region of residues 311–325, and that the interactions of APC with its substrate factor Va are remarkably extensive. Both peptide-(311–325) and peptide-(390–404) inhibit APC anticoagulant activity and APC inactivation of purified factor Va (Mesters et al., 1991). However, peptide-(311–325) has additional anticoagulant properties due to inhibition of prothrombinase activity, a property that peptide-(390–404) lacks. Both peptides most likely exert their inhibitory effect on APC activity by binding to factor Va. These sites on factor Va to which those peptides bind must be topologically distinct on factor Va because only peptide-(311–325) inhibits the expression of factor Va-dependent prothrombinase activity.

Factor Xa protects factor Va from inactivation by APC (Comp & Esmon, 1979; Walker et al., 1979; Nesheim et al., 1982; Suzuki et al., 1983), suggesting that both enzymes might share certain binding sites on factor Va. We therefore tested whether peptide-(311–325) binds to this hypothesized common binding site in factor Va and thereby blocks binding of either APC or factor Xa. Peptide-(311–325) inhibits binding of factor Va to either APC or factor Xa, providing support for this hypothesis. Inspection of the sequence of factor Xa for sequences homologous to residues 311–319 of APC yielded no sequence with four or more identities; the best identity for three residues involved the factor X sequence (residues 270–273), KHNR, compared to KRNR (APC residues 311–314). A synthetic peptide presenting the sequence of residues 263–274 of factor X inhibits interactions of factor Xa with factor Va and could be cross-linked to factor Va (Chattopadhyay et al., 1992). Thus, the tetrapeptide sequence, K-basic-N-R, likely provides a motif recognized by factor Va, and residues 263–274 of factor Xa are very likely to bind to factor Va close to the site for binding APC residues 311–319. Inspection of protein sequence alignments (Greer, 1990) and of three-dimensional homology models for the protease domains of APC and factor Xa and of the X-ray crystallographic structures of trypsin, chymotrypsin, and kallikrein (C. Fisher & J.H. Griffin, unpubl.) shows that residues 263–273 of factor X correspond to chymotrypsin residues 83–93 and that the KHNR sequence is in the so-called kallikrein loop. In contrast, APC residues 311–319 correspond to chymotrypsin residues 148–156 and the KRNR sequence is in the so-called chymotrypsin autolysis loop. Both the "kallikrein loop" and the "autolysis loop" are fairly near the active-site triad of serine proteases, but they are on opposite sides of the active site. Therefore, the binding of factor Va either to KHNR on factor Xa in the Xa:Va complex, or to KRNR on APC in the APC:Va complex, necessarily would result in very different relative orientations of factor Va and the active sites of APC or factor Xa.

For the region in factor VIIa structurally homologous to residues 311–325 of APC, a synthetic peptide comprising residues 285–305 of factor VIIa (Table 1) was reported that inhibited the factor VIIa–tissue factor-mediated conversion of factor X to factor Xa (Kumar et al., 1991) and a synthetic peptide derived from the homologous region in thrombin corresponding to residues 467–478 in prothrombin inhibited binding of thrombin to thrombomodulin and thrombin-induced clotting of fibrinogen (Suzuki et al., 1990). These findings, combined with our data here for residues 311–325 of APC, indicate the general importance of this region, the so-called autolysis loop of chymotrypsin, of the homologous vitamin K-dependent serine proteases APC, factor IXa, factor VIIa, factor Xa, and α-thrombin for recognition of macromolecular substrates or cofactors.

Materials and methods

Materials

Human PC was purified and activated as described (Gruber et al., 1989; Mesters et al., 1991). The specific anti-

