A Chimeric Protein C Containing the Prothrombin Gla Domain Exhibits Increased Anticoagulant Activity and Altered Phospholipid Specificity*

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Mikhail D. Smirnov‡, Omid Safa‡, Lisa Regan§, Timothy Mather‡,
Deborah J. Stearns-Kurosawa‡, Shinichiro Kurosawa‡, Alireza R. Rezaie‡,
Naomi L. Esmon‡, and Charles T. Esmon§**

From the ‡Cardiovascular Biology Research Program, Oklahoma Medical Research Foundation, the §Howard Hughes Medical Institute, and the Departments of Pathology and Biochemistry and Molecular Biology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73104

To determine the structural basis of phosphatidylethanolamine (PE)-dependent activated protein C (APC) activity, we prepared a chimeric molecule in which the Gla domain and hydrophobic stack of protein C were replaced with the corresponding region of prothrombin. APC inactivation of factor Va was enhanced 10–20-fold by PE. Protein S enhanced inactivation 2-fold and independently of PE. PE and protein S had little effect on the activity of the chimera. Factor Va inactivation by APC was approximately 5-fold less efficient than with the chimera on vesicles lacking PE and slightly more efficient on vesicles containing PE. The cleavage patterns of factor Va by APC and the chimera were similar, and PE enhanced the rate of Arg506 and Arg306 cleavage by APC, but not the chimera. APC and the chimera bound to phosphatidylserine:phosphatidylcholine vesicles with complement solely of phosphatidylethanolamine (PE) or cardiolipin potently enhanced the rate of inactivation. Subsequently, roles for PE in factor VIII binding (8), tissue factor-factor VIIa activation of factor X (9), and prothrombin activation (10, 11) have been reported. In the case of prothrombin activation, with PE present, the amount of PS required to support prothrombin activation was reduced severalfold. In the case of tissue factor, it was shown that the presence of PE enhanced activation primarily by decreasing the amount of PS required for optimal activation, and this was largely a K_d effect on the substrate. The impact of PE on the inactivation of factor Va was substantially greater than on the other systems. For prothrombin activation and tissue factor-mediated factor X activation, the augmentation by PE could be overcome simply by increasing PS concentration, whereas the PE impact on factor Va inactivation was not eliminated by similar increases in PS concentration (7).

Protein C, like the other vitamin K-dependent proteins, is composed of several domains (9). These include the protease domain, two epidermal growth factor-like domains, an aromatic stack, and the vitamin K-dependent Gla domain contain-

Assembly of multiprotein enzyme complexes on negatively charged phospholipid membrane surfaces is critical to both the amplification of the blood clotting process and its negative regulation. Zymogen activations occur rapidly when the enzyme, usually a vitamin K-dependent protein, binds to a cofactor, usually a non-vitamin K-dependent protein, to activate a substrate, usually a vitamin K-dependent protein (reviewed in Refs. 1–3). The enzymes and substrates interact with the membrane reversibly, while the cofactors may either bind reversibly or be integral membrane proteins. The nature of the phospholipid head group appears to contribute to catalytic and binding efficiency, with phosphatidylserine (PS) being generally accepted as the most important phospholipid (2, 4). The vast majority of biophysical and kinetic studies of the assembly of the vitamin K dependent complexes have used membranes composed solely of phosphatidylcholine (PC) and PS (1, 2, 5).

Recently, we observed that phosphatidylethanolamine (PE), a major constituent of the outer leaflet of the membrane of activated platelets (6), plays an important role in the function of one of these complexes, the activated protein C (APC)-dependent inactivation of factor Va (7). In this case, the presence of PE or cardiolipin potently enhanced the rate of inactivation. Subsequently, roles for PE in factor VIII binding (8), tissue factor-factor VIIa activation of factor X (9), and prothrombin activation (10, 11) have been reported. In the case of prothrombin activation, with PE present, the amount of PS required to support prothrombin activation was reduced severalfold. In the case of tissue factor, it was shown that the presence of PE enhanced activation primarily by decreasing the amount of PS required for optimal activation, and this was largely a K_d effect on the substrate. The impact of PE on the inactivation of factor Va was substantially greater than on the other systems. For prothrombin activation and tissue factor-mediated factor X activation, the augmentation by PE could be overcome simply by increasing PS concentration, whereas the PE impact on factor Va inactivation was not eliminated by similar increases in PS concentration (7).

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** An investigator of the Howard Hughes Medical Institute, To whom correspondence should be addressed: Oklahoma Medical Research Foundation, Cardiovascular Biology Research, 825 N.E. 13th St., Oklahoma City, OK 73104. Tel.: 405-271-6474; Fax: 405-271-3137.

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1 The abbreviations used are: PS, phosphatidylserine; APC, activated protein C; PC-PT Gla, a chimeric molecule in which the Gla domain and hydrophobic stack (residues 1–46) of protein C have been replaced with the corresponding regions of prothrombin; PC-PT Gla 1–22, a chimeric molecule in which the Gla domain and hydrophobic stack (residues 1–46) of protein C have been replaced with the corresponding region of prothrombin; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PPACK, S-Phenyl-O-Acetyl-Arg-chloromethyl ketone; X-CP, the factor X activator from Russell’s viper venom; BSA, bovine serum albumin; MOPS, 4-morpholinepropanesulfonic acid.
ing the Gla residues. These Gla residues are involved in Ca\(^{2+}\)-dependent membrane binding and are clustered within the amino-terminal 48 residues of the vitamin K-dependent plasma factors (1, 3, 12). The sequences of these proteins within this region are similar, but the number of Gla residues varies from 9 to 12 (3). Since the Gla domains are implicated in the membrane binding and membrane-dependent catalytic activity (1, 13, 14), it is possible that the differences in PE-dependent behavior between protein C and the other complexes might be mediated by the Gla domains. To test this possibility, we have prepared a chimeric form of protein C in which the entire Gla domain or only the portion from residue 1 to 22 has been exchanged with that of prothrombin in an effort to evaluate the regions of the molecules involved in the PE-dependent activities. Other chimeric proteins with protein C with the Gla domain of factor VII (15) and factor IX (16) have been prepared previously and are known to be active, but these have not been examined for their PE dependence or their ability to interact with protein S.

