Identification of a region in protein C involved in thrombomodulin-stimulated activation by thrombin: potential repulsion at anion-binding site I in thrombin

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During coagulation human protein C is activated by thrombin; however, this cleavage reaction is slow unless thrombin is complexed with a cofactor, thrombomodulin. Near the thrombin cleavage site in protein C is a cluster of basic residues, at positions P5' (Lys-174), P8' (Arg-177) and P9' (Arg-178). We have explored the role of this basic cluster in the activation of protein C by thrombin, and by thrombin–thrombomodulin complex, by substitution of glutamic acid at each position to generate the acidic protein C derivative P'-EEE. The activation rate of P'-EEE by free α -thrombin was approx. 12-fold faster than that observed for wild-type (wt) human protein C zymogen (HPC) in the presence of calcium, but unchanged in the absence of calcium. While the thrombin-catalysed activation of wt-HPC was stimulated approx. 300-fold by thrombomodulin, we observed no effect of thrombomodulin on thrombin-catalysed

activation of the P'-EEE derivative. Using synthetic peptides that bind to anion-binding site I of thrombin (thrombin-receptor sequence 52–66 and hirudin sequence 54–65-SO₄ Tyr), we found that the rate of thrombin-catalysed activation of wt-HPC in the presence of calcium could be increased severalfold in a dose-dependent manner. However, the enhanced rate of thrombin-catalysed activation of P'-EEE could be progressively reduced to wt-HPC levels with increasing concentrations of both synthetic peptides. Our data suggest that the P' basic cluster in protein C reduces interaction with free α -thrombin through electrostatic repulsion with anion-binding site I, a site that is masked when thrombomodulin binds thrombin. Further, the lack of thrombomodulin cofactor activity with thrombin-catalysed activation of P'-EEE suggests that the basic cluster in protein C forms a contact site with thrombomodulin.

INTRODUCTION

Human protein C zymogen (HPC) is an endogenous regulator of coagulation that is activated by thrombin [1–3]. Under physiological conditions, the conversion of HPC into activated protein C (aPC) by α -thrombin alone occurs slowly, primarily due to the inhibitory effect of a calcium-induced conformational change in protein C [4,5]. The efficiency of protein C activation in the presence of calcium is enhanced substantially when thrombin is complexed with the integral membrane protein, thrombomodulin (reviewed in [6]). The mechanism by which thrombomodulin enhances protein C activation by thrombin has not been totally elucidated, but it is assumed that thrombomodulin overcomes the inhibitory effect of suboptimal contact residues between protein C and thrombin [7]. Surrounding the thrombin cleavage site in HPC, particularly at both the P3 and P3' positions, are acidic residues that have been shown to contribute to the low rate of activation by free α -thrombin [5,8–11], an effect suggested to result from repulsive interactions with Glu-39 and Glu-192 in thrombin (reviewed in [7]). Thrombomodulin has been shown to induce a conformational change in the active-site region of thrombin and it has been suggested that this change reduces the inhibitory influence of these acidic P3/P3' residues in protein C [12]. While the thrombomodulin-induced conformational changes in thrombin's active site and the negative influence of the acidic P3/P3' residues are clearly important, they do not appear to completely account for the poor interaction of protein C with

free α -thrombin or for the dramatic increase in protein C activation following thrombomodulin binding to thrombin.

Thrombomodulin binds thrombin via anion-binding site I (reviewed in [13]), a basic cluster near the catalytic site. Anion-binding site I is an important exosite for the interaction of many thrombin substrates (e.g. fibrinogen) and inhibitors (e.g. hirudin) via positive electrostatic interactions with acidic residues P' to the cleavage site (reviewed in [14]). In protein C, the analogous region P' to the thrombin-cleavage site is not acidic, but contains several basic residues at P5' (Lys-174), P8' (Arg-177) and P9' (Arg-178). In this study we have explored the possibility that repulsion between this P' basic cluster and anion-binding site I reduces efficient interaction between protein C and thrombin. As it has been suggested that the electrostatic interaction between anion-binding site I and thrombin substrates/inhibitors utilizes a more general complementarity of electrostatic fields, rather than specific salt-bridges [14], we chose to simultaneously eliminate all three basic residues in the P' region. We show that substitution of acidic residues in the basic cluster increased the rate of activation by free α -thrombin by approx. 12-fold in the presence of calcium. This enhanced rate effect could be essentially eliminated by competition with a peptide that binds to anion-binding site I. In addition, we show that the rate of thrombin-catalysed activation of the acidic-substituted derivative could no longer be stimulated by thrombomodulin, suggesting that in addition to masking negative interactions at anion-binding site I, thrombomodulin may interact with the basic P' cluster in protein C.

