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A monoclonal antibody recognizing the activation domain of protein C in its calcium-free conformation

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Abstract A monoclonal antibody (mAb) binding to protein C (PC) heavy chain but not to activated PC was found to inhibit PC activation by free thrombin, suggesting that epitope involved the activation site. Using a set of overlapping synthetic peptides, we confirmed that this mAb recognizes the sequence encompassing the thrombin cleavage site (^{165}QVDPRLI^{171}). Surprisingly, epitope was only accessible in the absence of calcium, half-maximal inhibition of mAb binding occurring at 100 μM Ca^{2+}. Thus, our antibody provides direct evidence that conformation and/or accessibility of the activation site differ between the apo and Ca^{2+}-stabilized conformers of PC.

Key words: Protein C; Monoclonal antibody; Activation peptide; Epitope mapping; Calcium ion

1. Introduction

Protein C (PC) is a precursor of a vitamin K-dependent serine-protease [1]. It is composed of a light chain of 21 kDa and a heavy chain of 41 kDa, linked by a disulfide bridge. The light chain contains the Gla domain and two domains homologous with epidermal growth factor (EGF). The heavy chain contains the serine-protease domain, the catalytic triad of which consists of Ser-360, His-211 and Asp-257 [2]. Thrombin cleaves PC after Arg-169, releasing a dodecapeptide at the N-terminal end of the heavy chain. In vivo, PC activation requires thrombomodulin, an endothelial cell surface protein acting as cofactor [3,4]. Activated PC (APC) limits the coagulation process by inactivating factors Va and VIIIa. This inactivation occurs through specific proteolytic cleavage in the presence of a negatively charged phospholipid surface, Ca^{2+} and protein S, the latter being a non-enzymatic vitamin K-dependent protein that acts as cofactor.

Calcium ions are required for PC activation by the thrombin-thrombomodulin complex, whereas they have a strong inhibitory effect when thrombin alone is the activator, suggesting a change in conformation at the cleavage site upon calcium ion binding. Three types of Ca^{2+} binding sites have been identified in PC: (1) those of the Gla domain, involved in the interaction with phospholipid surfaces [5]; (2) that of the EGF domain which includes the β-hydroxy aspartate-71 and is involved in the interaction of PC with protein S, factor Va, factor VIIIa and the thrombin-thrombomodulin complex [6,7]; and (3) that of the Ca^{2+} binding loop encompassing amino acids 225–235 in the serine-protease domain, which is similar to that found in trypsin [8–11]. This 225–235 loop of the serine-protease domain is the only Ca^{2+} binding site required to inhibit activation by thrombin alone and rapid activation by the thrombin-thrombomodulin complex. Its interaction with Ca^{2+} induces a conformational change reflected by changes in intrinsic fluorescence attributable to Trp-231 and Trp-234 [12]. Occupancy of this site seems to correlate with the inability of thrombin to activate PC in the presence of Ca^{2+}.

Consistent with this hypothesis, we have characterized a monoclonal antibody, $3H_9E_7$, which recognizes the PC cleavage site only in the absence of Ca^{2+}.

2. Materials and methods

2.1. Reagents

Human thrombin was from Diagnostica Stago (Asnières, France); recombinant desulfate hirudin variant 1 (TM Revasc) was a generous gift from Dr. G.F. Pay (Ciba-Geigy, Basle, Switzerland); substrate S-2366 (Glu-Pro-Arg-pNA) was from Chromogenix (Mölndal, Sweden). Horseradish peroxidase-labelled rabbit anti-human-PC polyclonal immunoglobulin was from Dako (Glostrup, Denmark). Horseradish peroxidase-labelled rabbit anti-mouse-IgG and bovine serum albumin (BSA) were from Sigma (St. Louis, MO, USA). Nitrocellulose membrane and alkaline phosphatase-conjugated goat anti-mouse IgG polyclonal antibodies were from Bio-Rad (Hercules, CA, USA).

