Precursors of Novel Gla-Containing Conotoxins Contain a Carboxy-Terminal Recognition Site That Directs γ-Carboxylation[†,‡]

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ABSTRACT: Vitamin K-dependent γ-glutamyl carboxylase catalyzes the conversion of glutamyl residues to γ-carboxyglutamate. Its substrates include vertebrate proteins involved in blood coagulation, bone mineralization, and signal transduction and invertebrate ion channel blockers known as conotoxins. Substrate recognition involves a recognition element, the γ-carboxylation recognition site, typically located within a cleavable propeptide preceding the targeted glutamyl residues. We have purified two novel γ-carboxyglutamate-containing conotoxins, Gla-TxX and Gla-TxXI, from the venom of *Conus textile*. Their cDNA-deduced precursors have a signal peptide but no apparent propeptide. Instead, they contain a C-terminal extension that directs γ-carboxylation but is not found on the mature conotoxin. A synthetic 13-residue "postpeptide" from the Gla-TxXI precursor reduced the K_m for the reaction of the *Conus* γ-carboxylase with peptide substrates, including FLEEL and conantokin-G, by up to 440-fold, regardless of whether it was positioned at the N- or C-terminal end of the mature toxin. Comparison of the postpeptides to propeptides from other conotoxins suggested some common elements, and amino acid substitutions of these residues perturbed γ-carboxylation of the Gla-TxXI peptide. The demonstration of a functional and transferable C-terminal postpeptide in these conotoxins indicates the presence of the γ-carboxylation recognition site within the postpeptide and defines a novel precursor structure for vitamin K-dependent polypeptides. It also provides the first formal evidence to prove that γ-carboxylation occurs as a post-translational rather than a cotranslational process.

The vitamin K-dependent γ-glutamyl carboxylase is an integral membrane protein of the endoplasmic reticulum that catalyzes the conversion of glutamyl residues in a polypeptide substrate to γ-carboxyglutamate (Gla).[1] In a reaction that requires O_2, CO_2, and reduced vitamin K, a proton on the γ-carbon of a Glu residue is replaced with a CO_2 molecule (*1*). The two carboxyl groups on the Gla side chain confer calcium-binding properties on the modified protein (*2*). Historically, the γ-carboxylase has been studied in the context of vertebrate hemostasis, as several proteins involved in blood coagulation or its regulation must be γ-carboxylated to be biologically active. Proteins with roles in bone mineralization, the extracellular matrix, and signal transduction are also substrates for the γ-carboxylase (*3*, *4*). Moreover, the γ-carboxylase plays an important role in the biology of at least one invertebrate. In predatory marine snails of the genus *Conus*, small neuroactive peptides (conotoxins) in the venom are used to immobilize prey by targeting voltage- and ligand-gated ion channels (*5*, *6*). Some of these conotoxins have been shown to contain Gla residues that are important for biological activity (*7*−*12*).

The γ-carboxylase cDNA or gene has been cloned from vertebrates and invertebrates, including *Conus* and *Drosophila* (*13*−*17*). Substrates for the enzyme have been identified in many species, though only in *Conus* among the invertebrates. In almost all known cases, the nascent vitamin K-dependent polypeptide has a clearly defined prepropeptide structure comprising an N-terminal signal peptide, an intervening propeptide that is proteolytically removed after γ-carboxylation is complete, and an adjacent region contain-

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[1] Abbreviations: Gla, γ-carboxyglutamate; γ-CRS, γ-carboxylation recognition site; CHAPS, 3-[(cholamidopropyl)dimethylammonio]-1-propanesulfate; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; PMSF, phenylmethylsulfonyl fluoride; PTH, phenylthiohydantoin; TFA, trifluoroacetic acid.

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ing the glutamyl residues that are slated for γ-carboxylation. In most of the vertebrate vitamin K-dependent proteins, 10−13 Glu residues are modified by the γ-carboxylase. These reside within an ~45-residue stretch, termed the Gla domain, that is located at the N-terminus of the mature polypeptide (*3, 18*). Osteocalcin and matrix Gla protein are exceptions in that they do not have a Gla domain, and only 3−5 Glu residues become γ-carboxylated.

