A Novel Exosite in the Light Chain of Human Activated Protein C Essential for Interaction with Blood Coagulation Factor Va†

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ABSTRACT: Activated protein C (APC) exerts its physiologic anticoagulant role by proteolytic inactivation of the blood coagulation cofactors Va and VIIIa. To identify regions on the surface of the light chain of APC that mediate anticoagulant activity, 10 synthetic peptides were prepared and tested for their ability to inhibit APC anticoagulant activity. The synthetic peptide-(142–155) inhibited APC anticoagulant activity in Xa-1-stage coagulation assays in normal and protein S-depleted plasma with 50% inhibition at 5–25 μM peptide. In a system using purified clotting factors, peptide-(142–155) inhibited APC catalyzed inactivation of factor Va in the presence or absence of phospholipids with 50% inhibition at 50 μM peptide. However, peptide-(142–155) had no effect on APC amidolytic activity or on the reaction of APC with the serpin, recombinant [Arg^{358}]-α_1-antitrypsin. Moreover, peptide-(142–155) inhibited factor Xa clotting activity in normal plasma as well as in a prothrombinase assay in the presence of factor Va with 50% inhibition at 5 μM and 50 μM peptide, respectively, under the assay conditions. 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 that it does not directly inhibit these enzymes' active sites. Peptide-(142–155) was shown to bind directly to immobilized factor Va. These data are consistent with the hypothesis that the sequence of residues 142–155 in the light chain of APC provides a factor Va binding site and that peptide-(142–155) binds factor Va, thereby interfering with both APC inactivation of factor Va and expression of factor Xa activity in the prothrombinase complex.

Protein C (PC),[1] a vitamin K-dependent zymogen of a serine protease (Stenflo, 1976), is activated in blood by the thrombin–thrombomodulin complex (Kisiel, 1979a,b; Esmon et al., 1981, 1983a,b). Activated protein C (APC), 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 et al., 1980; Marlar et al., 1982). The important role of protein C 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; Bertina et al., 1982; Bovill et al., 1989) and that 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 (Esmon et al., 1983a,b; Sugo et al., 1985; Zhang et al., 1990, 1991) and the EGF-like regions (Johnson et al., 1983; Hill et al., 1987; Öhlin et al., 1987, 1988) for 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).

In previous studies, we identified the regions comprising residues 311–330 and 390–404 in the heavy chain of APC essential for anticoagulant activity and recognition of its substrate factor Va by using synthetic peptides and antipeptide antibodies (Mesters et al., 1991, 1993). In this study, we identify a sequence in the human PC light chain that is essential for anticoagulant activity and for the interaction of APC with its substrate factor Va.

MATERIALS AND METHODS

Materials. Human protein C (PC) was purified and activated as described by Gruber et al. (1989) and Mesters et al. (1991). The specific anticoagulant activity of APC was 250 unit/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-antitrypsin was a generous gift from Drs. Michael Courtney and Rainer Bischoff (Transgene, Strasbourg, France), human factor Va was a generous gift from Drs. Guido Tans and Jan Rosing (University of Limburg, Maastricht, The Netherlands), and they were purified and characterized as described (Heeb et al., 1990; Tans et al., 1991). Human thrombin (specific activity 2878 NIH unit/mg) and human factor Xa were obtained from Enzyme Research Laboratories (Southbend, IN), and the molar concentrations were determined by active-site titration as described (Chase et al., 1967). All proteins appeared to be >95% homogeneous judged by SDS–PAGE. The molecular weights and extinction coefficients ($E_{280\ cm}^{1mg/mL}$) used in calculating protein concentrations were as follows: prothrom-

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[1] Abbreviations: PC, protein C; APC, activated protein C; APTT, activated partial thromboplastin time; Gla, γ-carboxyglutamic acid; EGF, epidermal growth factor; NHP, normal human plasma; PSDP, protein S-depleted plasma; 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; BSA, bovine serum albumin; α_1-AT, α_1-antitrypsin; TBS, Tris-buffered saline; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SPDP, *N*-succinimidyl-3-(2-pyridyldithio)propionate; ELISA, enzyme-linked immunosorbent assay.

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bin, 72 000 and 1.44 (Francis et al., 1986); APC, 62 000 and 1.45 (Kisiel, 1979a,b; Plutzky et al., 1986); 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, OH); rabbit brain cephalin, rabbit serum albumin, bovine brain phosphatidylserine (CCl_4/CH_3OH 95/5 v/v solution), and soybean phosphatidylcholine (type III-S, CCl_4 solution) were from Sigma (St. Louis, MO); bovine serum albumin and human fibrinogen were from Calbiochem (La Jolla, CA); and the APTT-reagent Thrombosil was from Ortho-Diagnostics (Raritan, NJ). Normal human citrate-anticoagulated plasma (NHP) was purchased from George King Bio-Medical, Inc. (Overland Park, KS), and biotinylated goat–anti-rabbit–IgG, streptavidin alkaline phosphatase conjugate (SAAP), *p*-nitrophenylphosphate, and *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP) were from Pierce (Rockford, IL). Protein S-depleted plasma (PSDP) was prepared and characterized as previously reported (Mesters et al., 1991). All other reagents were of the highest quality available.

