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Recombinant human protein C derivatives: altered response to calcium resulting in enhanced activation by thrombin

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Calcium plays a dual role in the activation of protein C: it inhibits protein C activation by α-thrombin, whereas it is required for protein C activation by the thrombomodulin–thrombin complex. Available information suggests that these calcium effects are mediated through calcium induced structural changes in protein C. In this paper, we demonstrate that substitution of Asp167 (located in the activation peptide of human protein C, occupying position P3 relative to the peptide bond Arg169–Leu170 which is susceptible to hydrolysis by thrombin) by either Gly or Phe results in protein C derivatives which are characterized by an altered response to calcium. At 3 mM calcium, α-thrombin activated the derivatives 5- to 8-fold faster compared with the wild-type, an effect which was shown to be caused by a decreased inhibitory effect of calcium on the reaction. These same single amino acid substitutions enhanced the affinity of the thrombomodulin–thrombin complex for the substrate at 3 mM calcium 3-(Gly-substitution) to 6-(Phe-substitution) fold, either without influencing k_{cat} (Gly-substitution) or with a 2.5-fold decrease of k_{cat}. For both derivatives, the calcium concentrations resulting in half maximal inhibition of activation by α-thrombin and in half maximal stimulation of activation by the thrombomodulin–thrombin complex increased from 0.3 mM to 0.6 mM. It is concluded that Asp167 is involved in the calcium induced inhibition of protein C activation by thrombin. Moreover, our studies demonstrate that it is feasible to enhance the efficiency of enzymatic reactions by introducing point mutations in the substrate.

Key words: calcium/protein C/thrombin/thrombomodulin/zymogen activation

Introduction

Thrombin plays a critical role in the regulation of hemostasis by either triggering or inhibiting the coagulation cascade (Esmon, 1987, 1989). Downregulation of coagulation by thrombin is mediated through the activation of protein C, the zymogen of a vitamin K dependent plasma serine protease. As depicted in Figure 1, the mature human protein C zymogen is a disulphide linked heterodimer consisting of an NH_2-terminal light chain and a COOH-terminal heavy chain (Foster and Davie, 1984). The NH_2-terminal 12 amino acids of the heavy chain (resdues 158–169: Asp-Thr-Glu-Asp-Asn-Glu-Asp-Gln-Val-Asp-Pro-Arg) represent the activation peptide. Conversion of the zymogen to the active serine protease is accomplished through proteolytic removal of the activation peptide, which involves cleavage of the peptide bond between residues 169 and 170 (Arg-Leu). Activated protein C functions as an anticoagulant by degrading coagulation cofactors Va and VIIIa.

Under physiological conditions, human protein C and protein C of other mammalian species are activated by a complex of thrombin and its endothelial cell surface receptor thrombomodulin, which enhances the rate of activation >1000-fold over the rate achieved by thrombin alone, presumably by altering the macromolecular specificity of thrombin (Esmon and Owen, 1981). Calcium at physiologic extracellular concentrations inhibits protein C activation by thrombin alone, but is required for activation by the thrombomodulin–thrombin complex (Esmon *et al.*, 1983). The molecular basis for the difference in the substrate of free thrombin and the thrombomodulin–thrombin complex remains unknown. However, even in the absence of calcium, human protein C is a poor substrate for α-thrombin (Esmon *et al.*, 1983) compared with its natural substrate, the fibrinogen Aα-chain (Marsh *et al.*, 1982). Comparison of the amino acid sequences around the thrombin cleavage sites of human protein C (Beckmann *et al.*, 1985), the fibrinogen Aα-chain (Iwanaga *et al.*, 1967) and several other substrates sensitive or insensitive to thrombin (Chang, 1985) suggests that the site in human protein C is not optimal for efficient cleavage. Unlike most sensitive thrombin substrates, which have either neutral or hydrophobic residues close to the cleavage sites, Asp occupies position P3 relative to the thrombin cleavage site in human protein C. Thus, the primary structure around the thrombin cleavage site in human protein C may partially account for its ineffective activation by thrombin.

Site-specific mutagenesis was used to test the hypothesis that the replacement of Asp167 in human protein C (to make the substrate more fibrinogen Aα-chain like) by Gly (human protein C derivative D167G) or Phe (human protein C derivative D167F) may result in higher activation efficiency by α-thrombin. Therefore, recombinant human protein C wild-type and derivatives were expressed in a mammalian cell system that had been previously demonstrated to yield recombinant protein C with normal levels of biological anticoagulant activity (Ehrlich *et al.*, 1989). In the current study, we provide evidence that Asp167 is involved in the calcium induced inhibition of protein C activation by thrombin. The two derivatives in which Asp167 was replaced by Gly or Phe functioned as improved substrates for thrombin at physiological calcium concentrations. In addition, it is suggested that the NH_2-terminus of the heavy

chain of protein C, which does not contain a calcium binding site by itself, substantially contributes to the high affinity calcium binding properties of the epidermal growth factor homology domain, located on the light chain of protein C.

