Identification and Purification of Vitamin K-dependent Proteins and Peptides with Monoclonal Antibodies Specific for γ-Carboxyglutamyl (Gla) Residues*

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Novel monoclonal antibodies that specifically recognize γ-carboxyglutamyl (Gla) residues in proteins and peptides have been produced. As demonstrated by Western blot and time-resolved immunofluorescence assays the antibodies are pan-specific for most or all of the Gla-containing proteins tested (factors VII, IX, and X, prothrombin, protein C, protein S, growth arrest-specific protein 6, bone Gla protein, conantokin G from a cone snail, and factor Xa-like proteins from snake venom). Only the Gla-containing light chain of the two-chain proteins was bound. Decarboxylation destroyed the epitope(s) on prothrombin fragment 1, and Ca2+ strongly inhibited binding to prothrombin. In Western blot, immunofluorescence, and surface plasmon resonance assays the antibodies bound peptides conjugated to bovine serum albumin that contained either a single Gla or a tandem pair of Gla residues. Binding was maintained when the sequence surrounding the Gla residue(s) was altered. Replacement of Gla with glutamic acid resulted in a complete loss of the epitope. The utility of the antibodies was demonstrated in immunchemical methods for detecting Gla-containing proteins and in the immunopurification of a factor Xa-like protein from tiger snake venom. The amino acid sequences of the Gla domain and portions of the heavy chain of the snake protein were determined.

γ-Carboxyglutamic acid (Gla) is derived from glutamate in a vitamin K-dependent enzymatic reaction and is unique among amino acids in possessing two side-chain carboxyl groups. Since its discovery (1), ten human proteins that contain Gla have been isolated and extensively characterized. These comprise four procoagulant (factors VII, IX, and X and prothrombin) and two anticoagulant (protein C and protein S) proteins, which are synthesized primarily in the liver, two proteins of bone and extracellular matrix (bone Gla protein or osteocalcin and matrix Gla protein), and two proteins with functions that have not been fully defined (growth arrest-specific protein 6 (Gas6) and protein Z). Gla-containing proteins are synthesized in many tissues and, in addition to blood and bone, they occur in dentin, renal stones, atherosclerotic plaques, semen, lung surfactant, and urine (2). However, in many instances the proteins have not been isolated and chemically characterized. In all known cases, the Gla residues function as ligands for Ca2+ and are crucial for the biological activity of the proteins. Gla-containing proteins have been purified from a wide range of vertebrates and also from molluscs of the genus Conus. In the latter case, Gla is utilized in the synthesis of potent neurotoxic peptides that bear little structural resemblance to vertebrate Gla-containing proteins (3).

The biosynthesis of Gla is a post-translational modification catalyzed by γ-glutamyl carboxylase, a resident enzyme of the endoplasmic reticulum (4, 5). The nascent precursor polypeptide comprises a single chain with an N-terminal signal peptide that is cleaved off prior to the synthesis of Gla. An adjacent propeptide of 18–28 amino acids mediates binding of the substrate to the carboxylase and directly activates the enzyme, which then γ-carboxylates any glutamyl (Glu) residues located nearby in the primary structure of the substrate (6–8). In the vitamin K-dependent hemostatic proteins and their homologues (Gas6 and protein Z), 9–13 Glu residues C-terminal to the propeptide are converted to Gla and all are located within a stretch of ~45 amino acids known as the Gla domain. The conversion of these Glu residues to Gla is coupled to a vitamin K redox cycle in vivo (9). In a reaction that requires vitamin K dihydroquinone, CO2, and O2, the carboxylase abstracts a proton from the γ-carbon of a Glu residue, and CO2 is subsequently incorporated at the same position. The mechanism for proton abstraction has not been resolved, but it is assumed that the carboxylase converts vitamin K to a peroxide intermediate that reacts further to form a dialkoxide capable of abstracting the proton (10). Following collapse to a 2,3-epoxide the vitamin is recycled to the dihydroquinone form in two enzyme-catalyzed steps. Coumarin-based vitamin K antagonists such as warfarin inhibit the enzyme catalyzing the first reductive step (vitamin-K-epoxide reductase).