A γ-carboxylation recognition site (γ-CRS) that defines a polypeptide as a substrate for the γ-carboxylase has been identified in the propeptides of the vitamin K-dependent proteins from vertebrates (*3, 19*−*22*). Matrix Gla protein is unusual in that the γ-CRS resides within the mature protein (*23*). With the exception of osteocalcin, which contains additional recognition elements (*24*), the propeptide is both necessary and sufficient to promote efficient γ-carboxylation of an adjacent Glu-containing polypeptide (*25*). The propeptide is bound directly by the enzyme, thereby tethering the substrate. It also potently stimulates enzyme activity, even if it is not covalently attached to the substrate (*20*). Though no strict consensus prevails, the mammalian γ-CRS is best defined by the motif *Z*-Phe-*Z*-*X*-*X*-*X*-*X*-Ala, where *Z* is an aliphatic hydrophobic residue (Ile, Val, Leu) and *X* is any amino acid (*3*). Mutations, either synthetic or naturally occurring, affecting the conserved residues of the vertebrate γ-CRS result in poor carboxylation of substrates. A γ-CRS also resides within the prepro-region in the precursors of γ-carboxylated conotoxins, though the exact nature of the *Conus* γ-CRS has remained elusive (*26, 27*).

In this report, we describe two novel conotoxins, Gla-TxX and Gla-TxXI, that were isolated from the venom of *Conus textile*. Comparison of the amino acid sequences of the purified peptides to those predicted by their cognate cDNAs revealed that the precursors of these peptides have a signal sequence that is followed immediately by the mature peptide sequence. Thus, they are remarkable in lacking an intervening propeptide. However, both precursors are predicted to include a short C-terminal extension resembling a propeptide that is missing from the mature conotoxin. We hypothesized that despite its unusual location at the C-terminus, this "post-peptide" might contain a γ-CRS and function much like a conventional N-terminal propeptide, thus, providing evidence from nature to support the idea that γ-carboxylation proceeds as a post-translational rather than co-translational process.

EXPERIMENTAL PROCEDURES

Purification of Gla-TxX and Gla-TxXI. Frozen *C. textile* snails were obtained from Vietnam and live snails from Fiji. The venom was extracted and fractionated on a Sephadex G50 Superfine column, and Gla-containing peaks were purified by reversed-phase HPLC on a HyChrom C18 column as described previously (*11*). The peptide Gla-TxX was further purified using a similar reversed-phase HPLC protocol on a Vydac C18 column (0.46 mm × 250 mm, 5 μm particle size).

Amino Acid Sequence and Composition Analyses. The purified Gla-TxX and Gla-TxXI peptides were dissolved in 2 mL of 1 M Tris-HCl, 10 mM EDTA, pH 8.6, and 6 M guanidine HCl. The peptides were reduced with 20 mM dithiothreitol at 37 °C for 1.5 h and S-alkylated with 4-vinylpyridine at 37 °C for 30 min. The samples were then dialyzed against 20% (v/v) acetic acid overnight. The reduced and alkylated peptides were separated by reversed-phase HPLC on a HyChrom column that had been equilibrated with 0.1% (v/v) trifluoroacetic acid (TFA). The peptides were eluted at a flow rate of 0.5 mL/min using a 0−80% gradient of CH_3CN over 1 h. The peptides were digested with L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin, dried, and separated by HPLC on the Vydac column. The amino acid composition was determined following acid hydrolysis on a Beckman 6300 amino acid analyzer. Gla content was determined after alkaline hydrolysis (*28*) and Cys after oxidation of cysteine to cysteic acid with performic acid (*29*). The peptides were sequenced by automated Edman degradation on a Perkin-Elmer ABI Procise 494 sequencer both before and after reduction and S-alkylation of Cys with 4-vinylpyridine. Gla was identified in the sequences following methyl-esterification with methanolic HCl (*30*). Phenylthiohydantoin (PTH)-Cys was not quantitated as its vinylpyridine derivative is unstable. A standard for methyl-esterified PTH-Gla was not available; its elution position in the HPLC chromatogram was calibrated by sequencing the isolated Gla domain of Factor X.