Results

Expression of human protein C wild-type and derivatives

Recombinant human protein C wild-type and derivatives were expressed in adenovirus type 12-induced Syrian hamster tumor cells under the control of a tripartite regulatory element consisting of the SV40 and BK virus enhancer and the adenovirus type 2 major late promoter (Berg *et al.*, 1988). At the expression levels observed (0.2−0.5 μg/ml serum free media), this cell line is capable of performing the required post-translational modifications, resulting in the secretion of human protein C zymogen, which after activation possesses full biological (anticoagulant) activity (Ehrlich *et al.*, 1989).

Western blot analysis of the recombinant proteins

Immunoblot analyses of the partially purified wild-type protein C and the human protein C derivatives D167G and D167F under reducing conditions indicated that they were predominantly (>90%) two chain molecules; the M_r of the heavy and light chains of the derivatives were identical to those of the wild-type (Figure 2). Plasma derived protein C exhibits a higher M_r than the recombinant molecules, a finding which is assumed to be caused by differential glycosylation (Ehrlich *et al.*, 1989).

Specific amidolytic and anticoagulant activities of the recombinant proteins

After complete activation with immobilized thrombomodulin−thrombin (Vigano-D'Angelo *et al.*, 1986), the human protein C wild-type and derivatives exhibited relatively similar amidolytic and biological (anticoagulant) activities (Table I). Slightly (8−15%) higher specific amidolytic and anticoagulant activities of the derivatives as compared with the recombinant wild-type were observed. This finding may be related to a marginally (<15%) diminished recognition of these proteins in the enzyme linked immunosorbent assay (ELISA), for which two monoclonal antibodies against protein C were utilized. For that reason, the amount of the activated protein C generated from each recombinant derivative was established using separate calibration curves constructed from amidolytic activities of the fully activated derivatives (Table II and Figure 3). However, the alterations of residue 167 had no substantial effect on the secretion, the chain structure and the functionality of domains required for activated protein C activity (e.g. serine protease domain, γ-carboxyglutamic acid domain). A slightly higher specific biological (anticoagulant) activity of the recombinant proteins compared with human protein C purified from plasma was found as noted previously (Grinnell *et al.*, 1987) and is believed to be caused by differences in carbohydrate content (B.W.Grinnell and S.B.Yan, unpublished observation).

Initial rates of activation of protein C wild-type and derivatives by α-thrombin

To test the hypothesis that the human protein C derivatives might be better substrates for α-thrombin than the wild-type, initial rates of activation of the three proteins by α-thrombin were established. Recombinant human protein C wild-type and derivatives were incubated with thrombin, and the amount of activated protein C generated in the presence of either 8 mM EDTA or 3 mM calcium was determined

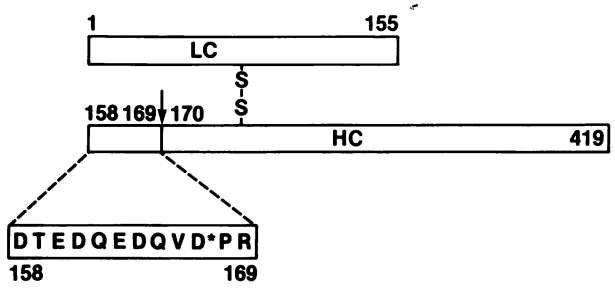

Fig. 1. Schematic representation of human protein C (Foster and Davie, 1984). The light chain (LC) is connected via at least one disulphide bridge to the heavy chain (HC). The number and localization of interchain disulphide linkages in human protein C are not known; however, the existence of a single interchain disulphide bridge is assumed based on homology with bovine chymotrypsin A (Dahlbäck *et al.*, 1986). Residues 158−169 comprise the activation peptide. Conversion of the zymogen to the active serine protease is accomplished through proteolytic removal of the activation peptide by the thrombomodulin−thrombin complex (↓). Numbers indicate the positions of NH_2- or COOH-terminal amino acid residues as derived from the cDNA sequence. A dipeptide comprising residues 156−157 (Lys-Arg) is released during post-translational processing. Abbreviations for the amino acid residues are: D, Asp; E, Glu; P, Pro; Q, Gln; R, Arg; T, Thr;V, Val. The asterisk (*) indicates the Asp in position 167 (P3 relative to the thrombin cleavage site) which was substituted by Gly or Phe.

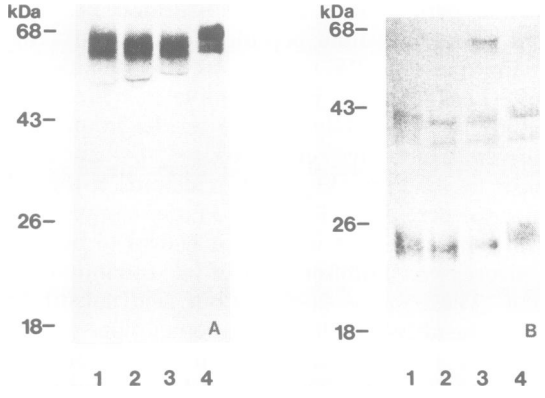

Fig. 2. Western blot analysis of human protein C and derivatives. **A**, non-reducing conditions; **B**, reducing conditions. **Lane 1**, protein C derivative D167F; **lane 2**, protein C derivative D167G; **lane 3**, recombinant human protein C wild-type; **lane 4**, human protein C purified from plasma.

