Discrimination of Normal and Abnormal Prothrombin and Protein C in Plasma Using a Calcium Ion-Inhibited Monoclonal Antibody to a Common Epitope on Several Vitamin K-Dependent Proteins

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Vitamin K deficiency or administration of vitamin K antagonists results in the biosynthesis of abnormal des-γ-carboxy forms of the vitamin K-dependent proteins. Monoclonal antibody H-11 binds several vitamin K-dependent proteins at a determinant that includes the first two residues of γ-carboxyglutamic acid. Antibody H-11 binds fully carboxylated prothrombin and protein C in the presence of EDTA but binding is inhibited by the divalent metal ions, calcium, magnesium, and manganese. By contrast, des-γ-carboxy prothrombin and protein C bind antibody H-11 the same in the presence of EDTA or calcium ion. Antibody H-11 thus appears to bind a conserved antigenic site containing γ-carboxyglutamic acid that in the presence of divalent metal ions undergoes a conformational transition. This ability of antibody H-11 to bind des-γ-carboxy prothrombin and protein C in the presence of calcium ion allowed the development of an immunoassay for these proteins in plasma. Prothrombin and protein C from stably anticoagulated individuals receiving warfarin were characterized by their ability to bind antibody H-11 in the presence of calcium ion. Binding of prothrombin and protein C to antibody H-11 in the presence of calcium correlated temporally with warfarin administration. The inability of calcium ion to inhibit binding of antibody H-11 to abnormal prothrombin and protein C in plasma suggests that the circulating forms of both proteins following warfarin administration cannot undergo the metal ion-dependent conformational transition that includes sequence residues 1 through 12.

The calcium-mediated conformational transition of the Gla domains of the vitamin K-dependent proteins is required for their normal function in blood coagulation.5-11 Both polyclonal and monoclonal antibodies have been used to probe the conformational transitions that occur on calcium binding to Gla and possibly other residues in the Gla domain.5,12-16 Recently, we have described a murine monoclonal antibody (MoAb, designated H-11) produced against protein C which binds a subset of vitamin K-dependent proteins including prothrombin, factors VII and X, and protein C.17 The antigenic determinant recognized by this antibody was localized to the amino terminal residues of the Gla domain at a site containing the conserved sequence Phe-Leu-Glu-Glu. This site contains the first two residues of γ-carboxyglutamic acid in the Gla domain of several vitamin K-dependent proteins. The binding of antibody H-11 to protein C was inhibited by divalent metal ions and occurred only following removal of divalent ions with EDTA or dilution into metal-free solutions. This differential binding of the antibody to protein C in the presence and absence of divalent metal ion suggested that such an antibody might prove useful in discriminating normal and abnormal vitamin K-dependent proteins. The following describes the use of antibody H-11 to detect abnormal prothrombin and protein C in plasma from individuals receiving warfarin.

MATERIALS AND METHODS

Isolation of proteins. Prothrombin and protein C were isolated from frozen plasma obtained from the Vermont/New Hampshire Red Cross Blood Center. Proteins were prepared from barium citrate precipitates of plasma by EDTA elution followed by ammonium sulfate fractionation and chromatography on either DEAE-Sephadex (prothrombin) or an anti-protein C MoAb immunoaffinity column.18-19 Purity of the proteins was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis.20 Prothrombin was decorboxylated by the method of Bajaj et al21 and the extent of decorboxylation was determined by amino acid analysis following base hydrolysis of the protein.21 For the prothrombin radioimmunoassay (see below) purified prothrombin was labeled with 125I using iodogen (Pierce, Rockford, IL).22 Immunoassays for total carboxylated and decorboxylated pro-
thrombin and protein C. Antibodies used in this study were provided by the Immunology Core Laboratory of the Vermont Specialized Center of Research (SCOR) in Thrombosis. These antibodies included burro anti-prethrombin 1, horse anti-protein C,23 and the murine anti-protein C MoAb designated aHPC-2.19 Purified antibody H-11 was generously provided by J. Amiral of Stago Diagnostica (Paris, France). Antibodies were purified either by gel filtration on ACA 34 resin (LKB) followed by chromatography on DEAE-cellulose24 or by DEAE-cellulose chromatography alone. Antibody H-11 was radiolabeled with 125I using Iodogen.25 Specific activities of 90 μCi/μg were obtained.

