The Interaction of a Ca$^{2+}$-dependent Monoclonal Antibody with the Protein C Activation Peptide Region

EVIDENCE FOR OBLIGATORY Ca$^{2+}$ BINDING TO BOTH ANTIGEN AND ANTIBODY*

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Protein C is a member of the vitamin K-dependent plasma zymogens, is activated in a Ca$^{2+}$-dependent reaction by the thrombin-thrombomodulin complex. A Ca$^{2+}$-dependent monoclonal antibody (HPC4) that blocks protein C activation was used to study conformational changes near the activation site in protein C. The half-maximal Ca$^{2+}$ dependence was similar for protein C and γ-carboxyglutamic acid-domainless protein C for binding to HPC4 (205 ± 23 and 110 ± 28 μM Ca$^{2+}$, respectively), activation rates (214 ± 22 and 210 ± 37 μM), and intrinsic fluorescence of γ-carboxyglutamic acid-domainless protein C (176 ± 34 μM). Protein C heavy chain binding to HPC4 was half-maximal at 36 μM Ca$^{2+}$, although neither the heavy chain nor HPC4 separately bound Ca$^{2+}$ with high affinity. The epitope was lost when the activation peptide was released. A synthetic peptide, P'(6–17), which spans the activation site, exhibited Ca$^{2+}$-dependent binding to HPC4 (half-maximal binding = 6 μM Ca$^{2+}$). Thus, each decrease in antigen structure resulted in a reduced Ca$^{2+}$ requirement for binding to HPC4. Tb$^{3+}$ and Ca$^{2+}$ binding studies demonstrated a Ca$^{2+}$-binding site in HPC4 required for high affinity antigen binding. These studies provide the first direct evidence for a Ca$^{2+}$-induced conformational change in the activation region of a vitamin K-dependent zymogen. Furthermore, Ca$^{2+}$ binding to HPC4 is required for antigen binding. The multiple roles of Ca$^{2+}$ described may be useful in interpretation of other metal-dependent antibody/antigen interactions.

Protein C, a member of the vitamin K-dependent plasma zymogens, is activated in a Ca$^{2+}$-dependent reaction by the thrombin-thrombomodulin complex to form an anticoagulant enzyme (1, 2). As with other proteins of this class, calcium plays a central role in the activation and function of protein C. Potential structures involved in Ca$^{2+}$-dependent reactions of protein C have been identified. These include two of the post-translational modifications: the γ-carboxyglutamic acid-residues (Gla) located in the amino-terminal portion of the light chain (3, 4) and the erythro-β-hydroxyaspartic acid located in the first region of the light chain showing homology to epidermal growth factor (5, 6). The Gla residues are known to participate in Ca$^{2+}$ binding (7, 8). Although no direct evidence exists, spectral evidence and immunologic data suggest a similar role for the β-hydroxyaspartic acid (9, 10).

The heavy chain of protein C contains the functional serine protease domain of the molecule and is linked to the Ca$^{2+}$-binding light chain by a single disulfide bond. Although activation of protein C is Ca$^{2+}$-dependent, the site of cleavage (Arg12-Leu13) is located on the heavy chain, far removed from known Ca$^{2+}$-binding sites.

Calcium has novel influences on protein C activation both on the membrane surface and in solution. Whereas Ca$^{2+}$ is required for activation by the thrombin-thrombomodulin complex, it inhibits activation by free thrombin (11). The Ca$^{2+}$ concentration dependence of these two processes is indistinguishable and correlates with changes in protein C conformation monitored by intrinsic protein fluorescence (12). At least two possible mechanisms could be responsible for these observations. One is that Ca$^{2+}$ alters protein C conformation at the cleavage site for the activator. An alternative is that Ca$^{2+}$-induced conformational changes occur distal to the cleavage site and are important for secondary binding interactions with the thrombin-thrombomodulin activation complex.

As one means of monitoring conformational changes in such complex systems, conformationally sensitive metal-dependent antibodies have often been employed. Distinct metal-induced structural transitions relating to function have been described for both prothrombin and Factor IX by the selective application of metal-dependent antibodies (13, 14). Ca$^{2+}$-dependent monoclonals to human protein C (HuPC) have been described which bind to epitopes on the light chain either in the Gla domain (15) or the epidermal growth factor homology domain (10, 16).

Using an analogous approach, we have isolated a Ca$^{2+}$-dependent monoclonal antibody to human protein C, termed HPC4, that blocks protein C activation by thrombin-thrombomodulin both in vivo and in vitro (17). In the presence of Ca$^{2+}$, this antibody binds to protein C, but not activated protein C, which permitted development of a functional assay for HuPC (18).