Table I. Comparison of amidolytic and anticoagulant activities of activated human protein C (activated HPC) derived from different zymogens

Activated HPC derived from	Functional activity (units/mg)	
	Amidolytic	Anticoagulant
Plasma HPC	25 ± 3	250
Recombinant HPC (wild-type)	25 ± 2	290 ± 40
HPC derivative D167G	29 ± 3	330 ± 20
HPC derivative D167F	27 ± 2	310 ± 20

Table II. Kinetic evaluation of thrombomodulin–thrombin catalyzed activation of recombinant human protein C (HPC) wild-type and derivatives[a]

Substrate	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} μM^{-1})
Recombinant HPC (wild-type)	5.0 ± 0.4	149 ± 5	30
HPC derivative D167G	1.5 ± 0.2	155 ±10	103
HPC derivative D167F	0.8 ± 0.1	61 ± 3	76

[a]Data represent mean ± 1 SD from three independent experiments, each carried out in duplicate.

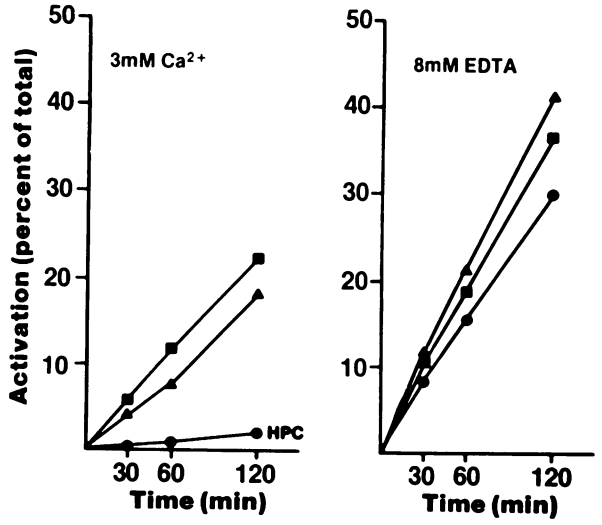

Fig. 3. Comparison of α-thrombin catalyzed activation of recombinant human protein C wild-type (●), D167G (▲) and D167F (■) in either 3 mM calcium or 8 mM EDTA. Human protein C wild-type or derivatives (0.33 μM) were incubated with α-thrombin (0.01 μM) in 20 mM Tris–HCl, pH 7.4, 0.1 M NaCl, 0.02% NaN_3, 0.1 mg/ml bovine serum albumin containing either 3 mM $CaCl_2$ (left panel) or 8 mM EDTA (right panel) at 37°C over time. The reaction was stopped at designated times by hirudin (60 U/ml final concentration). Activated protein C generated was measured as described in Materials and methods. Maximal activation observed by incubation of human protein C wild-type or derivatives with thrombomodulin–thrombin as described was taken as 100%.

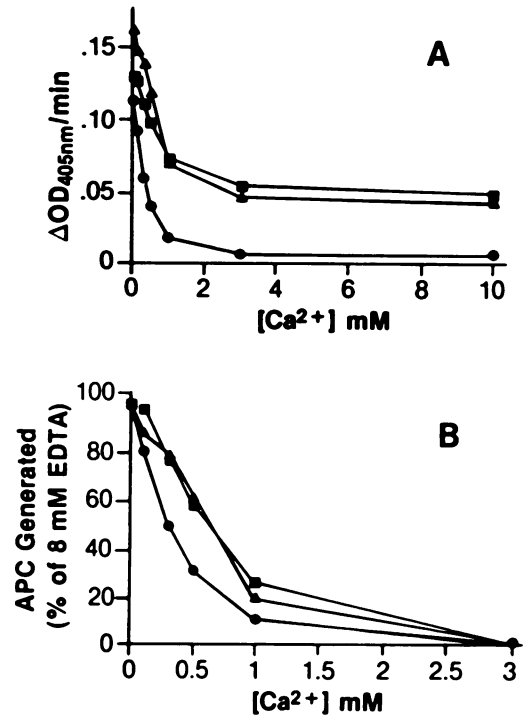

Fig. 4. Inhibitory effect of increasing calcium concentrations on the activation of recombinant human protein C wild-type (●), D167G (▲) and D167F (■) by α-thrombin. The recombinant proteins were passed over a Chelex column with quantitative protein recovery prior to the experiments. Gelatin (1 mg/ml) replaced the bovine serum albumin in the activation buffer. The reaction mixture contained 0.33 μM human protein C wild-type or derivatives in activation buffer as described in Figure 2 with varying $CaCl_2$ or, as a control, 8 mM EDTA. α-thrombin (0.01 μM final concentration) was added and the reaction was allowed to proceed for 90 min at 37°C, before it was terminated by the addition of hirudin (60 U/ml final concentration). Aliquots (50 μl) of the incubation mixes were then diluted 20-fold with 0.6 mM S2238 (in 20 mM Tris–HCl, pH 7.4, containing 100 mM NaCl and 0.1 mg/ml bovine serum albumin), and the release of *p*-nitroaniline was determined at 25°C by recording the increase in OD_{450} over 5 min in a Gilson RESPONSE spectrophotometer. **A.** *p*-nitroaniline generated (Δ OD at 405 nm/min). **B.** Replotting the data from A by arbitrarily taking the rate of activation observed with 8 mM EDTA as 100%. Half maximal inhibitory calcium concentrations were calculated on the basis of maximal inhibition (= 0% stimulation) at 3 mM calcium.