Mass Spectrometry. Mass spectra were acquired on a Voyager-Elite matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) mass spectrometer (PerSeptive Biosystems, Framingham, MA) equipped with delayed ion extraction technology and a N_2 laser. The spectra were acquired in positive-ion linear or reflector mode. On-target sample preparation was performed by either the thin-layer or dried-droplet method (*31*). Gla-TxX and Gla-TxXI were monitored before and after reduction, alkylation, and tryptic digestion. Gla-TxXI was also monitored after methyl-esterification of tryptic digests of the peptide. For thin-layer on-target sample preparation, a matrix solution was prepared by mixing nitrocellulose (15 mg/mL in acetone/2-propanol, 1:1, v/v) and 2,4,6-trihydroxyacetophenone (120 mg/mL in methanol) in a ratio of 1:4. Matrix solution (0.6 μL) was placed on the target and allowed to dry. Ammonium citrate (20 mM, pH 5, 0.3 μL) followed by sample solution (0.5 μL) was deposited on the matrix layer and dried. The samples were washed one to four times with 0.1% TFA (5−10 μL) by placing the droplet on the target and removing it with argon. For dried-droplet on-target sample preparation, the sample (0.5−0.6 μL) and matrix solution (0.2−0.3 μL of 2,5-dihyroxybenzoic acid/100 mM acetonitrile:0.3% TFA, 1:2, v/v) were mixed on the target and allowed to dry at room temperature.

cDNA Cloning. Degenerate oligonucleotide primers were designed based on the mature peptide sequences of Gla-TxX and Gla-TxXI. For Gla-TxX, the primers were 5′-ACICAY-ACITGYTGYCAY-3′ (forward) and 5′-YTGIGGYTTC-ATRCAYTG-3′ (reverse), where R = A or G, Y = C or T, and I denotes deoxyinosine. For Gla-TxXI, the primers were 5′-GGITCITGYTGYCAYAAR-3′ (forward) and 5′-GGYT-GRTTRCAIGTCCA-3′ (reverse). The primers were used in PCR reactions to amplify partial cDNAs from a λ phage library of *C. textile* venom duct cDNA. The PCR products (51 bp for Gla-TxX and 47 bp for Gla-TxXI) were purified and TA-cloned into the vector pCR2.1 (Invitrogen, Carlsbad, CA). After sequencing the inserts, nondegenerate oligonucleotide primers were designed for RACE (Rapid Amplification of cDNA Ends). For Gla-TxX, the primers were 5′-

CACTGATCCCTTCTGGCCCAGTG-3′ (5′ primer) and 5′-CACTGGGCCAGAAGGGATCAGTG-3′ (3′ primer), and for Gla-TxXI, the primers were 5′-CAGGTCCAACGACAGCAACTCTTG-3′ (5′ primer) and 5′-CAAGAGTTGCTGTCGTTGGACCTG-3′ (3′ primer). The RACE primers were used in conjunction with adapter primers to amplify the 5′ and 3′ cDNA sequences from a Marathon RACE library of *C. textile* venom duct cDNA (*27*) using a kit from Clontech (Palo Alto, CA). In each case, a single major PCR product was amplified, cloned, and sequenced as described above. The open-reading-frame sequences were verified by amplifying and sequencing the Gla-TxX- and Gla-TxXI-encoding cDNAs from the λ phage cDNA library. All oligonucleotides were synthesized by Life Technologies (Gaithersburg, MD).

Preparation of Microsomes. Live *C. textile* snails were maintained in a heated running seawater tank in the Marine Resources Center at the Marine Biological Laboratory, Woods Hole, MA. The snails were placed on ice, and the venom ducts were removed by dissection, then immediately frozen in liquid N_2 and stored at −80 °C. Venom ducts were thawed, ground in liquid N_2 with a mortar and pestle, and homogenized using a Tissue Tearor (Biospec Products, Inc., Bartlesville, OK) in 0.25 M sucrose, 0.5 M KCl, 25 mM imidazole, pH 7.2, 0.1% (w/v) 3-[(cholamidopropyl)dimethylammonio]-1-propanesulfate (CHAPS), and 0.1 mM phenylmethylsulfonyl fluoride (PMSF). The homogenates were centrifuged at 10 000*g* for 10 min at 4 °C and the supernatants frozen in aliquots at −80 °C. After thawing, the supernatants were centrifuged at 100 000*g* for 3 h at 4 °C. The pellets were resuspended in 25 mM 3-[*N*-morpholino]propanesulfonic acid (MOPS), pH 7.0, 0.5 M NaCl, 0.1% (w/v) phosphatidylcholine, 0.1% (w/v) CHAPS, 0.1 mM PMSF, and 20% (v/v) glycerol and sonicated, then frozen at −80 °C.