The propeptide is proteolytically removed from the γ-carboxylated proteins prior to their secretion, exposing the Gla do-

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main at the N terminus of the mature molecule (matrix Gla protein is an exception in that the propeptide-like region contains a Gla residue and remains attached to the secreted protein). This allows the Gla domain to fold into a stabilized active form that is required for interaction with cell membranes and other proteins (11). The active conformation is induced by the cooperative binding of Ca$^{2+}$ to Gla residues (12, 13), and the structure has been observed by x-ray crystallography (14). Whereas individual dicarboxylic acids are weak chelators of Ca$^{2+}$ ($K_{d}$ ~30 mM for malonic acid), concerted binding allows the Gla domain to bind Ca$^{2+}$ with an average $K_{d}$ of 0.3–0.7 mM (11). Thus, the Ca$^{2+}$-binding sites are essentially saturated at the concentration of free Ca$^{2+}$ in extracellular fluids, i.e., ~1.2 mM. Although neither osteocalcin nor matrix Gla protein possesses a Gla domain as such, the high affinity for hydroxyapatite crystals conferred by their Gla residues appears to be important for regulating mineralization in the skeletal extracellular matrix (15).

A wide variety of methods have been developed for the qualitative or quantitative identification of Gla, including specific stains, modified protein sequence analysis, isotopic labeling, mass spectrometry, and analysis of alkaline hydrolysates using capillary electrophoresis or anion-exchange, reversed-phase, gas, or thin layer methodologies (16–22). However, most of these methods are limited by a requirement for purified proteins or highly enriched preparations. Recent developments, including the demonstration of vitamin K-dependent synthesis of Gla by marine snails (23) and the isolation of human cDNAs including the demonstration of vitamin K-dependent synthesis (24), indicate that vitamin K has a broader role in biology than previously thought. Progress in this field would benefit from access to simple methods for the identification and purification of vitamin K-dependent proteins. Here we describe a novel approach that allows the sensitive identification of Gla-containing proteins in complex biological samples by utilizing monoclonal antibodies that specifically recognize γ-carboxyglutamyl residues. We also demonstrate the usefulness of such antibodies for the immunopurification of Gla-containing proteins.

### EXPERIMENTAL PROCEDURES

**Chemicals and Reagents—**All solutions were prepared using reagents of the highest quality and highly purified water. Fmoc-protected L-amino acids were purchased from PerSeptive Biosystems (Framingham, MA). Freund’s complete and incomplete adjuvants were from BioRad Laboratories (Dartmouth, MA). NBT and BCIP were purchased from Sigma.

**Proteins—**Human FVII, FIX, FXa, FX, PC, and PS and bovine BGP were purchased from Hematologic Technologies, Inc. (Essex Junction, VT). Synthetic conantokin G was from Bachem (Heidelberg, Germany). Chymotrypsin was from Roche Molecular Biochemicals. Synthetic OpfpL-amino acids at the appropriate fold were synthesized with a Milligen 9505 Plus peptide synthesizer (Perkin-Elmer Corp.) using standard Fmoc chemistry and purified by reversed-phase high pressure liquid chromatography before use. An eight-branched immunogenic complex was synthesized using the multiple antigen peptide (MAP) system (29) by coupling an octodecapeptide sequence (JS30; see Table I) to an octaventoyl polymer glycol-polylysine MAP support (PerSeptive Biosystems). The amino acids at all positions except those containing the Gla and terminal residues were varied by using an equimolar mixture of 5–7 Fmoc-OpfpL-amino acids at the appropriate cycle. The JS34 and JS45 peptides used for testing the monoclonal antibodies (Table I) were conjugated to BSA using m-maleimidobenzoyl-N-hydroxysulfosuccinimide (MBS) ester (Calbiochem) according to the manufacturer’s instructions. Briefly, BSA was derivatized with MBS followed by coupling to the JS34 and JS45 peptides, each equimolar mixture of 5–7 Fmoc-OpfpL-amino acids at the appropriate cycle, and the unconjugated ester was removed by chromatography on a PD-10 gel filtration column (Amersham Pharmacia Biotech). The peptides were coupled to the derivatized BSA via their C-terminal cysteine residue (using a maleimide reaction scheme), dialyzed exhaustively against TBS, and stored frozen at −20 °C.