(Figure 3). The initial rates of activation were similar for all the three proteins in the presence of EDTA (Figure 3, right panel), with D167G being 1.4-fold and D167F 1.2-fold faster activated than the wild-type, respectively. In contrast, the activation of protein C wild-type was inhibited far more extensively by 3 mM calcium than the activation of the human protein C derivatives (Figure 3, left panel). D167G was activated 5 ± 1 (mean ± 1 SD from three independent experiments) times faster than the wild-type, and D167F was activated 8 ± 2 times faster. Thus, Asp167 appears to be largely responsible for the calcium dependent inhibition of protein C activation by thrombin; however, this amino acid residue does not seem to mediate the relatively slow activation of protein C by thrombin in the absence of calcium. Considering the high K_m reported for the activation of protein C by α-thrombin in the presence of calcium (Esmon *et al.*, 1983), we did not attempt to establish whether these increased rates of activation were mainly due to a reduced K_m or an increased k_{cat}. To elucidate further the inhibitory effect of calcium on the activation of protein C wild-type and derivatives by α-thrombin, the reactions were studied as a function of the calcium concentration. The results are shown in Figure 4. The slightly faster rates of activation of the derivatives in 8 mM EDTA as compared with the wild-type have already been discussed above. Increasing calcium concentrations caused a much more pronounced decrease of the rate of activation of the wild-type (factor 15) than of the two derivatives (D167G: factor 3.5; D167F: factor 2.4). Half maximal inhibitory calcium concentrations derived from the curves in Figure 4B are 0.3 mM for the wild-type and ~0.6 mM for both D167G and D167F, indicating a different response of the derivatives to calcium in comparison with the wild-type.

Initial rates of activation of protein C wild-type and derivatives by the thrombomodulin–thrombin complex

Calcium dependent conformational changes in protein C have been detected by fluorescence spectroscopy (Johnson *et al.*, 1983). It has been suggested that the calcium induced

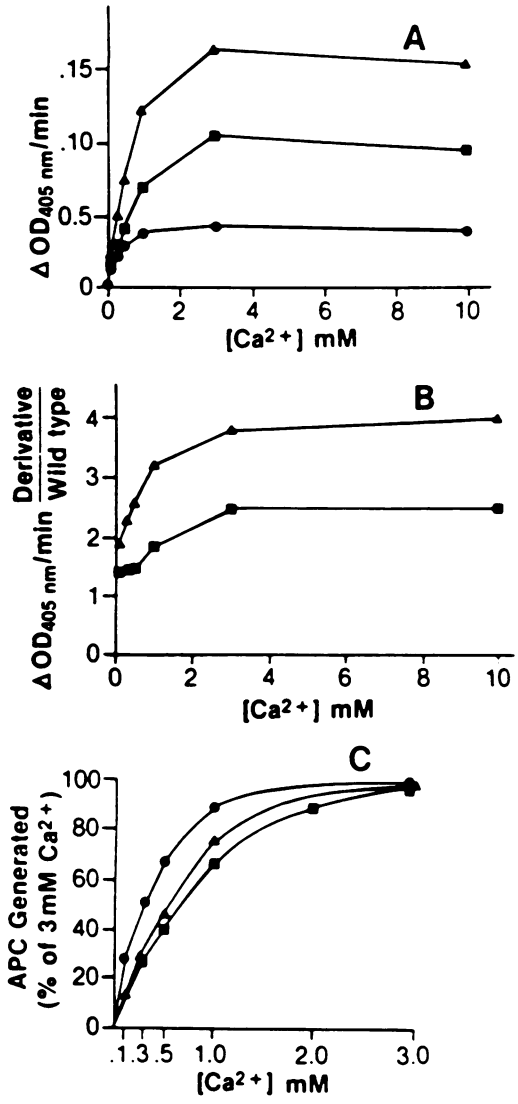

Fig. 5. Effect of increasing calcium concentrations on the activation of recombinant human protein C wild-type (●), D167G (▲) and D167F (■) by the thrombomodulin–thrombin complex. The reaction mixtures contained 0.33 μM human protein C wild-type or derivatives, 0.67 nM thrombin and 20 nM thrombomodulin in 20 mM Tris–HCl, pH 7.4, containing 100 mM NaCl and 1 mg/ml gelatin. After an incubation of 6 min at 37°C, the reaction was stopped by adding hirudin (60 U/ml final concentration) and EDTA (15 mM final concentration). Less than 15% of the total protein C was activated under these conditions. The amount of activated protein C generated was determined from the release of *p*-nitroaniline as described in the legend to Figure 4. **A.** *p*-nitroaniline generated (Δ OD at 405 nm/min). **B.** Factor of enhanced activation of protein C derivatives D167G (▲) and D167F (■) over wild-type as a function of the calcium concentration. **C.** Replotting the data from A by arbitrarily taking the rate of activation observed with 3 mM calcium as 100%.