Carboxylase Assays. The amount of $^{14}CO_2$ incorporated into the peptide substrates during a 30-min incubation at 25 °C was measured in 125-μL reaction mixtures containing 222 μM vitamin K dihydroquinone (Abbott Laboratories, North Chicago, IL), 28 mM MOPS, pH 7.0, 0.5 M NaCl, 0.16% (w/v) phosphatidylcholine, 0.16% (w/v) CHAPS, 0.8 M $(NH_4)_2SO_4$, and 1.48 mM (i.e., 10 μCi) $NaH^{14}CO_3$ (Amersham Biosciences, Piscataway, NJ), *C. textile* microsomes, and peptide, as described previously (*17*). Peptides were prepared in water, except for the TxXI peptides, which were prepared in 0.4 M dithiothreitol to reduce disulfide bonds. All kinetic experiments were repeated at least three times and included two to four replicates for each peptide concentration.

RESULTS

Purification and Sequence Analysis of Conotoxins Gla-TxX and Gla-TxXI. To isolate Gla-containing peptides from the venom of *C. textile*, venom duct extracts were subjected to size-exclusion chromatography, and Gla-containing fractions were identified by monitoring the amino acid composition of the eluate. Six major peaks and three minor peaks (those labeled 3, 7, and 9) were identified (Figure 1A). Peak 8, the most prominent Gla-containing peak, has been shown to contain the γ-carboxylated conotoxin ϵ-TxIX (*11*). Two pools of Gla-containing fractions were subjected to reversed-phase HPLC, and five Gla-containing fractions were identified in the eluates (Figure 1B,C). Homogeneous γ-carboxylated peptides could be prepared from two of them, and these were selected for structural characterization (Figure 1B,C). Determination of the amino acid compositions of the peptides revealed that each contained eight Cys residues (data not shown). In addition, amino acid analyses of alkaline hydrolysates indicated that Gla-TxX contained five Gla residues per molecule and Gla-TxXI contained one Gla residue per molecule. The intact peptides and an internal tryptic fragment of Gla-TxXI were subjected to automated Edman degradation. A sequence of 47 amino acids was obtained for the Gla-TxX peptide, which corresponds to the full-length mature conotoxin as demonstrated by mass spectrometric analyses (see below). A sequence of 29 amino acids was obtained for the Gla-TxXI peptide. The last two residues of Gla-TxXI (i.e., residues 30 and 31) were not observed during Edman degradation but were revealed by