The mechanisms by which APC inactivates factor Va in biological systems are complex and not fully understood. It is known that cleavage at Arg\(^{306}\) results in partial loss of activity (17), but the extent of inactivation is dependent on the assay conditions with high factor Xa levels resulting in lower apparent inactivation. Cleavage of Arg\(^{306}\) leads to total inactivation (18) and the release of a portion of the factor Va molecule (19). The function of protein S in this process is controversial. In purified systems, protein S enhances the rate of factor Va inactivation about 2-fold (Ref. 7 and references therein). In plasma, however, APC anticoagulant activity is strongly dependent on protein S. When protein S is removed, it requires approximately 10-fold more APC to achieve the same anticoagulant effect. One function of protein S appears to be to favor cleavage of Arg306 (20). Factor Xa can protect factor Va from inactivation by APC (21, 22), but this protection is primarily due to blocking cleavage at Arg\(^{306}\) (20). Protein S overcomes the factor Xa protection of factor Va (20, 22). It has been difficult to detect protein S interaction with APC in solution, but interaction has been detected with bovine APC by light scattering (23) and with human APC by fluorescence energy transfer approaches on membranes (24). The change in active site-membrane distance brought about through interaction with protein S (24) may contribute to the selective effect on cleavage at Arg\(^{306}\). Protein S and factor V have also been shown to work synergistically in the inactivation of factor Va by APC (25, 26). In addition, a wide variety of studies have suggested that protein S contributes to the plasma anticoagulant response by binding to factor Xa (27) or factor Va (28) and interfering with prothrombin activation (27–31). The importance of understanding the mechanisms by which the system is modulated stems from the clinical observations that protein C and protein S deficiency create thrombotic risk and that one of the most common risk factors related to venous thrombosis involves a substitution of Arg with Gln at position 506 in factor V, a trait referred to as APC resistance or factor V Leiden (32). Development of an APC mutant that does not interact with protein S would provide a useful approach for testing the importance of the direct inhibition of prothrombinase in the anticoagulant activity of APC in plasma and in our understanding of how protein S functions.

EXPERIMENTAL PROCEDURES

Proteins and Reagents—Human thrombin and prothrombin (33), human APC (34), human protein S (35), human factor Xa (36), bovine factor Va (37), and the factor X activator from Russell’s viper venom (X-CP) (38) were prepared as described previously. Meizothrombin labeled in the active site with fluorescein was prepared as described by Armstrong et al. (39) and Bock (40). Recombinant human protein C was a generous gift from Dr. Brian Grinnell (Lilly). Bovine serum albumin (BSA), Russell’s viper venom, ovalbumin, gelatin, MOPS, Tris-HCl, and salts were from Sigma. The chromogenic substrates Spectrozyme TH and Spectrozyme PCa were from American Diagnostica (Greenwich, Conn.). Palmitoyl-2-ethyl-sn-glycero-3-phosphoethanolamine (1 mM) (Sigma), phenylmethylsulfonyl fluoride (100 mM) (Sigma), β-Pro-Arg-chloromethyl ketone (PPACK, 5 mM) (Calbiochem), β-Glu-Gly-Arg-chloromethyl ketone (5 mM) (Calbiochem), leupeptin hemisulfate (1 mM) (Calbiochem), aprotinin (5 μg/ml) (Calbiochem), and benzamidine chloride (10 mM). PEG 8000 was used in place of the PEG 6000. After barium citrate elution, the material was immunopurified using a monoclonal antibody, V227, which was prepared by standard techniques (34). The antibody was linked to Affi-Gel 10 (Bio-Rad) at 5 mg/ml. The partially purified factor V from 2 liters of plasma in 0.1 M NaCl, 0.02 mM Tris-HCl, pH 7.5 (TBS), 10 mM benzamidine was batch-absorbed with 25 ml of antibody resin for 3 h, poured into a 2.5 × 10- cm column, and washed rapidly with TBS, 5 mM benzamidine (100 ml), 0.5 M NaCl, 0.02 mM Tris-HCl (7.5 ml) and TBS (100 ml) and TBS (100 ml) and TBS (100 ml) and TBS (100 ml). Factor Va was eluted with 50% ethylene glycol, 1 mM MOPS, pH 7.5, 1.5 mM Ca\(^{2+}\) at 5 μl/h. After dialysis into TBS, 1 mM Ca\(^{2+}\), the factor V was activated by incubation with 1 unit/ml bovine thrombin at 37 °C for 10 min. The thrombin was inactivated by the addition of p-(aminodiphenyl)-methanesulfonyl fluoride (5 mM) (Calbiochem). The factor Va for all other experiments was from Hematologics Technology. The specific activity of the two preparations was equivalent, but the commercial material had too many additional cleavage products within the preparation to allow gel time course analysis.

Preparation of Phospholipid Vesicles—Sonicated or extruded vesicles were prepared as described (7). Unless indicated, PS/PC vesicles were 75% PS, 20% PC, and PE/PS/PC vesicles were 50% PE, 20% PS, 30% PC. Sonicated vesicles were used for light scattering and adsorption to the latex beads. These vesicles were characterized by electron microscopy. PS/PC vesicles were 20–25 nm in diameter, and the PE/PS/PC vesicles were 100–200 nm (42). Briefly, lipids were mixed in the weight proportions indicated, dried under argon, and lyophilized overnight to remove organic solvents. They were then reconstituted in 0.15 M NaCl, 0.02 mM Tris-HCl, pH 7.4, 0.02% sodium azide to 2 mg of total lipid/ml and sonicated (Bronson Sonic Power Co., model 350) for 15 min in an ice-water bath under argon flow, centrifuged at 8000 × g for 15 min, and filtered through a 0.22-μm filter. For kinetic and clotting studies, 100-nm vesicles were prepared with a 100-nm Nucleopore membrane in an extruder (Avestin) to ensure comparable size. The vesicles were used immediately or stored at +20 °C. Storage did not alter vesicle activity.