The studies presented here have investigated the mechanism of this Ca$^{2+}$-dependent antibody/antigen interaction. One of the potential complications we considered, although seemingly unlikely, was that Ca$^{2+}$ binding to the antibody was an obligatory event in high affinity antigen binding. The evidence presented demonstrates multiple roles for Ca$^{2+}$ in this antibody/antigen interaction. One is Ca$^{2+}$-induced expression of the protein C epitope which overlaps the acti-
A Ca²⁺-binding Antibody Binds the Protein C Activation Site

**EXPERIMENTAL PROCEDURES**

**Materials**—The reagents used in this study and their respective suppliers were as follows: Immunobeads, Affi-Gel 10, Chelex 100, and Enzymobeads (Bio-Rad); Spectrozyme PCAs (American Diagnostica); MOPS, MES, pristane, and cyclophosphamide (Sigma); thrombomodulin (ICN Biomedicals); and CaCl₂ (ICN Biomedicals). and "CaCl₂ (ICN Biomedicals)."

**Solutions**—For experiments requiring metal-free conditions, buffers were dialyzed extensively in polypropylene containers with Chelex 100 in the dialysis to remove divalent cations. Protein solutions contained 1–2 μM EDTA and were dialyzed at 4 °C against the appropriate buffer containing Chelex.

**Protein Preparation**—Human protein C (18), activated protein C (18), bovine thrombin (19), and rabbit lung thrombomodulin (20) were purified by published methods. Human protein C was reduced and carboxymethylated as previously described (11). HPC4 monoclonal antibody (see below) was coupled to Affi-Gel 10 (final concentration, 5 mg/ml) or Enzymobeads (final concentration, 0.4 mg/ml) according to the manufacturer's directions. Proteins were radiolabeled with Na[125]I using Enzymobeads with a net specific activity of 10–40 μCi/ml. Bovine pancreatic chymotrypsin (Worthington) was treated with 10 μM p-aminophenylmethanesulfonyl fluoride (Behring Diagnostics) to inhibit potential tryptic contamination. Human Gla-domainless protein C (HuGDPC) was prepared essentially as described for bovine protein C (11) by chymotryptic proteolysis of HuPC (0.8 mg/ml HuPC, 1 μg/ml chymotrypsin, 10 min, 37 °C) in TBS (0.1 M NaCl, 0.02 M Tris-HCl, pH 7.5, 1 mM CaCl₂, pH 7.5). HuGDPC and were separated from residual HuPC by ion-exchange chromatography on FPLC Mono Q column (Pharmacia LKB Biotechnology Inc.).

**Extent of Enzyme and Molecular Weights for Proteins**—The following values were used for calculating protein concentration: thrombin, E₅₉₅ = 21, 37,000 (19); thrombomodulin, E₅₉₅ = 8.8, 74,000 (21); human protein C, E₅₉₅ = 14.5, 62,000 (3); Gla-domainless protein C, E₅₉₅ = 88,000 (11); and HPC4 antibody, E₅₉₅ = 15, 150,000 (22). The extinction coefficient for reduced carboxymethylated (RCM) heavy chain was estimated to be 19.7 based on a dye binding assay for protein content (Bio-Rad) using human protein C as the standard. The molecular weight used for the RCM heavy chain was 40,000 (3).

**Preparation of Synthetic Peptides**—Solid-phase synthesis of peptides was performed with an Applied Biosystems 430A peptide synthesizer using the t-butoxycarbonyl chemistry (23). Peptides were cleaved by treatment with anhydrous hydrogen fluoride. Purity of the synthesized peptides as assessed by reverse-phase high pressure liquid chromatography was >90%. Molecular weight of the peptides was estimated by summation of the individual anhydrous amino acid molecular weights with correction for peptide bond formation. Peptide concentrations were estimated by reference to the absorbance at 220 nm of 1 mM peptide solutions in purified water.

**Data Analysis**—Values for half-maximal metal ion concentrations were obtained by nonlinear least-squares regression analysis of the data.

**Monoclonal Antibody Production**—BALB/c mice were injected peritoneally with 50–100 μg of HuPC in complete Freund's adjuvant. The HuPC immunization was repeated after 3 weeks (emulified in incomplete Freund's adjuvant) and 6 weeks (in TBS). Four days later, spleen cells were fused with the mouse myeloma cell line P3X63AG8-653 according to the method of Kohler and Milstein (24) using 35% polyethylene glycol 1450.

**Preparation of Hybridoma Fluid**—B10.BR mice were initially primed with pristane and 14 days later injected peritoneally with 0.1 ml of 10 mg/ml cyclophosphamide. Twenty-four hours later, 3–6 x 10⁶ cells were injected intraperitoneally. After 7–10 days, ascites fluid was collected. The monoclonal antibody, HPC4, was purified from ascites fluid (50 ml) or N-Dodecyl β-D-maltoside (80,000) fractionation, followed by chromatography on QAE-Sephadex Q-50 (17) or by HuPC-Affigel affinity chromatography.

