Amino acids 225–235** of the protein C serine-protease domain are important for the interaction with the thrombin–thrombomodulin complex

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Abstract Protein C (PC) is a vitamin K-dependent zymogen that inactivates factors Va and VIIIa after its activation by thrombin complexed to thrombomodulin. We characterized a monoclonal antibody (mAb) against PC, whose only influence on PC functions was to inhibit PC activation by the thrombin–thrombomodulin complex. It recognized an epitope in the PC heavy chain, the conformation of which is calcium-dependent. The mAb did not recognize a natural PC variant that was not activated by the thrombin-thrombomodulin complex (mutation R229Q) and did recognize a synthetic peptide corresponding to PC amino acids 225–235 in an Elisa assay. The peptide inhibited PC activation by the thrombin–thrombomodulin complex. These data confirm that the calcium-binding loop of the serine-protease domain is involved in the interaction of PC with the thrombin–thrombomodulin complex.

Key words: Protein C; Activation; Monoclonal antibody; Thrombin–thrombomodulin complex

1. Introduction

Protein C is a vitamin K-dependent zymogen [1], which is activated on the endothelial cell surface by thrombin complexed to thrombomodulin [2,3]. In the presence of its nonenzymatic cofactor protein S, activated protein C inactivates factors Va and VIIIa by limited proteolysis [4,5], and is thus a potent anticoagulant [6].

Protein C consists of a light chain (M_r 21,000), which contains the Gla domain (nine γ-carboxyglutamic acids) and two domains homologous to the epidermal growth factor (EGF) precursor (EGF-like domains), and a heavy chain (M_r 41,000) linked to the light chain by a single disulfide bond and containing the serine protease domain [7].

Calcium ions are known to play an important role in the activation and physiological activity of protein C [8,9]. Calcium binding to the Gla domain is required for membrane association [10,11,12] and the first EGF-like domain may be involved in high-affinity calcium binding [13]. Recently, another high-affinity calcium-binding site has been located in the serine protease domain [14]; this site is involved in protein C activation by the thrombin–thrombomodulin complex [15].

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**The numbering used in this manuscript corresponds to the amino acid sequence of mature single-chain protein C.

Abbreviations: PC, protein C; mAb, monoclonal antibody; APC, activated protein C; Ig, immunoglobulin.

The PC functional domains governing its interactions with other macromolecular components of the protein C–protein S system remain to be identified. EGF-like domains have been implicated in its interaction with protein S [13], factors Va and VIIIa [16], and the thrombin-thrombomodulin complex in association with the Gla domain [17]. Mesters et al. [18] demonstrated that the sequence corresponding to amino acids 142–155 of the human PC light chain was a factor Va-binding site. In the heavy chain, two other domains have been shown to interact with macromolecules: the sequence corresponding to residues 311–325 is required for factor Va binding [19], and the sequence corresponding to residues 390–404 is required for factor Va and/or VIIIa binding [20].

Monoclonal antibodies (mAb) are useful tools to analyse protein functional domains [21,22,23]. Among 10 monoclonal antibodies directed against human protein C, we selected one which specifically inhibited protein C activation by the thrombin–thrombomodulin complex. The location of its epitope in the domain encompassing amino acids 225–235 suggests that thrombomodulin interacts with the heavy chain of protein C, at the site involved in its high affinity for calcium.

2. Materials and methods

2.1. Reagents

Protein C, thrombin and substrate CBS-4246 were from Diagnostica Stago (Asnières, France), hirudin was from Ciba-Geigy (Bâle, Switzerland), heparin was from Choay (Sanofi, France) and substrate S-2366 was from Chromogenix (Montpellier, France). Activated human protein C was purified by pseudo-affinity chromatography [24].

Horseradish peroxidase-labelled rabbit anti-human PC polyclonal immunoglobulin was obtained from Dako SA (Trappes, France) and rabbit thrombomodulin from American Diagnostica (Greenwich, USA). Horseradish peroxidase-labelled rabbit anti-mouse IgG polyclonal immunoglobulin and bovine serum albumin (BSA) were from Sigma Chimie (St. Quentin Fallavier, France).

Monoclonal antibodies and the synthetic peptide were prepared by bioMérieux (Marcy-l'Etoile, France).

