Proteolytic Formation and Properties of γ-Carboxyglutamic Acid-domainless Protein C*

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Protein C activation is catalyzed by a complex between thrombin and an endothelial cell surface receptor, thrombomodulin. To investigate the function of the γ-carboxyglutamic acid (Gla) residues in the calcium-dependent activation of protein C, residues 1-41 were removed from the protein C light chain by selective proteolysis with chymotrypsin, and the Gla-domainless protein C was purified to homogeneity. Activated Gla-domainless protein C and activated protein C hydrolyzed H-D-Phe-pipercolyl-Arg-p-nitroanilide at the same rate. Unlike activated protein C, Gla-domainless activated protein C did not function as an anticoagulant.

Calcium ions were required for Gla-domainless protein C activation by the thrombin-thrombomodulin complex. At 2 μM substrate, half-maximal rates of activation were achieved at 250 ± 50 μM Ca²⁺ for protein C and 50 ± 5 μM Ca²⁺ for Gla-domainless protein C. At saturating levels of Ca²⁺, the activation properties of these two substrates were identical; Vmax = 250 mol/min/mol and Kₘ = 8 μM. In the absence of thrombomodulin, calcium inhibits thrombin-catalyzed activation of both Gla-domainless protein C and protein C. Half-maximal inhibition of protein C activation occurs at 250 μM Ca²⁺, while half-maximal inhibition of Gla-domainless protein C activation occurs at 50 μM Ca²⁺.

Over the surface of rabbit endothelial cells, Gla-domainless protein C, at physiological concentrations, was activated at less than 5% the rate of protein C. Endothelial cell-mediated protein C activation exhibited sites with both high and low affinity for protein C, while only low affinity sites were observed for Gla-domainless protein C.

These results indicate that protein C possesses a Gla-independent high affinity calcium-binding site that is required for recognition by the thrombin-thrombomodulin complex and that metal ion interaction with this same site is responsible for Ca²⁺ inhibition of protein C activation by thrombin alone. These studies further show that the Gla domain of protein C is essential for optimal protein C activation at the endothelial cell surface.

The conversion of the protein C zymogen (1-4) to the biologically active anticoagulant enzyme (3-8) activated protein C is catalyzed by a complex between thrombin and a specific endothelial cell surface protein (9-11), thrombomodulin. In this complex, thrombomodulin appears to function as a cofactor or regulatory protein which augments the ability of thrombin to activate protein C (11). The availability of the isolated receptor has made it possible to more accurately describe the mechanism of protein C activation by the thrombin-thrombomodulin complex.

Previous studies (12, 13) have demonstrated that the activation of protein C by thrombin is inhibited potently by low levels of Ca²⁺. In contrast, the activation of protein C by the thrombin-thrombomodulin complex is dependent on the presence of Ca²⁺ (11). The calcium concentration dependence of these reactions led us to postulate that the Ca²⁺ influence on protein C activation is mediated through the Gla domain (11). Since the formation of the Gla residues is dependent on vitamin K and inhibited by coumarin anticoagulants, the role of the Gla domain in both the activation and expression of protein C activity is of particular interest.

A useful approach to study the functions of the Gla domain in vitamin K-dependent proteins was suggested by the recent studies of Morita and Jackson (14) who demonstrated that the Gla domain of factor X could be removed selectively by limited proteolysis with chymotrypsin. The resultant protein retained the ability to be converted to a fully active esterase but was without biological activity as a blood clotting factor. We have used this approach to investigate the function of the Gla domain in protein C.

EXPERIMENTAL PROCEDURES

Materials

Bovine protein C (7), activated protein C (7), thrombin (15), and rabbit lung thrombomodulin (11) were purified by published methods. Chymotrypsin was purchased from Worthington. p-Nitrophenyl-p'-guanidinobenzoate and QAE (quaternary aminoethyl)-Sephadex Q-50 were purchased from Sigma.