2.2. Preparation of monoclonal antibodies

Murine monoclonal antibodies (mAb) were prepared by bioMérieux (Marcy l'Etoile, France). MAbs were selected, isolated and characterized according to Köhler and Milstein [13]. BALB/c mice were immunized by intraperitoneal injections of 10 μg of purified PC emulsified in an equal volume of Freund's complete adjuvant. These injections were followed on days 28, 60 and 82 by injection of the same quantity of antigen in Freund's incomplete adjuvant. Spleen cells isolated from immunized mice were fused with Sp2/o-Ag 14 myeloma cells using polyethylene glycol. Culture supernatants were screened in an ELISA method and positive colonies recognizing immobilized human PC were cloned twice by the limiting dilution technique. Ascitic fluid was obtained from mice primed by intraperitoneal injection of 0.5 ml of pristane and then injected with 10^6 hybridoma cells. The IgG1 subclass was determined by using a commercial kit (Clonotyping System III, Fisher, Pittsburgh, PA, USA). MAbs were purified from ascitic fluid by means of protein G-Sepharose affinity chromatography (Pharmacia LKB, Uppsala, Sweden).

2.3. Protein C purification and activation

Human plasma PC was immunopurified by using Sepharose-immobilized mAb 10D1A3D11 (provided by Dr. Pittet, bioMérieux). The purification procedure was followed by anion-exchange chromatography (MonoQ, Pharmacia LKB) using a $CaCl_2$ gradient to elute PC. Purified human PC (0.45 μM) was activated for 0–300 min at 37°C by human thrombin (18 U/ml) in Tris-20 mM HCl, 0.15 M NaCl, 10 mM EDTA, buffer at pH 7.4. The amidolytic activity of the APC thus generated was quantified by measuring the initial velocity (V_i) of hydrolysis of substrate S-2366 (0.6 mM) at 405 nm on a microplate reader connected to a computer (MR 5000, Dynatech Laboratories Inc., Chantilly, VA, USA).

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2.4. ELISA assays

Polystyrene microtiter plates (Nunc Maxisorp) were coated overnight at 4°C with 130 μl of 10 μg/ml mAb $3H_9E_7$ solution in 50 mM sodium carbonate buffer pH 9. Plates were then washed thrice in Tris-20 mM HCl-Tween buffer (TTBS). Samples diluted in 20 mM Tris, 150 mM NaCl buffer, pH 7.4, containing 10 mM EDTA or 0.1 to 2.5 mM $CaCl_2$ were incubated in microplate wells (100 μl) for 1 h at room temperature. After three washes in TTBS, bound PC was measured by adding 100 μl of horseradish peroxidase-labelled rabbit anti-human PC polyclonal immunoglobulin diluted 1:1000. After 1 h of incubation, 100 μl of *o*-phenylenediamine (substrate) was added and the reaction was stopped 10 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).

2.5. Epitope mapping

Biotinylated peptides (14-mers, overlapping each other by 7-mers) homologous to the PC heavy chain were synthesized by Chiron Mimotopes Pty (Clayton, Australia). Another set of 15 peptides (7–14-mers) encompassing amino acids 161–174 was also synthesized. Microtiter plates (Nunc) treated with streptavidin (5 μg/ml, Sigma) were coated for 2 h at room temperature with each peptide diluted 1:1000 (5.5 μM). After three washes in PBS-Tween buffer and saturation with 5% bovine serum albumin (w/v, Sigma) for 2 h, plates were washed thrice and incubated with mAb (50 nM in PBS) for 1 h. After three washes bound mAb was measured by adding 100 μl of horseradish peroxidase-labelled rabbit anti-mouse IgG diluted 1:1000 (Sigma), and then revealed.

2.6. Determination of the K_d of mAb $3H_9E_7$-PC interaction

Polystyrene microtiter plates (Nunc) were coated overnight at 4°C with 120 μl of 62 nM PC solution in 50 mM sodium carbonate buffer, pH 9. After five washes in PBS-Tween the plates were saturated with 200 μl of 5% BSA (w/v) for 2 h at room temperature. After five washes, 100 μl of serial dilutions of mAb $3H_9E_7$ was incubated for 1 h at room temperature. Bound mAb was revealed as described above. The dissociation constant (K_d) of $3H_9E_7$ for PC was estimated by non-linear regression analysis of the A_{490} value as a function of the $3H_9E_7$ concentration, using the following equation [14]:

$$A_{490} = \alpha\Big\{K_d + B_{max} + 3H_9E_7 - [(K_d + B_{max} + 3H_9E_7)^2 - 4 \times B_{max} \times 3H_9E_7]^{1/2}\Big\} \quad (1)$$

where B_{max} is the apparent molar concentration of PC, and α is a parameter such that the concentration of the PC·$3H_9E_7$ complex is $\alpha \times A_{490}$.