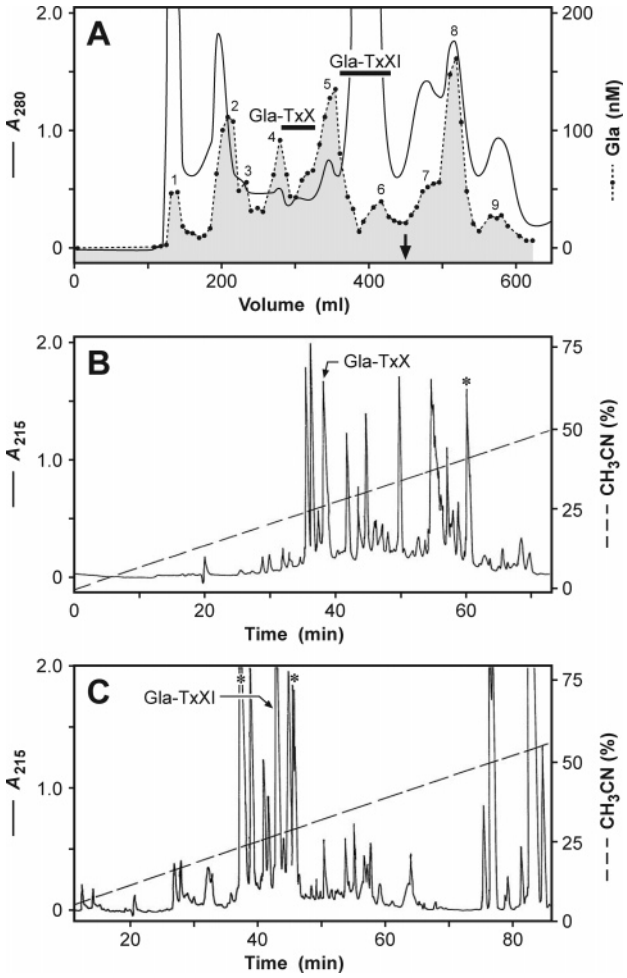

FIGURE 1: Purification of conotoxins Gla-TxX and Gla-TxXI. (A) Venom extract was chromatographed on a column of Sephadex G50 Superfine resin in 0.2 M ammonium acetate buffer, pH 7.5, and the Gla content in alkaline hydrolysates of the column fractions was determined. Gla-containing peaks have been numbered. The arrow denotes the position of one column volume of eluate. The Gla-containing fractions indicated by thick bars (A) were subjected to reversed-phase HPLC on a HyChrom C18 column using an acetonitrile gradient, which resulted in the isolation of the Gla-TxX (B) and Gla-TxXI (C) peptides. Peaks denoted with an asterisk in panels B and C contained γ-carboxylated peptides that could not be prepared in a homogeneous form and were not characterized.

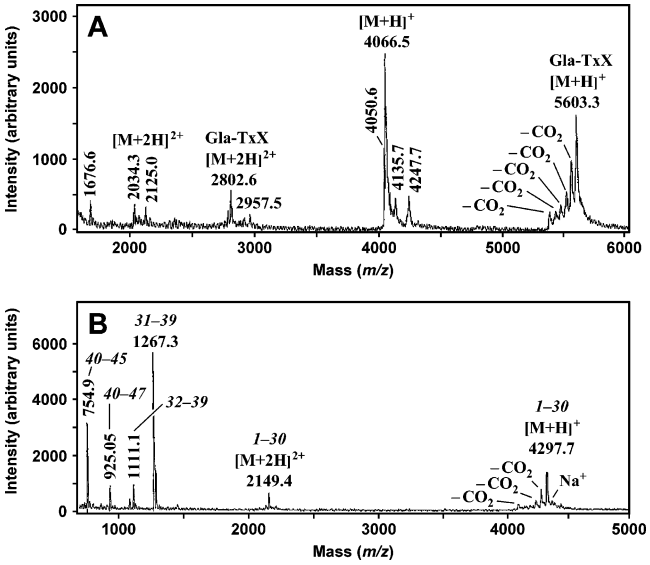

FIGURE 2: Mass spectrometric analysis of conotoxin Gla-TxX. MALDI-TOF MS spectra acquired in linear positive ion mode are shown for native Gla-TxX (A) and a tryptic digest of the reduced and alkylated peptide (B). Samples were applied using the thin-layer method, with 2,4,6-trihydroxyacetophenone/nitrocellulose (4:1) as the matrix. Partial decarboxylation of Gla residues from the peaks corresponding to the singly protonated Gla-TxX peptide (A) and the N-terminal tryptic peptide (B) is observed. The intense peak at *m*/*z* 4066.5 (A) is likely a peptide contaminant.

mass spectrometry and confirmed by the cloned cDNA sequence (see below).