Construction of the Protein C Prothrombin Gla Domain Chimer—The chimeric protein C was constructed in which the first three exons of prothrombin (i.e. coding for the signal peptide, the Gla domain, and the aromatic stack regions) replaced the corresponding regions of protein C. The construction, expression, and isolation of the chimera was done by standard methods as described previously (43). A separate chimera, PC-PT Gla 1–22, was prepared to map the PE dependence further. PC-PT Gla 1–22 is a chimera protein C molecule in which the signal peptide and residues 1–22 of the prothrombin Gla domain replaced the corresponding residues of protein C. This construct was prepared by two rounds of polymerase chain reaction amplification of protein C and prothrombin cDNA. In the first round, the protein C cDNA was amplified with the sense primer 5′-ctggctgaggaagactGACCTGCGAGGAGCCAAAGGA-3′ (primer 1), which contains 18 bases (lowercase) from the prothrombin Gla domain (coding for residues Cys\(^{17}\)-Cys\(^{25}\)) and starts from the Asp\(^{23}\) of protein C. The antisense primer 5′-CTGAGGCGATCTGACTAGTGGGCCAG-GTTCCTGG-3′ (primer 2) includes the stop codon of protein C (underlined) and contains an XhoI restriction site for cloning purposes. The prothrombin Gla (1–22) domain was amplified from the PC-PT Gla DNA using the sense primer 5′-CGATCGATGCGACTGCTGAGGCG-3′ (primer 3), which contains 18 bases (lowercase) from the prothrombin Gla domain and contains a HindIII restriction site and the antisense primer 5′-ctggctgaggaagactGACCTGCGAGGAGCCAAAGGA-3′ (primer 4), which extends from Gla\(^{16}\) (Glu\(^{16}\)) to Cys\(^{27}\) and also contains 18 bases from the protein C Gla domain starting at Asp\(^{23}\} (lowercase). The polymerase chain reaction-amplified DNA fragments were isolated by standard procedures. These fragments were combined...
and then used as a template for a second round of polymerase chain reaction amplification using primers 2 and 3. The final product was digested with HindIII and XhoI and subcloned into the identical sites of pRc/RSV (Invitrogen, San Diego, CA), expressed and isolated as described previously (43). The mutations were confirmed by DNA sequencing.

Gla residue determinations were kindly performed by Dr. Betty Yan and Cindy Payne at Eli Lilly Research Laboratory or in our laboratory (44). The Gla content per mol of protein obtained was as follows: 8.7 ± 0.3 for protein C, 10.9 ± 0.2 for prothrombin, 9.5 ± 0.2 for PC-PT Gla, and 10.3 ± 0.4 for APC-PT Gla.

Measurement of APC and Prothrombin Activity in the Purified System—Factor Va inactivation was analyzed with a three-stage assay essentially as described (see Ref. 7 for experimental details). All reactions were run at room temperature in 96-well polyvinyl chloride plates (Costar). All reagents were diluted in TBS containing 1 mg/ml gelatin, 1 mg/ml ovalbumin, and 10 mg/ml BSA (TBS-GOB). Briefly, factor Va (0.2 nM unless noted) was incubated with APC or the chimera in the first stage for 30 min with phospholipid in the presence or absence of protein S. The concentrations of reactants are indicated in the figure legends. In the second stage, after inhibition of APC with N-(α-aminodiphenyl)-methanesulfonyl fluoride (30 μM), residual factor Va activity was monitored by its ability to enhance prothrombin activation in the presence of excess factor Xa (1 nM), prothrombin (1.4 μM), and 5 mM CaCl2. In experiments utilizing 20 nM factor Va in the first stage, because dilution was necessary, additional lipid (PS/PC, 10 μg/ml) was also added in this stage. After 5 min of activation, the reaction was stopped by the addition of EDTA, and the resultant thrombin was measured in the third stage using a chromogenic assay. Remaining factor Va activity was calculated by dividing thrombin formation in the presence of APC by thrombin formation in its absence.

Clotting Assays—Clotting was initiated at the level of factor X activation by use of X-CP. All reagents were diluted in TBS containing 1 mg/ml gelatin. Assays were performed in 96-well plates. To dilutions of APC (20 μl) were added 10 μl of phospholipid at the concentrations indicated in the figure legends, 10 μl of 20 ng/ml X-CP, and 20 μl of normal pooled plasma. The entire mixture was incubated for 1 min. Clotting was initiated by the addition of 25 μl of 20 mM CaCl2. The clotting time was determined on a Vmax Kinetic Microplate Reader as the time to reach the threshold optical density value of 0.02 at 405 nm above the plasma reading before the initiation of clotting. This value corresponds to ~10% of the increase in optical density of fully clotted plasma. The progress curves were examined for all reactions to ensure that this cut off accurately reflected the clotting process.

Adsortion of Liposomes onto Latex—A 10% suspension of polystyrene latex beads (Sigma) (50 μl) was pelleted in Eppendorf tubes, washed three times with TBS by centrifugation at 8,000 × g for 1 min, and resuspended in 50 μl of TBS, 5 mM CaCl2. Liposomes (100 μl at 1 mg/ml total phospholipid in TBS) were added and incubated 2 h at 37°C with shaking. After two washes, the beads were resuspended in TBS-GOB and further incubated for 2 h at room temperature with shaking. An additional wash in TBS-GOB, the beads were resuspended in 500 μl of TBS. Total phospholipid concentration was determined by counting the 14C-PC tracer included in the phospholipid mixture (Beckman model LS 6000SE scintillation counter) and found to be 50 μg/ml latex suspension for both PS/PC and PE/PS/PC adsorbed liposomes. The beads could be stored at 4°C for at least 7 days without loss of adsorbed phospholipid.