3. Results

Seven of the 10 mAbs binding to PC on immunoblots recognized the heavy chain of PC after reduction. The mAb selected for this study ($3H_9E_7$) was one of the mAbs which did not bind to APC in either reducing or non-reducing conditions (Fig. 1), suggesting that epitope was lost after release of the activation peptide.

To confirm interaction of $3H_9E_7$ with the activation domain of PC, the loss of mAb $3H_9E_7$ binding to PC upon activation by thrombin was examined by ELISA. APC formation was determined at each time point in terms of the hydrolysis of a chromogenic substrate. As shown in Fig. 2A, there was a negative correlation between the amount of PC bound to immobilized mAb $3H_9E_7$ and the amount of APC generated. Almost complete disappearance of the mAb/PC interaction after 5 h showed that thrombin cleavage destroyed the mAb epitope on the PC molecule. Concentration-dependent inhibition of APC generation occurred when PC was incubated with the mAb prior to activation by thrombin (Fig. 2B). Taken together, these experiments strongly suggest that the epitope was located in the activation domain.

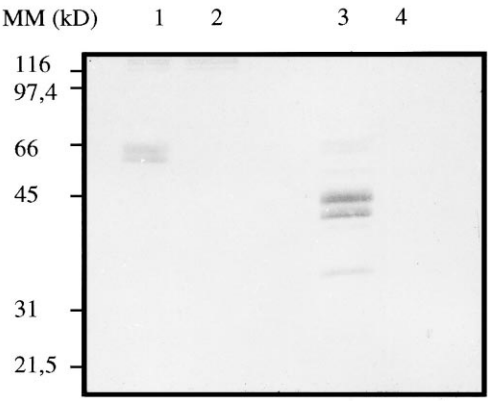

Fig. 1. Immunoblot analysis. PC (lanes 1 and 3) and APC (0.2 μM) (lanes 2 and 4) were electrophoresed on 5% stacking and 10% separating polyacrylamide slab gels in the presence of sodium dodecyl sulfate (SDS-PAGE) in non-reducing (lanes 1 and 2) and reducing (lanes 3 and 4) conditions, the latter in the presence of 4% β-mercaptoethanol. After protein transfer to a nitrocellulose membrane, the blot was allowed to react with mAb $3H_9E_7$ (20 nM).

The epitope appeared to be linear, as mAb $3H_9E_7$ still recognized the protein after denaturation and disulfide bond reduction. Using a bank of overlapping 14-mer peptides corresponding to the PC heavy chain sequence, we found that mAb $3H_9E_7$ recognizes two peptides only encompassing amino acids 158–171 (DTEDQEDQVDPRLI) and 165–178 (QVDPRLIDGKMTRR). We then used a set of peptides encompassing the sequence DQEDQVDPRLIDGK (amino acids 161–174) from which residues were deleted one by one at the amino-terminus and carboxy-terminus (Fig. 3). The minimal peptide sequence binding mAb $3H_9E_7$ was QVDPRLI (amino acids 165–171).

The effect of Ca^{2+} on PC binding to mAb $3H_9E_7$ was examined by incubating PC in Ca^{2+}-depleted buffer or in buffer containing increasing concentrations of Ca^{2+} and the immobilized mAb. As shown in Fig. 4, PC binding by the mAb decreased as calcium ion concentrations increased, with half-maximal inhibition at 100 μM. The mAb thus essentially recognized the activation domain in its Ca^{2+}-free conformation, corresponding to the optimal conformation for free thrombin activation. In our conditions, half-maximal inhibition of thrombin activation was observed at 300 μM (not shown), a value similar to that reported in the literature [5].