2.2. Preparation of monoclonal antibodies

Murine monoclonal antibodies (mAb) were selected, isolated and characterized essentially as described [25]. The subclass was determined with a commercial kit (Clonotyping System III, Fisher, Pittsburgh, PA) as IgG_1. MAbs were purified from ascitic fluid by means of protein G-Sepharose affinity chromatography (Pharmacia LKB Biotechnology).

2.3. Immunoblotting for protein C

Samples containing 10 μl of purified protein C or activated purified protein C (0.2 μM) were electrophoresed on 5% stacking and 12% separating polyacrylamide slab gels in the presence of sodium dodecylsulfate (SDS-PAGE) according to Laemmli [26], and with 4% β-mercaptoethanol when reduction was required. Protein was transferred onto a nitrocellulose membrane, using the Bio-Rad Minisystem (Ivry-sur-Seine, France). The blot was treated as recommended by the

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manufacturer, made to react with the mAb (20 nM), thoroughly washed, and incubated with a 1:1000 dilution of alkaline phosphatase-conjugated goat anti-mouse IgG (Bio-Rad) before revelation.

2.4. Plasma assay for protein C

The hereditary protein C abnormality was diagnosed by three types of assays: immunoenzymatic (Asserachrom Protein C, Diagnostica Stago, Asnières, France), amidolytic (Berichrom Protein C, Behring, Rueil-Malmaison, France), and coagulation (Staclot Protein C, Diagnostica Stago), all performed as recommanded by the manufacturers. In the amidolytic and coagulation assays, plasma protein C was activated by Protac snake venom. Once activated, it was made to react with a synthetic chromogenic substrate in the amidolytic assay and in the coagulation assay, with a natural substrate, i.e. plasma depleted in protein C as a source of factor V, factor VIII, and protein S in the presence of cephaline and calcium [28].

Protein C activity was also evaluated after activation by the thrombin–thrombomodulin complex according to Sala [30]. Briefly, 1 ml of plasma was treated with 80 μl of 1 M $BaCl_2$, 0.3 M Tris, pH 8, at 4°C for 30 min. The precipitate obtained was washed with buffer 50 mM Tris, 100 mM NaCl, pH 7.4, and eluted by 250 μl of 0.15 mM trisodium citrate, pH 7.5. The eluate was then added with thrombin (10 U/ml)–thrombomodulin (4.5 U/ml) complex (9:1) and allowed to react for 60 min at 37°C. The reaction was stopped by adding the same volume of hirudin (10 U/ml) and amidolytic activity of the activated protein C generated was measured by monitoring hydrolysis of the substrate CBS-4246 (1.25 mM) at 405 nm for 60 min.

2.5. Effects of $12H_{12}F_5$ mAb on protein C functions

2.5.1. Protein C activation. The effect of the monoclonal antibodies on protein C activation was analyzed after incubating protein C with the mAb (ratio 1:0 to 1:8) for 15 min prior to activation. Protein C (0.22 μM) was then activated for 30 min at 37°C with human thrombin (1.8 U/ml) in buffer A (20 mM Tris-HCl, 0.15 M NaCl, pH 7.0) or for 2 h with a human thrombin (0.036 U/ml)–rabbit thrombomodulin (7.5 U/ml) complex in buffer A containing 2.5 mM $CaCl_2$. The reaction was stopped by adding 10 μl hirudin (200 U/ml). The amidolytic activity of the activated protein C (APC) thus generated was measured by monitoring hydrolysis of the substrate S-2366 (Glu-Pro-Arg-pNA) (0.6 mM) at 405 nm for 15 min.

The effect of the monoclonal antibodies on APC amidolytic activity was tested as follows: APC was completely activated with human thrombin and then incubated with the mAb for 15 min at 37°C. Thrombin was then inhibited by hirudin and absorbance was measured at 405 nm for 15 min after adding S-2366.

2.5.2. APC anticoagulant activity. This experiment was adapted from the APC-resistance test [27].

Fifty μl of a pool of normal plasmas was incubated with 50 μl of APTT (Organon-Technika) at 37°C for 3 min. Clotting was initiated by adding 50 μl of a mixture of APC (25.6 nM), $CaCl_2$ and mAb (100 nM). The clotting time was measured on a ST4 analyser (Diagnostica Stago).