Peptide Synthesis and Characterization. Synthetic peptides were prepared, purified to homogeneity by reverse-phase high-performance liquid chromatography (rp-HPLC), and characterized as described including verification of the expected mass by mass spectrometry (Mesters et al., 1991). Peptide-(142–155) with an additional carboxyterminal cysteine was used in these studies and, unless otherwise specified, this peptide with an additionally introduced carboxyterminal cysteine is designated as peptide-(144–155). The sequence of the scrambled peptide with a randomized sequence of residue 142–155 in PC was obtained by blindly drawing from a bag 14 individually labeled folded pieces of paper, each paper bearing the name of one residue. The sequence of drawing each amino acid dictated the random order of amino acids. This peptide had the sequence, MHKLREGKWRPKSR, and is designated as a scrambled peptide. Mass spectroscopic analyses of peptide-(142–155) and the scrambled peptide using the previously reported method (Mesters et al., 1991) yielded in each case the exact expected molecular weight of 1911 for the single protonated form of peptide-(142–155) and 1809 for the scrambled peptide. Solutions of each peptide were prepared, and the concentrations were determined as described (Mesters et al., 1991) using a molar extinction coefficient of 5600 M^{-1} cm^{-1} for peptide-(142–155) as well as for the scrambled peptide.

Coagulation Assays. The effect of the synthetic peptides and the anti-(142–155) antibody on APC anticoagulant activity was determined using recently described protocols (Mesters et al., 1991). Peptides were assayed in Xa-1-stage assays using the same procedure previously used for antibodies (Mesters et al., 1991) with the preincubation of peptides with APC and/or factor Xa for 10 min. Since peptide-(142–155) and the scrambled peptide were 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 based on the double-logarithmic plot of clotting time versus factor Xa concentration in the absence of peptide. Therefore, in order to determine the inhibition of APC by the peptide based on the observed APC-induced prolongation of clotting time, different standard curves for each peptide concentration were generated based on the actual factor Xa activity that was observed at the corresponding peptide concentration.

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 as reported for APC (Mesters et al., 1991). Kinetic studies of inhibition of APC by recombinant [Arg^{358}]-α_1-antitrypsin in the absence or presence of peptides, or the anti-(142–155 antibodies were performed and based on measurement of the APC amidolytic activity.

Fibrinogen Clotting. A total of 0.5 unit/mL human thrombin was preincubated with 0–1 mM peptide at five different concentrations in 200 μL of 0.05 M Tris/HCl, 0.1 M NaCl, 0.5% BSA, 0.02% NaN_3 (TBS-BSA), and 2.5 mM $CaCl_2$ for 10 min at 37 °C in polystyrene cuvettes. Reaction was initiated by the addition of 200 μL of 5 mg/mL human fibrinogen in TBS-BSA and 2.5 mM $CaCl_2$, and time (in s) from the addition of fibrinogen to the clot formation was measured in an Electra 700 automatic coagulation timer (Medical Laboratory Automation, Inc., Mount Vernon, NY).

Preparation, Purification, and Characterization of Polyclonal Anti-Peptide-(142–155) Antibody. The polyclonal anti-(142–155) antibodies were obtained by immunizing rabbits with the synthetic peptide-(142–155) coupled to SPDP-activated rabbit serum albumin. After screening antisera for immunoreactivity toward PC, APC, and peptide-(142–155), the antibodies were subsequently immunoaffinity purified on PC-Sepharose employing recently described protocols (Mesters et al., 1991). Unless otherwise specified, only immunoaffinity-purified anti-peptide antibodies are designated as anti-(142–155) antibody.

The specificity of anti-(142–155) antibody toward synthetic peptides, APC, or PC was evaluated by measuring the binding of the antibodies to immobilized peptides, PC, APC, factor IX, factor X, and prothrombin using the previously described ELISA assays (Mesters et al., 1991).

Phospholipid Preparations. Phospholipid vesicles consisting of 20% phosphatidylserine/80% phosphatidylcholine (M/M) were prepared by sonication as described (Mesters et al., 1991).

Inactivation of Factor Va by APC. The APC-catalyzed inactivation of purified factor Va in the presence and absence of phospholipid vesicles was carried out as recently reported (Mesters et al., 1991).

Prothrombinase Assays. Prothrombinase assays were performed as recently described for the factor Va assay using 0.4 nM factor Va (Mesters et al., 1991). In the absence of factors Va, 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. Synthetic peptide 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.

Binding of Peptide-(142–155) to Immobilized Factor Va. Factor Va was coated to the wells of microtiter plates, and the plates were blocked with 0.5% gelatin-TBS and then with 2% bovine serum albumin-TBS, and washed as previously described (Heeb et al., 1993). Wells without factor Va were blocked to be used as controls for nonspecific binding. Peptide-(142–155) and, as a negative control, peptide-(170–184) were incubated at 37 °C for 50 min in the wells at various concentrations in duplicate in 0.5% gelatin, 0.05 M Tris, 0.2 M NaCl, 5 mM $CaCl_2$, 0.1 mM $MnCl_2$, and 0.02% azide, pH