coagulant activity of APC was 250 U/mg. The human vitamin K-dependent factors prothrombin, factor X, and factor IX were isolated as previously reported (Stenflo, 1976; van der Graaf et al., 1983). Recombinant [Arg^{358}]α_1-AT was a gift from Drs. Michael Courtney and Rainer Bischoff (Transgene, Strasbourg, France); human factor Va was a gift from Drs. Guido Tans and Jan Rosing (University of Limburg, Maastricht, The Netherlands). These were purified and characterized as described (Heeb et al., 1990; Tans et al., 1991). Human thrombin (specific activity 2,878 NIH U/mg), protein S, and human factor Xa were obtained from Enzyme Research Laboratories (Southbend, Indiana), and the molar concentrations were determined by active-site titration as described (Chase & Shaw, 1967). All proteins appeared to be >95% homogeneous judged by SDS-PAGE. The molecular weights and extinction coefficients ($E_{280nm}^{1mg/mL}$) used in calculating protein concentrations were as follows: prothrombin, 72,000 and 1.44 (Francis et al., 1986); APC, 62,000 and 1.45 (Kisiel, 1979b); and human factor X, 65,300 and 1.16 (DiScipio et al., 1977). The chromogenic substrates S-2222, S-2238, and S-2366 were purchased from Kabi-Vitrum (Franklin, Ohio); rabbit brain cephalin, rabbit serum albumin, bovine brain phosphatidylserine (CCl_4/CH_3OH solution, 95:5, v/v), and soybean phosphatidylcholine (Type III-S, CCl_4 solution) from Sigma (St. Louis, Missouri); BSA and human fibrinogen from Calbiochem (La Jolla, California); and the APTT reagent Thrombosil from Ortho-Diagnostics (Raritan, New Jersey). Normal human citrate-anticoagulated plasma (NHP) was purchased from George King Bio-Medical, Inc. (Overland Park, Kansas), and biotinylated goat anti-rabbit IgG, streptavidin alkaline phosphatase, conjugated (SAAP), *p*-nitrophenylphosphate, and SPDP came from Pierce (Rockford, Illinois). PSDP was prepared and characterized as previously reported (Mesters et al., 1991). All other reagents were of the highest quality available.

Synthesis and characterization

Synthetic peptides were prepared, purified to homogeneity by reverse-phase high performance liquid chromatography, and characterized as described (Mesters et al., 1991). The sequence of the control peptide with a randomized sequence of residue 311–325 was obtained by randomly drawing 15 individually labeled folded pieces of paper, each paper representing one residue. The sequence of drawing each amino acid dictated the random order of amino acids. This peptide had the sequence, VKFTIRVFNPRNLKI, and was designated as control peptide. Mass spectroscopic analyses of peptide-(311–325) and the control peptide using the previously reported method (Mesters et al., 1991) yielded in each case single peaks and the exact expected molecular weight of 1,845 for the single protonated form of peptide-(311–325) and the control peptide, respectively. Solutions of each peptide were prepared and the concentrations determined as described (Mesters et al., 1991) using a molar extinction coefficient of 400 M^{-1} cm^{-1}.

Coagulation assays

The effect of the synthetic peptides on APC anticoagulant activity was determined using recently described protocols (Mesters et al., 1991). Peptides were assayed in APTT and Xa-1-stage assays using the same procedure previously used for antibodies (Mesters et al., 1991), in which peptides were preincubated for 10 min with APC and/or factor Xa. Because peptide-(311–325) was anticoagulant in the Xa-1-stage coagulation assays even in the absence of APC, the residual factor Xa clotting activity in the presence of each different peptide concentration was calculated from a standard curve derived from the double-logarithmic plot of clotting time versus added factor Xa. Therefore, in order to determine the inhibition of APC by the peptide, different standard curves for APC-induced prolongation of clotting time for each peptide concentration were generated using the residual factor Xa activity that was observed at the corresponding peptide concentration. Because factor Xa activity versus inhibitory peptide concentration was linear on log–log plots, the additional inhibition by APC was greater than twofold at each peptide concentration, and the effect of APC was readily determined as units or percentage of factor Xa activity. All clotting times were determined in duplicate.

APC, factor Xa, and thrombin amidolytic assays

Amidolytic activities of APC, factor Xa, and thrombin toward the appropriate respective peptide substrates S-2366, S-2222, and S-2238 were assayed in the presence or absence of 5 mM calcium ions as reported for APC (Mesters et al., 1991). Kinetic studies of inhibition of APC by recombinant [Arg^{358}]α_1-AT in the absence or presence of peptides were performed and based on measurement of APC amidolytic activity as described (Mesters et al., 1991).

Fibrinogen clotting

A total of 0.5 U/mL human thrombin was preincubated with 0–1 mM peptide at six different concentrations in 200 μL of 0.05 M Tris/HCL, 0.1 M NaCl, 0.5% BSA, 0.02% NaN_3 (TBS-BSA), 2.5 mM $CaCl_2$ for 10 min at 37 °C in polystyrene cuvettes. Reaction was initiated by addition of 200 μL of 5-mg/mL human fibrinogen in TBS-BSA, 2.5 mM $CaCl_2$, and time in seconds from addition of fibrinogen to clot formation was measured in an Electra 700 Automatic Coagulation Timer (Medical Laboratory Automation, Inc., Mount Vernon, New York).