2.5.3. Enzyme-linked immunosorbent assay (Elisa).

(a) Recognition of plasma PC by mAbs. Polystyrene microtiter plates (MaxiSorp, Immuno Nunc, Roskilde, Denmark) were coated overnight at 4°C with 130 μl of 10 μg/ml mAb in 50 mM sodium carbonate buffer, pH 9; the plates were then washed thrice in PBS-Tween buffer and blocked with 2% bovine serum albumin in buffer A for 2 h at room temperature. A pool of normal control plasmas and a plasma sample from a patient heterozygous for mutation R229Q [28] were diluted 1:5 to 1:40 in buffer A. The samples were incubated in a volume of 100 μl for 2 h at room temperature. After 3 washings, bound protein C was measured by adding 100 μl of horseradish peroxidase-labelled rabbit anti-human PC polyclonal immunoglobulin diluted 1:1000. After one hour of incubation at room temperature, 100 μl of *o*-phenylene diamine (substrate) was added and the reaction was stopped 30 min later by adding 50 μl of 1 M H_2SO_4. Absorbance was measured at 490 nm on a microplate reader (Dynatech MR 5000, Saint Cloud, France).

(b) Recognition of peptide 225–235 (Glu-Tyr-Asp-Leu-Arg-Arg-Trp-Glu-Lys-Trp-Glu). The specificity of $12H_{12}F_5$ for the peptide was evaluated by studying mAb binding to the immobilized peptide in the ELISA assay, by comparison with a control mAb, $2E_2A_8D_2$, which inhibits PC activation by both thrombin and the thrombin–thrombomodulin complex.

The plates were coated with 120 μl of 25 μM peptide in sodium carbonate buffer and blocked with 4% BSA in PBS buffer. Monoclonal antibodies were diluted from 20 nM to 2.5 nM and incubated in a volume of 100 μl for 1 h at 20°C. Bound mAb was determined by adding 100 μl of horseradish peroxidase-labelled rabbit anti-mouse IgG polyclonal immunoglobulin diluted 1:1000. After 1 h of incubation, bound antibodies were quantified as described above.

2.5.4. Inhibition of PC activation by the thrombin–thrombomodulin complex in the presence of peptide 225–235. PC was activated by the thrombin–thrombomodulin complex in the presence of peptide 225–235, as described above. The peptide (0–0.54 μM) was incubated for 15 min at 37°C with thrombin–thrombomodulin (0.036–7.5 U/ml) complex before the addition of PC (0.22 μM).

3. Results

3.1. Characterization of monoclonal antibody $12H_{12}F_5$ directed against human protein C

Among the 10 mAbs tested, $12H_{12}F_5$ was the only one with a selective inhibitory effect on the activation of protein C by thrombin–thrombomodulin complex. $12H_{12}F_5$ mAb did not interfere with the activation of protein C by thrombin alone and did not inhibit the amidolytic or anticoagulant activity of preactivated protein C (data not shown). This mAb recognized both forms of protein C (zymogen and activated) and bound the heavy chain in reducing conditions (not shown).

Fig. 1 clearly shows that the inhibition of protein C activation by thrombin–thrombomodulin complex was concentration-dependent.

As PC has been shown to undergo calcium-induced conformational changes required for activation by the thrombin–thrombomodulin complex [29], we evaluated the calcium dependence of PC recognition by the mAb. As shown in Fig. 2, $12H_{12}F_5$ binding to protein C was more efficient in the absence of calcium than in its presence.

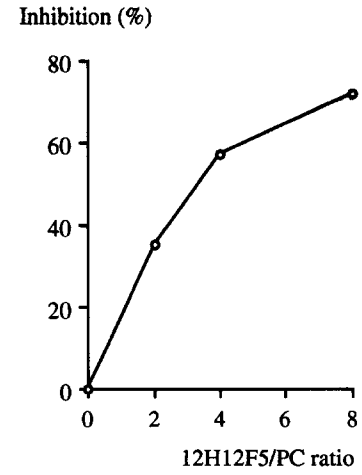

Fig. 1. Effect of mAB $12H_{12}F_5$ on PC activation by thrombin–thrombomodulin complex. PC (0.22 μM) was activated by thrombin–thrombomodulin (0.036–7.5 U/ml) complex in the presence of 2.5 mM calcium chloride and increasing mAb concentrations. The reaction was stopped with hirudin and amidolytic activity was measured by the hydrolysis of the substrate S-2366. Results are the mean of 3 experiments. The graph represents the percentage (%) inhibition of PC activation by thrombin–thrombomodulin complex versus the PC:mAb ratio (1:0 to 1:8).