Immunassays for total prothrombin and protein C were performed by the Assay Core Laboratory of the Vermont SCOR. The concentration of prothrombin in plasma was determined using a solid-phase radioimmunoassay. Tissue culture 96-well plates (Nunc Immunoplates, Roskilde, Denmark) were coated with 10 μg/mL purified burro anti-human prothrombin 1 antibody diluted in 16 mmol/L sodium carbonate, 34 mmol/L sodium bicarbonate, pH 9.5, buffer overnight with 0.2 mL per well. The plate was washed with 10 mm sodium phosphate, 0.15 mol/L NaCl, pH 7.4 (PBS) containing 0.1% Tween 20 and then blocked with 0.3 mL of 5% bovine serum albumin (BSA) for 1 h. Samples were diluted in PBS containing 1% BSA, 0.1% Tween 20, and 2 mmol/L EDTA to a final volume of 0.1 mL/well. After addition of diluted sample to the well, 0.1 mL 125I-labeled prothrombin diluted in assay buffer was added. The plates were routinely incubated overnight at 4°C. The wells were washed three times with PBS-Tween buffer and then 0.25 mL of 0.1 mol/L NaOH was added to each well for 1.5 hours. An aliquot of the NaOH wash (0.2 mL) was transferred to a small tube and the amount of radioactivity determined using a Beckman Biogamma gamma-counter (Fullerton, CA). The sensitivity of this assay was approximately 60 ng/mL prothrombin and the interassay coefficient of variation was near 8%.

Total protein C concentration in plasma was determined using a sandwich-type enzyme-linked immunosorbent assay (ELISA). The wells of 96-well plates were coated with 0.2 mL of equine anti-human protein C antibody (10 μg/mL) diluted in carbonate/bicarbonate buffer overnight at 4°C. Non-specific protein binding sites were blocked with 5% BSA-PBS for 1 hour at room temperature. Samples were diluted in PBS containing 3% BSA, 0.01% Tween 20 and 0.2 mL sample was incubated in the antibody-coated wells for 3 hours at room temperature. The wells were then washed with PBS-Tween and 0.2 mL of an appropriately diluted solution of aHPC-2 conjugated with horseradish peroxidase in 1% BSA-PBS-Tween buffer was added to each well. Following an overnight incubation at 4°C, the wells were washed six times with PBS-Tween and 0.1 mL of o-phenylenediamine (OPD) substrate (0.4 mg/mL in 75 mmol/L citrate-phosphate buffer, pH 5.0, containing 0.012% H2O2) was added to the wells. The plates were incubated at room temperature in the dark and the development of color in the wells was monitored. The absorbance of the wells were made with a BioTek Microtiter plate spectrophotometer (BioTek Inc, Burlington, VT). The sensitivity of the assay was approximately 10 ng/mL with a coefficient of variation of 3%.

Immunassays using antibody H-11. Assays were performed in plastic 96-well microtiter plates (Removewells, Dynatech, Chantilly, VA). For prothrombin, plates were coated overnight at 4°C with burro anti-prethrombin 1 antibody diluted to 10 μg/mL in sodium carbonate/bicarbonate buffer, 0.1 mL/well. The plates were then aspirated and blocked with 0.2 mL/well 1% BSA in 0.02 mol/L Tris, 0.15 mol/L NaCl, pH 7.4 buffer (BSA-TBS) for 1 hour at room temperature. Plates were also stored at −20°C with BSA-TBS and thawed when needed. After washing the plates once with TBS containing 0.1% Tween 20, purified prothrombin or plasma samples (0.1 mL) diluted in 0.1% BSA-TBS were added and the plates incubated for 1 hour at room temperature. Plates were then washed three times with TBS-Tween followed by addition of antibody H-11 (0.1 mL) at a concentration of 5 μg/mL in 0.1% BSA-TBS containing either 10 mmol/L calcium ion or 10 mmol/L EDTA. Following a 1-hour incubation at room temperature the wells were washed three times with TBS-Tween. The antibody H-11/prothrombin/anti-prethrombin 1 complexes were detected using a goat anti-mouse immunoglobulin G antibody conjugated with horseradish peroxidase (Hyclone, Ogden, UT). Conjugated antibody was added to the wells for 1 hour at room temperature, washed five times with TBS-Tween followed by addition of 0.1 mL of OPD substrate. The absorbance measurements were made after 10 minutes using an ELISA microtiter plate spectrophotometer.