conformational change, which inhibits the activation of protein C by free thrombin, is the same conformational change which is required for activation by thrombomodulin–thrombin. To test this hypothesis, the initial rates of activation of recombinant protein C wild-type and derivatives by thrombomodulin–thrombin were established as a function of increasing calcium concentrations. As depicted in Figure 5A, both derivatives were better substrates for the complex at all calcium concentrations studied. However, as shown in Figure 5B, this effect was much more pronounced at high calcium concentrations (3 and 10 mM) than at low calcium concentrations (0.1–0.5 mM). From this data, it appeared that the derivatives needed more calcium to reach maximal velocity of the activation reaction than the wild-type. Therefore, the data was replotted such that calcium concentrations causing half maximal acceleration of the thrombomodulin–thrombin catalyzed activation of recombinant protein C wild-type and derivatives could be established (Figure 5C). The values were 0.3 mM for human protein C wild-type, consistent with previously reported data on bovine protein C (Esmon *et al.*, 1983), and ~0.6 mM for both derivatives. Thus, the substitution of Asp167 results in an identical shift in the calcium concentration required for half maximal stimulation of thrombomodulin–thrombin induced zymogen activation and half maximal inhibition of zymogen activation catalyzed by thrombin.

To establish whether the increased rates of activation by thrombomodulin–thrombin of the derivatives as compared with the wild-type were due mainly to an enhanced affinity of the enzyme–cofactor complex for the substrate or to an increased turnover rate, kinetic analysis was performed at a calcium concentration of 3 mM. Data were processed for the determination of K_m and k_{cat} utilizing a weighted, non-linear least squares fit analysis (Cleland, 1979). The results are summarized in Table II. The data obtained with the recombinant wild-type are in good agreement with data reported earlier for the activation of protein C from bovine plasma, also using bovine thrombin and rabbit thrombomodulin under similar conditions (Esmon *et al.*, 1983). As has been noted before (Esmon *et al.*, 1983), the K_m observed for the protein C wild-type (5 μM) is much higher than the concentration of protein C in plasma (~0.07 μM). However, it should be emphasized that these experiments were performed in solution with purified bovine thrombin and rabbit thrombomodulin, the latter being a detergent-solubilized membrane protein with a typical transmembrane domain. This *in vitro* system is not entirely representative of the *in vivo* situation, where protein C activation is initiated by assembling all necessary factors and cofactors on the endothelial cell surface. Indeed, it was shown that the activation of protein C by the enzyme–cofactor complex is accelerated almost 100-fold upon reconstitution of thrombomodulin into phospholipid vesicles, an effect entirely due to reduction of K_m (Galvin *et al.*, 1987).

Comparing wild-type and derivatives based on k_{cat}/K_m, D167G and D167F are 3.5-fold and 2.5-fold better substrates for the thrombomodulin–thrombin complex than the wild-type, consistent with data shown in Figure 5B. The effect on D167G appears to be solely due to an increased affinity, since a reduction of the K_m, but no effect on the k_{cat} was observed. The effect of the mutation in derivative D167F is more complex: a 6-fold reduction of K_m is observed concomitantly with a 2.5-fold reduction of k_{cat}. Thus, the activator complex has indeed a 6-fold higher affinity for this molecule; however, this is accompanied by a reduced rate of cleavage of the susceptible peptide bond.

Discussion

One of the striking features of the protein C activation complex is the important role played by calcium. With free thrombin, calcium functions as a potent inhibitor, whereas

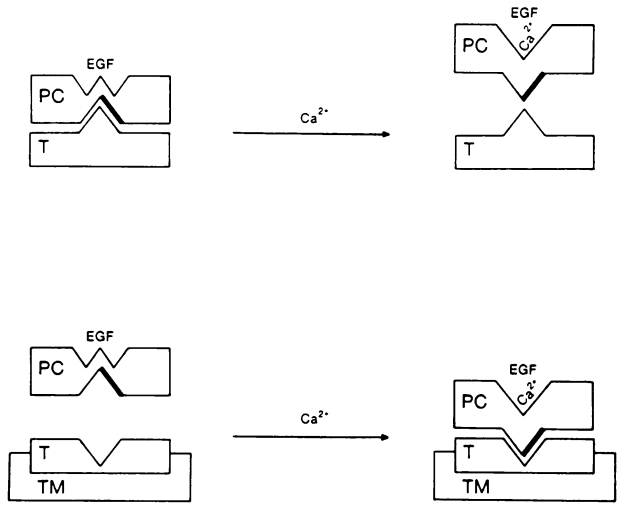

Fig. 6. Model for the conformational change of protein C resulting from high affinity calcium binding and the effect of this conformational change on the interaction of the substrate (protein C) with thrombin and thrombomodulin–thrombin. The model is based on published data (Johnson *et al.*, 1983; Öhlin *et al.*, 1988b) and incorporates the following observations: (i) in the absence of calcium, protein C interacts with thrombin, but not with thrombomodulin–thrombin, (ii) high affinity calcium binding to the epidermal growth factor homology domain results in a conformational change of protein C that inhibits the interaction with thrombin and facilitates the interaction with thrombomodulin–thrombin, (iii) independent of the presence of calcium, complex formation with thrombomodulin modifies the macromolecular substrate specificity of thrombin. The data presented in this paper is most consistent with Asp167 (relative to Gly or Phe) increasing the affinity for calcium ions, thereby facilitating the conformational change responsible for the decreased affinity of thrombin for protein C as well as the increased affinity of thrombomodulin–thrombin for protein C which is observed in the presence of calcium. PC = protein C; EGF = epidermal growth factor homology domain in protein C; T = thrombin; TM = thrombomodulin. The thick line in protein C is representative of the activation peptide.