Mass Spectrometric Analyses. The primary structures of the mature Gla-TxX and Gla-TxXI conotoxins were confirmed by mass spectrometric data. The MALDI-TOF MS spectrum for Gla-TxX acquired in positive linear ion mode shows two main groups of peaks (Figure 2A). The singly charged molecular ion corresponding to Gla-TxX is at *m*/*z* 5603.3. This peak is accompanied by a number of decarboxylated forms indicating that all five Glu residues in the peptide are γ-carboxylated. The peak observed in the spectrum at *m*/*z* 4066.5, which is accompanied by a doubly charged peak at *m*/*z* 2034.3, is presumed to represent a peptide contaminant. This component disappears after reduction, alkylation, and subsequent purification by HPLC (data not shown). The reduced and alkylated Gla-TxX peptide produced a single sequence during Edman degradation. The observed average mass of 5602.3 Da indicates that all eight Cys residues participate in disulfide bonds and is consistent (within the expected mass accuracy) with the theoretical mass of 5601.0 Da. The MALDI-TOF MS peptide map obtained from a tryptic digest of S-pyridylethylated Gla-TxX contains six main peaks (Figure 2B). The peak at *m*/*z* 925.05 corresponds to the C-terminal peptide (residues 40−47) and confirms amidation of the C-terminal asparagine residue (theoretical *m*/*z* 925.1). Amidation was further confirmed by recording a fully isotope-resolved spectrum in reflector mode, which gave a monoisotopic molecular mass of 923.49 Da, in agreement with the theoretical value of 923.47 Da. The peak at *m*/*z* 4297.7 corresponds to the N-terminal peptide (residues 1−30), verifying that all five Glu residues are γ-carboxylated. As expected, decarboxylated forms of this peptide are apparent in the spectrum. The amino acid sequence of the mature 47-residue Gla-TxX peptide was

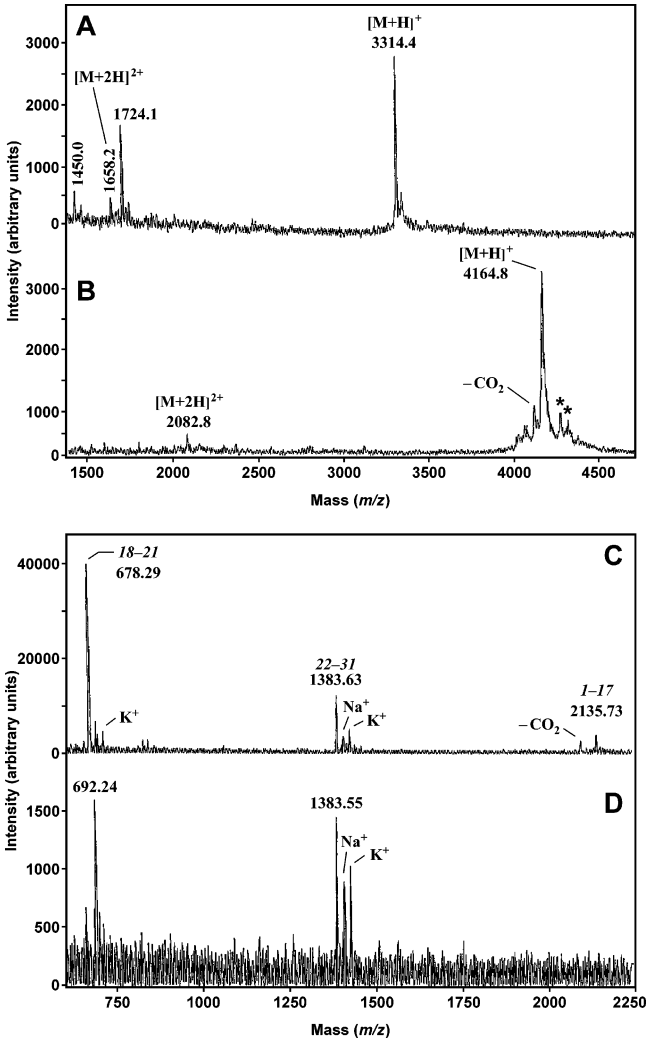

FIGURE 3: Mass spectrometric analysis of conotoxin Gla-TxXI. (A) The MALDI-TOF MS spectrum of the native peptide acquired in linear positive ion mode. The sample was applied using the thin-layer method, with 2,4,6-trihydroxyacetophenone/nitrocellulose (4:1) as the matrix. The peaks observed at *m*/*z* 1724.1 and 1450.0 represent impurities rather than fragment ions. (B) The MALDI-TOF MS spectrum of S-pyridylethylated Gla-TxXI acquired in linear positive ion mode. The sample was applied using the dried-droplet method with 2,5-dihyroxybenzoic acid as the matrix. Peaks labeled with an asterisk represent matrix adducts. MALDI-TOF MS spectra of a tryptic digest of S-pyridylethylated Gla-TxXI were acquired in reflector positive ion mode before (C) and after (D) methyl-esterification. The samples were applied using the dried-droplet method with 2,5-dihyroxybenzoic acid as the matrix. After methyl-esterification, a 14-Da increase in mass was observed for the peak at *m*/*z* 678.29 that represents the internal peptide (amino acids 18−21), whereas no mass-shift was observed for the C-terminal peptide (*m*/*z* 1383.63), which has an amidated C-terminus.