**Production of Monoclonal Antibodies—**Each BALB/c mouse was immunized intracutaneously with 10 μg of the JS30-MAP complex emulsified in Freund’s complete adjuvant. Boosters comprising 10 μg of the complex emulsified in Freund’s incomplete adjuvant were given subsequently at 1 and 3 weeks, and plasma from the immunized mice was tested by ELISA for the presence of cross-reactive antibodies (see below). Two additional boosters were given at 7 and 10 weeks, each with 50 μg of the peptide complex in Freund’s incomplete adjuvant. 4, 3, 2, and 1 day prior to harvesting splenocytes, each mouse was injected intraperitoneally with 200 μg of the JS30-MAP complex dissolved in 50 mM Tris-HCl, pH 7.4. Splenocytes were extracted and fused with SP2/AG14 myeloma cells using 45% (w/v) polyethylene glycol 1540 and 7% (v/v) dimethyl sulfoxide under standard conditions (30). Fused cells in Dulbecco’s modified Eagle’s medium supplemented with hypoxanthine, aminopterin, and thymidine were seeded into 96-well microtiter plates at a density of ~10$^5$ cells per well together with ~10$^4$ feeder cells per well. After about 10 days the hybridoma supernatants were screened by ELISA, and cells producing antibodies with the desired properties (i.e., specific for Gla-containing peptides/proteins) were cloned twice by the limiting dilution method in 96-well plates (0.5–1.0 cell per well) using mouse peritoneal macrophages as feeder cells. For antibody production stable clones were grown to a high cell density, and 5 × 10$^5$ cells (0.2 ml) were injected intraperitoneally into mice that had been primed 1–4 days beforehand with 0.2 ml of pristane (2, 6, 10, 14-tetramethylpentadecane). The ascites fluid was tested by ELISA. Antibodies were also produced on a preparative scale by culturing the hybridoma cells in a Technomouse apparatus (Integra Biosciences, Wallisellen, Switzerland). Hybridoma cell lines were maintained in 95% fetal bovine serum/5% dimethyl sulfoxide in liquid N2. The monoclonal antibodies were purified from ascites fluid or conditioned hybridoma cell culture medium by affinity chromatography on a 5-ml HitTrap Protein G column (Amersham Pharmacia Biotech) according to the manufacturer’s instructions. Binding was performed in 0.1 M sodium acetate buffer/150 mM NaCl, pH 5.0, with elution employing 0.1 M glycine-2HCl buffer, pH 2.7. After neutralization with 1 M Tris-HCl buffer, pH 9.0, the antibodies were dialyzed into 20 mM Tris-HCl, pH 7.4, containing either 154 mM or 0.5 mM NaCl and concentrated. Concentrations were determined using an Easy-Titer™ mouse IgG assay kit (Pierce), and the antibodies were stored at −20 °C. The purified antibodies appeared to be homogeneous by SDS-PAGE.

**ELISA—**Plasma and ascites from immunized mice and hybridoma supernatants were screened by ELISA with six peptide-BSA conjugates (Table I), bovine PT, and a proteolytic fragment of bovine FXI comprising the Gla and N-terminal EGF-like domains (31). The proteins were coated in 96-well microtiter plates (0.5 μg per well in 0.1 M bicarbonate buffer, pH 9.6) overnight at 4 °C. After washing the wells three times with 10 mM sodium phosphate buffer/0.5 mM NaCl, pH 1.0 (v/v) Tween 20, pH 8.0 (PBS/T), they were blocked for 15 min with 10 mg/ml BSA in 50 mM Tris-HCl/0.1 mM NaCl, pH 7.4 (100 μl per well). The wells were washed three times with PBS/T, and 50 μl samples of plasma, ascites fluid, or hybridoma supernatants (serially diluted with 1 mg/ml BSA in 154 mM NaCl) were added, and the plates were incubated for 30 min. The wells were washed three times as above, horseradish peroxidase-conjugated rabbit anti-mouse IgG (DAKO A/S, Glostrup, Denmark) was added, and the plates were incubated for 30 min. The plates were then washed three times, developed with ABTS solution (Sigma), and the A$_{405}$ was measured in a microtiter plate reader (Molecular Devices Corp., Menlo Park, CA).

**Western Blot Analysis—**Protein samples were denatured, reduced, and resolved in SDS-12 or 15% (v/v) polyacrylamide gels (32). After electrophoresis the gels were either stained with Coo massie Blue R-250
or electrobotted (33) to ProBlott polyvinylidene difluoride membrane (Applied Biosystems, Inc., Stockholm, Sweden). The membrane was blocked with 10% (v/v) skim milk powder in TBS for 2 h and then incubated for 1 h with 5 μg/ml purified monoclonal antibody in TBS containing 0.2% (v/v) Tween 20 (i.e., TBS/T). After washing with TBS/T, the membrane was incubated for 30 min with the monoclonal antibody and a conjugated rabbit anti-mouse IgG (DAKO A/S, Glostrup, Denmark), washed with TBS/T, and developed with BCIP and NBT.