Inactivation of factor Va by APC

The APC-catalyzed inactivation of factor Va in the presence and absence of phospholipid vesicles was carried out as recently reported (Mesters et al., 1991). Phospholipid vesicles consisting of phosphatidylserine/phosphatidylcholine (20:80%, M/M) were prepared by sonication as described (Mesters et al., 1991).

Prothrombinase assays

Prothrombinase assays were performed as recently described for the factor Va assay using a final concentration of 0.4 nM factor Va (Mesters et al., 1991). In the absence of factor Va or phospholipid vesicles, the time intervals for quenching the prothrombinase by adding aliquots of the prothrombinase mixture to a solution containing 10 mM EDTA were 5 min instead of 1 min as for the presence of factor Va and phospholipid vesicles. Synthetic peptides were preincubated with factor Va for 20 min at 22 °C or, in the case of assays lacking factor Va, with prothrombin in the presence of phospholipid vesicles for 20 min at 22 °C. Prothrombinase activity, expressed as the amount of generated thrombin per unit of time, was based on thrombin amidolytic activity toward the chromogenic substrate S-2238.

Sequence alignment of various serine proteases was performed as described (Greer, 1990; Mesters et al., 1991).

Binding assays

Peptides at different concentrations were coated to the wells of microtiter plates, and the wells were blocked as described (Heeb et al., 1993). Factor Va (3 nM) in the absence or presence of peptide was incubated in the wells at 37 °C for 50 min. The wells were washed, and bound factor Va was immunologically detected as described (Heeb et al., 1993). Uncoated, blocked wells in the same plate were used as controls for background and the absorbance values for these wells were subtracted from the appropriate data points.

In other experiments, APC or factor Xa were coated at 4 μg/mL to the wells of microtiter plates, and the wells were blocked. Factor Va (3 nM) was preincubated with various concentrations of peptide in the wells of a separate low-binding microtiter plate for 30 min at 23 °C before transferring to the plates coated with APC or factor Xa. Following incubation at 37 °C for 50 min, bound factor Va was detected (Heeb et al., 1993).

Acknowledgments

We are grateful to Dr. András Gruber for preparation of APC, to Drs. Richard Houghten and Hernan Cuervo from the Torrey Pines Institute for Molecular Studies for synthesis and purification of peptides, and to Benjamin Montoya for assistance in binding experiments. We also thank Dr. Rainer Bischoff and Transgene for the recombinant [Arg^{358}]α_1-AT, Drs. Guido Tans and Jan Rosing for providing the purified factor Va, and Drs. José Fernández and Zaverio Ruggeri for their helpful discussions. This work was done during the tenure of a fellowship (R.M.M.) from the Deutsche Forschungsgemeinschaft (Federal Republic of Germany). The study was supported in part by grant HL-31950 from the National Institutes of Health. A preliminary report of this work was presented at the American Society of Hematology meeting (1991, *Blood 78*, 277a).

References

Beckmann, R.J., Schmidt, R.J., Santerre, R.F., Plutzky, J., Crabtree, G.R., & Long, G.L. (1985). The structure and evolution of a 461 amino acid human protein C precursor and its messenger RNA, based upon the DNA sequence of cloned human liver cDNAs. *Nucleic Acids Res. 13*, 5233–5247.

Branson, H.E., Katz, J., Marble, R., & Griffin, J.H. (1983). Inherited protein C deficiency and a coumarin-responsive chronic relapsing purpura fulminans syndrome in a neonate. *Lancet ii*, 1165–1168.

Chase, T. & Shaw, E. (1967). *p*-Nitrophenyl-*p'*-guanidinobenzoate HCl: A new active site titrant for trypsin. *Biochem. Biophys. Res. Commun. 29*, 508–514.

Chattopadhyay, A., James, H.L., & Fair, D.S. (1992). Molecular recognition sites on factor Xa which participate in the prothrombinase complex. *J. Biol. Chem. 267*, 12323–12329.

Comp, P.C. & Esmon, C.T. (1979). Activated protein C inhibits platelet prothrombin-converting activity. *Blood 54*, 1272–1281.

DiScipio, R.G., Hermodson, M.A., Yates, S.G., & Davie, E.W. (1977). A comparison of human prothrombin, factor IX (Christmas factor), factor X (Stuart factor), and protein S. *Biochemistry 16*, 698–705.

Esmon, C.T. & Owen, W.G. (1981). Identification of an endothelial cell cofactor for thrombin-catalyzed activation of protein C. *Proc. Natl. Acad. Sci. USA 78*, 2249–2252.