Table I: Effect of Synthetic Peptides on APC Anticoagulant Activity[a]

peptide	amino acid sequence	inhibition of APC (%)[b]
peptide-(36–50)	DTLAFWSKHVDGDQG	0
peptide-(43–57)	KHVDGDQSLVLPLEH	0
peptide-(48–62)	DQGLVLPLEHPGASL	0
peptide-(65–79)	GHGTSIDGIGSFSSD	0
peptide-(81–95)	RSGWEGRFSQREVSF	40
peptide-(110–124)	LEEVGWRRSSSAPGY	0
peptide-(121–135)	APGYKLGDDLLQSHP	0
peptide-(125–139)	KLGDDLLQSHPAVKF	30
peptide-(134–148)	HPAVKFPSGRPWKRM	66
peptide-(142–155)	GRPWKRMEKKRSHLC	80

[a] Inhibition of APC anticoagulant activity was determined in Xa-1-stage coagulation assays in NHP using a final concentration of 500 μM of each of the synthetic peptides. Amino acids are numbered according to Plutzky et al. (1986). Underlined letters in the amino acid sequence indicate substitutions of cysteines in the native sequence by serine or glycine in the peptide. [b] 0 is defined as no observable effect ±10%.

7.4. The wells were washed, and bound peptide was detected as previously described (Heeb et al., 1993), except that the primary antibodies were either rabbit antipeptide-(142–155) antibody, rabbit antipeptide-(170–184) antibody, or rabbit nonimmune IgG controls. The antipeptide antibodies specifically recognized the respective immobilized peptides as well as native PC and APC in controls (data not shown), and they were immunoaffinity purified on a column of PC coupled to Sepharose (Mesters et al., 1991). Nonspecific binding from corresponding duplicate wells that were not coated with factor Va was subtracted from each average of duplicate data points.

RESULTS

Ten synthetic PC peptides (15-mers) derived from the light chain of PC were screened for their ability to inhibit APC anticoagulant activity in Xa-1-stage coagulation assays in NHP at a final peptide concentration of 500 μM. The peptides are numbered according to Plutzky et al. (1986). In order to prevent multiple disulfide formation and subsequent potential polymerization among peptides with multiple cysteines, in some peptides the naturally occurring cysteine was substituted by serine or glycine as indicated in Table I. Table I presents the sequence of the assayed peptides and summarizes the percentage of inhibition of APC activity calculated from the APC standard curve as determined in Xa-1-stage coagulation assays in NHP. Six of 10 peptides had no effect on APC anticoagulant activity at all (Table I). The most potent peptide from the initial screening, peptide-(142–155), inhibited APC anticoagulant activity in a Xa-1-stage coagulation assay in protein S-depleted plasma with 50% inhibition at 5 μM peptide (Figure 1). A similar dose–response of peptide-(142–155) on APC anticoagulant activity was obtained in Xa-1-stage coagulation assay in NHP with 50% inhibition at 25 μM (data not shown). Peptide-(134–148) that shares parts of its sequence with peptide-(142–155) (Table I) inhibited 50% of APC anticoagulant activity in a Xa-1-stage assay in NHP at 250 μM peptide. The scrambled peptide with a randomized sequence of the 142–155 residues showed very little if any significant inhibition of APC anticoagulant activity (Figure 1).

Antipeptide antibodies were prepared and used to assess the implications of the peptide inhibition data. The immunoaffinity purified polyclonal anti-peptide antibody, anti-(142–155), was capable of binding native APC when the antibody was immobilized in the wells of microtiter plates (Figure 2).

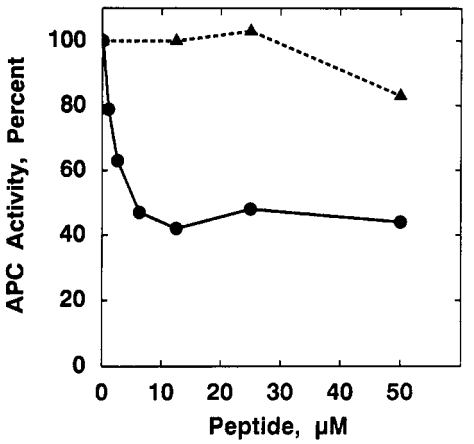

FIGURE 1: Inhibition of APC anticoagulant activity by peptide-(142–155) from the PC sequence in Xa-1-stage coagulation assays. The assays were performed as described under Materials and Methods. Solid lines with solid circles represent inhibition of anticoagulant activity of 30 nM APC by peptide-(142–155) in a Xa-1-stage assay in PSDP; dashed lines with solid triangles represent the activity of 30 nM APC in a Xa-1-stage assay in NHP in the presence of the scrambled peptide. All clotting times were done in duplicate.

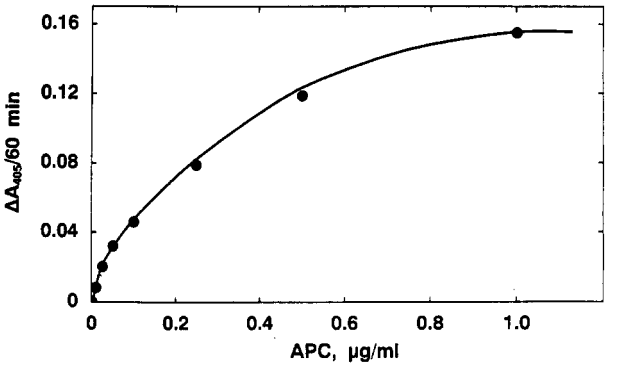

FIGURE 2: Binding of APC to immobilized anti-(142–155) antibody. 10 μg/mL of anti-(142–155) was coated on microtiter wells. After blocking with 10% BSA, wells were incubated with 0–10 μg/mL APC. After washing, bound APC was detected by adding the substrate S-2366 and measuring the change in absorbance at 405 nm over time. All data are mean values of duplicate measurements.