**Time-resolved Immunofluorescence Assays—**Human PT (hPT) was labeled with the Eu$^{3+}$ chelate of N$_2$N-$\text{N'}$-diisothiocyanatobenzyl)-diethylentriamine-N$_2$N$_2$N$_2$-tetraacetic acid using a DELFIA Eu-labeling kit (Wallac Oy, Turku, Finland). The labeled hPT (1–1.5 μg/ml) was added to the plates (100 μl/well) and was purified according to Wallac’s instructions and stored in stabilizer solution (Wallac) at ~70 °C. Wash steps were performed using a DELFIA research model 1296–024 plate washer (Wallac), and fluorescence measurements were made with a DELFIA research model 1 2 3 4 fluorometer (Wallac) with excitation at 320 nm, emission at 615 nm, and a pulse rate of 1000/second. Fluorescence was measured for 400 ms between flashes after a delay of 400 ms. All steps were performed at room temperature unless stated otherwise. For competitive assays, the purified monoclonal antibodies or purified polyclonal mouse IgG (IgG) were coated on MaxiSorp FluoroNunc 96-well microtiter plates (Nunc A/S, Roskilde, Denmark) at a concentration of 20 μg/ml in TBS (50 μl per well) overnight at 4 °C. The coating solution was removed, and plates were blocked with Assay buffer (50 mM Tris-HCl/154 mM NaCl/20 mM diethylentriaminepentaacetic acid/0.01% (v/v) Tween 40/0.5% (v/v) BSA/0.05% (v/v) Na$_2$EDTA, pH 7.8) for 2 h. The plates were washed twice with Wash buffer (5 mM Tris-HCl/154 mM NaCl/0.005% (v/v) Tween 20/0.1% (v/v) Germal II, pH 7.8), and 1.5 nM Eu$^{3+}$-labeled hPT that had been premixed with the competitor samples in Assay buffer was added to the plates (100 μl per well). After incubation for 1 h the wells were washed four times, and Enhancement solution (Wallac) was added (200 μl per well). The plates were vortexed gently for 5 min, and the fluorescence intensity of the samples (up to ~450,000 counts per second) was measured. The background fluorescence in control wells coated with polyclonal mouse IgG was always less than 2000 counts per second and was subtracted before plotting the data. All assays were performed at least in duplicate. Non-competitive assays to assess the effect of Ca$^{2+}$ on the binding of Eu$^{3+}$-labeled hPT were performed as above except that competitor proteins were omitted, and Assay buffer supplemented with either 5 mM CaCl$_2$ or 15 mM NaCl (to maintain an equivalent ionic strength) was used for the label-binding step. An additional control comprised wells coated with a murine monoclonal antibody against human PC inhibitor (hPCI) (unpublished data) and employed Eu$^{3+}$-labeled hPCI.

**Surface Plasmon Resonance Spectroscopy—**Purified hPT, BSA, and BSA-conjugated peptides (Table I) were immobilized on CM5 sensor chips (Biacore AB, Uppsala, Sweden) using an amine coupling kit (Biacore AB) according to the manufacturer’s instructions. Amounts representing 1600–3600 response units were immobilized. A BIACORE 2000 biosensor (Biacore AB) was used to study the association and dissociation kinetics of the interaction of the purified monoclonal antibodies with the immobilized proteins. Measurements were made using a flow rate of 30 μl/min at 25 °C, and the flow buffer was TBS. Antibodies were diluted in TBS, and 120 μl injections were employed to monitor the association phase followed by a return to flow buffer to monitor dissociation. The chip was regenerated with either 2 15-μl pulses of TBS containing 50 mM CaCl$_2$ (in the case of interactions with hPT) or with 3 30-μl pulses of TBS containing 100 mM CaCl$_2$ (in the case of interactions with the BSA peptide conjugates). Regeneration was followed by a 15-μl pulse of TBS containing 2 mM EDTA. The bulk sample effect was subtracted online by using a flow cell containing immobilized BSA as a reference channel for interactions with hPT and immobilized JS44(E)-BSA (Table I) as the reference for interactions with the Gla-containing BSA peptide conjugates. Data were evaluated with BIAevaluation 3.0 software (Biacore AB).

**Immunooaffinity Chromatography—**All procedures were performed at room temperature unless stated otherwise. Purified monoclonal antibody M3B (100 mg) was coupled to 9 ml (drained bed volume) of UltraLink™ immobilized hydrazide resin (Pierce) according to the manufacturer’s instructions. The coupling efficiency (determined spectrophotometrically) was estimated to be 95%. The resin was stored in 20 ml Tris-HCl/0.5 mM NaCl, pH 7.4, containing 0.05% (w/v) NaN$_3$ at 4 °C. An aliquot of the M3B-coupled resin was used to prepare an immunoaffinity column with a settled bed volume of 3.5 ml, and the column was equilibrated in Binding buffer (19 ml Tris-HCl/146 mM NaCl/10 mM EDTA, pH 7.4). Lypophilized tiger snake venom (10 mg) was dissolved in 2.5 ml of Binding buffer and chromatographed on a PD-10 gel filtration column (Amersham Pharmacia Biotech) that had been pre-equilibrated with Binding buffer. The sample was loaded onto the immunoaffinity column at a flow rate of 0.5 ml/min, and the A$_{280}$ of the effluent was monitored. After the A$_{280}$ decreased to near the baseline the column was washed with 19 ml Tris-HCl/0.1 M NaCl/10 mM EDTA, pH 7.4, at 2 ml/min, and then 19 ml Tris-HCl/146 mM NaCl/50 mM CaCl$_2$, pH 7.4, was applied at 2 ml/min. The Ca$^{2+}$-eluted fraction wasdialed against TBS, concentrated, and stored at ~20 °C.