**Calcium Dependence of HuPC, HuGDPC, and RCM Heavy Chain Binding to HPC4**—HuPC4 coupled to Immunobeads was incubated overnight at 4 °C in TBS, 0.1% gelatin, 1 mM EDTA, pH 7.5, and then washed extensively with TBS, 0.1% gelatin, pH 7.5 (Chelex-treated). Radiolabeled proteins were added to 10 μl of HPC4-coupled beads with increasing concentrations of Ca²⁺ in TBS, 0.1% gelatin, pH 7.5. Total volume was 200 μl. The solutions were incubated with mixing (2 l, 25 °C) and washed with gelatin buffer containing the appropriate amount of Ca²⁺, and the beads were counted in an NE 1600 γ counter (Nuclear Enterprises, Ltd.). Control samples included solutions with no added Ca²⁺ and 1 mM EDTA, respectively. Final antigen concentrations ranged from 0.04 to 1 μM in several experiments, which were sufficiently low to ensure an excess of antibody-binding sites. Base-line counts/minute determined in the absence of 1 mM EDTA (5-15% of total counts added) were subtracted from the total counts/minute bound. Maximal binding of the 125I-labeled RCM heavy chain was 80–90% of the total amount added and 60–70% of the added 125I-labeled HuPC or Gla-domainless protein C.

**Fluorescence Studies**—All fluorescence spectra were obtained on an SLM-8000 fluorescence spectrophotometer (SLM-Aminco, Urbana, IL) equipped for stirring and temperature control. The temperature of the cuvette was maintained at 23 °C, and shutters were kept closed except during scans to minimize photodegradation of the sample. Correction for wavelength response of the photomultiplier tube was performed using correction factors supplied by the manufacturer. Integration of all emission spectra was performed in wave numbers.

Tryptophan was excited at 285 nm (4-nm slit widths), and emission was recorded at 2-nm intervals (4-nm slit widths). Emission peak intensity was quantified by integration of the fluorescence from 305 to 500 nm. In initial experiments, background subtraction of the buffer solvent was negligible relative to the sample intensity (<1%). Therefore, background correction of the tryptophan emission spectra was not done.

Samples for the terbium titration studies were excited at 285 nm (2-nm slit widths) using an SB 300 UV band-pass filter (Oriel) in the 360-nm excitation path. Tb³⁺ emission intensity was measured over 10-nm intervals and integrated from 538 to 552 nm. Contributions from light scattering were measured as the harmonic of the excitation wavelength measured at 570–580 nm. For each Tb³⁺ concentration, the emission intensity of the solvent blank titrated in parallel with the sample was subtracted from that of the protein solution to give only the protein-dependent fluorescence.

**Titrations for the Fluorescence Studies**—For the intrinsic fluorescence studies, HPC4 antibody (1 μM) in TBS, pH 7.5 (Chelex-treated), in the presence or absence of 2 μM P(6–17) was titrated with CaCl₂ or MgCl₂ diluted in the same buffer. HPC4 titrated with the human peptide P(1–12), P(6–17), or P(15–27) at 0.1 μM CaCl₂ or MgCl₂ before addition of peptides. The emission intensity (305–400 nm) was recorded 5 min after each addition of titrant. Under all experimental conditions, sample dilution due to addition of titrant contributed <5% of the observed change in the signal.

**Equilibrium Dialysis**—Calcium binding to RCM heavy chain, HPC4 antibody, or a mixture of the two proteins was determined by equilibrium dialysis using 50 mM CaCl₂ as described elsewhere (25). The dialysis experiments were performed at room temperature in 0.25-mM wells for 24 h, a time sufficient for the system to reach equilibrium.

**Ca²⁺ Dependence of HuPC and HuGDPC Activation by Thrombin-Thrombomodulin**—HuPC or HuGDPC (1 μM) was incubated at 37 °C with 20 nM thrombomodulin, 0.4 nM thrombin, and Ca²⁺ at the indicated concentrations. The reaction was stopped by making the solution 5 μM in antithrombin III in a final volume of 90 μl. Activated protein C was measured using the Vmax kinetic microparticle readout (Molecular Devices Corp., Palo Alto, CA). Each sample (10 μl) was mixed with Spectrozyme PCa synthetic substrate (final concentration 250 μM) in a total volume of 1 ml. Data analysis was done using the Soft Max software program (Molecular Devices Corp.) internated with the microparticle reader.
Interaction of HPC4 Antibody with HuPC—One of the monoclonal antibodies, HPC4, demonstrated Ca\(^{2+}\)-dependent binding to solid-phase HuPC using standard enzyme-linked immunosorbent assay screening procedures. HPC4 did not bind to bovine protein C either in the presence or absence of Ca\(^{2+}\) (data not shown). To determine which portion of HuPC contained the antibody-binding epitope, HuPC was reduced, carboxymethylated, and chromatographed on an HPC4-Affi-Gel 10 affinity column. One peak was observed in the breakthrough fractions, and a single peak was eluted with 2 mM EDTA (Fig. 1A). Contrary to expectations, the bound protein corresponded to the RCM heavy chain (Fig. 1B) which exhibited the characteristic doublet (3).