For protein C, the anti-protein C MoAb aHPC-2 was immobilized in the wells. After washing, plates were blocked with 0.3 mL of 1% BSA in TBS for 1 hour at room temperature. Plasma samples diluted in 0.1% BSA-TBS were added (0.1 mL/well) and incubated for 1 hour at room temperature. After washing the plate three times with TBS-Tween, 125I-labeled antibody H-11 in 0.1% BSA-TBS containing 10 mmol/L calcium ion or EDTA were added to the wells (50,000 cpm/well). Following a 1-hour incubation at room temperature, the plates were washed three times, aspirated to dryness, and the amount of radioactivity remaining in the individual wells determined.

Patient samples. This study was approved by the Institutional Review Board of the University of Vermont, Burlington, VT. Normal patient samples were obtained from the Vermont/New Hampshire Regional Blood Center of the American Red Cross. Warfarin-treated patient's blood samples (warfarin plasma) were obtained from the Clinical Coagulation Laboratory of the Medical Center Hospital of Vermont, Burlington, VT. All plasma samples from warfarin-treated patients were from individuals who had been stably anti-coagulated for at least 1 month. A pool of warfarin plasmas (n = 6) was also created from these same samples for use in plasma mixing experiments. Patient blood samples were collected into vacutainer tubes (Becton-Dickinson, Research Triangle Park, NC) containing 0.01 mol/L sodium citrate and centrifuged at 3,000 × g for 10 minutes. The plasmas were separated from the cells and stored at −70°C.

RESULTS

Inhibition of antibody H-11 binding to prothrombin by divalent metal ions. MoAb H-11 was produced in mice by immunizing with protein C. Characterization of this antibody showed that it reacted with a conserved antigenic site in the Gla domain of several vitamin K-dependent proteins.17 Furthermore, antibody binding to protein C was inhibited by several divalent metal ions including calcium, manganese, and magnesium; the antibody was able to bind protein C only in the presence of EDTA or in the absence of added divalent metal ions. As shown in Fig 1, binding of 125I-labeled antibody H-11 to human prothrombin immobilized onto plastic 96-well plates is inhibited by increasing concentrations of calcium, magnesium, and manganese ions similarly to that previously reported with protein C. Both calcium and magnesium ions over a concentration range of 10 μmol/L to 40 mmol/L inhibited approximately 80% to 90% of 125I-labeled antibody H-11 binding to prothrombin with a transition midpoint near 1 mmol/L. Magnesium ion inhibited approximately 40% of antibody binding to prothrombin over an identical range of concentration. Inhibition of antibody
Inhibition by divalent metal ions of $^{125}$-antibody H-11 binding to human prothrombin. Increasing concentrations of CaCl$_2$ (■), MnCl$_2$ (△), or MgCl$_2$ (□) were incubated with $^{125}$-labeled antibody H-11 (50,000 cpm) in 96-well microtiter plates coated with prothrombin. After incubation at room temperature, the wells were washed and the amount of radioactive antibody bound to the wells was determined using a gamma-counter.

H-11 binding by divalent metal ions is, thus, also observed with prothrombin and is not exclusive to protein C.

Immunoassay for des-$\gamma$-carboxy prothrombin. An assay was designed as outlined in Fig 2 to exploit the unique metal ion-dependence of antibody H-11 for binding several vitamin K-dependent proteins. Our previous data with antibody H-11 indicated that the antigenic site was masked by the presence of calcium ion due, presumably, to a metal ion-dependent conformational change within the $\gamma$-carboxyglutamic acid containing domain.1,7,14,17,25,26 Conversion of the $\gamma$-carboxyglutamic acid residues to glutamic acid by heating the protein,3 did not destroy the antigenic site recognized by the antibody but did abolish the calcium-mediated inhibition of antibody binding. Similar behavior would be predicted for the des-$\gamma$-carboxy vitamin K-dependent proteins resulting from warfarin therapy. Figure 2A represents the possible structural transition of the Gla domain of prothrombin and des-$\gamma$-carboxy prothrombin in the presence of calcium ion.