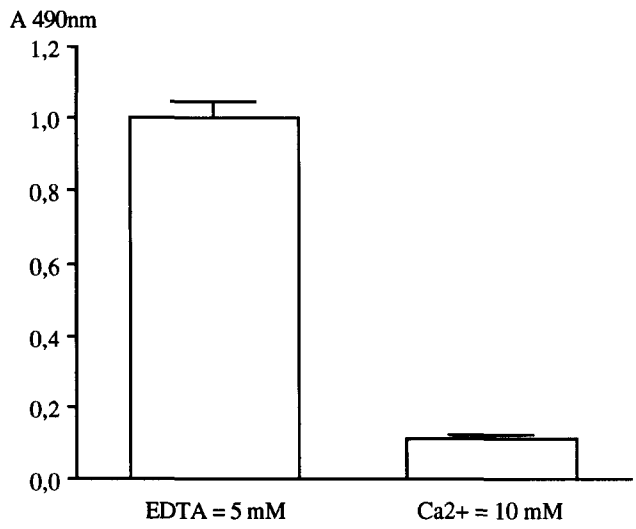

Fig. 2. Effect of $CaCl_2$ concentration on protein C binding to $12H_{12}F_5$. MAb $12H_{12}F_5$ (10 μg/ml) was coated on wells of microtiter plates by overnight incubation in 50 mM sodium carbonate buffer, pH 9.0. After washings, protein C (100 μl of normal plasma diluted 1:40) was incubated for 1 hour at room temperature in the presence of 5mM EDTA or 10 mM $CaCl_2$ and 2000 UI/l hirudin in 20 mM Tris, 0.15 M NaCl. Bound protein C was measured as described in section 2. The results are the mean (± S.D.) of six experiments.

3.2. *Binding of $12H_{12}F_5$ to PC in the plasma of a patient heterozygous for the Arg-229 to Gln mutation*

The patient carying the Arg-229 to Gln mutation belongs to a family having a qualitative deficiency previously described [28]. Protein C concentration was 100% in an immuno-assay using a polyclonal antibody. Protein C activity after activation by the Protac was 96% in an amidolytic assay, and 57% in a coagulation assay. Protein C activity after activation by the thrombin-thrombomodulin complex was 44% (see section 2). These data point to a role of Arg-229 in the activation of protein C by thrombin in the presence of thrombomodulin.

A pool of normal plasmas and the patient's plasma were made to react with the mAb in microtiter plates and the amount of bound protein C was measured. As shown in Fig. 3, $12H_{12}F_5$ bound to the patient's protein C less efficiently than to normal plasma PC. The patient plasma PC concentration measured with this monoclonal antibody based assay was 50%, showing that half the patient plasma PC molecules were not recognized by $12H_{12}F_5$. Since the patient was heterozygous, this indicate that the PC molecules bearing the Arg-229 to Gln mutation were not recognized by $12H_{12}F_5$.

3.3. *Binding of $12H_{12}F_5$ to peptide 225–235*

We used a synthetic peptide to confirm that $12H_{12}F_5$ binds to an epitope absent from the mutated protein C. The peptide consisted of amino acids 225–235 of human PC, which are involved in the interaction of calcium with the serine-protease domain. Recognition of the peptide by the mAb $12H_{12}F_5$ was concentration-dependent (Fig. 4), whereas the control mAb $2E_2A_8D_2$, which inhibits PC activation by both thrombin and thrombin–thrombomodulin complex, did not recognize the peptide. The $12H_{12}F_5$ epitope is thus at least partly located within the amino acid domain 225–235.

3.4. *Inhibition of PC activation by peptide 225–235*

PC activation by the thrombin–thrombomodulin complex was inhibited by the peptide in a concentration-dependent manner (Fig. 5).

These results support the location of a thrombin–thrombomodulin complex binding site in the region containing amino acids 225–235 of the PC serine-protease domain.

4. Discussion

Previous studies with protein C lacking the Gla domain have identified a high-affinity, Gla-independent calcium-binding site within the serine-protease domain [14]. Calcium binding to this site elicits a conformational change that could play a critical role in PC activation. Rezaie et al. [15] demonstrated that, when PC Glu-235 was replaced by Lys, calcium ions were no longer required for optimal activation by the thrombin–thrombomodulin complex, showing that Glu-235 was involved in the calcium-dependent conformation.