Methods

Assay of Protein C Activation—Initial rates of protein C activation with purified thrombomodulin and thrombin were determined by stopping the activation reaction and measuring activated protein C formed in the reaction mixture at selected time points. All activations were performed in 0.1 M NaCl, 0.02 M Tris-HCl, 10 mg/ml of bovine serum albumin, pH 7.5, at 37 °C. Gelatin (0.1%) replaced the bovine serum albumin in those cases where the effect of calcium was being determined. In these experiments, all proteins were dialyzed against Chelex-treated buffer (Bio-Rad) before use and the buffer was also

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The abbreviations used are: Gla, γ-carboxyglutamic acid; PTH, phenylthiohydantoin; PBS, physiological buffered saline, HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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Properties of Gla-domainless Protein C

Proteins used to construct the standard curves were prepared by total activation of protein C and Gla-domainless protein C with thrombomodulin. The molar extinction coefficient of 16,000 was used for p-nitrophenyl hydrolysis was monitored at 405 nm in a Beckman DU8 spectrophotometer. The active site-(3-150 equilibrated in 0.1 M Tris-HCl, pH 7.4, was determined with the active site-dependent hydrolysis of the titrant was determined by extrapolation between 95 and 103%. The molecular weight of Gla-domainless protein C was decreased approximately 25% and the floating adipocytes and supernatant discarded. The vascular wall was washed in PBS and suspended in complete medium containing 111 pg/ml (final concentration) plus 5 mg of Polybrene as carrier. The conversion of anilinothiazolyl chloromethyl ketone to remove excess NH4HCO3.

Extinction Coefficients and Molecular Weights for Proteins—The extinction coefficients and molecular weights used for calculating protein concentration were: thrombin, e405 = 21,900 (15); protein C, e405 = 13.7, 62,000 (2); Gla-domainless protein C, e405 = 13.7, 58,000. Although the extinction coefficient for Gla-domainless protein C was not determined independently, the extinction coefficient could be estimated from the distribution of products on the preparative ion exchange column (Fig. 2). The yield on this column was consistently between 95 and 103%. The molecular weight of Gla-domainless protein C was decreased approximately 7% and the distribution of 280-nm absorbing material was 95% in the Gla-domainless protein C peak and 7% in the peak tail. Therefore, the extinction coefficient of protein C was used for calculations of Gla-domainless protein C concentration.

Active Site Determination—The number of active sites/mol of activated protein C or activated Gla-domainless protein C was determined with the active site tritiated p-nitrophenyl-p'-guanidinobenzoate as described by Chase and Shaw (16). The titration was performed in 0.1 M barbital buffer, pH 8.3, and nitrophenyl release was monitored at 405 nm in a Beckman DU8 spectrophotometer. The active site-dependent hydrolysis of the titrant was determined by extrapolation to zero time for spontaneous hydrolysis and deacylation of the enzyme. A molar extinction coefficient of 30,000 was used for p-nitrophenyl (16).

Disc Gel Electrophoresis—Disc gel electrophoresis was performed by the method of Davis (17). Disc gel electrophoresis in the presence of sodium dodecyl sulfate was performed by the method of Laemmli (18). The gels were made with 10% sodium dodecyl sulfate and suspended in a boiling water bath for 1 min prior to electrophoresis. Samples which were reduced prior to electrophoresis were treated as described above except that 10% 2-mercaptoethanol was present during the heating process.

Preparation of the Gla-domainless Protein C Light Chain and Sequence Analysis—Gla-domainless protein C (18.7 mg) in 2 ml of 6 M guanidine HCl, 6.5 M Tris-HCl, 5 mM EDTA, pH 8.0, was incubated at 37 °C for 2 h with 25 mM dithiobiotin. The sample was then reacted in the dark with an equal volume of 0.5 M Tris-HCl, 5 mM EDTA, 6 M guanidine HCl, 60 mM iodoacetic acid, pH 8.5, at 22 °C for 2 h. The sample was applied to a column (1.5 × 94 cm) of Sephadex G-150 equilibrated in 0.1 M NH4HCO3. The carboxymethylated light chain was found in the second protein peak to emerge from the column. The recovery was 102% and 2.3-ml fractions were collected. The chains were homogeneous on sodium dodecyl sulfate gel electrophoresis.