The assay format designed to measure abnormal prothrombin using antibody H-11 is shown in Fig 2B. Plates were coated using a capture antibody directed to antigenic determinants not contained in the Gla domain that are able to bind prothrombin and abnormal prothrombin in the presence of both calcium ion and EDTA. Binding of antibody H-11 to prothrombin is detected using an enzyme-linked antibody detection system.

Using the assay format represented in Fig 2B, purified fully carboxylated prothrombin (10 Gla) and prothrombin that had been heat decarboxylated (0 Gla) were measured in the H-11 assay. The data in Fig 3A (left panel) indicate that in the presence of calcium ion, there is a decrease in the binding of antibody H-11 to normal prothrombin compared with the level of antibody H-11 binding to prothrombin in the presence of EDTA. Previous experiments (data not shown) had established that binding of prothrombin to the anti-prethrombin 1 antibody was identical in the presence of 10 mmol/L calcium or EDTA. Antibody H-11 binding to prothrombin that had been decarboxylated by heating was equivalent in the presence of both calcium ion and EDTA (Fig 3A, right panel). These data suggest that decarboxylation of $\gamma$-carboxyglutamic acid destroys the calcium ion binding sites in the Gla domain of prothrombin resulting in the loss of the calcium ion-dependent inhibition of antibody H-11 binding to prothrombin due to inability of the protein to undergo the conformational transition.
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A comparison of serially diluted normal plasma and warfarin plasma in the H-11 assay is summarized in Fig 3B. The concentration of prothrombin in both samples was determined using a radioimmunoassay for total prothrombin antigen and the plasma in the assay samples were adjusted to give the concentration of total prothrombin shown along the abscissa in Fig 3B. As seen in Fig 3B, left panel, prothrombin from normal plasma showed the characteristic decreased binding of antibody H-11 to prothrombin in the presence of calcium ion when compared with the amount of antibody H-11 bound in the presence of EDTA. Purified prothrombin at the same concentrations showed similar binding curves in the assay (data not shown). On the other hand, prothrombin in plasma from individuals receiving warfarin bound antibody H-11 identically in the presence of calcium ion or EDTA (Fig 3B, right panel). These data suggested that des-γ-carboxyprothrombin in plasma from individuals receiving warfarin cannot undergo the metal ion-dependent conformational transition and therefore express the antibody H-11 antigenic site in a manner similar to normal prothrombin that had been stripped of calcium ion by treatment with EDTA or decarboxylated by heating.

Immunoassay for des-γ-carboxy protein C. The ability of antibody H-11 to bind more than one vitamin K-dependent protein increases its versatility to detect vitamin K-dependent proteins in plasma. In the H-11 assay for protein C, the capture antibody is a MoAb to protein C that binds to a determinant on the heavy chain of protein C distant from the Gla domain. The detection system for the captured protein C was 125I-labeled antibody H-11. After incubating dilutions of the test sample in the antibody-coated wells, 125I-labeled antibody H-11 in the presence of either 10 mmol/L calcium ion or 10 mmol/L EDTA was added to the wells. After a second incubation at room temperature the wells were washed and the amount of bound 125I-labeled antibody H-11 determined using a gamma counter.

The results of a comparison of protein C in a normal plasma sample with a plasma sample from a warfarin-treated individual are shown in Fig 3C. As with the H-11 immunoassay for prothrombin, the concentration of protein C in both plasma samples was previously determined using an ELISA for total protein C. Binding of 125I-labeled antibody H-11 to plasma protein C as a function of protein C concentration in the presence of EDTA or calcium ion is shown in Fig 3C. In the presence of EDTA the binding of 125I-labeled antibody H-11 to protein C is similar in both plasma samples and increases in a linear fashion as the protein C concentration increases. Antibody H-11 binding to protein C from normal plasma was markedly decreased in the presence of calcium ion when compared with the level of antibody H-11 binding seen in the presence of EDTA (Fig 3C, left panel). This result is similar to the behavior of the binding curves seen with prothrombin (Fig 3A and B). By contrast, and in distinction from the prothrombin H-11 assay, protein C in the sample from the warfarin-treated individual, in the presence of calcium, showed a higher level of antibody H-11 binding than in the presence of EDTA (Fig 3C, right panel). This increased binding of antibody H-11 to protein C from warfarin plasma results most likely from the calcium dependence of the capture antibody.23