Moreover, the antibody bound to immobilized PC and APC in a saturable manner with the same apparent affinity for immobilized PC or APC (data not shown). Studies were made to investigate whether the anti-(142–155) antibody was specific for PC and APC compared to other vitamin K-dependent serine proteases. The binding of anti-(142–155) antibody to immobilized PC, factor IX, factor X, and prothrombin was measured using solid-phase ELISA methods. When incubating anti-(142–155) antibody in microtiter plate wells coated with PC, peptide-(142–155), factor IX, factor X, or prothrombin, the amount of antibody detected bound to the wells containing PC or peptide-(142–155) was at least 26-fold higher than to the wells coated with factor IX, factor X, or prothrombin (Table II), even though solutions in the latter wells contained 100-fold higher antibody concentrations. Positive controls for the wells with immobilized factor IX, factor X, or prothrombin with the appropriate respective specific antisera confirmed that these proteins were bound to the plate (data not shown). This suggests that anti-(142–155) was greater than several orders of magnitude more specific for PC compared to the other vitamin K-dependent factors tested. The anti-(142–155) antibody inhibited APC anticoagulant activity in APTT assays in NHP as well as in PSDP at 2.5 and 5.0 nM APC final concentration, respectively, with

Table II: Binding of Anti-(142–155) Antibody to Immobilized PC, Peptide-(142–155), Factor IX, Factor X, or Prothrombin[a]

immobilized protein or peptide	anti-(142–155) antibody added (μg/mL)	anti-(142–155) bound (ΔA_{405}/5 min, OD)
protein C	0.5	0.341
peptide-(142–155)	0.5	0.426
factor IX	50.0	0.007
factor X	50.0	0.013
prothrombin	50.0	0.009

[a] 10 μg/mL of each protein or 50 μM of peptide-(142–155) were coated on microtiter wells. After blocking with 10% BSA, wells coated with either PC or the peptide were incubated with 0.5 μg/mL anti-(142–155) antibody, and wells coated with factor IX, factor X, or prothrombin were incubated with 50 μg/mL anti-(142–155) antibody followed by biotinylated–goat–anti–rabbit–IgG and streptavidin–alkaline–phosphatase. Bound IgG was detected by addition of 5 mg/mL *p*-nitrophenylphosphate in 0.1 M diethanolamine, pH 9.6, and measuring change in absorbance over time (ΔA_{450}/5 min). Positive controls with the appropriate respective specific antiserum confirmed that factor IX, factor X, or prothrombin were bound to the plate. Negative controls revealed less than 5% nonspecific binding. All data are mean values of duplicate measurements.

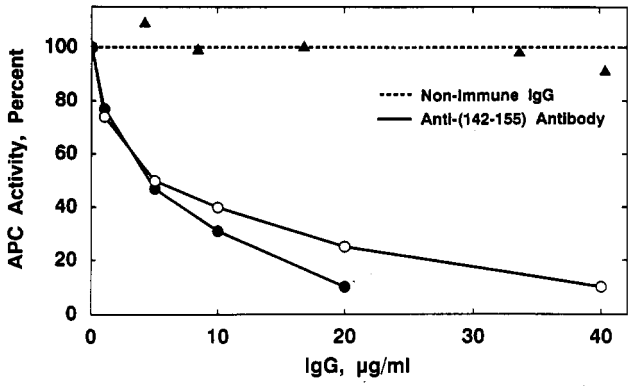

FIGURE 3: Inhibition of APC anticoagulant activity by anti-(142–155) antibody in APTT assays. The assays were performed as described under Materials and Methods, and the anti-(142–155) antibody was preincubated with APC for 30 min at 37 °C. Inhibition of the APC anticoagulant activity by anti-(142–155) antibody was tested in an APTT assay using NHP and 2.5 nM APC (solid circles) and in an APTT assay using PSDP and 5.0 nM APC (open circles). The control indicates the anticoagulant activity of 2.5 nM APC in an APTT in NHP in the presence of nonimmune rabbit-IgG (solid triangles, dashed line).

50% inhibition at 33 nM antibody (Figure 3). A similar dose–response of anti-(142–155) antibody on APC anticoagulant activity was obtained in a Xa-1-stage assay in factor VIII-deficient plasma at 30 nM APC with 50% inhibition of APC activity at 187 nM antibody (data not shown). The antipeptide antibody had no effect on APTT or Xa-1-stage coagulation assays performed in the absence of added APC. Control nonimmune polyclonal rabbit IgG that failed to recognize APC or PC as judged by ELISA assays had no significant effect on APC anticoagulant activity (Figure 3).

Studies were performed to test if the inhibitory peptide or antibody inhibited other enzymatic activities of APC. In contrast to the observations of APC inhibition in the coagulation assays, neither peptide-(142–155) nor anti-(142–155) antibody, when preincubated with APC for 30 min at 37 °C prior to the addition of the chromogenic substrate, had any significant effect on APC amidolytic activity toward the chromogenic substrate S-2366 (Table III). Since 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 et al., 1990), we investigated the effect of

Table III: APC Amidolytic Activity in Presence of Peptide-(142–155) and Anti-(142–155) Antibody toward Chromogenic Substrate S-2366[a]

reagent added	ΔA_{405}/5 min
none	0.374
peptide-(142–155)	0.344
anti-(142–155) antibody	0.376
scrambled peptide	0.364

[a] The assays were performed as described under Materials and Methods. The final concentrations used were 500 μM of peptide-(142–155) and 330 nM anti-(142–155) antibody with 10 nM APC. The amidolytic activity of APC was monitored by the change in absorbance at 405 nm over time at 37 °C. All data are mean values of duplicate measurements.