**Purification of the Gla Domain of Tiger Snake Fxa-LP—**The immunoaffinity-purified tiger snake Fxa-LP (~450 μg) was digested with 1 μg of chymotrypsin for 75 min at 37 °C in TBS containing 5 mM EDTA in 10 mM vautized pH 7. The digestion mixture was resolved by size-exclusion chromatography in TBS using a 2.4-ml Superdex 75 PC 3.2/30 column and a SMART fast protein liquid chromatography system (both from Amersham Pharmacia Biotech). A flow rate of 20 μl/min was employed, and 50-μl fractions were collected while monitoring A$_{280}$ and A$_{214}$. The Gla domain fragment, which was eluted in two consecutive fractions after 65 min (as determined by SDS-PAGE), was further purified by reversed-phase chromatography in a 0.35-ml Sephasil C8 SC 2.1/10 column (Amersham Pharmacia Biotech) using the SMART fast protein liquid chromatography system. The column was pre-equilibrated with Buffer A (aqueous 0.1% (v/v) trifluoroacetic acid), and after loading the sample, proteins were eluted with a linear gradient of 0–100% Buffer B (0.1% (v/v) trifluoroacetic acid in acetonitrile) over 90 min at a flow rate of 100 μl/min. The Gla domain peptide, which was eluted around 30% Buffer B, was collected with a large increase in A$_{214}$, was lyophilized and stored at ~20 °C.

**Amino Acid Sequence Analysis—**Immunooaffinity-purified tiger snake Fxa-LP was reduced with dithiothreitol and alkylated with 4-vinylpyridine (34). The sample was resolved by SDS-PAGE, electroblotted to ProBlott polyvinylidene difluoride membrane (Applied Biosystems, Inc.) (33), and the polypeptides were stained with Coomassie Blue R-250. Bands were excised and sequenced by employing Edman degragation chemistry and an ABI Procise 494 sequencer (Perkin-Elmer Corp.). The purified Gla domain peptide was sequenced both without prior chemical modification and after treatment with methanolic HCl to methyl esterify Gla residues, thus allowing their identification (19).

**RESULTS**

**Production of Gla-specific Antibodies—**Preliminary tests in which the JS30-MAP complex (Table I) was used to immunize rabbits indicated that apparently Gla-specific polyclonal antibodies could be fractionated from the immune serum by peptide affinity chromatography (data not shown). We therefore decided to generate monoclonal antibodies against the same complex. A good immune response against the complex was elicited in immunized mice after 4–5 months. Clonal hybridoma cell plex. A good immune response against the complex was elicited in immunized mice after 4–5 months. Clonal hybridoma cell proliferation was monitored. After the 2 immunoaffinity column was eluted in 2 ml of TBS-T.

**TABLE I Synthetic peptides**

| Peptide | Amino acid sequence |
|---------|---------------------|
| JS30    | NH$_2$-KADYDAFAYAAALYAYDA-COOH |
| JS44(γ) | NH$_2$-LTGKYLTDG-COOH |
| JS44(ΔE) | NH$_2$-LTGKELTDG-COOH |
| JS44(γγ) | NH$_2$-LTGKYLLTDG-COOH |
| JS45(γ) | NH$_2$-ASRVYTFAGC-COOH |
| JS45(ΔE) | NH$_2$-ASRVETAFGC-COOH |
| JS45(γγ) | NH$_2$-ASRVYTFAGGC-COOH |

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the corresponding positions (Table I). In this way, 16 hybridoma cell lines were chosen for further analysis, and the antibodies were purified and characterized according to their IgG subclass, mobility in agarose and SDS-polyacrylamide gels, cross-reactivities on Western blots and in competitive and cross-over immunofluorescence assays, and relative rates of binding and dissociation as assessed by surface plasmon resonance spectroscopy. All of the antibodies were of the subclass IgG1, except M3B (IgG2b, k) and M42 (IgG3, k). However, clear differences among the IgG1 antibodies were observed in their electrophoretic mobilities, cross-reactivities, and binding/dissociation characteristics with a range of antigens, and this allowed a total of seven groups of antibodies with different characteristics to be distinguished. Data are presented for one antibody from each group (Table II).