The role of the activation peptide region was examined by affinity chromatography on an HPC4-Affi-Gel 10 column. As expected, HuPC bound to the antibody column in a Ca\(^{2+}\)-dependent manner (Fig. 2A). Following activation, the protein C no longer bound (Fig. 2B). The RCM heavy chain also bound in the presence of Ca\(^{2+}\) and was eluted with EDTA (Fig. 2C). When the eluate was treated with thrombin, the heavy chain no longer bound to HPC4 (Fig. 2D). By sodium dodecyl sulfate gel analysis, the thrombin-treated RCM heavy chain was indistinguishable from activated protein C heavy chain (data not shown). These results suggested that the Ca\(^{2+}\)-dependent HuPC epitope was in or near the activation peptide region of the heavy chain. This is supported by the previous observation that HPC4 inhibits HuPC activation by thrombin-thrombomodulin in the presence of Ca\(^{2+}\) (17).

Ca\(^{2+}\) Dependence of Antigen Binding to HPC4 Antibody—The Ca\(^{2+}\) dependence of the HPC4/antigen binding was examined by incubating radiolabeled antigens with immobilized HPC4 (Fig. 3). The Ca\(^{2+}\) concentration resulting in half-maximal binding to HPC4 was 36 ± 5 μM for the RCM heavy chain, 110 ± 29 μM for HuGDPC, and 205 ± 23 μM for HuPC.

Ca\(^{2+}\)-induced Conformational Changes in HuPC and HuGDPC—If binding of human protein C/GDPC to HPC4 were determined solely by a Gla-independent, Ca\(^{2+}\)-induced neo-

**Fig. 1.** HPC4 affinity chromatography of RCM human protein C. Protein C was reduced and carboxymethylated, dialyzed against TBS, 2 mM CaCl\(_2\), pH 7.5, and applied to an HPC4-Affi-Gel 10 column (1.5 × 18 cm, 0.2 ml/min, 2-ml fractions) equilibrated in the same buffer. A, elution profile of RCM protein C. Bound protein was eluted with TBS, 2 mM EDTA, pH 7.5, at the fraction indicated by the arrow. B, peak fractions from the chromatogram shown in A were analyzed by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (26). Lane 1, fraction 11; lane 2, fraction 41; lane 3, sample applied to the affinity column; lane 4, HuPC. The positions of the RCM heavy chain (HC) and the RCM light chain (LC) are indicated.

**Fig. 2.** Loss of HuPC and RCM heavy chain epitope after thrombin cleavage. Proteins (0.1 M NaCl, 2 mM CaCl\(_2\), 0.1 M MOPS, pH 7.5) were diluted to 0.2 absorbance unit/ml, and 1-ml samples were applied to an HPC4-Affi-Gel 10 column (0.5 × 6 cm) equilibrated in the same buffer. Bound protein was eluted with 0.1 M NaCl, 2 mM EDTA, 0.1 M MOPS, pH 7.5, at the fraction indicated by the arrow. Each fraction contained 0.7 ml. A, human protein C; B, thrombin-activated protein C; C, RCM heavy chain; D, thrombin-cleaved RCM heavy chain. Fractions 23–25 of the RCM heavy chain eluted in C were pooled, and the activation peptide was released by thrombin proteolysis (10%, w/w, 4 h, 37 °C). Excess antithrombin III was added to stop the reaction; the solution was recalcified to 5 mM CaCl\(_2\) and applied to the same column. Recovery of proteins for each chromatogram was >98% based on recovery of A\(_{280}\) absorbing material.

**Fig. 3.** Calcium dependence of antigen binding to HPC4. \(^{125}\)I-Labeled RCM heavy chain (X), HuPC (O), or Gla-domainless HuPC (□) was incubated with HPC4-coupled Immunobeads at the indicated Ca\(^{2+}\) concentrations as described under "Experimental Procedures."
epitope, we would have expected half-maximal binding to the antibody to occur at a Ca\(^{2+}\) concentration similar to the high affinity site (K\(_D \approx 60 \mu M\)) in bovine GDPC (12). The observation that HuPC displayed half-maximal binding to HPC4 at 205 \(\mu M\) Ca\(^{2+}\) seemed inconsistent with this hypothesis. Therefore, we examined the influence of Ca\(^{2+}\) on the initial rate of HuPC and HuGDPC activation and on the conformation of these proteins. Analysis of the Ca\(^{2+}\) dependence of the activation rates by thrombin-thrombomodulin (Fig. 4A) revealed half-maximal activation at 214 \(\pm 22 \mu M\) Ca\(^{2+}\) for HuPC and at 210 \(\pm 37 \mu M\) Ca\(^{2+}\) for HuGDPC. Fig. 4B depicts Ca\(^{2+}\)-induced quenching of the intrinsic fluorescence of the proteins. For HuGDPC, the half-maximal change in tryptophan fluorescence occurred at 176 \(\pm 34 \mu M\) Ca\(^{2+}\), in reasonable agreement with that found for activation (210 \(\mu M\)), but somewhat higher than that observed for binding to HPC4 (110 \(\mu M\)). HuPC showed a maximal decrease in tryptophan fluorescence of 14\% with the half-maximal change occurring at 390 \(\pm 77 \mu M\) Ca\(^{2+}\), which is higher than that required for activation (214 \(\mu M\)) or binding to HPC4 antibody (205 \(\mu M\)).