Automated Edman degradation was performed on a Beckman Sequencer model 890C. A dilute Quadril program (19) was employed with 5 mg of Polybrene as carrier. The conversion of anilinothiazolines to PTH was accomplished with anhydrous methanolic HCl (20) in a Sequest P-6 Auto Converter. The PTH derivatives were identified using Waters Associates gradient high pressure liquid chromatography, with a Waters Associates 5 μm, (8 × 100 mm) radial compression column and module. The PTH derivatives were independently identified by thin layer chromatography (21).

γ-Carboxyglutamateran Determination—γ-Carboxyglutamate was determined on alkaline hydrolysates of protein C (22) by a modification of the high performance liquid chromatographic analytical procedure described by Hill et al. (23) for separating amino acids on C-18 columns after derivatization with p-phthalaldehyde deacylation. Lowering the initial concentration of acetone from 9 to 5% completely resolved γ-carboxyglutamate from aspartate and glutamate on a Dupont Zorbax ODS column (46 × 250 mm). The extinction coefficient could not be determined independently, the extinction coefficient of p-nitrophenyl hydrolysis was monitored at 405 nm in a Beckman DU8 spectrophotometer, and the concentration of activated protein C was determined by preparing a standard curve which related the rate of p-nitroanilide formation to the concentration of activated protein C. The activated proteins used to construct the standard curves were prepared by total activation of protein C and Gla-domainless protein C with the thrombin-thrombomodulin complex immediately before use. The slopes of the standard curves for activated protein C and activated Gla-domainless protein C were indistinguishable.

RESULTS

Formation of Gla-domainless Protein C—The Gla-containing domain was removed from protein C by selective proteolysis with chymotrypsin (1/400, w/w) (Fig. 1). Proteolysis was localized to the light chain of protein C and the reaction was complete in 10 min. A major and minor protein appeared in the region of the modified light chain. The minor component was shifted significantly to the trailing shoulder of the peak. This material was not used in the kinetic studies.

Isolation and Characterization of Gla-domainless Protein C—The chymotryptic digest was made 2 mm in disopropylfluorophosphate and chromatographed on a column of QAE-Sephadex Q-50. The Gla-domainless protein was eluted from the column as a single symmetrical peak (Fig. 2). At the end of the gradient, the NaCl concentration was increased to 1 M and an additional protein peak emerged from the column.

FIG. 1. Time course of Gla-domainless protein C formation. Proline C (2.0 mg/ml) was incubated at 37 °C with chymotrypsin (5 pg/ml) in 0.1 M NaCl, 0.02 M Tris-HCl, pH 7.5. Samples (20 μg) were removed at the times indicated and prepared for sodium dodecyl sulfate acrylamide gel electrophoresis (12%) (18) as described under "Methods." Disulfide bonds were reduced prior to electrophoresis.
The NH₂-terminal sequence was Gln-Cys which is identical with the sequence reported for the light chain, Gla-domainless protein C was reduced and prepared for sequence analysis (see under "Methods").

Acrylamide gels (7%) without sodium dodecyl sulfate (1%) and prepared for sequence analysis (see under "Methods"). The sample (25 mg) was applied to a column (0.9 cm) of QAE-Sephadex. The reaction mixture shown in Fig. 1 was treated with dithiothreitol to reduce disulfide bonds. Gla-domainless protein C (15 μg); protein C before disulfide bond reduction; D) protein C (15 μg) following disulfide bond reduction. Acrylamide gels (7%) without sodium dodecyl sulfate (1%) of: E) Gla-domainless protein C (15 μg); F) protein C (15 μg).