Abbreviations used: aPC, activated human protein C; HPC, human protein C zymogen; Hyg, hygromycin B; wt, wild type; EGF, epidermal growth factor; TR52–66, hirudin-like domain of the human thrombin receptor residues 52–66 (YEPFWEDEEKNEGSG); HR54–65, hirudin peptide (GDFEIEPEYLQ with sulphated Tyr-63); BCA, bicinchoninic acid; P'-EEE, protein C derivative in which residues 174, 177 and 178 are replaced by glutamic acid residues.

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EXPERIMENTAL

Materials

Materials utilized included: Muta-Gene M13 *in vitro* Mutagenesis kit (170-3580) and 4-chloro-1-naphthol (170-6534) from Bio-Rad; PAGE 1 protein gel mix (100-681) from Boehringer-Mannheim; hygromycin B (Hyg) (400051) from Calbiochem; chromogenic substrate S-2366 (Glu-Pro-Arg-*p*-nitroanilide; cat. no. 82-10-90) from Chromogenix; fetal bovine serum (A-1111-L) from HyClone; Pentex FAF BSA (82-002) and human transferrin (82-316) from Miles Scientific; Immobilon-P (IPVH-151-50) and Ultrafree-CL 30000 NMWL filtration units (UFC4-LTK-25) from Millipore; restriction endonucleases, T4 polynucleotide kinase, and T4 DNA ligase from either New England Biolabs or Boehringer-Mannheim; fast-flow Q-Sepharose (17-0510-01) from Pharmacia; bicinchoninic acid (BCA) protein assay reagent (23225-B) from Pierce; ampicillin (A-9518), benzamidine hydrochloride (B-6506), sulphated hirudin peptide 54-65 (HR54-65) (cat. no. H-7445) from Bachem Bioscience Inc. Thrombin-Sepharose, coupled at 2 mg of thrombin per ml of resin, was obtained from J. Secnik and Dr. W. Prouty (Eli Lilly and Co.). All other chemicals used were of the highest purity commercially available.

Site-directed mutagenesis and expression of protein C

Changes in the serine protease domain of human protein C were made by site-directed mutagenesis using M13mp19-NHPC.730 [15] as the source of single-stranded DNA template. The oligonucleotide primer for the generation of derivative P'-EEE (Glu substituted for residues 174, 177 and 178 of protein C) was made with an Applied Biosystems model 380 DNA synthesizer and had the following sequence: CTGCCAGGGGCTTCCCTCC-TCAGTCATCTCCCCATCAATGAGCCGCGG, with the changed residues at positions of native human protein C shown underlined. Mutagenesis was performed by the method originally outlined by Kunkel [16], with modifications to the Bio-Rad protocol as described previously [15]. Preliminary screening of resultant phage was by DNA miniprep procedure and restriction endonuclease digestion. Candidate positives were then verified by dideoxy-nucleotide sequencing using an ABI 370A Automated Fluorescent Sequencer. Following sequence confirmation of mutant phage, double-stranded phage DNA was prepared and used to isolate the protein C insert, a 730 bp *SalI*/*SacI* fragment, which was used to reconstruct plasmid pLPC [17]. The 1425 bp *BclI* fragment, containing the mutated HPC sequence, was excised and inserted into the eukaryotic expression vector pGT-h [18] at its unique *BclI* site, linearized with *FspI*, and used to transfect 293 cells (A.T.C.C. CRL 1573). Stable recombinant cell lines secreting wild-type (wt) and mutant protein C were isolated as described in Berg et al. [18]. Stable clones were cultured in roller bottles, conditioned serum-free medium was collected, and protein C purified by fast-flow Q-Sepharose anion-exchange chromatography by the method of Yan et al. [19] as described previously [15].