Ca\(^{2+}\) Binding to Components of the Antigen-HPC4 Complex—When equilibrium dialysis experiments with \(^{45}\)Ca\(^{2+}\) at 205 \(\mu M\) Ca\(^{2+}\), assuming a 2:1 stoichiometry of RCM heavy chain to protein (at 0.8 mM Ca\(^{2+}\), 0.36 site in RCM heavy chain and 0.32 site in HPC4). However, when dialyzed together (15 \(\mu M\) RCM heavy chain, 30 \(\mu M\) HPC4), the results indicated between 2 and 3 mol of Ca\(^{2+}\) bound per mol of complex at 2 mM Ca\(^{2+}\), assuming a 2:1 stoichiometry of RCM heavy chain to HPC4 in the complex (data not shown; see below). This suggested that the Ca\(^{2+}\) dependence of complex formation was mediated not only by the antigen conformation, but also by contributions directly from the antibody. Due to the complexity of this system and the expense and scarcity of the RCM heavy chain, further analysis of this system was not attempted.

Identification of the Protein C Epitope—Since the activation peptide region was required for antibody binding, three synthetic peptides were prepared which span this region of the heavy chain (Fig. 5). These peptides were then assayed for their effect on \(^{125}\)I-labeled HPC4 binding to solid-phase HuPC. Polyvinyl chloride plates (96-well) were coated with HuPC (50 \(\mu l\) of 10 \(\mu M/ml\)) overnight at 4 \(^\circ\)C and rinsed; and residual reactive sites were blocked with TBS, 1\% gelatin, pH 7.5 (6 h, 25 \(^\circ\)C). \(^{125}\)I-Labeled HPC4 (final concentration, 10 \(nM\)) was added with the synthetic peptides (final concentration, 0.1 \(\mu M\) to 1 \(mM\)) in TBS, 5 mM Ca\(^{2+}\), 0.1\% gelatin, 0.02\% NaN\(_3\), pH 7.5, in a total volume of 50 \(\mu l\). The plates were incubated overnight at 25 \(^\circ\)C with mixing. After washing (TBS, 5 mM Ca\(^{2+}\), 0.1\% gelatin, pH 7.5), the wells were cut out and counted for radioactivity. Each data point represents the average of triplicate determinations with the standard deviations indicated by the error bars. O, P(6-17); \(
\), P(15-27); \(\square\), P(1-12).

The interaction of HPC4 with the synthetic peptides was also studied by monitoring intrinsic protein fluorescence in the presence of absence of Ca\(^{2+}\) (27, 28). This approach was used because any changes observed would be directly attributable to changes in the antibody resulting from peptide binding since the antigen does not contain aromatic amino acids. The intrinsic fluorescence of HPC4 increased when titrated with P(6-17) in the presence of 1 mM Ca\(^{2+}\), reaching a maximum by the expected 2:1 ratio of peptide to antibody (Fig. 7). In 1 mM EDTA, the fluorescence also increased, but this required higher peptide concentrations. The other two peptides did not significantly change the HPC4 fluorescence in the presence of Ca\(^{2+}\). The combined data of Figs. 6 and 7 demonstrate that P(6-17) contains the Ca\(^{2+}\)-dependent epitope.

Role of Ca\(^{2+}\) Binding to HPC4 in Complex Formation—The Ca\(^{2+}\) dependence of HPC4/P(6-17) binding was also studied using fluorescence methods. The intrinsic fluorescence of HPC4 in the presence of P(6-17) increased when titrated with Ca\(^{2+}\) with the half-maximal change occurring at 6.5 \(\pm 1.2 \mu M\) Ca\(^{2+}\) (Fig. 8). Mg\(^{2+}\) had no effect on HPC4 intrinsic fluorescence.

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**Fig. 5.** Relative sequence position of synthetic peptides. The amino-terminal residues of human protein C heavy chain are shown with the standard one-letter code. The sequence position of each peptide is indicated, and the appropriate residues are in parentheses. The thrombin cleavage site is shown by the arrow between the Arg\(_{12}\), Leu\(_{13}\) bond.