Fluorescent Labeling—Active site fluorescein-labeled enzymes were prepared according to the method of Bock (40). Briefly, to 300 μl of enzyme (1 mg/ml) were added 40 μl of 1 M Hepes, pH 7.4, 1 μl of 0.2% EDTA, and 2× 5 μl of N-(α-acetylthioacetyl)-Phe-Pro-Arg-CH2Cl (4 mM), 10 μl of EDA, to form ATTA-PPF-enzyme. After overnight dialysis, 45 μl of hydroxyamine (1 M in 1 M Hepes, pH 7.4) and 50 μl of 5-(iodoacetamido)fluorescein (Molecular Probes; 1 mg/ml in 1 M Hepes, pH 7.4) were added to the ATA-PPF-enzymes and incubated for 1 h at 4°C. Free fluorescein was removed by gel filtration on a PD-10 column (Amersham Pharmacia Biotech), and the samples were then dialyzed overnight at 4°C. With this method, each molecule of labeled enzyme contains a single dye at the same location; thus, all of the fluorescence behave equivalently.

Binding of Fluorescein-labeled Proteins to Liposomes Absorbed on Latex—Liposome-adsorbed latex beads (0.5 μg of total phospholipid/ml) were suspended in TBS-GOB containing 2.5 mM CaCl2. Appropriate protein(s) were added at the concentrations indicated and incubated at 25°C for 20 min in the dark with occasional mixing. Binding was analyzed on a FACScan flow cytometer (Becton Dickinson). To determine the calcium-independent, irreversible component of the fluorescence-labeled protein binding, 50 mM EDTA was added in 200 mM MOPS, pH 7.4, to a final concentration of 10 mM, and samples were reanalyzed after a 20-min incubation in the dark. This component accounted for 20% of the observed binding.

Binding parameters were determined by fitting the calcium-dependent binding to the equation for single site binding model using the ENZFITTER program (Elsevier Biosoft, Cambridge, UK).

Liposome-Protein Interactions Measured by Right Angle Light Scattering—Right angle light scattering was performed as described by the Nelsestuen (5) and Castellino (45) laboratories on an SLM 8000 fluorometer (SLM Instruments, Urbana, IL) with the wavelength set at 320 nm. Protein C in the presence of APC had an elasticity of 50 mN/m. Binding experiments were performed in TBS, pH 7.4, containing 5 mM CaCl2. The prothrombin and protein C concentrations were varied from 0 to 3 μM, and the PC-PT Gla concentration was varied from 0 to 1.2 μM. To avoid possible differences in liposome preparations, all binding experiments were performed on the same day with the same lot of liposomes. Similar results were obtained on separate experiments performed on two separate days. Binding parameters were determined by fitting the irreversible calcium-dependent binding to the equation for single-site binding model using the ENZFITTER program. The free concentration of proteins was calculated as described by Nelsestuen's laboratory (5).

Electrophoresis—SDS-polyacrylamide gel electrophoresis was performed with either 10% or 6–20% gradient acrylamide gels with the Laemmli buffer system (46). For Western blots, gels were transferred to polyvinylidene difluoride membranes (Millipore Corp.) in a semidydry apparatus (Bio-Rad). Membranes were blocked with 2% BSA in TBS and reacted with an affinity-purified goat anti-bovine factor Va heavy chain immunoglobulin preparation followed by rabbit anti-goat IgG-alkaline phosphatase conjugate (Pierce). The blots were developed with 3-APPhos reagent (Vistra fluorescence Western kit; Amersham Pharmacia Biotech) and imaged on a Storm PhosphorImager (Molecular Dynamics).

MODELING—Homology models of the Gla domains of human prothrombin and protein C were constructed using the x-ray crystal structures of bovine prothrombin (Protein Data Bank accession number 2pf2 (47)) and factor VII (1dan; Ref. 48). In cases of residue substitution, the peptide was taken from the crystal structure of the vicinity. Residues 4033–9033 were included and the Gla domain, we felt that the small differences in the Gla domain sequence between protein C and prothrombin might account for the differences in PE dependence. To test this possibility, we prepared a chimeric form of protein C in which residues 1–46 of protein C were replaced with the corresponding residues in prothrombin to form the chimera, PC-PT Gla. Purified PC-PT Gla was subjected to SDS gel electrophoresis before and after disulfide reduction. As expected, the protein migrated as a doublet of approximately 62 kDa before reduction. After reduction, the heavy chain migrated as a doublet with a mass of approximately 40-kDa, and a light chain was observed (Fig. 1). The masses of the chains with and without reduction were similar to those of protein C. A small amount of single chain chimera was also present. Previous studies with mutations of the cleavage site in protein C have shown that the single chain and two-chain protein C have similar activities (49). As anticipated, PC-PT Gla could be activated to form an enzyme with amidolytic activity toward Spectrozyme PCa equivalent to wild type APC (data not shown).

We next compared the concentration dependence of inactivation of factor Va between APC (Fig. 2A) and APC-PT Gla (Fig. 2B) on vesicles with or without PE supplementation. On extruded vesicles composed solely of PS/PC, APC-PT Gla was approximately 5 times more active than wild type APC. PE had
APC-PT Gla.

Lanes 1 and 5, plasma-derived protein C; lanes 2 and 4, recombinant PC-PT Gla; lane 3, molecular weight markers.

Little effect on factor Va inactivation by APC-PT Gla, although in some experiments, approximately a 50% increase in activity could be observed. In addition, protein S inhibited factor Va inactivation by the APC-PT Gla, whereas protein S enhanced factor Va inactivation by APC. These effects were not PE-dependent. Therefore, it would appear that much of the PE dependence of APC is mediated by the Gla domain and that at least some portion of the Gla domain is important for protein S-mediated effects in purified systems. To ensure that differences between the APC-PT Gla and plasma-derived APC were not due to the processing of the recombinant protein, we compared the anticoagulant activities and phospholipid binding characteristics of the recombinant and plasma-derived APC. Recombinant and plasma-derived APC had exactly the same anticoagulant activity and PE dependence in plasma coagulation tests (data not shown) and bound phospholipids indistinguishably (see below). Because the plasma-derived material is much less expensive, all results shown in this paper were performed with plasma-derived protein C or APC.