with the thrombomodulin–thrombin complex, calcium is required for efficient activation. All of the available information suggests that this calcium effect is mediated through conformational changes in the substrate, i.e. the protein C molecule. Specifically, high affinity calcium binding to the substrate correlates well with the calcium dependence of protein C activation (Esmon *et al.*, 1983) and with a conformational change monitored by protein fluorescence (Johnson *et al.*, 1983). The influence of calcium ions on protein C activation is largely reflected by a decreased affinity of thrombin for protein C and, at the same time, by an increased affinity of the thrombomodulin–thrombin complex for protein C. Occupancy of the high affinity calcium binding site also correlates well with the calcium dependence of binding of a calcium dependent monoclonal antibody to an epitope that overlaps the activation site (Stearns *et al.*, 1988). Recent studies by Öhlin and coworkers have demonstrated that the high affinity calcium binding site in protein C is located in the epidermal growth factor domain (Öhlin and Stenflo, 1987; Öhlin *et al.*, 1988a). Taken together, these results suggest a conformational coupling between a high affinity calcium binding site in the epidermal growth factor homology region of the light chain and the activation peptide, located at the NH_2-terminus of the heavy chain, such that calcium binding in the epidermal growth factor domain changes the conformation of protein C at the activation site. This conformational change is essential for the correct presentation of the Arg169–Leu170 peptide bond for efficient cleavage by thrombomodulin–thrombin, tentatively explaining why, in the presence of calcium, protein C is a good substrate for the thrombomodulin–thrombin complex, but a poor substrate for α-thrombin. A schematic model, consistent with this information, is presented in Figure 6.

The present study provides insights into the mechanism by which calcium inhibits α-thrombin induced activation of protein C. Substitution of Asp167 by either Gly or Phe to a large part eliminates the inhibitory effect of calcium, while hardly influencing the activation in the absence of calcium. Thus, it is apparent that Asp167 plays a critical role in the calcium induced inhibition of protein C activation by thrombin. Our initial hypothesis, i.e. that better thrombin substrates are generated by replacing the negatively charged residue in position P3 relative to the thrombin cleavage site, is therefore only valid when calcium is present. The failure of calcium, even at high concentrations, to block thrombin activation of the derivatives as completely as it blocks thrombin activation of the protein C wild-type may suggest that the conformational change is less complete with the derivatives. In addition to decreasing substantially the magnitude of the calcium inhibition, these single amino acid substitutions also increase the calcium concentration dependence of the inhibition ~2-fold, since the calcium concentration at which half maximal inhibition is observed shifted from 0.3 mM to 0.6 mM. A similar shift is observed for the half maximal stimulatory calcium concentration when the derivatives are activated by the thrombomodulin–thrombin complex. Given that the amino acid substitutions influence the calcium requirements for both thrombin and thrombomodulin–thrombin to the same extent and in the same direction, it is likely that these substitutions affect the affinity of the substrate for calcium. The influence of the single amino acid substitutions on zymogen activation by thrombomodulin–thrombin, compared with thrombin alone, appears to be more complex. From the combined effects of (i) a lower affinity for calcium of the derivatives and (ii) a less complete conformational change, a less efficient rate of activation of the derivatives by thrombomodulin–thrombin (which requires the calcium induced conformational change) would be expected. Consistent with this prediction, the derivatives need more calcium to reach maximal velocity of the activation by the thrombomodulin–thrombin complex than the wild-type. The finding that the maximal velocity of thrombomodulin–thrombin catalyzed activation of the derivatives is higher than that of the wild-type suggests that, in addition to the affinity for calcium and the extent of the calcium induced conformational change, the nature of the primary structure of the activation peptide in the area of the susceptible (Arg169–Leu170) peptide bond may determine the affinity of the enzyme–cofactor complex for the substrate.

Considering the striking homology in domain stucture of protein C and other zymogens of vitamin K dependent serine proteases of the coagulation system (Patthy, 1985; Furie and Furie, 1988), it is not surprising that high affinity calcium binding to an epidermal growth factor homology domain has also been described for factor IX (Morita *et al.*, 1984; Morita and Kisiel, 1985) and factor X (Sugo *et al.*, 1984). Specifically, it has been demonstrated that the first of the