SCDSγFSSγFCγRP$\gamma\gamma$SCSCSTHTCCHWARRDQCMKP-QRCISAQKGN-NH_2, where γ designates a Gla residue.

The MALDI-TOF MS spectrum for Gla-TxXI acquired in positive linear ion mode shows three main peaks (Figure 3A). The peak at *m*/*z* 3314.35 represents the singly charged Gla-TxXI molecular ion. The observed average mass that was determined for the native species (3313.4) is consistent with the theoretical mass (3312.8 Da). Two other peaks (*m*/*z* 1724.1 and 1450.0) represent impurities since the corresponding peaks are not present after reduction, alkylation, and subsequent purification by reversed-phase HPLC (Figure

3B). The mass spectrum of the S-pyridylethylated species (Figure 3B) contains a peak at *m*/*z* 4164.8 that is accompanied by the loss of CO_2, thus, indicating the presence of a single Gla residue. The shifts in mass after reduction and alkylation with 4-vinylpyridine (850.4 Da for average mass) are in accordance with the presence of eight disulfide-linked Cys residues. The MALDI-TOF MS peptide map obtained after tryptic digestion of S-pyridylethylated Gla-TxXI (Figure 3C) has three main peaks and covers the complete sequence: *m*/*z* 2135.73 (the N-terminal peptide, residues 1−17), *m*/*z* 678.29 (residues 18−21), and *m*/*z* 1383.63 (the C-terminal peptide, residues 22−31). The latter value reveals that the C-terminal proline residue is amidated (theoretical *m*/*z* 1383.7). The monoisotopic molecular mass of Gla-TxXI, as based on the sum of the molecular masses of the three tryptic peptides, was measured as 4158.61 Da, in accordance with the theoretical monoisotopic value of 4158.76 Da. The peak at *m*/*z* 2135.73 is accompanied by a loss of CO_2 from a Gla residue present in the N-terminal peptide. After methyl-esterification of the tryptic peptide mixture and subsequent analysis by MALDI-TOF MS, the peak at *m*/*z* 678.29 exhibited a 14-Da increase in mass (Figure 3D), consistent with methyl-esterification of the C-terminal α-carboxyl group in the internal peptide. No mass-shift was observed for the C-terminal peptide (*m*/*z* 1383.63), thus, confirming amidation of the C-terminal proline. The N-terminal peptide was not observed for the methyl-esterified sample due to a low signal-to-noise ratio. The amino acid sequence of the mature 31-residue Gla-TxXI peptide was CIPγGSSCSSSGSCCHKSCCRWTCNQPCLIP-NH_2, where γ designates a Gla residue.

Cloning of cDNAs Encoding Gla-TxX and Gla-TxXI. The amino acid sequences obtained for the Gla-TxX and Gla-TxXI peptides were used to design degenerate oligonucleotide primers for cloning the cognate cDNAs. A combined PCR and RACE approach allowed us to clone the cDNAs encoding the precursors of both peptides from venom duct cDNA libraries. A 696-bp cDNA was obtained for Gla-TxX. This sequence contains an open-reading-frame of 249 bp that is predicted to encode a tripartite precursor peptide of 82 amino acids. The deduced translation product has a 25-residue presequence followed by a 47-residue stretch identical to the Gla-TxX peptide isolated from venom. Notably, a 10-residue C-terminal extension not found in the venom peptide is encoded before the termination codon is encountered (Figure 4). For Gla-TxXI, a 463-bp cDNA containing an open-reading-frame of 210 bp was cloned. The deduced precursor peptide (69 amino acids) contains a 25-residue presequence followed by a 31-residue sequence identical to the Gla-TxXI venom peptide. Similar to the Gla-TxX precursor, a C-terminal extension of 13 amino acids is observed which is missing from the mature venom peptide (Figure 4). In both cases, the region preceding the mature conotoxin is rich in hydrophobic amino acids and is predicted to contain an N-terminal signal sequence by the PSORT II algorithm (*32*). The predicted site of signal peptide cleavage in the Gla-TxX precursor is between residues 27 and 28, close to the experimentally determined N-terminus of the mature peptide (residue 26). The cleavage site predicted for the Gla-TxXI precursor is between residues 25 and 26, which corresponds exactly to the experimentally determined N-terminus of mature Gla-TxXI. No marked similarity to known propeptide sequences is observed in the presequence of either peptide, but in both cases, the C-terminal extension encoded by the cloned cDNA bears some resemblance to a typical γ-CRS-containing propeptide (see below).