These comparisons in enzyme activity are valid only if the rate of factor Va inactivation is directly dependent on APC concentration. This is the anticipated situation under these experimental conditions, since APC inactivation of factor Va occurs on the membrane surface and since the concentration of the enzyme is far below the \( K_{d(app)} \) for the liposomes. Factor Va inactivation is complex, however, and a simple rate calculation is difficult. Therefore, we examined the time course of factor Va inactivation with different APC concentrations on PS/PC vesicles that either did or did not contain PE (Fig. 3A). A 20-fold increase in APC concentration was required to achieve a time course of factor Va inactivation similar to that on PE-containing vesicles when PE was not present. In contrast, the time courses of factor Va inactivation by the APC-PT Gla at the same enzyme concentration were quite similar with or without PE in the liposomes (Fig. 3B). Thus, PE enhances the rate of factor Va inactivation by APC to a much greater extent than APC-PT Gla, and comparisons of the enzyme concentration dependence of factor Va inactivation at a set time give a valid indication of the relative activities of different enzyme forms.

To determine if the sequence of bond cleavage or the nature of the cleavage sites was altered by PE or APC-PT Gla, we examined the time course of factor Va inactivation by APC or the APC-PT Gla by SDS gel electrophoresis (data not shown). PE increased the rate of cleavage of the factor Va by APC. The band corresponding to cleavage at position 506 appeared first, followed by the appearance of a band corresponding to the 306 cleavage product. Protein S also increased the rate of appearance of the 306 cleavage product, and PE altered the cleavage pattern by APC-PT Gla. In our hands, the transfer variability was too large to allow accurate quantitation of the blots.

To ascertain whether the increased factor Va inactivation observed with the APC-PT Gla relative to APC on PS/PC vesicles was due to differences in phospholipid affinity, we performed light scattering experiments with prothrombin, protein C, or PC-PT Gla and the activated forms of these proteins on...
binding to phospholipid membranes. Proteins were added to sonicated liposomes (50 μg/ml) in TBS, pH 7.4, containing 5 mM CaCl₂. Protein binding was measured by right angle light scattering as described under “Experimental Procedures.” A, binding of prothrombin and binding of the protein C zymogens were compared on 20% PS, 80% PC liposomes (open symbols) and 50% PS, 50% PC liposomes (closed symbols). ○ and ●, prothrombin; ▼ and ▲, protein C; △ and ◆, PC-PT Gla. B, the binding of PC-PT Gla (●), PPACK-APC-PT Gla (◆), plasma-derived protein C (○), PPACK-APC (▲), recombinant protein C (◇), and PPACK-recombinant APC (▼) was compared on 20% PS, 80% PC vesicles.

Fig. 4. Comparison of protein C, PC-PT Gla, and prothrombin binding to phospholipid membranes. Proteins were added to sonicated liposomes containing either 20 or 50% PS (Fig. 4A). More prothrombin than protein C or PC-PT Gla bound to the vesicles containing 20% PS. The Kₐ(app) values, however, were similar for all of these proteins. Increasing the PS concentration to 50% increased the amount of protein C and PC-PT Gla binding more than 2-fold but did not change the affinity of protein C appreciably. Increasing the PS content of the liposomes had a relatively small effect on prothrombin binding. PC-PT Gla bound to membranes at least as well as wild type protein C, but the affinity was not significantly better than wild type and hence cannot account for the increased activity on PS/PC vesicles. The differences in maximum binding between protein C and prothrombin presumably reflect the maximum number of molecules bound per liposome and the approximately 20% larger molecular mass of prothrombin. On a separate vesicle preparation, the binding of recombinant and plasma-derived APC were compared as were the binding of activated and zymogen forms (Fig. 4B). The recombinant and plasma-derived protein C binding were indistinguishable. As expected, the activation of protein C or the PC-PT Gla did not influence phospholipid binding.

It is possible that the differences in activity between APC and APC-PT Gla reflect differences in interaction with other protein components. Light scattering approaches are not suitable to investigate these possibilities. Furthermore, PE-containing vesicles are too large to utilize in light scattering approaches. Therefore, we had to employ different binding methodologies that would allow the presence of PE and/or other protein components. This was accomplished by flow cytometry. Liposomes adsorbed to latex were employed, and binding was monitored as a function of increasing fluorescent enzyme concentration. On PS/PC vesicles, the concentration dependence of binding of protein C by light scattering and the concentration dependence of APC binding to latex-adsorbed vesicles were indistinguishable, thereby validating this approach (Fig. 5). Furthermore, the Kₐ(app) values for prothrombin and meizothrombin were similar, as determined by light scattering, and the meizothrombin binding was equivalent by the two methods, further validating this approach (Table I).

We then utilized this approach to compare the affinities of APC and APC-PT Gla on vesicles with and without PE and in the presence of protein S and factor Va. These data are summarized in Table I. The major feature distinguishing wild type APC and APC-PT Gla is the degree to which protein S and factor Va synergize to augment membrane binding. Comparison of APC-PT Gla and APC reveals that the binding affinity of APC is higher than that of the APC-PT Gla on PE-containing vesicles when both factor Va and protein S are present and weaker when binding is examined on phospholipid devoid of PE. Factor Va alone and protein S alone had relatively little influence on the binding affinity of wild type APC. In contrast, factor Va alone enhanced APC-PT Gla binding significantly, especially in the absence of PE, probably accounting for the increased activity of APC-PT Gla on vesicles in the absence of PE.

To determine whether these differences in properties between APC and the APC-PT Gla were retained under more physiological conditions, we compared their ability to anticoagulate plasma. Concentrations of the liposomes of different composition were adjusted to obtain the same base-line clotting time in the absence of added APCs. As expected, APC was much more active on vesicles containing PE. Surprisingly, the APC-PT Gla exhibited much higher anticoagulant activity than APC on vesicles with or without PE (Fig. 6). Unlike APC, the anticoagulant activity of the PC-PT Gla chimera was not strongly influenced by the presence of PE.