Dependence of the H-11 immunoassay on percent abnormal prothrombin and protein C in plasma samples. A series of sample plasmas prepared by mixing normal and warfarin plasma were measured in the H-11 assay. Total prothrombin concentrations in both the normal plasma sample and in a plasma sample from a pool of warfarin-treated individuals were determined using the prothrombin radioimmunoassay. The plasma mixtures were then adjusted to give a final concentration of 100 ng/mL. The data, shown in Fig 4, summarize the analysis of the plasma mixtures in the antibody H-11 immunoassay. The percent abnormal pro-
The $^{125}$I-labeled antibody H-11 binding to protein C was also examined by a plasma mixing experiment similar to that used for prothrombin. The total protein C concentration in both samples was measured using a protein C ELISA and the samples were mixed to give a final protein C concentration of 100 ng/mL in the assay. Two matched sets of mixed samples were examined. One set contained $^{125}$I-labeled antibody H-11 in the presence of 10 mmol/L EDTA while the other set contained 10 mmol/L calcium ion. The data summarized in Fig 4B indicate that in the presence of calcium ion, as the percent warfarin plasma in the presence of calcium ion is increased from 0% to 100%, binding of antibody H-11 to protein C increases. This is similar to the finding with prothrombin. In the presence of EDTA both normal and des-$\gamma$-carboxy-protein C bind equivalently as shown by the horizontal line. Crossover of the EDTA line by the calcium values as the percent abnormal protein C increases is different than the data in Fig 4A for prothrombin but is the expected result due to the calcium-dependent effects on the capture antibody. An additional result of the crossover of the EDTA and calcium ion curves is that the difference of the counts per minute of antibody bound in the presence of EDTA, minus the counts per minute in the presence of calcium, may be a negative number. The data in Figs 3 and 4 demonstrate that the H-11 immunoassay is dependent in a linear fashion on the fraction of total prothrombin or protein C that is fully carboxylated. The measurable difference in antibody H-11 binding to prothrombin and protein C in the presence of EDTA or calcium ion thus appears to directly correlate with the percent of the respective des-$\gamma$-carboxy protein in plasma.

The ability of the H-11 immunoassay to detect undercarboxylated prothrombin and protein C was demonstrated by examining a panel of normal plasma samples ($n = 18$) and comparing these with a panel of plasma samples from stably warfarin-treated individuals ($n = 17$). To account for differences in prothrombin and protein C levels between patients, prothrombin and protein C antigen levels were first determined by immunoassay. The amount of plasma for each sample in the antibody H-11 immunoassay was adjusted to a final concentration of 100 ng/mL. The data in Fig 5 represent the difference in antibody H-11 binding to prothrombin and protein C in the presence of EDTA versus calcium ion. The plotted values are determined by subtracting the amount of antibody H-11 bound in the presence of calcium ion from that bound in the presence of EDTA. The data summarized in Fig 5 clearly indicate that the H-11 immunoassays for prothrombin and protein C can distinguish between normal prothrombin and protein C in normal plasma and abnormal prothrombin and protein C in plasma from individuals given warfarin. The means of the two groups were significantly different ($P < .0005$).

Fig 4. Linearity of antibody H-11 immunoassay response to percent abnormal prothrombin and protein C in plasma. Normal pooled plasma and plasma from a warfarin plasma pool were mixed to give a final concentration of prothrombin or protein C of 100 ng/mL in the assay. (A) Binding of antibody H-11 to prothrombin in the presence of EDTA ($) or calcium ion (O) was determined by H-11 immunoassay. The absorbance data of duplicate samples are plotted versus the percent of total prothrombin contributed by the plasma from the individual receiving warfarin. (B) Binding of $^{125}$I-labeled antibody H-11 (50,000 cpm/well) to protein C in the presence of 10 mmol/L EDTA ($) or 10 mmol/L calcium ion (O).
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Fig 5. Antibody H-11 binding to abnormal prothrombin and protein C in plasma from normal individuals and individuals receiving warfarin. The data is represented as the difference of antibody H-11 binding in the presence of EDTA minus binding in the presence of calcium. Duplicate samples were averaged to give the indicated results for normal plasma samples (A, B) and for plasma samples from individuals receiving warfarin (C, D). The amount of plasma in each sample was adjusted to prothrombin and protein C concentrations of 100 ng/mL in the antibody H-11 assay.