To determine the affinity of $3H_9E_7$ for PC, increasing concentrations of mAb were incubated with immobilized purified PC in buffer containing EDTA. Analysis of the data by non-linear regression according to Eq. 1 gave a K_d value of 0.55 ± 0.19 nM (Fig. 5), implying strong affinity of the mAb for PC. The binding curve was not influenced by the Ca^{2+} concentration (not shown), indicating that Ca^{2+} had no effect on mAb $3H_9E_7$/PC interaction when the antigen was immobilized on microplates. This might be due to the loss of calcium binding site(s) in such a conformation. This experiment also suggested that Ca^{2+} binding by the mAb did not play a major role in its interaction with PC. To rule out an influence of Ca^{2+} in the antibody reaction, we studied the effect of Ca^{2+} on mAb $3H_9E_7$ binding to the immobilized peptide DQEDQVDPRLIDGK, which encompasses the epitope. There was no change in binding on exposure to Ca^{2+} concentrations up to 1 mM (not shown).

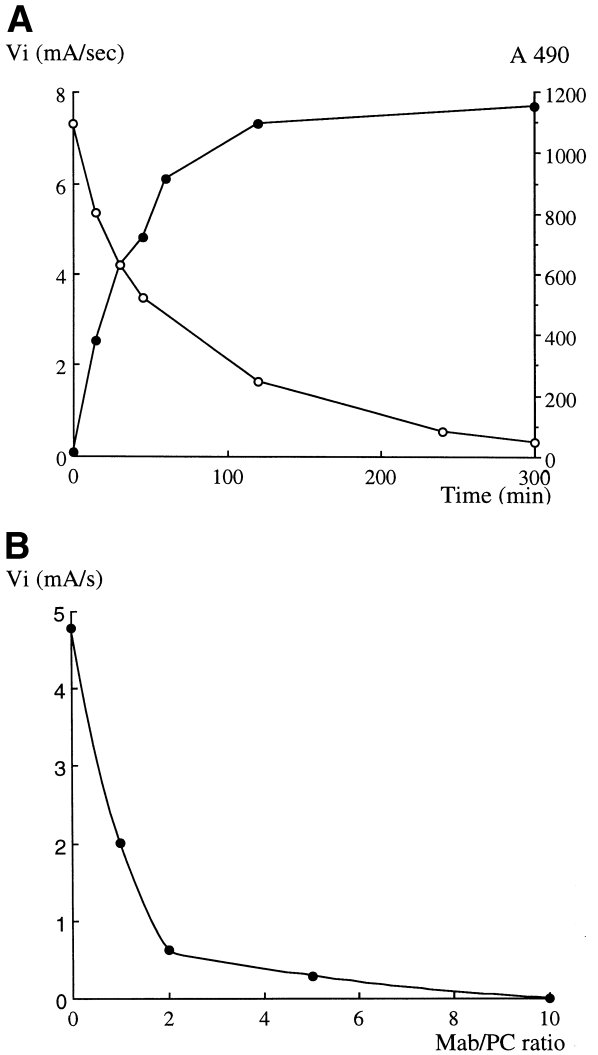

Fig. 2. A: Time course of PC activation by thrombin and subsequent inhibition of PC binding to $3H_9E_7$. Purified PC (0.45 μM) was activated by thrombin (18 U/ml) in 20 mM Tris, 150 mM NaCl, 10 mM EDTA. Aliquots were removed from the activation mixture at 0, 15, 30, 45, 60, 120, 240 and 300 min and the reaction was stopped by adding 130 U/ml hirudin. Amidolytic activity was determined by adding 0.6 mM S-2366. Samples were diluted 1:4 in Tris-20 mM HCl, 150 mM NaCl, 10 mM EDTA, pH 7.4 buffer and added to $3H_9E_7$-coated microplate wells (100 μl). The graph represents the initial velocity V_i (●) of substrate hydrolysis (mA/s) and absorbance at 490 nm in the ELISA assay (A_{490}) (○) versus the activation time. Mean of three duplicate experiments. B: Effect of mAb $3H_9E_7$ on PC activation by thrombin. Purified PC (0.31 μM) was incubated with $3H_9E_7$ (0–3.1 μM) for 15 min at 37°C prior to activation. PC was then activated with human thrombin (18 IU/ml) for 120 min at 37°C. The amidolytic activity of the APC thus generated was measured by monitoring hydrolysis of the substrate S-2366 (0.6 mM) at 405 nm on a microplate reader (Dynatech MR 5000). The mAb/PC ratio is plotted against the amidolytic activity of generated APC (V_i: initial velocity of the substrate hydrolysis reaction (mA/s)).