Modelling studies have suggested that this high-affinity calcium-binding site is located in amino acid domain 225–235 corresponding to the trypsin calcium-binding loop; three acidic residues within this region were thought to be crucial: Glu-225, 232 and 235, which carboxylate oxygen could ligate calcium [31,32].

Monoclonal antibodies against human PC have been used to study conformational changes [21,22]. Using this approach, we characterized a calcium-dependent monoclonal antibody to human PC, $12H_{12}F_5$, which binds to the heavy chain and only blocks PC activation by the thrombin-thrombomodulin complex (no effect on PC activation by thrombin, or on the amidolytic and anticoagulant activities of PCa).

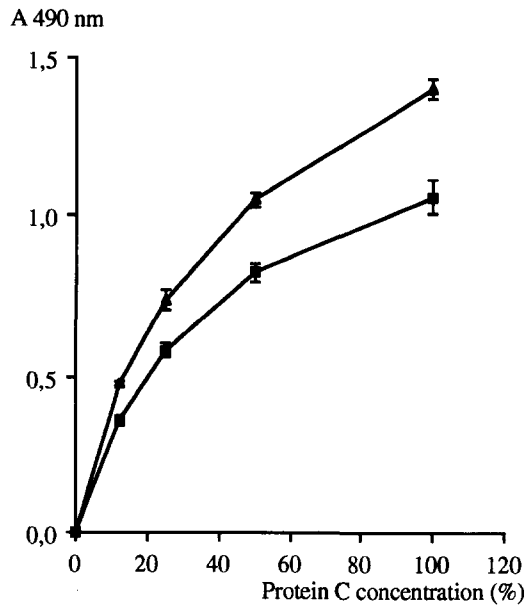

Fig. 3. Comparison of the binding curves of normal and mutant (R229Q) protein C to mAb $12H_{12}F_5$. $12H_{12}F_5$ was coated on the wells of microtiter plates as described in section 2. The wells were incubated with various dilutions of normal (▲) or R229Q mutant (■) protein C (dilution 1:10 corresponding to 100%) in 20 mM Tris, 0.15 M NaCl. Bound protein C was measured as described above. The results are the mean (± S.D.) of six experiments. The graph represents the absorbance at 490 nm ($A_{490\ nm}$) versus the protein C concentration (%).

Optimal binding of $12H_{12}F_5$ to the heavy chain in the absence of calcium, and the sole inhibitory effect on protein C activation by the thrombin-thrombomodulin complex, suggested that the epitope was located in the putative calcium-binding loop.

To characterize the $12H_{12}F_5$ mAb epitope, we used plasma from a patient who is heterozygous for the mutation Arg-229 to Gln. This mutated PC, characterized by reduced anticoagulant activity and normal amidolytic activity, was not activated by the thrombin–thrombomodulin complex, whereas activation by Protac was normal [28]. Residue Arg-229 is located within the high-affinity calcium-binding loop. Substitution of Arg-229 abrogates both anticoagulant activity and PC activation by the thrombin–thrombomodulin complex, confirming the functional importance of this region for PC activity [31].

$12H_{12}F_5$ mAb did not recognize the mutated PC, indicating that Arg-229 is required for the binding of protein C to $12H_{12}F_5$ and is included within the epitope. Moreover, the reduced binding of the mAb to PC in the presence of calcium suggested that it recognized the calcium-binding loop. This was confirmed by the use of a synthetic peptide comprising residues 225–235 of human protein C (Glu-Tyr-Asp-Leu-Arg-Arg-Trp-Glu-Lys-Trp-Glu). In the ELISA method, the mAb recognized this peptide in a concentration-dependent manner, whereas another mAb ($2E_2A_8D_2$), which inhibited PC activation by both thrombin and thrombin–thrombomodulin complex, did not.

The intact Gla-EGF fragment has been reported to inhibit PC activation [17]. The properties of $12H_{12}F_5$, which recognized the calcium-binding loop and only inhibited PC activation by the thrombin–thrombomodulin complex, together with the characteristics of PC with Arg-229 to Gln [28] and Glu-235 to Lys mutations [15], show that the high-affinity calcium-binding site of the serine-protease domain is involved in PC activation by the thrombin–thrombomodulin complex.