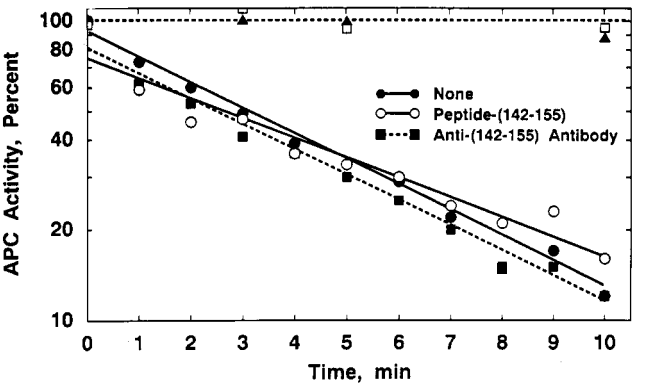

FIGURE 4: Inhibition of APC by recombinant [Arg^{358}]-α_1-AT in the absence or presence of peptide-(142–155) or anti-(142–155) antibody. The assay was performed as described under Materials and Methods. Solid lines with solid circles denote inhibition of APC by [Arg^{358}]-α_1-AT in the absence of peptides. Solid lines with open circles denote inhibition of APC by [Arg^{358}]-α_1-AT in the presence of 500 μM peptide-(142–155), and dashed lines with solid squares denote inhibition of APC by [Arg^{358}]-α_1-AT in the presence of 450 nM anti-(142–155) antibody. APC activity in the absence of [Arg^{358}]-α_1-AT but in the presence of 500 μM peptide-(142–155) is indicated by closed triangles and in the presence of 450 nM anti-(142–155) antibody is indicated by open squares. The APC concentration was 18 nM, and the concentration of [Arg^{358}]-α_1-AT was 100 nM. Linear regression analysis was performed from each set of data (with the exception of the controls), and the correlation ranged from –0.97 to –0.99.

peptide-(142–155) and anti-(142–155) on the interaction of APC with the recombinant mutant [Arg^{358}]-α_1-antitrypsin (AT). Substitution of Met^{358} by Arg in the reactive center of recombinant α_1-AT results in an increase of over 4400-fold in the association rate constant for inhibition of APC (Heeb et al., 1990). Peptide-(142–155) (500 μM final concentration) as well as anti-(142-155) antibody (450 nM final concentration) had no significant effect on the time course of inhibition of APC by recombinant [Arg^{358}]-α_1-AT (Figure 4). Controls showed no significant effect of peptide-(142–155) and anti-(142–155) antibody on APC activity in the absence of [Arg^{358}]-α_1-AT over the course of the experiment (Figure 4).

To see if peptide-(142–155) directly affected recognition of factor Va by APC, studies were made using purified proteins. Peptide-(142–155) was tested for its ability to inhibit APC-catalyzed inactivation of purified factor Va in the presence of phospholipid vesicles. As shown in Figure 5, peptide-(142–155) inhibited the APC-catalyzed inactivation of factor Va with half-maximal inhibition at 50 μM peptide. Moreover, the peptide-(142–155) inhibited the APC-catalyzed inactivation of factor Va in the absence of phospholipids. For example, at 5 nM APC and 40 nM factor Va, the rate of factor Va inactivation under conditions described in Materials and Methods was –4.1% V_a/min in the absence of peptide and –0.5% V_a/min in the presence of 500 μM peptide-(142–155),

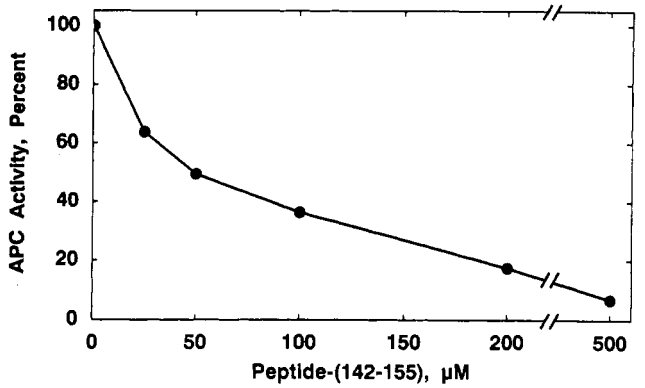

FIGURE 5: APC-catalyzed inactivation of factor Va in the presence of peptide-(142–155) and phospholipids. The assay was carried out in TBS-BSA and 2.5 mM $CaCl_2$, pH 7.4, using 0.25 nM APC, 10.0 nM factor Va, 50 μM phospholipid vesicles, and 0–500 μM peptide-(142–155) that had been preincubated for 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.