**Western Blot Analysis of Antibody Specificities**—In Western blot analyses, three of the antibodies (M3B, M27, and M55) proved to be pan-specific for all of the human vitamin K-dependent proteins that were tested (i.e. FVII, FIX, FX, PC, PS, PT, and Gas6) (Table II). In addition, all seven antibodies cross-reacted with three elapid snake FXa-like proteins that are presumed to contain a Gla domain homologous to that of the mammalian hemostatic proteins. Only the Gla-containing light chain of the two-chain proteins (FVIIa, FIXa, FX, and PC) was recognized (Fig. 1). However, four antibodies failed to give a detectable response against PC under Western blot conditions, and only M3B produced a detectable cross-reaction with bovine BGP. γ-Carboxylated PT F1 was recognized by all of the antibodies, whereas the decarboxylated form was not. Five of the antibodies bound the JS44 and JS45 (BSA-conjugated) peptides containing single or paired Gla residues, but peptides in which Gla was replaced with Glu were not recognized.

**Specificities toward Solution-phase Antigens**—Competitive immunofluorescence assays employing Eu³⁺-labeled hPT were
used to assess the reactivities of the immobilized antibodies toward solution-phase proteins (Fig. 2 and Table II). In most cases, the reactivities matched those observed with Western blots. However, all seven antibodies recognized PC under these conditions, and antibody M27 cross-reacted weakly with two of the Gla-containing synthetic peptides, whereas no reaction was evident on Western blots. Five antibodies also recognized co-nantokin G, a 17-amino acid cone snail neurotoxin containing five Gla residues and no disulfide bonds (3). Antibody M3B recognized all of the Gla-containing peptides and proteins tested in both Western blot and immunofluorescence assays. Binding of Eu$^{3+}$-labeled hPT was strongly inhibited by normal plasma (PT assay giving 104% versus normal) at a dilution of 1:100 and less so by plasma from patients on long-term warfarin treatment (8% versus normal) or with liver dysfunction (<5% versus normal) (Fig. 2).

**Effect of Ca$^{2+}$ on Binding to Prothrombin**—Ca$^{2+}$ strongly inhibited binding of the monoclonal antibodies to hPT in non-competitive immunofluorescence assays (Fig. 3). Inclusion of 5 mM CaCl$_2$ during the binding step almost completely abolished binding by all seven antibodies. This was not because of a direct effect of Ca$^{2+}$ on the assay per se, because the binding of hPCI to an anti-hPCI monoclonal antibody was actually enhanced in the presence of Ca$^{2+}$. Neither was the effect due to an increase in ionic strength; this was maintained with NaCl in the control samples.

**Surface Plasmon Resonance Analysis of Antibody-Antigen Interactions**—The interaction of the purified monoclonal antibodies with hPT and BSA-conjugated Gla-containing peptides was followed in real time with a BIACORE biosensor. Qualitative differences were apparent among the antibodies in both the magnitude of the interaction with hPT and the relative rates of association and dissociation (Fig. 4A). However, equilibrium constants could not be determined as binding did not reach the steady state during injection, and attempts to determine rate constants from the association and dissociation phase data were hampered by poor curve fits. Analysis of the binding behavior may have been complicated by the presence of multiple binding sites in the Gla domain of hPT, each with a potentially different affinity for the antibody. Consistent with this, differences were observed in the interaction of the antibodies with three different Gla-containing peptides (Fig. 4B, Table II). Binding to all three peptides exhibited a fast apparent association rate and a slow dissociation rate (indicative of a high affinity interaction), particularly with the JS44(γγ) pep-
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The injection of 120 μl of purified antibody (333 nM in TBS buffer) followed by a return to flow buffer (TBS) to monitor dissociation. A flow rate of 30 μl/min was used. A, interaction of seven of the antibodies with human prothrombin (RU, response units). B, interaction of antibody M5 with BSA-conjugated peptides containing either Gla (γ) or glutamic acid (see Table I). The curves have been normalized to the maximum observed response (100% represents 2900 response units).