Polyacrylamide gels of the major protein peak are shown in Fig. 3. Before reduction, the Gla-domainless protein C appeared as a single molecular species (Mᵦ = 58,900). After reduction, the protein appeared as two bands (Mᵦ = 40,000 and Mᵦ = 15,900). Only the light chain of protein C was altered by the chymotryptic digestion and the product isolated from the QAE-Sephadex was indistinguishable from that formed during digestion (Fig. 1). The Gla-domainless protein C migrated as a single species on alkaline disc gels in the absence of detergent, and the migration was considerably slower than that of protein C. Amino acid analysis following alkaline hydrolysis failed to detect Gla residues in the modified protein C (<0.01 residue/mol compared to 11 Gla residues/mol of protein C) (27). The NH₂-terminal sequence of the peptide eluted from the QAE-Sephadex by 1 M NaCl was determined to be Ala-Asn-Ser-Phe which is identical with the reported sequence (28) of the first 4 residues of the light chain of protein C. To identify the site of chymotryptic cleavage in the light chain, Gla-domainless protein C was reduced and carboxymethylated as described by Stenflo (29) for protein C and prepared for sequence analysis (see under "Methods").

The NH₂-terminal sequence was Ser-Lys-Tyr-Ser-Asp-Gly-Gln-Cys which is identical with the sequence reported for residues 42-49 of the protein C light chain. Thus, the chymotryptic cleavage is at residue 41 (Trp) of the light chain and the cleavage peptide, derived from the NH₂-terminal end of the light chain, contains the Gla residues.

The Gla-domainless protein C could be converted to an enzyme by either thrombin or the thrombin-thrombomodulin complex. The resultant enzyme hydrolyzed H-μ-Phe-piperoc-Arg-p-nitroanilide at the same rate as intact activated protein C. The active site titrant p-nitrophenyl-p'-guanidinobenzoate revealed 1.0 ± 0.05 mol of active site/mol of enzyme. Unlike activated protein C, the activated Gla-domainless protein C had no anticoagulant activity at 10 μg/ml in bovine plasma. By contrast, 0.1 μg/ml of activated protein C is readily detected in the plasma anticoagulant assay (7).

The Role of Calcium Ions in Protein C Activation—Before initiating studies on the role of the Gla domain in protein C activation, it was necessary to establish the influence of Ca²⁺ on the kinetic parameters of protein C activation by thrombin and the thrombin-thrombomodulin complex (Table I). In both instances, the effect of Ca²⁺ was greater on the Keₜₐᵦ (Kₐ) of the reaction than on Vₜₐᵦ (Vₐᵦ maximum). Initial velocity studies indicated that Ca²⁺ increases the Kₐ of thrombin for protein C from 1.2 μM

![Fig. 2: Chromatography of Gla-domainless protein C on QAE-Sephadex](http://www.jbc.org/) The reaction mixture shown in Fig. 1 was treated with dithiothreitol to reduce disulfide bonds. Acrylamide gels (7%) without sodium dodecyl sulfate (1%) and prepared for sequence analysis (see under "Methods"). The sample (25 mg) was applied to a column (0.9 cm) of QAE-Sephadex. The reaction mixture shown in Fig. 1 was treated with dithiothreitol to reduce disulfide bonds. Acrylamide gels (7%) without sodium dodecyl sulfate (1%) of: E) Gla-domainless protein C (15 μg); F) protein C (15 μg).

![Fig. 3: Polyacrylamide disc gels of Gla-domainless protein C](http://www.jbc.org/) Sodium dodecyl sulfate acrylamide gels (12%) of A) Gla-domainless protein C (15 μg) following disulfide bond reduction; B) Gla-domainless protein C (15 μg) following disulfide bond reduction; C) protein C before disulfide bond reduction; D) protein C (15 μg) following disulfide bond reduction. Acrylamide gels (7%) without sodium dodecyl sulfate (17%) of: E) Gla-domainless protein C (15 μg); F) protein C (15 μg).