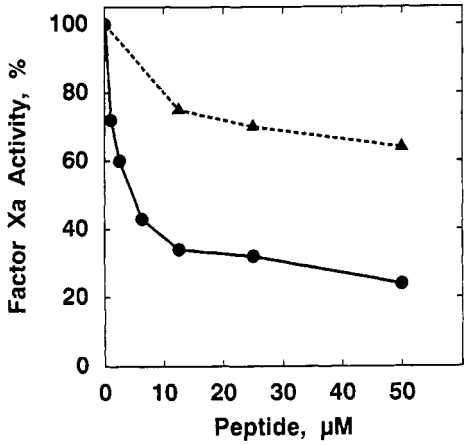

FIGURE 6: Factor Xa clotting activity in the presence of peptide-(142–155). The assay was performed as described under Materials and Methods. Solid circles with solid lines depict factor Xa activity of 0.15 nM factor Xa in NHP in the presence of peptide-(142–155); solid triangles with dashed lines depict factor Xa activity in the presence of the scrambled peptide.

corresponding to 87% inhibition of APC activity in this assay. Thus, the peptide-(142–155) 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-(142–155) was itself anticoagulant. For example, peptide-(142–155) inhibited factor Xa clotting activity in NHP with 50% inhibition at 10 μM peptide. The scrambled peptide with the randomized sequence slightly affected factor Xa activity with 37% inhibition at 50 μM peptide (Figure 6). In order to test whether peptide-(142–155) interferes with the interaction of factor Xa or prothrombin with the cofactor Va, studies using purified proteins were done. Peptide-(142–155) inhibited the generation of thrombin in the prothrombinase assay in the presence of factor Va with 50% inhibition of prothrombinase activity at 40 μM peptide (Figure 7). However, in the absence of factor Va even at concentrations up to 250 μM of peptide-(142–155), no significant effect on prothrombinase activity occurred (Figure 7 and data not shown). A small amount of inhibition of prothrombinase activity was also observed for the scrambled peptide with a randomized sequence in the presence of factor Va but not in the absence of factor Va

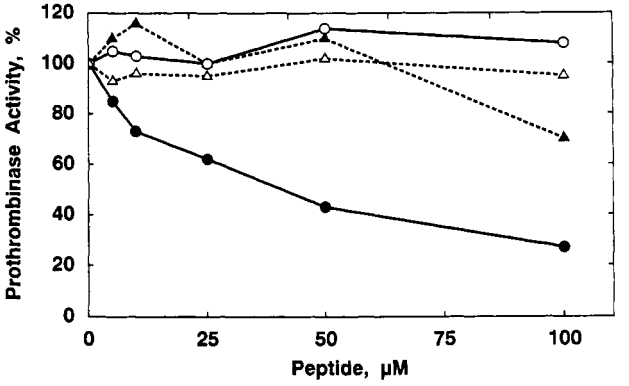

FIGURE 7: Prothrombinase activity in the presence of peptide-(142–155). The assay was performed using 1 nM factor Xa, 0.4 nM factor Va, 1.2 μM prothrombin, and 2.5 nM factor Xa in the absence of factor Va in TBS-BSA, 2.5 mM $CaCl_2$, and 50 μM phospholipid vesicles, pH 7.4. In all cases solid symbols 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-(142–155); solid triangles with dashed lines indicate prothrombinase activity (with factor Va) in the presence of the scrambled peptide. Open circles with solid lines denote prothrombinase activity (without factor Va) in the presence of peptide-(142–155), and open triangles with dashed lines denote prothrombinase activity (without factor Va) in the presence of the scrambled peptide.

Table IV: Factor Xa and Thrombin Amidolytic Activity in Presence of Peptides toward Chromogenic Substrates S-2222 and S-2238, Respectively[a]

enzyme	peptide added	ΔA_{405}/5 min
factor Xa	none	0.223
factor Xa	peptide-(142–155)	0.219
factor Xa	scrambled peptide	0.206
thrombin	none	0.178
thrombin	peptide-(142–155)	0.176
thrombin	scrambled peptide	0.170

[a] The assays were performed as described under Materials and Methods. The final concentrations used were 500 μM of each peptide with 2 nM thrombin and 4 nM factor Xa. The amidolytic activities of either factor Xa or thrombin were monitored by the change in absorbance at 405 nm over time at 37 °C. All data are mean values of duplicate measurements.

(Figure 7). Thus, the ability of peptide-(142–155) to inhibit prothrombinase activity was strictly dependent on the presence of factor Va.

To test if peptide-(142–155) directly inhibited the enzymatic activity of factor Xa or thrombin, further studies were made. Peptide-(142–155) as well as the scrambled peptide did not inhibit the amidolytic activity of factor Xa or thrombin toward the chromogenic substrates S-2222 and S-2238, respectively (Table IV). Furthermore, peptide-(142–155) and the scrambled peptide had no significant effect on the clotting of purified fibrinogen by thrombin when tested at five different peptide concentrations (0–500 μM final) (data not shown).