**Fig. 6.** Synthetic peptide inhibition of \(^{125}\)I-labeled HPC4 binding to solid-phase HuPC. Polyvinyl chloride plates (96-well) were coated with HuPC (50 \(\mu l\) of 10 \(\mu M/ml\)) overnight at 4 \(^\circ\)C and rinsed; and residual reactive sites were blocked with TBS, 1\% gelatin, pH 7.5 (6 h, 25 \(^\circ\)C). \(^{125}\)I-Labeled HPC4 (final concentration, 10 \(nM\)) was added with the synthetic peptides (final concentration, 0.1 \(\mu M\) to 1 \(mM\)) in TBS, 5 mM Ca\(^{2+}\), 0.1\% gelatin, 0.02\% NaN\(_3\), pH 7.5, in a total volume of 50 \(\mu l\). The plates were incubated overnight at 25 \(^\circ\)C with mixing. After washing (TBS, 5 mM Ca\(^{2+}\), 0.1\% gelatin, pH 7.5), the wells were cut out and counted for radioactivity. Each data point represents the average of triplicate determinations with the standard deviations indicated by the error bars. O, P(6-17); \(
\), P(15-27); \(\square\), P(1-12).

**Fig. 4.** Influence of Ca\(^{2+}\) on activation rates and changes in intrinsic fluorescence of HuPC and HuGDPC. Protein C (O) and Gla-domainless protein C (C) were present at 1 \(\mu M\) final concentration for both the kinetic and fluorescence experiments. A, HuPC and HuGDPC activation by the thrombin-thrombomodulin complex in the presence of increasing Ca\(^{2+}\) concentration. The activation rates at 5 mM Ca\(^{2+}\) were taken as maximal. B, Ca\(^{2+}\)-induced changes in intrinsic protein fluorescence. Proteins were titrated with CaCl\(_2\), and tryptophan fluorescence was monitored as described under "Experimental Procedures." Data are represented as the ratio of the emission peak area at that Ca\(^{2+}\) concentration (F) to the peak area in the absence of added metal (F\(_0\)).
proteins because its ionic radius and coordination properties are similar to those of Ca\(^{2+}\). When the ion binds to a site on a protein close enough to a tryptophan or tyrosine residue(s) to allow efficient singlet-singlet energy transfer, TCP binding to HPC4 provides a unique system to monitor Tb\(^{3+}\) binding since only the anti-Tb\(^{3+}\) antibody contained donor aromatic amino acids for enhanced Tb\(^{3+}\) fluorescence. The 17-fold increase in affinity for Tb\(^{3+}\) by the peptide-HPC4 complex directly demonstrates the metal ion-dependent changes in intrinsic fluorescence of HPC4 in the presence and absence of P(6-17). HPC4 (1 mM) was titrated with \(^{34}\)Ca\(^{4+}\) in the presence (O) and absence ( ) of 4 mM P(6-17). The buffer used was 0.1 M MES, 0.1% gelatin, 0.02% NaN\(_3\), pH 6.0. HPC4 tryptophan was excited at 285 nm, and Tb\(^{3+}\) emission intensity was integrated from 528 to 552 nm as described under "Experimental Procedures." At each Tb\(^{3+}\) concentration, fluorescence due to buffer background was subtracted from the total emission peak area to give only protein-dependent Tb\(^{3+}\) fluorescence (\(F_m\)). Half-maximal Tb\(^{3+}\) concentrations were determined after correction for bound Tb\(^{3+}\) assuming one Tb\(^{3+}\) ion/antibody-antigen complex.

![Fig. 7](image_url)

**Fig. 7.** Effect of synthetic peptides on intrinsic fluorescence of HPC4 in the presence or absence of metal ions. HPC4 (5 \(\mu\)M) in TBS containing 1 mM Ca\(^{2+}\) or 1 mM EDTA was titrated with peptides at the indicated ratios. HPC4 tryptophan fluorescence was monitored as described under "Experimental Procedures." The data are expressed as the ratio of emission peak area at each titration (\(F_0\)) relative to the peak area in the absence of peptide (\(F_p\)). \(\bullet\) and \(\circ\), P(6-17) in Ca\(^{2+}\) or EDTA, respectively; \(\square\) and \(\Delta\), P(1-12) or P(15-27) in Ca\(^{2+}\), respectively.

![Fig. 8](image_url)

**Fig. 8.** Metal ion-dependent changes in intrinsic fluorescence of HPC4 in the presence and absence of P(6-17). HPC4 (1 \(\mu\)M) was titrated with metal ions in the presence or absence of 2 \(\mu\)M P(6-17). Tryptophan emission changes were monitored as described under "Experimental Procedures." \(F_0\) represents the HPC4 emission peak area (\(\varphi\)peptide) in the absence of added metal. \(\bullet\) and \(\circ\), Ca\(^{2+}\) titration of HPC4 or HPC4 + peptide, respectively, \(\square\) and \(\Delta\), Mg\(^{2+}\) titration of HPC4 or HPC4 + peptide, respectively.