Esmon, N.L., DeBault, L.E., & Esmon, C.T. (1983). Proteolytic formation and properties of γ-carboxyglutamic acid domainless protein C. *J. Biol. Chem. 257*, 5548–5553.

Esmon, N.L., Owen, W.G., & Esmon, C.T. (1982). Isolation of a membrane-bound cofactor for thrombin-catalyzed activation of protein C. *J. Biol. Chem. 257*, 859–864.

Fernlund, P. & Stenflo, J.A. (1982). Amino acid sequence of the light chain of bovine protein C. *J. Biol. Chem. 257*, 12170–12179.

Francis, R.T., McDonagh, J., & Mann, K.G. (1986). Factor V is a substrate for the transamidase factor XIIIa. *J. Biol. Chem. 261*, 9787–9792.

Greer, J. (1990). Comparative modeling methods: Application to the family of the mammalian serine proteases. *Proteins Struct. Funct. Genet. 7*, 317–334.

Griffin, J.H., Evatt, B., Zimmerman, T.S., Kleiss, A.J., & Wideman, C. (1981). Deficiency of protein C in congenital thrombotic disease. *J. Clin. Invest. 68*, 1370–1373.

Gruber, A., Griffin, J.H., Harker, L., & Hanson, S.R. (1989). Inhibition of platelet-dependent thrombus formation by human activated protein C in a primate model. *Blood 73*, 639–642.

Heeb, M.J., Bischoff, R., Courtney, M., & Griffin, J.H. (1990). Inhibition of activated protein C by recombinant $\alpha_1$antitrypsin variants with substitution of arginine or leucine for methionine^{358}. *J. Biol. Chem. 265*, 2365–2369.

Heeb, M.J., Mesters, R.M., Tans, G., Rosing, J., & Griffin, J.H. (1993). Binding of protein S to factor Va associated with inhibition of prothrombinase that is independent of activated protein C. *J. Biol. Chem. 268*, 2872–2877.

Hill, K.A., Kroon, L.M.E., & Castellino, F.J. (1987). The effect of divalent cations on the amidolytic activity of bovine plasma activated protein C and des-1-41 light chain-activated protein C. *J. Biol. Chem. 262*, 9581–9586.

Janin, J. & Chothia, C. (1990). The structure of protein–protein recognition sites. *J. Biol. Chem. 265*, 16027–16030.

Johnson, A.E., Esmon, N.L., Laue, T.M., & Esmon, C.T. (1983). Structural changes required for activation of protein C are induced by Ca^{2+} binding to a high affinity site that does not contain γ-carboxyglutamic acid. *J. Biol. Chem. 258*, 5554–5560.

Kisiel, W. (1979a). Human plasma protein C. Isolation, characterization and mechanism of activation by α-thrombin. *J. Clin. Invest. 64*, 761–769.

Kisiel, W. (1979b). Molecular properties of the factor V-activating enzyme from Russell's viper venom. *J. Biol. Chem. 254*, 12230–12234.

Kisiel, W., Canfield, W.M., Ericsson, L.H., & Davie, E.W. (1977). Anticoagulant properties of bovine plasma protein C following activation by thrombin. *Biochemistry 16*, 5824–5831.

Kumar, A., Blumenthal, D.K., & Fair, D.S. (1991). Identification of molecular sites on factor VII which mediate its assembly and function in the extrinsic pathway activation complex. *J. Biol. Chem. 266*, 915–921.

Marlar, R.A., Kleiss, A.J., & Griffin, J.H. (1982). Mechanism of action of human activated protein C, a thrombin-dependent anticoagulant enzyme. *Blood 59*, 1067–1072.

Mesters, R.M., Houghten, R.A., & Griffin, J.H. (1991). Identification of a sequence of human activated protein C (residues 390–404) essential for its anticoagulant activity. *J. Biol. Chem. 266*, 24514–24519.

Mikes, O., Holeysovsky, V., Tomasek, V., & Sorm, F. (1966). Covalent structure of bovine trypsinogen. The position of the remaining amides. *Biochem. Biophys. Res. Commun. 24*, 346–352.

Mitchell, C.A., Rowell, J.A., Hau, L., Young, J.P., & Salem, H.H. (1987). A fatal thrombotic disorder associated with an acquired inhibitor of protein C. *N. Engl. J. Med. 317*, 1638–1642.