![Fig. 4: Influence of Ca²⁺ on the initial rate of Gla-domainless protein C activation by the thrombin-thrombomodulin complex](http://www.jbc.org/) Either protein C (2 μM) or Gla-domainless protein C (2 μM) was incubated with 0.2 nM thrombin-thrombomodulin complex. To ensure complete complex formation, the reaction complex was formed in the presence of a 20-fold excess of thrombomodulin (4 nM). Calcium was included in the mixtures at the concentrations indicated. The rate of protein C activation was quantitated as described under "Methods." The rates of activation observed in 5 mM Ca²⁺ were taken as maximal. Inset, Lineweaver-Burk plots of the calcium dependence of protein C and Gla-domainless protein C activation by the thrombin-thrombomodulin complex. x-axis, 1/[Ca²⁺]; y-axis, 1/ rate of activation (arbitrary units). Five independent experiments have indicated half-maximal rates of activation of 50 ± 20 μM for Gla-domainless protein C and 250 ± 50 μM for protein C.
to \( \sim 60 \, \mu M \). Conversely, \( Ca^{2+} \) decreases the \( K_m \) of the thrombin-thrombomodulin complex from \( >100 \, \mu M \) to \( 8 \, \mu M \).

Role of Metal Ions in the Activation of Gla-domainless Protein C—The initial rate of activation of Gla-domainless protein C by the thrombin-thrombomodulin complex was studied as a function of \( Ca^{2+} \) concentration and compared to that of protein C (Fig. 4). The activation of Gla-domainless protein C by the thrombin-thrombomodulin complex was dependent on the presence of \( Ca^{2+} \). At saturating \( Ca^{2+} \) concentration, the activation rates of Gla-domainless and normal protein C were identical. The initial rate data were transformed into double reciprocal plots (Fig. 4, inset). These plots indicate that the half-maximal activation rate of Gla-domainless protein C occurs at \( 50 \, \mu M \) \( Ca^{2+} \) whereas that for protein C occurs at \( 250 \, \mu M \) \( Ca^{2+} \).

To determine if removal of the Gla domain influences other kinetic parameters, the \( K_m \) and \( V_{max} \) for Gla-domainless and normal protein C were determined at saturating \( Ca^{2+} \) (Fig. 5). The \( K_m \) (8 ± 2 \( \mu M \)) and \( V_{max} \) (250 ± 50 mol/min/mol complex) for these substrates were indistinguishable.

Calcium ions inhibit the activation of protein C by thrombin (12). To determine if this \( Ca^{2+} \)-dependent inhibition could still be observed with Gla-domainless protein C, initial velocity studies were performed (Fig. 6). The activation of Gla-domainless protein C was still inhibited by \( Ca^{2+} \). Half-maximal inhibition occurred at \( 50 \pm 10 \, \mu M \) \( Ca^{2+} \) for Gla-domainless protein C and \( 250 \pm 50 \, \mu M \) \( Ca^{2+} \) for protein C. Although the influence of \( Ca^{2+} \) on the activation properties of Gla-domainless protein C was readily observed, this protein was a poor substrate for thrombin, which precluded accurate assessment of the kinetic constants. The \( K_m \) was greater than 40 \( \mu M \) and no estimate of \( V_{max} \) was obtained.

Direct comparisons were made of the activation rates of protein C and Gla-domainless protein C by both thrombin and the thrombin-thrombomodulin complex. Each substrate was studied with both activators in the presence and absence of \( Ca^{2+} \) (Table II). To simplify comparisons, the rates of activation with both substrates and activators were normalized to that of protein C in 5 mM \( Ca^{2+} \) with the thrombin-thrombomodulin complex. In 5 mM \( Ca^{2+} \), protein C and Gla-domainless protein C activate at the same rate when either thrombin or the thrombin-thrombomodulin complex is employed as the activator species. However, in the absence of \( Ca^{2+} \), Gla-domainless protein C is activated by thrombin much more slowly than is protein C. In contrast, in the absence of \( Ca^{2+} \), Gla-domainless protein C is activated by the thrombin-thrombomodulin complex more rapidly than is protein C.