Experiments were performed to test the hypothesis that peptide-(142–155) binds directly to factor Va. The results in Figure 8 show that peptide-(142–155) bound specifically to immobilized factor Va, as detected by the binding of immunoaffinity purified antipeptide-(142–155) antibodies as described in Materials and Methods. In control studies, no specific binding of peptide-(170–184) to factor Va was detected by antipeptide-(170–184) antibodies. The latter finding is consistent with the observation of a lack of inhibition of APC activity by peptide-(170–184) (Mesters et al., 1991). No binding of either peptide was detected when rabbit IgG was used in place of antipeptide antibodies, and nonspecific binding

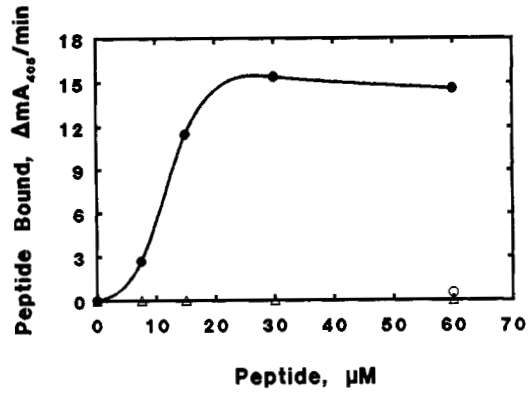

FIGURE 8: Binding of peptide-(142–155) to immobilized factor Va. Factor Va was coated to the wells of a polystyrene microtiter plate, and the plate was blocked. Control blocked wells contained no factor Va. Peptide-(142–155) was incubated in the wells at various concentrations as indicated. Bound peptide was detected with rabbit antipeptide-(142–155) antibody (closed circles) or with rabbit nonimmune IgG (open circle) as a control as indicated in Materials and Methods. As an additional control, peptide-(170–184) (triangles) was incubated in wells coated with factor Va and in control blocked wells. Binding to blocked wells containing no factor Va gave a small background which was subtracted from the observed color change for corresponding wells containing factor Va. Details are given in Materials and Methods.

in the absence of factor Va was subtracted from the average of each duplicate set of data points.

DISCUSSION

The antithrombotic enzyme, APC, a vitamin K-dependent serine protease, exerts its physiologic role by proteolytic inactivation of the blood coagulation cofactors, factors Va and VIIIa (Stenflo, 1976; Kisiel, 1979a,b; Kisiel et al., 1977; Walker et al., 1979; Vehar et al., 1980; Marlar et al., 1982). In this study using synthetic peptides and a specific antipeptide antibody, we have identified a sequence, residues 142–155, in the light chain of human APC that may be important for APC anticoagulant activity and for its interaction with factor Va. The synthetic PC peptide-(142–155), containing the sequence GRPWKRMEKKRSHLC, inhibits APC anticoagulant activity in Xa-1-stage coagulation assays with 50% inhibition at 5 μM peptide in PSDP. Peptide-(134–148) that overlaps with peptide-(142–155) inhibits 50% of APC activity at 250 μM peptide. Peptide-(142–155) inhibits APC anticoagulant activity in NHP as well as in PSDP, suggesting that the inhibitory effect of this peptide is not dependent on the presence of protein S.

Several reports suggest the functional importance of the light chain or the EGF-like regions of APC for the biological activity of APC using different approaches (Johnson et al., 1983; Hill et al., 1987; Öhlin et al., 1987, 1988, 1990). Notably, Ohlin et al. (1990) demonstrated that a light-chain polypeptide fragment could inhibit APC action but that neither the Gla-containing peptide fragment of residues 1–43 nor the fragment containing only the EGF domains could inhibit APC action. However, in this study the synthetic peptides comprising residues from the EGF-like domains either do not significantly or only moderately inhibit APC anticoagulant activity. This finding does not necessarily contradict previous studies since the peptides used herein might not be able to assume appropriate conformations that resemble native EGF-like domains, especially considering the very complex tertiary structure due to multiple disulfide bond formation and the β-hydroxyaspartic acid residue at position 71 in the EGF-1 domain (Öhlin et al., 1990). Nonetheless, it may be noteworthy that peptides-(81–95) and -(125–139) at 0.5 mM gave reproducible though moderate inhibition of APC activity.

Polyclonal antibodies raised against the peptide-(142–155) were immunoaffinity-purified on a PC-Sepharose column, suggesting that at least parts of the region represented by this peptide in PC are exposed and available for interaction with antibodies at the solvent-accessible surface of PC. The immunoaffinity purified anti-(142–155) antibody recognizes the corresponding peptide and PC as well as APC. Similar to peptide-(142–155), the anti-(142–155) antibody potently inhibits APC anticoagulant activity in APTT and Xa-1-stage assays in NHP and PSDP with 50% inhibition at 33 nM antibody in APTT assays. The inhibition of APC by anti-(142–155) antibody is specific since nonimmune rabbit-IgG shows no effect on APC anticoagulant activity and the anti-(142–155) antibody has no effect on the coagulation assays in the absence of APC. Thus, based on these data together with the data obtained using the peptide-(142–155), we conclude that the region of APC comprising residues 142–155 is essential for activity and that this region is not involved in APC interactions with protein S.

Furthermore, peptide-(142–155) and the anti-(142–155) antibody do 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-(142–155) and anti-(142–155) antibody do not affect the reactivity of the active site serine, histidine, and aspartic acid residues and do not exert their inhibition of APC anticoagulant activity by blocking primary substrate binding sites close to the active site serine that are responsible for normal cleavage of a small tripeptide substrate like S-2366 or for recognition of the recombinant 55 000 M_r α_1-AT.