Activation of recombinant HPC

Thrombin-Sepharose 4B was washed extensively with Buffer A-EDTA (150 mM NaCl, 20 mM Tris/HCl, pH 7.4 and 1 mM EDTA) and 300 μ g of HPC in 1 ml of Buffer A-EDTA was incubated with 200 μ l of packed thrombin-Sepharose 4B for 4 h at 37 °C on a rotating platform. During the course of this

incubation, the degree of HPC activation was monitored by briefly pelleting the thrombin-Sepharose 4B and assaying an aliquot of the supernatant for aPC amidolytic activity using the chromogenic substrate S-2366. After complete activation, the thrombin-Sepharose 4B was pelleted, the supernatant collected, its protein concentration verified by Pierce BCA assay, and aPC either assayed directly or frozen in aliquots at -20 °C. The amidolytic activity of the aPC was determined by the hydrolysis of a tripeptide substrate Glu-Pro-Arg-*p*-nitroanilide (S-2366) at pH 7.4, 25 °C. One unit of aPC was defined as the amount of aPC required to release 1 μ mol of *p*-nitroanilide in 1 min at 25 °C, pH 7.4, using an absorbance coefficient for *p*-nitroanilide at 405 nm of 9620 M⁻¹·cm⁻¹.

Activation rates

Activation rates were determined at 37 °C using human α -thrombin (10 nM) in a reaction mix containing 20 mM Tris, pH 7.4, 150 mM NaCl, 0.1 mg/ml BSA, and 3 mM CaCl₂. Purified protein C, both wild-type and the P'-EEE derivative, were at a concentration of 0.4 μ M in the activation reaction. Activation rates were determined by removing aliquots from the activation-reaction mix at various time points to a 96-well plate, adding the chromogenic substrate (S-2366) to a final concentration of 0.75 mM, and measuring the change in absorbance units/min at 405 nm in a ThermoMax kinetic microtitre-plate reader (Molecular Devices). Rates were determined by converting change in absorbance/min into amount of aPC generated, using the specific activities determined for each protein, and plotted versus activation time. For rate determinations, the amount of aPC generated was less than 10% of the initial zymogen in all experiments. In some experiments, the rates were determined in the absence of calcium by the addition of 5 mM EDTA. Determination of activation rates by thrombin in complex with thrombomodulin was performed at 10 nM thrombin and a 15-fold molar excess of human recombinant soluble 75 kDa thrombomodulin at 37 °C [20]. Rate determinations for wt and P'-EEE protein C were also performed in the presence of a peptide representing the anion-binding region of the thrombin receptor amino acids 52-66 (TR52-66) and with a sulphated hirudin peptide, amino acids 54-65 (HR54-65).

RESULTS AND DISCUSSION

Expression and analysis of protein C derivatives

By site-directed mutagenesis of the protein C coding sequence, we changed the three basic residues C-terminal to the thrombin cleavage site at the P5' (Lys-174), P8' (Arg-177) and P9' (Arg-178) each to glutamic acid to form derivative P'-EEE (Figure 1). Both wt-HPC and P'-EEE were expressed in the human kidney cell line 293, which has been previously demonstrated to efficiently perform the post-translational modifications of γ -carboxylation and β -hydroxylation required for functional activity [17,19,21,22]. Stable recombinant 293 cell lines secreting wt-HPC and P'-EEE were created by the isolation of Hyg-resistant clones as previously described [17], and the protein C from the recombinant 293 lines was purified from serum-free conditioned culture medium using the procedure of Yan et al. [19]. On the basis of Coomassie Blue-stained gels, the purity of the preparations were > 95%. Although our studies were focused on protein C as a substrate for thrombin, we determined the functional activities of the P'-EEE derivative after activation by thrombin. The functional protease activity of P'-EEE was not substantially reduced, as measured by cleavage of synthetic tripeptide substrate S-2366;

Figure 1 Schematic representation of processed protein C with the location of site-specific amino acid changes

Numbers refer to amino acid positions in the mature protein (following removal of the signal sequence and propeptide). The site for thrombin cleavage of the activation peptide (AP) is indicated. The designations P1 and P1' refer to the first position N-terminal and C-terminal respectively to the thrombin cleavage site. Each amino acid change was made by site-directed mutagenesis using the cDNA coding sequence previously described [27]. The light chain contains two EGF-like regions and a region of γ -carboxyglutamic acid (GLA) residues that is required for calcium-dependent membrane binding and functional activity (reviewed in [28]).

the amidolytic activities for activated wt-HPC and P'-EEE were 35 ± 5 and 28 ± 4 units/mg respectively.