Role of the Gla Domain in Protein C Activation by Endothelial Cells—The above observations suggest that interaction of protein C with the thrombin-thrombomodulin complex is not mediated through the Gla domain of the molecule. Physiological activation occurs on the endothelial cell surface rather than in solution. Studies (30) of platelet involvement in prothrombin activation have shown that the Gla domain of the substrate is required for optimal activation. To determine if the protein C activation system shares this property with

![Fig. 5. Substrate concentration dependence of protein C activation by the thrombin-thrombomodulin complex. Thrombin-thrombomodulin complex (0.2 nM) was formed by adding 4 nM thrombomodulin to 0.2 nM thrombin in the presence of 3 mM calcium. Protein C (■) and Gla-domainless protein C (●) concentrations were varied between 0.5 and 20 μM. Activated products were assayed as described under “Methods.” Data points represent the numerical average of 3 independent experiments. Bars indicate 1 S.D.](http://www.jbc.org/)

![Fig. 6. Calcium inhibition of thrombin-catalyzed protein C and Gla-domainless protein C activation. Either protein C (1 μM) (■) or Gla-domainless protein C (1 μM) (●) was incubated at 37°C with thrombin (20 nM) at the Ca²⁺ concentrations indicated. Assays were performed as described under “Methods.” The respective rates of activation at 5 mM Ca²⁺ were set to 100% inhibition of the rates observed in 0.1 mM EDTA.](http://www.jbc.org/)
The prothrombin activation system, parallel activation studies of protein C and Gla-domainless protein C were performed over rabbit endothelial cells. Initial velocity experiments were performed at the concentrations of Gla-domainless protein C and protein C shown in Fig. 7. Near physiological levels, Gla-domainless protein C activated much more slowly than protein C. Most of this difference disappeared at high concentrations of the two substrates. Protein C activation appeared biphasic, indicating a high affinity for Ca²⁺ and many low affinity binding sites. The concentration dependence of Gla-domainless protein C activation was not compatible with simple Michaelis-Menten kinetics and appeared cooperative. Given the complexity of this system, no interpretation of the shape of this curve was attempted.

**DISCUSSION**

The characteristics of the soluble and cellular protein C activation complexes indicate that there are two distinct Ca²⁺-dependent processes required for optimal protein C activation. One of these is a Ca²⁺-induced effect which is independent of the Gla domain and is the only metal-induced transition required for activation by the soluble complex. The second of these is inferred from the cellular activation system which exhibits an almost total dependence on the presence of the Gla domain in protein C. While we have not proven that this dependence on the Gla domain involves Ca²⁺, the Ca²⁺-binding sites on the Gla residues and the Ca²⁺ dependence of the other Gla-dependent reactions (31) make it likely that the differential recognition of protein C and Gla-domainless protein C by the cell surface activation complex is also Ca²⁺-dependent.

Recognition of protein C by the purified thrombin-thrombomodulin complex is not dependent on the Gla residues. At saturating levels of Ca²⁺, the protein C and Gla-domainless protein C have the same activation properties, i.e. the same Vₘₐₓ and Kₐₙ values. The only difference observed in these substrates is the Ca²⁺ dependence of the activation, with half-maximal activation rates obtained at 50 μM Ca²⁺ for Gla-domainless protein C and 250 μM Ca²⁺ for protein C. These results demonstrate that modification of the substrate alters the Ca²⁺ dependence of the activation reaction. Since the activation reaction involves thrombin, thrombomodulin, and protein C it is not possible to ascertain the site of Ca²⁺ involvement from these studies alone. To investigate the involvement of Ca²⁺ in this process further, we examined the influence of Ca²⁺ on thrombin-catalyzed protein C activation in the absence of thrombomodulin. Unlike the activation of protein C or Gla-domainless protein C by the thrombin-thrombomodulin complex where Ca²⁺ is required for the activation process, Ca²⁺ inhibits thrombin-catalyzed activation of protein C (12). Although this could reasonably be assumed to correlate with Ca²⁺ binding to the Gla residues, this assumption is not consistent with the observation that Gla-domainless protein C activation is also inhibited by Ca²⁺.