4. Discussion

Many mAbs against PC are selected on the basis of their calcium ion dependence, meaning that Ca^{2+} ions are required for the antibody-antigen interaction. Most recognize epitopes located in the PC light chain, which bears Ca^{2+} binding sites

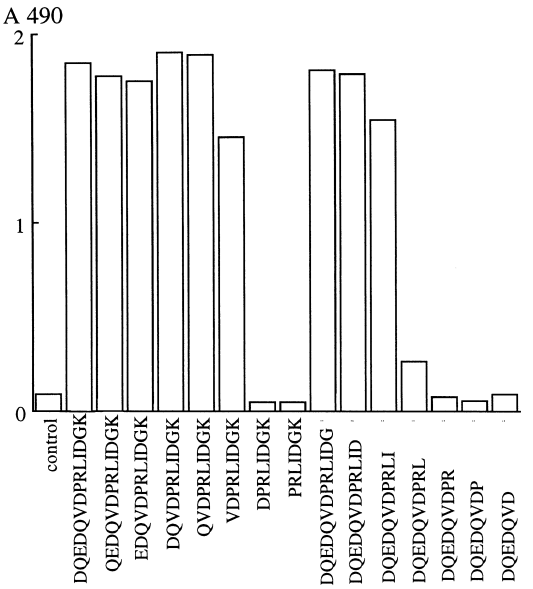

Fig. 3. ELISA for $3H_9E_7$ epitope mapping. Peptides (7–14-mers) encompassing the PC amino acid sequence 161–174 (DQEDQVDPRLIDGK) had their residues deleted one by one at the amino-terminus or carboxy-terminus. Streptavidin-treated microplates were coated with each of the peptides diluted 1:1000 (5.5 μM) as described in Section 2 before addition of mAb $3H_9E_7$ (50 nM in PBS) and incubation for 1 h. Bars represent the binding of mAb to each peptide, in terms of absorbance at 490 nm (A_{490}). The amino acid sequence of the control peptide was IKDFHVYFRESRDG.

in the GLA domain and the first EGF-like domain [15–17]. MAb HPC4, the epitope of which is a 12-residue peptide encompassing the activation site, requires Ca^{2+} to interact with both the antibody domain and the PC target peptide sequence [18].

In an attempt to map other epitopes, we selected mAbs binding to PC on immunoblots after denaturation by SDS. Seven recognized the PC heavy chain, and three of these in-

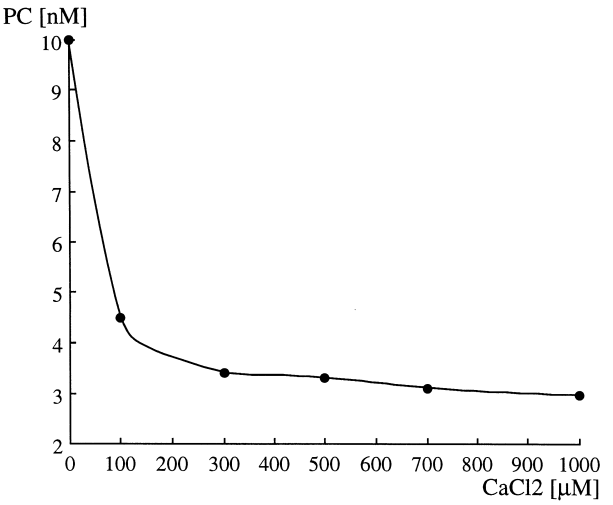

Fig. 4. Effect of calcium ions on PC/$3H_9E_7$ interaction. Samples containing purified PC (10 nM) in Tris-20 mM HCl, 0.15 M NaCl, pH 7.4 buffer containing 0–1 mM $CaCl_2$ were incubated for 1 h at room temperature. After washes, PC was detected as described in Section 2. Absorbance was converted into bound PC from a standard curve based on serial dilutions of purified PC (0.62–10 nM) in Tris-20 mM HCl, 0.15 M NaCl, pH 7.4 buffer pretreated with Chelex. Mean of three duplicate experiments.