One possible explanation for the increased activity of the APC-PT Gla was that it inhibited coagulation by a mechanism independent of the proteolytic inactivation of factor Va. In this case, either the zymogen or the enzyme form with the active site blocked might function as an anticoagulant. Neither the zymogen nor the inactivated enzyme forms of the proteins had significant anticoagulant activity in the concentration ranges where the enzymes were very effective. We also considered the possibility that the APC-PT Gla might interact in a synergistic fashion with protein C. Somewhat surprisingly, protein C partially blocked the anticoagulant activity of APC but not that of...
Calcium-dependent reversible binding was measured by flow cytometry and by light scattering as described under “Experimental Procedures.” The final concentration of phospholipid was 0.5 μg/ml, and, when present, protein S (Pro S) was 100 nM and factor Va (FVa) was 10 nM. All flow cytometric measurements were done with the enzymes labeled in the active site with fluorescein. All light scattering experiments were performed with the zymogens except the meizothrombin experiments, in which the enzyme activity was blocked with n-Phe-Pro-Arg chloromethylketone as described (39). Wild type, protein C or APC; ND, not done.

**TABLE I**

Comparison of binding affinities (K_d values) determined by fluorescence-activated cell sorting (FACS) analysis and right angle light scattering

|                | FACS analysis | Light scattering (PS/PC) |
|----------------|---------------|--------------------------|
|                | PS/PC         | PE/PS/PC                 |
| Wild type      | 500 ± 100     | 140 ± 15                 |
| PC-PT Gla      | 450 ± 72      | 160 ± 25                 |
| Wild type + FVa| 500 ± 120     | 160 ± 25                 |
| PC-PT Gla + FVa| 63 ± 8        | 45 ± 7                   |
| Wild type + FVa + Pro S | 140 ± 20 | 14 ± 2                   |
| PC-PT Gla + FVa + Pro S | 38 ± 6  | 41 ± 6                   |
| Wild type + Pro S | 390 ± 50 | 75 ± 11                  |
| PC-PT Gla + Pro S | ND         | ND                       |
| Prothrombin    | ND            | 700 ± 120                |
| Meizothrombin  | 600 ± 100     | 630 ± 150                |

**FIG. 6.** Comparison of the plasma anticoagulant activity of APC and APC-PT Gla. Plasma clotting was initiated with X-CP as described under “Experimental Procedures,” and the anticoagulant response was measured as a function of increasing zymogen concentration. Open symbols, PS/PC liposomes, 8 μg/ml; closed symbols, PE/PS/PC liposomes, 8 μg/ml. ○, APC; ● and ■, APC-PT Gla.

**FIG. 7.** Influence of the zymogen forms of protein C and PC-PT Gla on clotting and the anticoagulant activity of APC and APC-PT Gla. Plasma clotting was initiated as described under “Experimental Procedures” using 40 μg/ml PE/PS/PC, and the anticoagulant response was measured as a function of increasing zymogen concentration. Open symbols, protein C; closed symbols, PC-PT Gla. □ and ■, no APC added; ○ and ●, in the presence of 2 μg/ml APC; △ and ▲, in the presence of 0.2 μg/ml APC-PT Gla. The clots are indicated by bars with each bar representing the clotting time as determined by light scattering and right angle light scattering.

role in the anticoagulant activity of APC. For instance, previous studies have shown that protein S can block the ability of factor Xa to protect factor Va from inactivation (22) and that protein S can interfere directly with the assembly of the thrombinase complex (28). To test the possibility that one or more of these influences of protein S was operative with APC-PT Gla, we blocked protein S with an inhibitory monoclonal antibody and repeated the experiments. APC anticoagulant activity was decreased about 10-fold in the presence of this antibody (i.e. it required 10 times more APC to give the same anticoagulant response) (Fig. 8). With APC-PT Gla, the antibody increased anticoagulant activity slightly, probably because the antibody prevented protein S inhibition of chimera function in plasma, consistent with the observation that protein S slows the inactivation of factor Va by the APC-PT Gla (Fig. 2). Thus, protein S functions in plasma appear to be largely dependent upon interaction with APC, and this interaction is disrupted by substitution of the prothrombin Gla domain into APC.

To address the role of PE and the APC-PT Gla in factor Va inactivation in plasma more completely and to eliminate the possibility that the PE or APC-PT Gla effects were due to factor V processing, factor Va was added to factor V-deficient plasma, and the time course of factor Va inactivation was analyzed. A standard curve relating factor Va concentration to clotting time was established. APC or APC-PT Gla was then added to the plasma in the presence of vesicles with or without PE, and the clotting time was determined at various times after the addition of APC or APC-PT Gla (Fig. 9A). The clotting times were then converted to residual factor Va activity as a function of time under the various conditions (Fig. 9B). APC inactivated factor Va 2–4-fold faster on vesicles with PE than on those devoid of PE. The APC-PT Gla exhibited little PE dependence. The APC-PT Gla at 10% of the APC concentration inactivated factor Va about twice as fast as APC on vesicles with PE and 5–6 times faster than APC on vesicles devoid of PE. When the APC-PT Gla concentration was increased to that of APC, inactivation was too fast to measure. Decreasing the concentration of APC to that of APC-PT Gla resulted in rates of inactivation too slow to measure conveniently (data not shown). Thus, in this plasma setting, the inactivation of factor Va by APC-PT
Gla is approximately 20–50-fold faster than with wild type APC (combining the observed enhancement and the relative enzyme concentrations), and PE enhances inactivation 5–6-fold by APC but not APC-PT Gla.

Most studies focusing on the Gla domain in relationship to membrane binding have examined Gla residues and residues in the N-terminal half of the Gla domain (1), particularly hydrophobic residues thought to insert into the membrane (13, 14) and Arg15 (51). To map the region of the Gla domain involved in PE and protein S dependence more completely, we prepared another mutant in which only the first 22 residues of the Gla domain were replaced with those of prothrombin (see Fig. 10 for a molecular model of differences between prothrombin and protein C Gla domains and the sequence comparisons in this region). This chimera, APC-PT Gla 1–22, retained the PE-dependent enhancement of factor Va inactivation characteristic of APC and missing in APC-PT Gla (Fig. 11).

In plasma, APC-PT Gla 1–22 was intermediate between APC and the PC-PT Gla chimera. Like APC, APC-PT Gla 1–22 exhibited much less plasma anticoagulant activity when protein S function was blocked with an inhibitory antibody (Fig. 12A). APC-PT Gla 1–22 and APC activity were enhanced similarly by including PE in the liposomes (Fig. 12B). APC-PT Gla 1–22 activity was stimulated by PE almost as much as APC. It also displayed increased anticoagulant activity compared with APC.