Discussion

Antibody H-11 is the first immunochemical reagent to a coagulation protein that binds to a limited, defined region of polypeptide and has been shown to be inhibited by divalent metal ions. This inhibition is most likely due to the conformational transition of the polypeptide that masks the antigenic site. The recent x-ray crystallographic data for the calcium-fragment 1 complex indicates that the side chain of the Phe residue at position 4 is buried in the metal-protein complex. This residue has been proposed as a key residue within the antigenic determinant recognized by antibody H-11 and loss of antibody binding may be explained in part by the inaccessibility of the side chain in the folded calcium-protein complex. Recent studies using synthetic peptides synthesized with Gla have shown that calcium ion alone is not sufficient to block binding of antibody H-11 to peptides representing residues 1 through 12. This evidence would support the contention that inhibition of antibody H-11 binding by divalent calcium ion is not due simply to charge neutralization of negative Gla residues but most likely involves a conformational transition that includes amino acid residues removed from the H-11 antigenic site. To a first approximation, des-γ-carboxy prothrombin does not bind sufficient calcium ion to either stabilize or induce the folded structure observable from crystallographic studies. The unfolded polypeptide is, therefore, in a conformational state recognizable by antibody H-11 in the presence of calcium.

The disparate binding of antibody H-11 in the presence or absence of calcium ion allows quantitation of the fraction of plasma prothrombin and protein C that is able to undergo the calcium-dependent conformational change. The differential antibody binding in the presence of metal ion is similar to earlier work by Stenflo who reported analogous behavior of a polyclonal antiserum to normal prothrombin in the presence and absence of calcium ion. The particular antisera used in these earlier studies presumably contained a high percentage of antibodies directed to determinants expressed by the divalent metal ion-protein complex. This ability of antibody H-11 to bind to selected des-γ-carboxy vitamin K-dependent proteins forms the basis for the discrimination of normal and abnormal proteins.

The capture antibody is an important component of the assay for des-γ-carboxy proteins. Both the polyclonal anti-prethrombin 1 and the anti-human protein C MoAbs do not interfere with the binding of antibody H-11 to its determinant. The observable increase in binding of protein C to αHPC-2 in the presence of calcium ions is similar to the behavior of the antibody HPC-4 described by Stearns et al. Preliminary evidence suggests that the epitope recognized by αHPC-2 is in the heavy chain of protein C but the exact peptide site has not been localized.

Following warfarin administration various undercarboxylated forms of prothrombin and most likely other vitamin K-dependent proteins are synthesized and secreted into the plasma.
circulation. Earlier work from several groups has shown that the plasma pool of prothrombin consists of partially and completely decarboxylated forms of the protein and it is unclear how such partially carboxylated prothrombin, protein C, or any of the other vitamin K-dependent proteins function as competing substrates and enzymes in the important macromolecular complexes of hemostasis. Malhotra et al have shown that loss of as few as 3 of 10 Gla residues in prothrombin abruptly disrupts phospholipid binding, calcium binding, and prothrombin activation. Similar information on the other vitamin K-dependent proteins is not available. Our data using antibody H-11 binding indicates that the circulating des-γ-carboxy forms of prothrombin and protein C in stably anticoagulated individuals do not show a graded response but form a two-group distribution with fully carboxylated proteins (Fig 5). Both the data reported here and the previous reported observations of Malhotra et al support the simple conclusion that loss of a single or a few Gla residues following administration of warfarin results in the loss of the calcium ion-dependent conformational transition required for membrane binding and detectable by antibody H-11 binding.

Several reports have described the immunologic measurement of normal and des-γ-carboxy prothrombin and protein C using both monoclonal and polyclonal antibodies directed against the calcium-stabilized structure of human prothrombin. Biochemistry 22:948, 1983

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Discrimination of normal and abnormal prothrombin and protein C in plasma using a calcium ion-inhibited monoclonal antibody to a common epitope on several vitamin K-dependent proteins

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