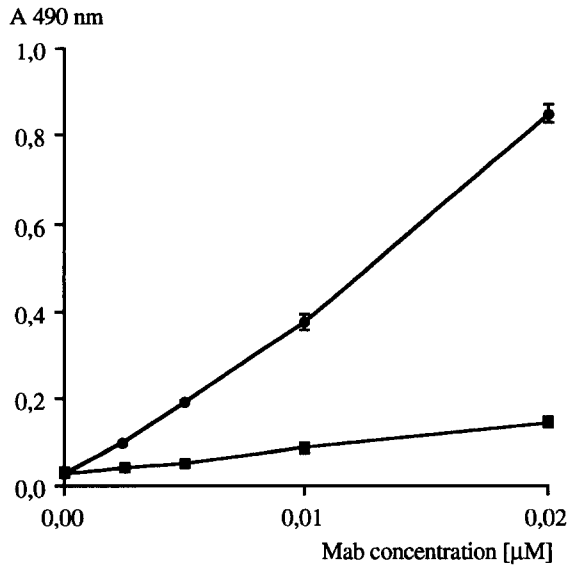

Fig. 4. Recognition of peptide 225–235 by mAbs $12H_{12}F_5$ and $2E_2A_8D_2$. Peptide 225–235 (25 μM) was coated on the wells of microtiter plates as described in section 2. The wells were incubated with increasing concentrations of mAb (2.5–20.0 nM). Bound mAb was measured by using horseradish peroxidase-labelled rabbit anti-mouse-IgG polyclonal immunoglobulins (diluted 1:1000) and the reaction was developed as described in section 2. The results are the mean (± S.D.) of six experiments. The graph represents the absorbance at 490 nm ($A_{490\ nm}$) versus the monoclonal antibody concentration: (●) $12H_{12}F_5$, (■) $2E_2A_8D_2$.

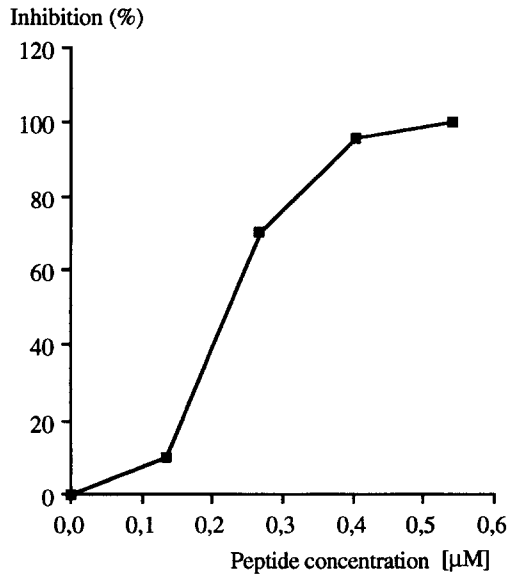

Fig. 5. Effect of peptide 225–235 on PC activation by the thrombin–thrombomodulin complex. Increasing concentrations of peptide (0–0.54 μM) were incubated for 15 min at 37°C with thrombin–thrombomodulin complex in the presence of calcium ions. PC (0.22 μM) was then activated by this mixture as described in Fig. 1. The results are the mean of three experiments. The graph represents percentage inhibition of protein C activation versus peptide concentration [μM].

We thus explored the effect of the synthetic peptide 225–235 on PC activation by the thrombin–thrombomodulin complex. As expected, the peptide inhibited activation in a concentration-dependent manner. Inhibition was complete with peptide concentrations over 0.54 μM (peptide:PC ratio = 4000:1).

In conclusion, anti-protein C monoclonal antibody $12H_{12}F_5$ did not bind the PC mutant R229Q, which is not activated by the thrombin–thrombomodulin complex, and recognized the serine-protease domain calcium-binding loop. Moreover, it only inhibited PC activation by the thrombin–thrombomodulin complex. The synthetic peptide 225–235, which encompasses the mAb $12H_{12}F_5$ epitope, also inhibited PC activation by the complex. These results provide direct evidence that the thrombin–thrombomodulin complex interacts with amino acid sequence 225–235 of human protein C. Further experiments with different peptides and/or PC mutants are necessary to determine the exact role of each residue of the loop in calcium and/or thrombomodulin binding.

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