Activation rate of wt-HPC and P'-EEE with α -thrombin

We determined the effect of the acidic substitutions in the P' region on thrombin-catalysed activation in the presence and absence of calcium ions. As shown in Figure 2(a), the rate of activation of the P'-EEE derivative by α -thrombin in the presence of calcium was approx. 12-fold higher than wt-HPC (11.6 ± 1.7 , $n = 7$). In the absence of calcium (5 mM EDTA) the rate of activation of wt-HPC was substantially higher, as expected. However, P'-EEE was not activated more rapidly; the rates of activation were the same for wt-HPC and P'-EEE in the absence of calcium (Figure 2b). These results suggest that the P' basic cluster is involved in inhibitory interactions with thrombin under physiological calcium ion concentrations, and that the protein C conformation in the absence of calcium masks these inhibitory residues. Because of the very high K_m for protein C with free α -thrombin in the presence of calcium [23], we were not able to directly compare affinities.

Activation rate of wt-HPC and P'-EEE with thrombin-thrombomodulin complex

In contrast with the increased rate of activation of P'-EEE by free α -thrombin, the rate of activation of this derivative by thrombin in complex with thrombomodulin was substantially reduced compared with wt-HPC (Figure 3a). In the experiment shown, wt-HPC was almost completely activated at the 10 min time point compared with approx. 1% activation of the P'-EEE derivative. As shown in Figure 3(b), the rate of thrombin-catalysed activation of wt-HPC was stimulated up to approx. 300-fold with increasing amounts of cofactor thrombomodulin, whereas the rate of activation of P'-EEE was unchanged even at saturating concentrations of thrombomodulin. These results indicate that the P' basic region of protein C is required for activation by thrombin-thrombomodulin complex. While thrombomodulin interacts with thrombin via residues in its epidermal growth factor (EGF)-5 and EGF-6 domains, residues which are

Figure 2 Rate of thrombin-catalysed activation of wt-HPC (●) and derivative P'-EEE (○)

(a) Activation rates with human α -thrombin in calcium (3 mM). Purified protein C, both wild type and derivatives, were at concentrations of $0.4 \mu\text{M}$ in the activation reaction. The amount of aPC generated was determined at various time points as described in the Materials and methods section. (b) Activation of wild-type and derivative protein C by α -thrombin in the absence of calcium (EDTA).

important for the interaction between protein C and thrombomodulin appear to reside in the EGF-4 domain (reviewed in [13,24]). Further studies will be required to determine whether basic substitutions in EGF-4 might compensate for the apparent lack of interaction between P'-EEE and thrombomodulin.

Effect of anion-binding site-I peptides on thrombin-catalysed activation

As residues P' to the cleavage site of thrombin substrates and inhibitors form positive electrostatic interactions with anion-binding site I, we hypothesized from the data above that poor

Figure 3 Activation of wt-HPC (●) and derivative P'-EEE (○) by thrombin in complex with thrombomodulin

(a) Determination of amount of aPC generated at various times following incubation of wt-HPC and P'-EEE with thrombin–thrombomodulin complex in the presence of calcium. Purified protein C was incubated with 24 nM thrombin in complex with a 15-fold molar excess of soluble recombinant thrombomodulin and the amount of aPC generated was determined at various time points as described in the Materials and methods section. (b) Comparison of the thrombin-catalysed activation of wt-HPC and P'-EEE in 3 mM calcium after 5 min incubation with increasing amounts of soluble recombinant thrombomodulin.

interaction between protein C and thrombin in the presence of calcium might be due, in part, to electrostatic repulsion between the P' basic cluster in protein C and anion-binding site I. To obtain additional evidence for such an interaction, we examined the rates of α -thrombin-catalysed activation in the presence of increasing amounts of two peptides, TR52–66 and HR54–65, both of which bind to thrombin's anion-binding site I. TR52–66 (YEPFWEDEEKNESGC) contains the hirudin-like domain of

Figure 4 Effect of peptides that bind thrombin's anion-binding site I on wt-HPC (●) and P'-EEE (○) activation by thrombin

The rates of activation of wt-HPC and P'-EEE in 3 mM calcium were determined in the presence of increasing concentrations of synthetic peptides (a) TR52–66, representing the hirudin-like domain of the human thrombin receptor residues 52–66 (YEPFWEDEEKNESGC), and (b) hirudin peptide HR54–65 (GDFEEIPEEYLQ with sulphated Tyr-63). The change in the rate of activation is shown relative to the rate of wt-HPC in the absence of peptide, with the rate of P'-EEE activation being approx. 12-fold higher without added peptide.