The observation that peptide-(142–155) inhibits APC-catalyzed inactivation of purified factor Va in the presence as well as in the absence of phospholipids with 50% inhibition at 50 μM suggests that the region of residues 142–155 represents an exosite on APC essential for its anticoagulant activity and for the recognition of its macromolecular substrate factor Va. This observation furthermore excludes the possibility that peptide-(142–155) inhibits binding of APC or its substrate factor Va to phospholipids. This proposed exosite is not involved in small substrate or serpin recognition immediately near the active site or in APC interactions with protein S. The data showing that the anti-(142–155) antibody inhibits APC anticoagulant activity is consistent with the existence and importance of the suggested exosite involving residues 142–155.

A surprising finding was that peptide-(142–155) alone inhibits factor Xa clotting activity, either in clotting assays in plasma with 50% inhibition at 5 μM peptide or in prothrombinase assays with 50% inhibition at 40 μM peptide under the conditions tested. 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. The scrambled peptide with a randomized sequence of peptide-(142–155), although less potent than peptide-(142–155), observably inhibits factor Xa clotting activity and prothrombinase activity in the presence of factor Va but not in the absence of factor Va with 40% inhibition at 50 and 100 μM peptide, respectively. In this regard, it should be mentioned that the synthetic peptide-(182–196) derived from the heavy chain of PC and containing the sequence PWQVVLLDSKKKLAC as well as the peptide with the reverse sequence inhibited factor Xa clotting activity

with 50% inhibition at 12 and 100 μM peptide, respectively (Mesters and Griffin, unpublished data). This might suggest some degree of nonspecific interactions of certain synthetic peptides with relatively high positive net charge with factor Va. Peptide-(142–155) as well as the scrambled peptide, however, have 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 peptides have anticoagulant properties because they bind to the active site of factor Xa or thrombin or because they interfere with thrombin interaction with fibrinogen or fibrin polymerization. These data in combination with the inhibitory effect of peptide-(142–155) on APC inactivation of factor Va suggest that the sequence of residues 142–155 in APC provides a site for binding factor Va and that the synthetic peptide-(142–155) binds to factor Va, thereby interfering with both inactivation of factor Va by APC and expression of factor Xa activity in the prothrombinase complex. Consistent with this hypothesis was the demonstration that peptide-(142–155) binds to immobilized factor Va (Figure 8).

Previously we suggested that the regions comprising residues 390–404 and residues 311–325 represent exosites for the binding of APC to the macromolecular substrate, factor Va, and possibly factor VIIIa (Mesters et al., 1991, 1993). Thus, it seems that APC may have at least three spatially distinct exosites for recognition of factor Va, namely, the regions of residues 390–404, 311–325, and 142–155. This suggests that the interactions of APC with its substrate factor Va are remarkably extensive. The three peptides representing these sequences inhibit both APC anticoagulant activity and APC inactivation of purified factor Va. However, peptide-(311–325) (Mester & Griffin, 1991; Mesters et al., 1993) and peptide-(142–155) remarkably exhibit potent anticoagulant properties both in clotting assays and in prothrombinase assays, a property that peptide-(390–404) lacks. Studies with purified proteins showed inhibition of prothrombinase activity requires factor Va. Since these two peptides inhibit inactivation of purified factor Va by APC, they most likely exert their inhibitory effect on APC activity by binding to factor Va, and studies indeed demonstrated that factor Va binds peptides-(142–155) and -(311–325) (Figure 8 and Mesters et al., 1993). The regions on factor Va to which peptides -(142–155) or -(311-325) and peptide-(390–404) bind must be topologically distinct on factor Va since peptides-(142–155) and -(311–325) but not -(390–404) inhibit the expression of factor Va-dependent prothrombinase activity. Factor Xa protects factor Va from inactivation by APC (Comp et al., 1979; Walker et al., 1979; Nesheim et al., 1982; Suzuki et al., 1983), suggesting that both enzymes bind, in part, to identical or adjacent regions on factor Va. In this case, peptides-(142–155) and -(311–325) presumably bind to such hypothesized common binding sites in factor Va, thereby blocking both binding of APC and binding of factor Xa. Albeit less likely, it is also possible that peptide-(142–155) or -(311–325) blocks binding of prothrombin to factor Va in the prothrombinase complex or that either peptide induces a conformational change in factor Va, thus rendering it much less active as a cofactor.

Although some binding sites on factor Va for APC and factor Xa must be identical or closely adjacent since molecules as small as pentadecapeptides compete for each interaction (Mesters et al., 1993; Chattopadhyay et al., 1992), the modes of binding of APC and factor Xa are certainly distinct and nonidentical since APC bound to factor Va proteolytically inactivates factor Va, whereas factor Xa bound to factor Va proteolytically activates prothrombin. A synthetic peptide comprising residues 263–274 of factor X inhibits interactions between factors Xa and Va and could be cross-linked to factor Va (Chattopadhyay et al., 1992), and a peptide comprising residues 404–418 of factor X inhibited prothrombinase activity only in the presence of factor Va (Mesters & Griffin, unpublished data). Thus, we speculate that very positively charged sequences of APC or factor Xa may bind to factor Va, e.g., part of the KRMEKKRSH sequence (APC residues 142–155), the KRNR sequence (APC residues 311–314), the KHNR sequence (factor X residues 270–273), and the RKGK sequence (factor X residues 406–409). These observations imply that a sequence of basic–basic–*x*–basic residues may provide a tetrapeptide motif in APC and factor Xa for binding factor Va (Mesters et al., 1993).

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