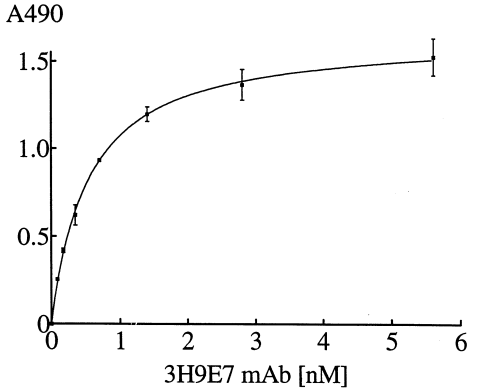

Fig. 5. Determination of the K_d of $3H_9E_7$ for PC. Microtiter plates were coated overnight at 4°C with 60 nM purified PC. After washes, serial dilutions of mAb $3H_9E_7$ (0–6 nM) were incubated for 1 h at room temperature. Plot of A_{490} as a function of the total $3H_9E_7$ concentration. The solid line represents the best fit to Eq. 1. The estimated K_d value was 0.55 ± 0.19 nM; estimated PC concentration less than 1 pM.

hibited PC activation by thrombin and recognized the same peptide encompassing the thrombin cleavage site (data not shown). Further studies were performed with one of the three mAbs, designated $3H_9E_7$. Several mAbs reacting with synthetic peptides encompassing the cleavage site have already been described [15–18], indicating that this domain probably forms a loop at the surface of the molecule that can be cleaved by thrombin, in keeping with computer models based on known crystal structures of serine-proteases [19]. The minimal sequence recognized by $3H_9E_7$ was ^{165}QVDPRLI^{171}, showing the importance of Q-165 and I-171. As the aligned sequence in mouse PC is EPDPRIV, the epitope is probably QVXXXLI. In the crystal structure of activated PC, L-170 forms an ion pair with D-359, burying the new amino-terminus in the core of the molecule and explaining the loss of recognition of the epitope after cleavage by thrombin [20]. This property of mAb $3H_9E_7$ may be useful for determining whether purified PC concentrates can be fully activated.

One unique feature of mAb $3H_9E_7$ is that it binds PC only in the absence of Ca^{2+}. Interestingly, the concentration of Ca^{2+} required to inhibit its binding to PC was of the same order as that inhibiting PC activation by thrombin (respective half-maximal inhibitory concentrations of 100 and 300 μM). This indicates that a conformational change occurs in the activation domain upon Ca^{2+} binding that prevents both recognition by mAb $3H_9E_7$ and proteolytic attack by thrombin. The high-affinity Ca^{2+} binding site important for PC activation is located within the serine protease domain and involves a loop sequence encompassing amino acids 225–235 [8,12,21]. The present findings suggest that the QVDPRLI sequence in the region of the scissile bond undergoes a conformational change upon Ca^{2+} binding to the calcium binding loop. Ca^{2+} binds neither to the activation peptide itself nor to the antibody, as the QVDPRLI peptide alone bound the mAb both in the presence and in the absence of Ca^{2+}. This contrasts with mAb HPC4, which binds the sequence EDQVPRLIDGK only in the presence of Ca^{2+} [18]. Thus, mAb $3H_9E_7$ provides us with a useful tool for PC immunopurification from Ca^{2+}-free media. The only other anti-PC mAb displaced from its epitope by Ca^{2+} ions is H11, which recognizes a sequence (FLEEXR/K) in the Gla domain that is shared by several vitamin K-dependent coagulation proteins [22].

The sequence ^{165}QVDPRLI^{171} is recognized in both denatured PC and the synthetic peptide, implying that the epitope is non-conformational and easily accessible in Ca^{2+}-free PC. The loss of the PC/mAb interaction in the presence of Ca^{2+} suggests a different structure or a different location of the activation domain after occupancy of the Ca^{2+} binding loop in the serine protease domain. Several amino acids in the vicinity of the P1-P1′ bond, such as Asp-167 (P3), Pro-168 (P2) and Asp-172 (P3′), are also important for the adoption of a calcium-dependent conformation precluding PC cleavage by thrombin, as their mutation lifts the inhibitory effect of Ca^{2+} [10,23]. Replacement of these residues by Gly, Val and Gly, respectively, seems to stabilize the conformation of the activation peptide in the absence of Ca^{2+}.

The release of mAb $3H_9E_7$ on Ca^{2+} binding to PC provides direct evidence that a conformational change occurs at the cleavage site in the Ca^{2+}-stabilized form of the molecule.

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