**DISCUSSION**

The PE dependence of APC anticoagulant activity is clearly mediated in large part by the Gla domain of protein C. APC-PT Gla exhibited little dependence on the presence of PE in the liposomes in purified systems. Furthermore, the factor Va-inactivating activity of APC-PT Gla was inhibited rather than stimulated by protein S. These differences were not due to defects in membrane binding, since the PC-PT Gla bound to membranes at least as well as wild type protein C and was more active than wild type on vesicles devoid of PE while being slightly less active on vesicles containing PE. Much of this difference appears to lie in the capacity of protein S and factor Va to synergistically augment APC binding to vesicles, especially those containing PE. Interestingly, although factor Va alone could enhance binding of the APC-PT Gla (in contrast to little effect on APC), protein S failed to enhance binding of APC-PT Gla.

The PE used in the present study has polyunsaturated fatty acids at both positions. Unlike other phospholipids, PE often has unsaturated fatty acids in the 1- and 2-positions (52) and/or an ether linkage to form plasmalogens. Optimal PE effects on factor Va inactivation require unsaturated fatty acids at both positions. These PE effects, however, cannot be mimicked by other phospholipids with both positions occupied with unsaturated fatty acids.

The plasma anticoagulant activity of the APC-PT Gla was relatively insensitive to protein S or to the presence of PE in the vesicles. In contrast, protein S increased plasma anticoagulant activity of wild type APC approximately 10-fold, and the presence of PE in the vesicles increased activity approximately 3–5-fold. These observations support the concept that the major anticoagulant activity associated with protein S is as a cofactor for APC rather than serving a direct anticoagulant function as has been suggested (28). If the protein S were serving an important direct anticoagulant function, this should have been observed with APC-PT Gla as well as with APC.

Why the APC-PT Gla is more active as a plasma anticoagulant in comparison to wild type APC is uncertain, but this activity probably reflects diminished competition with other components. Preliminary results suggest that prothrombin may be responsible for inhibiting APC anticoagulant activity to a greater extent than that of APC-PT Gla (53). Analysis of the PC-PT Gla 1–22 chimera, however, revealed that the increased activity was associated with the amino-terminal portion of the Gla domain, while the PE and protein S dependence was associated with the carboxyl-terminal portion of the Gla domain. The factors that inhibit APC function in plasma are not known, but the chimeras described here will serve as a useful probe to identify these putative inhibitors.

Assuming that APC interacts with the membrane by insert-

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2 D. A. Ford, personal communication.

3 M. D. Smirnov, D. A. Ford, C. T. Esmon, and N. L. Esmon, manuscript in preparation.
ing the hydrophobic loop in the amino-terminal portion of the Gla domain as suggested by mutagenesis (13) and structural studies (48), the regions of the Gla domain involved in protein S and PE dependence probably lie on top of the membrane. This assumes that the membrane surface approaches the Ca\(^{2+}\) ions seen in Fig. 10. The top portion of the presumptive interface between protein C and the membrane surface is formed by the carboxyl-terminal portion of the Gla domain. This region is filled with residues that differ between prothrombin and protein C including Phe at position 26, Gla at position 32, and Ser at position 23. These are only some of the differences that occur at this putative interface, but the nature of the changes would allow ionic interactions to be replaced by hydrogen bonding interactions at position 32 and the opposite effect at position 23. At position 26, the potential of an ion pair is present in protein C, replacing the possible hydrophobic contribution from the Phe in prothrombin. By changing the nature of this interface, the angle between the Gla domain and the remainder of the protein C molecule may be altered as indicated by the change in distance between the active site of APC and the membrane surface compared with the distance with the APC-PT Gla chimera (54).

The binding studies performed with APC complexes employed flow cytometric analysis of binding to membranes im-

**Fig. 10.** Homology models of the prothrombin and protein C Gla domains. The human prothrombin and protein C Gla domains were modeled as described under “Experimental Procedures.” Structural models of the Gla domains of human prothrombin and protein C are depicted with the main chain traced as a ribbon and the nonidentical residue side chains shown. Red, acidic residues; blue, basic residues; green, polar uncharged residues; yellow, hydrophobic residues. Residues that differ substantially between prothrombin and protein C are labeled. Calcium ions are depicted as small gray spheres. The sequences of human prothrombin and protein C Gla domains were from SwissProt (P00734 for prothrombin and P04070 for protein C). Sequence identities are identified by the line between the identical residues.

**Fig. 11.** The influence of PE on factor Va inactivation by APC-PT Gla 1–22. Factor Va (0.2 nM) was inactivated for 30 min as described under “Experimental Procedures” by the concentrations of APC (open symbols) or APC-PT Gla 1–22 (closed symbols) shown. PS/PC (circles) or PE/PS/PC (squares) vesicles were present at 10 \(\mu\)g/ml.

**Fig. 12.** Comparisons of the anticoagulant activities of APC, APC-PT Gla, and APC-PT Gla 1–22. A, plasma clotting was initiated as described under “Experimental Procedures” using 8 \(\mu\)g/ml PE/PS/PC vesicles in the presence and the absence of an inhibitor monoclonal antibody to protein S (S155, 300 \(\mu\)g/ml). Closed symbols, no antibody added; open symbols, antibody present. Diamonds, APC; triangles, APC-PT Gla 1–22; squares, APC-PT Gla. B, the influence PE on plasma anticoagulant activity of APC, APC-PT Gla, and APC-PT Gla 1–22. Clotting was initiated as described under “Experimental Procedures” with the concentrations of the anticoagulant enzymes indicated in the figure. Phospholipid concentrations were adjusted to give the same clotting times in the absence of the anticoagulant enzymes. Open symbols, 24 \(\mu\)g/ml PS/PC vesicles; closed symbols, 9 \(\mu\)g/ml PE/PS/PC vesicles. Diamonds, APC; triangles, APC-PT Gla 1–22; squares, APC-PT Gla.
mobilized on polystyrene beads. We utilized this approach because PE-containing vesicles are too large to allow light scattering measurements, and these measurements are not feasible in the presence of the other components involved in complex formation. Although not the normal method for binding analysis, this approach has been used previously to describe PE-dependent factor VIII interaction with membranes (8). Several lines of evidence in the present study support the concept that this approach is valid, at least for comparative purposes. In simple systems, the binding constants obtained with the flow cytometric analysis were in good agreement with the values from the more traditional light scattering methods. In the more complex systems involving protein S and factor Va, the differences in binding affinity between the APC-PT Gla and APC were consistent with the differences observed for requirements for factor Va inactivation.