two adjacent epidermal growth factor like domains from bovine factor X (Persson *et al.*, 1989) and bovine protein C (Öhlin *et al.*, 1988a) contains this calcium binding site. In addition, the first epidermal growth factor like domain from human factor IX (residues 46–84), expressed in yeast and purified to homogeneity, bound calcium with high affinity (Handford *et al.*, 1990). Recombinant factor IX derivatives, carrying point mutations in the epidermal growth factor homology domain, exhibited a reduced rate of factor X activation as compared with the wild-type only in the presence of the cofactors factor VIII, calcium and phospholipids (Rees *et al.*, 1988). The authors suggest that calcium binding to this region, presumably to β-hydroxyasp64 and to adjacent carboxylate residues (Asp47, Asp49 and Glu78), may result in the correct conformation of factor IXa necessary for interaction with factor VIIIa. Similar findings were reported for a derivative of protein C, in which β-hydroxyasp was substituted by Glu, resulting in a reduction of the biological anticoagulant activity to ~10% of normal (Öhlin *et al.*, 1988b). By contrast, in view of our findings presented here, the contribution of Asp167 to high affinity calcium binding is not crucial for expression of protein C anticoagulant activity in biological systems. This is supported by data from earlier experiments, in which the entire activation peptide (including Asp167) of human protein C was replaced by an eight amino acid sequence (Pro-Arg-Pro-Ser-Arg-Lys-Arg-Arg), involved in the proteolytic processing of the human insulin receptor precursor (Ebina *et al.*, 1985), resulting in the direct secretion of fully activated, biologically active protein C by mammalian cells (Ehrlich *et al.*, 1989).

The application of site specific mutagenesis has improved our knowledge of structure–function relationships of enzyme specificity and catalysis (Knowles, 1987). However, attempts to increase the catalytic efficiency of enzymatic reactions through mutagenesis have, for the most part, proven elusive: redesigning enzymes by changing residues in or near the active site has not as yet resulted in improved catalytic properties towards natural substrates (Craik *et al.*, 1985; Graf *et al.*, 1987; Knowles, 1987). The results presented here suggest that the overall activity of an important proteolytic pathway (the protein C anticoagulant pathway) can be enhanced by altering the rate of activation of a zymogen by changing the substrate (protein C) rather than redesigning the enzyme (thrombin or the thrombomodulin–thrombin complex).

Materials and methods

Materials

Restriction endonucleases were purchased from New England Biolabs or Bethesda Research Laboratories. T4 DNA ligase was purchased from New England Biolabs. T4 polynucleotide kinase was from Pharmacia and the Klenow fragment of DNA polymerase I from Boehringer Mannheim Biochemicals. M13mp18 RF and a 15-mer M13 sequencing primer (# 1200) were obtained from New England Biolabs. Dulbecco's modified Eagle medium, Ham's F12 medium and gentamicin were from Gibco; hygromycin was from Eli Lilly and Co., fetal calf serum was from Hyclone and bovine serum albumin (low endotoxin) was from Miles. Assayed reference plasma, activated partial thromboplastin time (APTT) reagent, and the substrate H-D-Phe-Pip-Arg-*p*-nitroanilide (S2238) (Kabi Vitrum) were purchased from Helena. Q-Sepharose Fast Flow was from Pharmacia. Aquamephyton, an aqueous colloidal solution of vitamin K1, was obtained from Merck Sharp and Dohme. Chelex was from BioRad. EDTA, Na-bicarbonate, Na-selenite, 2-aminoethanol, hirudin, bovine insulin and human transferrin were from Sigma. Murine monoclonal antibodies against human protein C used in the sandwich-ELISA were obtained from Dr J.H.Griffin, Scripps Institute (α-HPC-1, directed against the heavy chain of protein C and α-HPC-3, directed against the light chain of protein C). Rabbit thrombomodulin and anti-rabbit thrombomodulin antibody covalently coupled to Affigel 10 beads were a gift from Dr N.L.Esmon, Howard Hughes Medical Institute, Oklahoma Medical Research Foundation. The adenovirus type 12-induced Syrian hamster tumor cell line AV12-664 (deposited with ATCC, # CRL9595) was obtained form Dr J.P.Burnett, Lilly Research Laboratories.

General methods

Plasmid DNA was isolated by a modification of the Triton X-100 lysis procedure (Kahn *et al.*, 1979), followed by two consecutive CsCl equilibrium centrifugations. Enzyme reactions were carried out using standard conditions (Maniatis *et al.*, 1982). Nucleotide sequence determinations were performed using the dideoxy method of Sanger *et al.* (1977). Synthetic oligonucleotides were synthesized by solid-phase phosphoramidite chemistry on an automated synthesizer (Applied Biosystems, model 380B).

Purification of plasma proteins

Human protein C (Grinnell *et al.*, 1987), bovine thrombin (Owen *et al.*, 1974) (specific activity 2700 NIH-U/mg) and bovine antithrombin III (Owen, 1975) were purified according to published methods.

Oligonucleotide-directed mutagenesis and plasmid construction

A 710 bp *SstI–SalI* fragment from a full length copy of the human protein C coding sequence (Beckmann *et al.*, 1985) was used for oligonucleotide-directed, site specific mutagenesis in M13 (Zoller and Smith, 1984). The oligonucleotides used for the mutagenesis were: D167G, 5′-GACCAAGAA-GACCAAGTAGGCCCGCGGCTCATTGATG-3′ and D167F, 5′-GAC-CAAGAAGACCAAGTATTCCCGCGGCTCATTGATG-3′ (underlined nucleotides indicate the altered amino acid codons). The mutated fragments were substituted for the wild-type sequence in the mammalian cell expression plasmid for human protein C as described (Ehrlich *et al.*, 1989).