Imunoaffinity Purification of Tiger Snake FXa-like Protein—To assess the efficacy of the antibodies for purifying Gla-containing proteins, an M3B-coupled immunoaffinity resin was made and tested for its ability to specifically isolate the FXa-like protein from venom of the tiger snake, Tropidechis carinatus. The FXa-like protein was a 54 kDa two-chain FXa-like protein that contains 8 Gla residues based on its amino acid composition (35). Immunoaffinity chromatography was performed employing 50 mM CaCl₂ as the eluting agent (lower concentrations were not tested) (Fig. 5A). Examination of the flowthrough fraction (protein that did not bind to the resin during loading) and Ca²⁺-eluted fraction by SDS-PAGE and Western blot analysis revealed that a protein comprising two disulfide-linked subunits had been isolated (Fig. 5, B and C). The light chain, but not the heavy chain, cross-reacted with M3B on Western blots. No cross-reaction was observed with the flowthrough fraction, indicating that most or all of the FXa-like protein had been bound during a single passage through the column. CaCl₂ appeared to efficiently dissociate the bound protein from the column as little or no additional cross-reactive material was released if the column was washed with 0.1 M glycine-HCl, pH 2.7, after the CaCl₂ elution step (not shown). Amino acid sequence analysis of the immunopurified FXa-LP confirmed that the cross-reactive light chain polypeptide contained Gla. The Gla domain was isolated by cleavage with chymotrypsin and sequential size-exclusion and reversed-phase chromatography steps, and its sequence was determined (Fig. 6). Although the amino acid sequence of this protein has not been previously reported, the sequence of the Gla domain is identical to that recently reported for the prothrombin activator in venom of another elapid snake, Tropidechis carinatus (36), and the Gla domain is clearly conserved with respect to human FX (Fig. 6). The sequence of the N terminus of the heavy chain (37 residues) also matched that for T. carinatus, consistent with a close evolutionary relationship between the two snakes (Fig. 6). Although the heavy chain was resolved into two or three closely migrating bands by SDS-PAGE under reducing conditions (Fig. 5B) a single N-terminal sequence was obtained. The microheterogeneity may reflect the presence of FXαα, FXαβ, and other subforms, as observed in preparations of mammalian FXα, that arise from autocatalytic cleavages (37, 38). A minor band (~19 kDa) evident in reduced samples of the immunoaffinity-purified material was also sequenced. The band yielded two sequences; one (17 residues) corresponded exactly to the Gla domain sequence, and the other (20 residues)
carboxylic acid molecules such as Gla bind Ca2+ above the venom of the rough-scaled snake (T. carinatus) (36) and human FXa (56). Amino acids are given in single-letter code, and Gla residues are denoted N. scutatus scutatus protein purified from venom of the tiger snake (T. carinatus) are aligned with those of the FXa-like protein (prothrombin activator) from band observed in non-reduced samples (Fig. 5 B carinatus matched amino acids 271–290 of the heavy chain of theitory in competitive immunofluorescence assays than normal going treatment with warfarin, which inhibits the pool of peptide antigens would be unlikely to bear much, if any, resemblance to the Gla domains of known vitamin K-dependent proteins, increasing the chance of eliciting a response by Gla-specific antibodies. Furthermore, unlike the Gla domain in intact proteins or their fragments, the peptides would not be predicted to possess a defined secondary structure or to undergo any marked conformational change when exposed to plasma Ca2+, making it unlikely that antibodies specific for a Ca2+-dependent structure would be generated (individual dicarboxylic acid molecules such as Gla bind Ca2+ with a Kd well above the ~1.2 mM concentration of free plasma Ca2+). In this way, antibodies were obtained that were shown to be pan-specific for all or most of the vitamin K-dependent proteins tested.

**DISCUSSION**

The protocol used here to generate Gla-specific antibodies employed an ~17-kDa branched immunogen comprising eight degenerate octodecapeptides attached to a lysine core matrix. The peptides each contained two Gla residues and a tandem pair at fixed positions with variable amino acids encompassing them, achieved by using an equimolar mixture of 5–7 amino acids at appropriate cycles during peptide synthesis. Thus the theoretical number of different peptide sequences in the immunogenic pool exceeded 8 × 10^8. It was reasoned that the pool of peptide antigens would be unlikely to bear much, if any, resemblance to the Gla domains of known vitamin K-dependent proteins, increasing the chance of eliciting a response by Gla-specific antibodies. Furthermore, unlike the Gla domain in intact proteins or their fragments, the peptides would not be predicted to possess a defined secondary structure or to undergo any marked conformational change when exposed to plasma Ca2+, making it unlikely that antibodies specific for a Ca2+-dependent structure would be generated (individual dicarboxylic acid molecules such as Gla bind Ca2+ with a Kd well above the ~1.2 mM concentration of free plasma Ca2+). In this way, antibodies were obtained that were shown to be pan-specific for all or most of the vitamin K-dependent proteins tested.