An interesting relationship exists between the Ca²⁺ dependencies of the activation of protein C by the thrombin-thrombomodulin complex and the inhibition of protein C activation by thrombin alone. In both cases half-maximal changes in the rate of activation occur at 250 ± 50 μM. Removing the Gla domain shifts the half-maximal Ca²⁺ dependence for both processes to 50 μM, strongly suggesting that a single transition in one of the components of the activation system is responsible for both effects. Since thrombomodulin is present in only one of the two activation systems influenced by Ca²⁺, it does not appear likely that the functional site observed in these studies resides on thrombomodulin. Despite the observation that Gla²⁺ influences the activity of thrombin toward other substrates (32), to our knowledge no Ca²⁺-binding site on thrombin has been demonstrated. Our attempts to measure Ca²⁺ binding to thrombin by equilibrium dialysis indicate that if thrombin possesses a Ca²⁺-binding site, the dissociation constant must be greater than 500 μM. Thus, the most likely site for Ca²⁺ involvement is on the substrate.

Removal of the Gla domain from protein C reduces the calcium concentration dependence of both the thrombin and the thrombin-thrombomodulin activation systems. This suggests that the energy required for the Ca²⁺-dependent conformational change has been reduced. One interpretation of this observation is that even in the absence of Ca²⁺, the Gla-domainless protein C adopts a conformation closer to the calcium form of the substrate than that exhibited by protein C. This proposal is supported by two independent observations. First, activation of Gla-domainless protein C by the thrombin-thrombomodulin complex in the absence of Ca²⁺ is more rapid than that of protein C. Second, the rate of activation of Gla-domainless protein C by thrombin in the absence of Ca²⁺ is much slower than that of protein C. In both activation systems, the absolute rates of activation for these two substrates are identical in the presence of saturating Ca²⁺. Thus, the substrate appears to these two different activators to be shifted toward the Ca²⁺ form. This shift may account for the decreased requirements for Ca²⁺.

Similarities in the activation properties of protein C and Gla-domainless protein C with the soluble activator make these two substrates useful for initial studies comparing the nature and requirements of the soluble activator complex to those of the cell surface complex. Whereas the two substrates are identical with the soluble activator, protein C is a much better substrate over the endothelial cell surface. At physiological concentrations, the activation rate of protein C is approximately 50 times that observed for Gla-domainless protein C. At least two conclusions can be derived from this experiment. First, the Gla domain is required for optimal activation by the cellular activation complex. This requirement for the Gla domain is probably mediated through the Gla residues in a Ca²⁺-dependent reaction. Thus, it is probable that forms of protein C without Gla residues, such as those formed during warfarin anticoagulant therapy, cannot be activated by the cell surface complex. Until studies equivalent
to those shown in Fig. 7 are performed with the form of protein C present during warfarin anticoagulation, the possibility that the cell surface has a direct recognition site for this 41-residue peptide cannot be excluded totally.

The second conclusion that can be derived from these experiments is that the endothelial cell surface contributes protein C present during warfarin anticoagulation, the possibility that the cell surface has a direct recognition site for this sequence determinations and Dr. Robert Delaney for performing the required for optimal protein C activation. Although the chemical nature of this cell surface component is unknown, its influence on the protein C activation system is very similar to the influence of phospholipid on the prothrombin activation system (31). In the absence of phospholipid, prethrombin I (the proteolytically derived thrombin precursor lacking the Gla residues) and prothrombin are activated at similar rates by a complex between factor Xa, factor Va, and Ca2+. In the presence of either phospholipid or platelets (30, 31), prothrombin is the preferred substrate. The analogy between the two systems is complicated by the fact that protein C activation results in inhibition of blood coagulation (5) and stimulation of fibrinolysis (33), whereas prothrombin activation results in blood clot formation. Unlike platelets, endothelial cells do not support blood coagulation. Thus, the recognition site for protein C on the endothelial cell must be functionally distinct from the recognition site for prothrombin on the platelet. Certainly some of this specificity resides in the protein cofactors, factor Va, and thrombomodulin, but it is possible that additional specificity may reside in the cell surface component. Studies on the nature and function of this second site will be helpful in further understanding the anticoagulant nature of the endothelial cell.

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