Mammalian cell transfection, screening of recombinant clones and expression and partial purification of recombinant proteins

The human protein C expression vectors were cotransfected into the AV12 cell line with pSV2hyg (Grinnell *et al.*, 1987), which codes for the dominant selectable marker hygromycin phosphotransferase. Recombinant cells resistant to hygromycin (200 μg/ml) were isolated and grown in the presence of vitamin K1 (10 μg/ml). These clones were screened for the presence of human protein C antigen in the conditioned media using an ELISA described previously (Ehrlich *et al.*, 1989). Two litres of conditioned serum-free media (3 parts Dulbecco's modified Eagle medium and 1 part Ham's F12 medium, containing 1 mM Na-selenite, 100 μM 2-aminoethanol, 20 mM HEPES, 2.4 g/l Na-bicarbonate, 1 μg/ml transferrin, 1 μg/ml insulin, 100 μg/ml bovine serum albumin, 50 μg/ml gentamicin, and 10 μg/ml vitamin K1), containing either human protein C wild-type, D167G or D167F from recombinant AV12 cells grown in roller-bottles, were centrifuged (10 000 *g*, 20 min, +4°C) to remove cell debris. The supernatants were then adjusted to 4 mM EDTA and adsorbed batchwise (2 h, +4°C) onto 2 ml of Q-Sepharose Fast Flow, equilibrated with 20 mM Tris–HCl, pH 7.4, 100 mM NaCl, 4 mM EDTA. After decanting the supernatant, the resin was packed into a column (1.0 × 6.0 cm), washed with 5 column volumes of equilibration buffer and eluted with 20 mM Tris–HCl, pH 7.4, containing 0.5 M NaCl and 10 mM $CaCl_2$. The recombinant proteins were further concentrated and buffer exchanged to 20 mM Tris–HCl, pH 7.4, 100 mM NaCl using Centricon™ 10 microconcentrators (Amicon).

Western blot analysis

Human protein C, purified from plasma (Ehrlich *et al.*, 1989), as well as recombinant protein C wild-type or derivatives, partially purified as described above, were subjected to SDS–PAGE (0.5 μg/lane) according to Laemmli (Laemmli, 1970), using a 10% (acryl: bis, 30:0.8) separating gel. After the proteins had been transferred to nitrocellulose, the filter was blocked for 1 h (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 5% nonfat dried milk) and then incbuated for 1 h with polyclonal α-human protein C antiserum, raised in a goat. After washing three times wth 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, the sheet was incubated for 1 h with biotinylated anti-goat IgG, then washed extensively and finally developed utilizing horseradish peroxidase conjugated avidin D.

Activation of human protein C and derivatives

Human protein C purified from plasma and recombinant protein C wild-type or derivatives (0.33 μM as determined by ELISA) were activated by immobilized thrombomodulin–thrombin (50 nM) for 2 h at 37°C on a rocker

platform as described (Vigano-D'Angelo, 1986). After centrifugation, the supernatant was removed and incubated for 15 min at 20°C with bovine antithrombin III (4 U/ml) prior to assaying for functional activity.

Determination of amidolytic and anticoagulant activity

Amidolytic activity was measured by hydrolysis of the synthetic substrate S2238 (H-D-Phe-Pip-Arg-p-nitroanilide) at 25°C, pH 7.4 as described (Ehrlich *et al.*, 1989). One unit of activated protein C was defined as the activity that released 1 μmol of *p*-nitroaniline in 1 min at 405 nm, using an extinction coefficient for *p*-nitroaniline of 9620 M^{-1} cm^{-1} (Pfleiderer, 1970). Anticoagulant activity was determined by measuring the prolongation of the activated partial thromboplastin time assay, resulting from inactivation of coagulation cofactors Va and VIIIa as described (Ehrlich *et al.*, 1989). After recording the time required for clot formation, a standard curve was prepared using serial dilutions of activated protein C from plasma. Duplicate determinations were run for each test sample and converted to the concentration of functionally active protein. The equivalent of 4 μg plasma human protein C, present normally in 1 ml of plasma, was defined as 1 unit of anticoagulant activity. For both assays, activated protein C derived from human plasma served as the standard.

Kinetic analysis of activation of recombinant human protein C wild-type and derivatives by thrombomodulin – thrombin

Initial rates of protein C activation were determined by stopping the activation reaction and measuring the amount of activated protein C formed. All kinetic measurements were performed at 37°C in 20 mM Tris–HCl, pH 7.4, containing 100 mM NaCl, 3 mM $CaCl_2$, 0.1 mg/ml bovine serum albumin and 0.02% NaN_3. Recombinant human protein C wild-type (1.6–20 μM) or derivatives (0.16–2.0 μM) were incubated with thrombomodulin (20 nM) and thrombin (0.67 nM) at 37°C. The incubation time (2 min) was chosen such that $< 10\%$ of the substrates (recombinant protein C wild-type or derivatives) were converted into activated protein C. Incubation was terminated by the addition of hirudin (60 U/ml final concentration) and EDTA (15 mM final concentration). Activated protein C generated was measured utilizing S2238 as described above. The concentration of activated protein C was determined from standard curves relating the rate of *p*-nitroaniline generated to the concentration of activated protein C. The data was analyzed using a computer program that carries out a weighted non-linear least squares fit analysis (Cleland, 1979).

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