Binding studies of protein C/APC to membrane surfaces have resulted in large discrepancies in values from different laboratories. Many of the studies were performed with bovine proteins. Studies from Nelsestuen’s group (5), using right angle light scattering, reported a $K_d$ of 17 $\mu$M for protein C binding to PS/PC vesicles. Krishnaswamy, et al. (55) using fluorescence polarization, found that the $K_d$ for bovine APC was 0.073 $\mu$M and was reduced to 7 nM by the presence of factor Va. Walker (23), using the right angle light scattering approach, measured a $K_d$ of 0.23 $\mu$M for bovine protein C; 0.15 $\mu$M for APC; 1.4 nM for APC plus protein S; and 3.5 nM for APC, protein S, and factor Va. All of these studies were performed with PS/PC vesicles and bovine proteins and hence cannot be compared directly with the results of the present study. A few studies have examined the affinity of human protein C/APC for phospholipid vesicles. Colpitts and Castellino (56) have reported a $K_d$ of 1.9 $\mu$M for human protein C binding to PS/PC vesicles with 50% PS. Recently, Nelsestuen’s group (57) has compared the affinity of binding of bovine protein C and human protein C to PS/PC vesicles. In direct comparisons, they observed that bovine protein C bound much more weakly than human protein C and concluded that the affinity of human protein C for PS/PC vesicles was $\leq 1.7$ $\mu$M.

Membrane binding of the vitamin K-dependent proteins to phospholipid requires the Gla residues. In protein C, a detailed analysis of the function of each of these Gla residues has been performed (reviewed in Ref. 1). Gla residues 7, 20, 26, and 29 are critical for function, since mutation from Gla to Asp results in almost total loss of anticoagulant activity. Gla25 mutation results in the loss of about 75% of the anticoagulant activity, and was reduced to 7 nM by the presence of factor Va. Walker (23), using the right angle light scattering approach, measured a $K_d$ of 0.23 $\mu$M for bovine protein C; 0.15 $\mu$M for APC; 1.4 nM for APC plus protein S; and 3.5 nM for APC, protein S, and factor Va. All of these studies were performed with PS/PC vesicles and bovine proteins and hence cannot be compared directly with the results of the present study. A few studies have examined the affinity of human protein C/APC for phospholipid vesicles. Colpitts and Castellino (56) have reported a $K_d$ of 1.9 $\mu$M for human protein C binding to PS/PC vesicles with 50% PS. Recently, Nelsestuen’s group (57) has compared the affinity of binding of bovine protein C and human protein C to PS/PC vesicles. In direct comparisons, they observed that bovine protein C bound much more weakly than human protein C and concluded that the affinity of human protein C for PS/PC vesicles was $\leq 1.7$ $\mu$M.

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A different model of membrane binding has recently been advanced by Nelsestuen’s laboratory and associates (57). Using an analysis of changes in binding affinity between different vitamin K-dependent proteins, they noted that the absence of Gla25 (protein C numbering) correlated with lower affinity interaction with membranes and that Asp at position 33 correlated with high affinity interactions. They propose a model in which residues 10, 32, and 33 (protein C numbering) are on the surface near a metal in the strontium-prothrombin fragment 1 structure (60, 61). Ca$^{2+}$ does not occupy this position in the Ca$^{2+}$ structure; hence, they propose this site to be a candidate for head group-specific interaction. These authors did not compare human prothrombin membrane binding affinity with that of human protein C; therefore, the analysis is not strictly related to the current study. It does, however, indicate that residues outside of the N-terminal 22 residues of the Gla domain might play a direct role in membrane affinity or specificity. The model differs from that proposed by Castellino’s studies in that the hydrophobic interactions would not appear to play a dominant role.

The PE effects on catalysis of the APC anticoagulant complexes have both cell biological and pathophysiological ramifications. PE has been reported to be present on the surface of unactivated cells (62) and following activation may constitute nearly 40% of the outer leaflet membrane phospholipid (6). Furthermore, PE has been reported to have a higher $K_m$ for the flippase and hence is likely to be more slowly transported to the inner membrane leaflet (63). Therefore, if the different coagulation complexes were to exhibit widely different PE/PS dependences, this time-dependent change in membrane composition could selectively favor clot-promoting or clot-inhibiting reactions.

Many patients with thrombotic or inflammatory diseases have low functional levels of protein S (64). The observation that APC-PT Gla is a potent anticoagulant that, unlike APC, does not depend on protein S for its function makes it an attractive candidate for antithrombotic therapy. Indeed, preliminary studies have indicated that protein C or APC may be useful in treating thrombotic disease including disseminated intravascular coagulation in septic shock (reviewed in Ref. 65). In these sepsis patients, protein S is very low, providing a rationale for the use of this chimera in this acute, life-threatening coagulation disorder.

The observation that lupus anticoagulants and some antiphospholipid antibodies block the function of coagulation and anticoagulation complexes preferentially on PE-containing membranes (42, 66) also adds to the potential interest in the effects reported here. These antibodies are associated with an increased risk of thrombosis (67–69), and there is no detailed understanding of the best in vitro assays to detect the antibodies responsible for the pathogenic response. The development of an APC molecule with no PE dependence will allow the exploration of the mechanisms of PE-dependent lupus anticoagulant effects on the APC system in vitro and in vivo. This in turn could provide insights into the mystery of why patients with lupus anticoagulants have increased risk of thrombosis.

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