γ-Carboxylated PT F1 was recognized by all of the antibodies, localizing the epitope(s) to residues 1–156 of PT. Similarly, only the light chain subunit of the two-chain proteins (FVIIa, FIXa, FX, PC, and tiger snake FXa-LP), which contains the Gla domain, was recognized on Western blots. We have also observed that removal of the Gla domain (residues 1–41) from PT by digestion with chymotrypsin results in a complete loss of binding (data not shown). Decarboxylation, but not denaturation and reduction, caused a loss of the epitope(s) on PT F1, strongly implicating that Gla residues contributed to the antigenic site. Consistent with this, plasma from a patient undergoing treatment with warfarin, which inhibits γ-carboxylation, and from a patient with liver disease was markedly less inhibitory in competitive immunofluorescence assays than normal plasma. However, the nature of the antibody epitope was most clearly demonstrated by use of BSA-conjugated synthetic peptides. All seven antibodies bound short peptides (see JS44 and JS45 peptides, Table I) that contained only a single Gla residue or a tandem pair, although binding by antibodies M27 and M55 was relatively weak. Replacement of the dicarboxylic Gla residue(s) with monocarboxylic Glu resulted in a complete loss of the epitope. Thus, the antibodies were able to discern between peptides that differed only in the presence or absence of a carbonyl group on the γ-carbon of glutamic acid. More convincingly, binding was maintained when the sequence surrounding the Gla residue(s) was completely altered, clearly demonstrating the Gla-specific nature of the antibodies.

However, most of the antibodies exhibited limitations in their ability to bind certain Gla-containing proteins or peptides. For example, four of the antibodies failed to produce a detectable response with reduced PC under Western blot conditions (although binding was observed in immunofluorescence assays), and antibody M55 only weakly bound to one Gla-containing synthetic peptide, although it was pan-specific with regard to the vitamin K-dependent proteins. Thus it appears that binding by at least some of the antibodies is subject to constraints imposed by the primary sequence surrounding the Gla residues, steric hindrances due to local structural elements, or local electrostatic forces incompatible with the antibody-binding site. This was indicated especially by surface plasmon resonance analyses, where marked differences in binding characteristics were observed for each of three different Gla-containing peptides, particularly during the dissociation phase. In most instances a higher affinity was apparent for tandem Gla residues than a single Gla residue. It should also be noted that we have observed that the free (tricarboxylic) form of Gla has no inhibitory effect on the binding of PT in competitive immunofluorescence assays, even when present at more than a 600-fold molar excess. This may not be surprising when it is considered that during immunization Gla was presented to the mice in a dicarboxylic peptidyl form. Likewise, efficient binding of free phosphoamino acids to anti-phosphoamino acid antibodies requires acylation of the amino acid to provide a structure more similar to that of the form used as the immunogen; binding is up to three orders of magnitude higher with the acylated derivatives (39).

The Gla domain region is highly immunogenic (40), and previous studies employing site-directed mutagenesis have

| Light chain (Gla domain) |
|-------------------------|
| N. s. scutatus           |
| T. carinatus            |
| Human FXa               |
| +01                     |
| +10                     |
| +20                     |
| +30                     |
| +40                     |

| Heavy chain |
|-------------|
| N. s. scutatus |
| T. carinatus |
| Human FXa |
| +185       |
| +205       |
| +215       |
| +225       |

| Gla domain |
|------------|
| N. s. scutatus  |
| T. carinatus  |
| Human FXa  |
| +271        |
| +291        |

**Fig. 6. Amino acid sequences of snake FXa-like proteins and human FXa.** N-terminal amino acid sequences derived from the FXa-like protein purified from venom of the tiger snake (N. scutatus scutatus) are aligned with those of the FXa-like protein (prothrombin activator) from the rough-scaled snake (T. carinatus) (36) and human FXa (56). Amino acids are given in single-letter code, and Gla residues are denoted by shaded boxes. Residues that are identical to the tiger snake protein are boxed and shaded. Amino acids are numbered according to the sequence of mature human FX.
provided strong evidence that Gla residues can constitute part of an epitope (41–44). Nevertheless, the defined specificity of the antibodies reported here is more akin to those recognizing the O-phosphorylated forms of tyrosine, serine, threonine, and histidine (39, 45). The antibodies described here also appear to be unique in the breadth of their cross-reactivities, which in some cases includes BGP and conantokin G, both of which have structures very different from the Gla domain of hemostatic proteins. Monoclonal antibodies that are pan-specific for several of the hemostatic vitamin K-dependent proteins have been reported previously (46–48), but like the majority of antibodies raised against vitamin K-dependent proteins, they are specific for a metal ion-induced conformation (see Ref. 49 for a review). In contrast, Ca\(^{2+}\)-strongly inhibited binding of PT to the Gla-specific antibodies reported here. This effect is consistent with structural studies demonstrating that in the presence of Ca\(^{2+}\), PT adopts a conformation in which the Gla residues are folded for a metal ion-induced conformation (see Ref. 49 for a review).

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