Nesheim, M.E., Canfield, W.M., Kisiel, W., & Mann, K.G. (1982). Studies of the capacity of factor Xa to protect factor Va from inactivation by activated protein C. *J. Biol. Chem. 257*, 1443–1447.

Öhlin, A.-K., Bjork, I., & Stenflo, J.A. (1990). Proteolytic formation and properties of a fragment of protein C containing the γ-carboxyglutamic acid-rich domain and the EGF-like region. *Biochemistry 29*, 644–651.

Öhlin, A.-K., Landes, G., Bourdon, P., Oppenheimer, C., Wydro, R., & Stenflo, J.A. (1988a). β-Hydroxyaspartic acid in the first epidermal growth factor-like domain of protein C. *J. Biol. Chem. 263*, 19240–19248.

Öhlin, A.-K., Linse, S., & Stenflo, J.A. (1988b). Calcium binding to the epidermal growth factor homology region of bovine protein C. *J. Biol. Chem. 263*, 7411–7417.

Öhlin, A.-K. & Stenflo, J.A. (1987). Calcium-dependent interaction between the epidermal growth factor precursor-like region of human protein C and a monoclonal antibody. *J. Biol. Chem. 262*, 13798–13804.

Plutzky, J., Hoskins, J.A., Long, G.L., & Crabtree, G.R. (1986). Evolution and organization of the human protein C gene. *Proc. Natl. Acad. Sci. USA 83*, 546–550.

Seligsohn, U., Berger, A., Abead, M., Rubin, L., Attias, D., Zivelin, A., & Rapaport, S.I. (1984). Homozygous protein C deficiency manifested by massive venous thrombosis in the newborn. *N. Engl. J. Med. 310*, 559–562.

Stenflo, J.A. (1976). A new vitamin K-dependent protein: Purification from bovine plasma and preliminary characterization. *J. Biol. Chem. 251*, 355–363.

Stenflo, J.A. & Fernlund, P. (1982). Amino acid sequence of the heavy chain of bovine protein C. *J. Biol. Chem. 257*, 12180–12190.

Stenflo, J.A. & Suttie, J.W. (1977). Vitamin K-dependent formation of γ-carboxyglutamic acid. *Annu. Rev. Biochem. 46*, 157–172.

Sugo, T., Persson, U., & Stenflo, J.A. (1985). Protein C in bovine plasma after warfarin treatment. *J. Biol. Chem. 260*, 10453–10457.

Suzuki, K., Nishioka, J., & Hayashi, T. (1990). Localization of thrombomodulin-binding site within human thrombin. *J. Biol. Chem. 265*, 13263–13267.

Suzuki, K., Stenflo, J.A., Dahlbäck, B., & Teodorsson, B. (1983). Inactivation of human coagulation factor V by activated protein C. *J. Biol. Chem. 258*, 1914–1920.

Tans, G., Rosing, J., Thomassen, M.C., Heeb, M.J., Zwaal, R.F.A., & Griffin, J.H. (1991). Comparison of anticoagulant and procoagulant activities of stimulated platelets and platelet-derived microparticles. *Blood 77*, 2641–2648.

van der Graaf, F., Greengard, J.S., Bouma, B.N., Kerbiriou, D.M., & Griffin, J.H. (1983). Isolation and functional characterization of the active light chain of activated human blood coagulation factor XI. *J. Biol. Chem. 258*, 9669–9675.

Vehar, G.A. & Davie, E.W. (1980). Preparation and properties of bovine factor VIII. *Biochemistry 19*, 401–410.

Walker, F.J. (1980). Regulation of activated protein C by a new protein. A possible function for bovine protein S. *J. Biol. Chem. 255*, 5521–5524.

Walker, F.J., Scandella, D., & Fay, P.J. (1990). Identification of the binding site for activated protein C on the light chain of factors V and VIII. *J. Biol. Chem. 265*, 1484–1489.

Walker, F.J., Sexton, P.W., & Esmon, C.T. (1979). The inhibition of blood coagulation by activated protein C through the selective inactivation of activated factor V. *Biochim. Biophys. Acta 571*, 333–342.

Zhang, L. & Castellino, F.J. (1990). A γ-carboxyglutamic acid variant (γ^6D, γ^7D) of human activated protein C displays greatly reduced activity as an anticoagulant. *Biochemistry 29*, 10828–10834.

Zhang, L. & Castellino, F.J. (1991). Role of the hexapeptide disulfide loop present in the γ-carboxyglutamic acid domain of human protein C in its activation properties and in the in vitro anticoagulant activity of activated protein C. *Biochemistry 30*, 6696–6704.