![Fig. 9](image_url)

**Fig. 9.** Terbium binding by HPC4 in the presence and absence of P(6-17). HPC4 (1 \(\mu\)M) was titrated with \(^{34}\)Ca\(^{4+}\) in the presence (O) or absence ( ) of 4 \(\mu\)M P(6-17). The buffer used was 0.1 M MES, 0.1% gelatin, 0.02% NaN\(_3\), pH 6.0. HPC4 tryptophan was excited at 285 nm, and Tb\(^{3+}\) emission intensity was integrated from 528 to 552 nm as described under "Experimental Procedures." At each Tb\(^{3+}\) concentration, fluorescence due to buffer background was subtracted from the total emission peak area to give only protein-dependent Tb\(^{3+}\) fluorescence (\(F_m\)). Half-maximal Tb\(^{3+}\) concentrations were determined after correction for bound Tb\(^{3+}\) assuming one Tb\(^{3+}\) ion/antibody-antigen complex.

![Fig. 10](image_url)

**Fig. 10.** Calcium binding by HPC4-P(6-17) complex as determined by the Hummel-Dreyer technique (31). HPC4, P(6-17), and mixtures of the two were gel-filtered on a Sephadex G-15 column (0.6 x 30 cm) equilibrated in TBS, 0.1 mM CaCl\(_2\), and a trace amount of \(^{45}\)Ca\(^{4+}\). Column flow rate was controlled by FPLC pumps at 0.1 ml/min (0.5-ml fractions). Absorbance of each fraction at 280 or 220 nm was determined as well as \(^{45}\)Ca\(^{4+}\) content by counting 100- \(\mu\)l samples in 2 ml of Aquasol-2 in a liquid scintillation counter using the \(^{14}\)C channel. Specific activity (counts/minute/micromolar Ca\(^{4+}\)) of \(^{45}\)Ca\(^{4+}\) was determined for each experiment by averaging the counts/minute in the first 10 fractions. The chromatograms are shown with absorbances at 220 nm ( ) (peptide) or 280 nm ( ) (HPC4) and \(^{45}\)Ca\(^{4+}\) counts/minute/milliliter; A, P(6-17) (1 mM); B, HPC4 (48 \(\mu\)M); C, HPC4 (42 \(\mu\)M) + P(6-17) (143 \(\mu\)M).
transformation of a low affinity metal ion-binding site in HPC4 to a high affinity site.

To determine if Ca$^{2+}$ binding to the antibody-peptide complex had similar requirements, Ca$^{2+}$ binding studies were done using the Hummel-Dreyer gel filtration technique (31). P(6–17), HPC4, and mixtures of the two were applied to the column, and their ability to bind Ca$^{2+}$ was determined. P(6–17) did not bind Ca$^{2+}$ (Fig. 10A), as evidenced by the lack of either a "Ca$^{2+}$" peak eluting with the peptide or a trough at the inclusion volume (22.23 ml). HPC4 alone also did not show significant Ca$^{2+}$ binding (Fig. 10B). There was some indication of a "Ca$^{2+}$" peak coincident with the A$_{280}$ peak (0.3 mol of Ca$^{2+}$/mol of HPC4), but no discernible trough. When the experiment was performed with HPC4 and a 3.5-fold molar excess of peptide (Fig. 10C), Ca$^{2+}$ binding increased to 1.76 mol of Ca$^{2+}$/mol of HPC4. The Ca$^{2+}$ binding was constant across the A$_{280}$ peak (1.72–1.77 mol of Ca$^{2+}$/mol of HPC4), and analysis of the trough data indicated an average of 1.65 mol of Ca$^{2+}$/mol of HPC4. Mg$^{2+}$ had no effect on Ca$^{2+}$ binding by the peptide-HPC4 complex (1.41 and 1.52 mol of Ca$^{2+}$/mol of HPC4 in 0.02 mM Ca$^{2+}$ or 0.02 mM Mg$^{2+}$, respectively). This was consistent with the inability of Mg$^{2+}$ to affect the intrinsic fluorescence of HPC4 ± peptide.