the human thrombin receptor residues 52–66 shown by Thien-Khai et al. [25] to interact at anion-binding site I, and HR54–65 (GDFEEIPEEYLQ with sulphated Tyr-63) is a hirudin peptide that binds anion-binding site I with relatively high affinity. Neither peptide had any effect on aPC amidolytic activity. Using wt-HPC we observed a dose-dependent increase in the rate of activation by α -thrombin in the presence of peptide TR52–66 (Figure 4a). However, using P'-EEE we did not observe an increase in activation rate in the presence of TR52–66, but instead observed a substantial decrease in rate. As was observed

with TR52–66, increasing concentrations of HR54–65 resulted in an approx. 2.5-fold increase in activation rate with wt-HPC and a substantial decrease in the activation rate with P'-EEE (Figure 4b). As expected, based on known affinities for anion-binding site I [26], HR54–65 was more potent than TR52–66. The increase in rate of activation of wt-HPC by both peptides would be consistent with our hypothesis, i.e. anion-binding-site occupancy masks the acidic residues, enhancing interaction with wt-HPC in the presence of calcium. However, the previously described effect of anion-binding-site-I occupancy on active-site catalysis [7,13,26] could also be contributing to the small increase in rate with wt-HPC. Nevertheless, our data with P'-EEE show that the increase in thrombin-catalysed activation rate obtained by reversing the charge in the P' basic cluster could be substantially reversed by anion-binding-site-I occupancy.

In a previous study, several anion-binding site peptides were shown to decrease the rate of thrombin-catalysed protein C activation by approx. 50% when analysed in the absence of calcium [26], and this effect was attributed to allosteric changes in thrombin, not to direct competition with interactions of protein C. Our results above indicate that the effect of the P' basic cluster on inhibiting activation only occurs in the presence of calcium, as there was no effect on thrombin-catalysed activation in the absence of calcium. Therefore, we might expect a slight reduction in activation rate with both wt-HPC and P'-EEE by anion-binding-site occupancy in the absence of calcium. When examined, we observed a 30–40% decrease in rate with both wt-HPC and P'-EEE at high concentrations of HR54–65 peptide (above 10 μ M).

The P' regions of thrombin substrates such as fibrinogen and the thrombin receptor, and inhibitors such as hirudin, contain clusters of acidic residues shown to act as charge centres for interaction at thrombin's anion-binding site. From our results with the poor thrombin substrate protein C, we suggest that the basic residues in the P' sequence KMTRR destabilize interaction of protein C by preventing interaction at anion-binding site I. When thrombomodulin interacts with thrombin via anion-binding site I, it masks this basic thrombin exosite and possibly forms a new surface for interaction with the basic cluster in protein C. In support of this latter point, the rate of thrombin-catalysed activation of the P'-EEE derivative was no longer stimulated by thrombomodulin (Figure 3), strongly suggesting poor interaction between protein C and the thrombin-thrombomodulin complex. Also, consistent with this model, the P'-EEE derivative interacted more efficiently with free α -thrombin (Figure 2a). The fact that the anion-binding site peptides enhanced the interaction of wt-HPC and inhibited the interaction of P'-EEE with thrombin, as measured by changes in activation rates, further supports the hypothesis that the P' basic cluster plays an important negative role in controlling the interaction of protein C with thrombin. While the most straightforward explanation for the inhibition of P'-EEE activation rate by the peptides seems to be competitive interaction at anion-binding site I, we cannot rule out the possibility that occupancy of anion-binding site I results in the exposure of new contact sites on thrombin for protein C that involve the P' basic region.

As described in the Introduction, the acidic residues at the P3 and P3' position appear to reduce the affinity of protein C for

thrombin through repulsive interactions with acidic residues in thrombin. Furthermore, when thrombomodulin interacts with thrombin, it appears to induce conformational changes that mask these repulsive interactions and/or allows the presence of an acid residue near the active site. These results, along with the data presented here, suggest that the relatively poor affinity of protein C for thrombin results from unfavourable charge interaction at several sites; at the P3 and P3' thrombin subsites, and at anion-binding site I. Likewise, the relatively high affinity of protein C for thrombin-thrombomodulin complex results from a combination of conformational change reducing repulsive effects at P3/P3', masking of anion-binding site I and possibly formation of a new surface for protein C interaction.

We gratefully thank Bruce Glover for the synthesis of oligonucleotides, Ivan Jenkins for DNA sequencing and Pat Fouts for preparation of mammalian cell culture media. We also thank Kwan Hui and Eddie Angleton for providing the TR52–66 peptide.

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