**DISCUSSION**

During the course of these studies, the evolving characteristics of the Ca$^{2+}$-dependent HPC4/antigen interaction posed some interesting problems. The observation that the Ca$^{2+}$-dependent HuPC epidermis was independent of the light chain Gla domain was compatible with known Ca$^{2+}$ binding characteristics of bovine protein C and GDPC (11, 12). The apparent $K_d$ of this site on the bovine molecule is ≈60 μM, well below the observed Ca$^{2+}$ dependence for HuPC/HPC4 binding (205 μM Ca$^{2+}$). Analysis of the Ca$^{2+}$ dependence of activation and conformational changes in human GDPC revealed that this site has a lower affinity (≈200 μM Ca$^{2+}$) than the bovine protein. The observation that HuGDPC required less Ca$^{2+}$ for antibody binding (110 μM) than for activation (210 μM) may be related to the fact that the antibody binds the Ca$^{2+}$-stabilized form of GDPC with high affinity, thereby favoring the Ca$^{2+}$-complexed conformer of GDPC. The resulting shift in equilibrium could reduce the Ca$^{2+}$ concentration dependence for this process. Overall, these results show that Ca$^{2+}$ binding to the Gla-independent site, probably in the light chain epidermal growth factor homology domain (10), alters the conformation of the active peptide region of the heavy chain and suggests that this change in conformation is critical for substrate presentation to the thrombin-thrombomodulin complex. In addition, these studies provide the first direct evidence for a Ca$^{2+}$-induced conformational change in the activation site of a vitamin K-dependent zymogen.

What remained unresolved was the observed Ca$^{2+}$ dependence of RCM heavy chain binding to HPC4. The half-maximal binding decreased to 36 μM Ca$^{2+}$ for a protein with less secondary structure than the native molecule. Furthermore, neither the RCM heavy chain nor the HPC4 antibody had the ability to bind Ca$^{2+}$ with the expected high affinity. Our working model for the system was the classical case, wherein Ca$^{2+}$ binding to the antigen results in a conformation change exposing the epitope on the antigen. However, none of the data for the RCM heavy chain/HPC4 interaction could be explained by this mechanism. In addition, the observation that Ca$^{2+}$ binding increased when both the RCM heavy chain and HPC4 were present together suggested that the role of Ca$^{2+}$ in this system was more complicated than anticipated.

The availability of the synthetic peptide P(6–17) permitted studies to resolve the paradoxical results obtained with the RCM heavy chain. This peptide, which spans the activation site on protein C, contains the epitope recognized by HPC4. Like the native molecules, the HPC4/P(6–17) interaction was Ca$^{2+}$-dependent. Furthermore, the peptide alone did not bind Ca$^{2+}$, but in the presence of the antibody, Ca$^{2+}$ binding increased to ≈2 mol of Ca$^{2+}$/mol of HPC4. These observations, in combination with the ability of the peptide to interact weakly with HPC4 in the presence of EDTA, led to the possibility that the antibody itself contained a Ca$^{2+}$-binding site required for high affinity binding to the antigen. This was directly demonstrated by Tb$^{3+}$ binding experiments. The antibody alone bound Tb$^{3+}$, with half-maximal binding occurring at 34 μM Tb$^{3+}$. In the presence of P(6–17), the apparent affinity for Tb$^{3+}$ increased 17-fold. In this system, the only possible donor for energy transfer to the Tb$^{3+}$ ion was the antibody. The simplest interpretation of these results is that binding the antigen transforms the metal-binding site in the antibody from low to high affinity. Further studies are necessary to establish exactly where the ion binds in HPC4 and the mechanism of antigen-induced changes in metal ion affinity. It is possible that the site is directly in the HPC4 combining region of the variable domains or, alternatively, that metal binding to HPC4 affects antigen binding by allosteric mechanisms.

By comparing the Ca$^{2+}$ dependence of HuPC binding with that of the peptide, at least two roles have emerged for Ca$^{2+}$ in mediating antigen binding to HPC4. The first incorporates the classical mechanism where Ca$^{2+}$ binds to HuPC or HuGDPC and stabilizes a particular conformation. This results in a conformation recognized by both the thrombin-thrombomodulin complex and HPC4. The second role for Ca$^{2+}$ is stabilization of the antigen-HPC4 complex. This is mediated by a metal ion-binding site in the antibody. The two Ca$^{2+}$-dependent events are clearly related, but can be discriminated. As the antigen structure decreases, the Ca$^{2+}$ requirement for antigen binding decreases, presumably because the epitope becomes less conformationally constrained. These studies provide the first evidence to our knowledge of a Ca$^{2+}$ requirement for antibody/antigen binding where a critical role for Ca$^{2+}$ interaction with the antibody is implicated.

Like protein C, the other vitamin K-dependent proteins require metal ions for optimal expression of function. Recently, several laboratories (13–10) have described the role of cations in structure/function relationships of prothrombin, Factor IX, and protein C by using various metal-dependent antibodies. Whereas these immunological approaches are ideally suited to the study of ion specificity in these complex systems, interpretation of metal involvement in the antigen/antibody interactions may be more complex than has previously been appreciated. This study demonstrates that the role of the metal ion is not necessarily limited to expression of an epitope or stabilization of a particular protein conformation. Furthermore, the inability of Mg$^{2+}$ to support HPC4-P(6–17) complex suggests that metal ion specificity may be related to requirements of the antibody. Whether HPC4 constitutes a unique antibody or represents a class of antibodies remains to be determined.

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