Demonstration of a γ-Carboxylation Recognition Site in the Postpeptide of Gla-TxXI. To investigate whether the 13-residue C-terminal "postpeptide" encoded by the Gla-TxXI cDNA could direct γ-carboxylation of an adjacent peptide, a series of synthetic peptides was tested in carboxylase assays using microsomes prepared from *C. textile* venom ducts as a source of the *Conus* γ-carboxylase. The peptide TxXI-Pp, which had the native postpeptide attached at the C-terminus of the region corresponding to the mature toxin, was found to be an excellent substrate exhibiting an apparent K_m of 7.8 μM (Table 1). This peptide would be expected to constitute the natural substrate for the *Conus* γ-carboxylase. By contrast, it was not γ-carboxylated to any appreciable extent by the bovine γ-carboxylase when assayed at concentrations up to 250 μM (data not shown). The Pp-TxXI construct, in which the postpeptide was shifted to the N-terminus to mimic a conventional protoxin structure, was also found to be a low-K_m substrate for the *Conus* γ-carboxylase (Table 1).

Our attempts to solubilize a peptide comprising the very hydrophobic mature region of the Gla-TxXI precursor lacking the postpeptide (i.e., amino acids +1 to +31) were frustrated by its insolubility in aqueous solutions. Clearly, the solubility of the Gla-TxXI precursor is influenced by the highly basic postpeptide. Unfortunately, this precluded the determination of a K_m for the substrate lacking a post- or propeptide sequence, and we were thus unable to rule out the possibility that the mature region contributes to substrate recognition.

We therefore tested whether the Gla-TxXI postpeptide could direct γ-carboxylation of a more water-soluble natural *Conus* substrate, conantokin-G (*7*). Consistent with earlier studies (*16*, *26*), the uncarboxylated conantokin-G peptide lacking its normal propeptide was a poor substrate for the *Conus* γ-carboxylase, having an apparent K_m in the millimolar range (Table 1). Addition of the Gla-TxXI postpeptide to the C-terminus resulted in an ~40-fold reduction in the apparent K_m. Moreover, addition of the postpeptide at the N-terminus reduced the apparent K_m by more than 400-fold. Conjugation of the Gla-TxXI postpeptide to the C-terminus of the synthetic substrate FLEEL lowered the K_m about 6-fold (Table 1). Thus, the Gla-TxXI postpeptide was able to function as a γ-carboxylation recognition site and enhance γ-carboxylation of an adjacent substrate, regardless of whether it was linked to the C- or N-terminus.

The functionally transferable nature of the postpeptide indicated that it contains a γ-CRS with a function akin to that of the N-terminal propeptides of other vitamin K-dependent proteins. We therefore compared the amino acid sequences of the Gla-TxX and Gla-TxXI postpeptides to the precursors of other γ-carboxylated conotoxins to see whether a conserved motif could be identified. In all cases, the region of the precursors that is supposed to constitute the propeptide was found to be rich in basic residues, a property shared by the postpeptides of Gla-TxX, Gla-TxXI, and another conotoxin, κ-BtX (*33*) (Table 2). This basic region was not observed in the precursors of conotoxins that do not undergo γ-carboxylation (see Discussion). The average combined frequency of Arg and Lys residues in the propeptides is

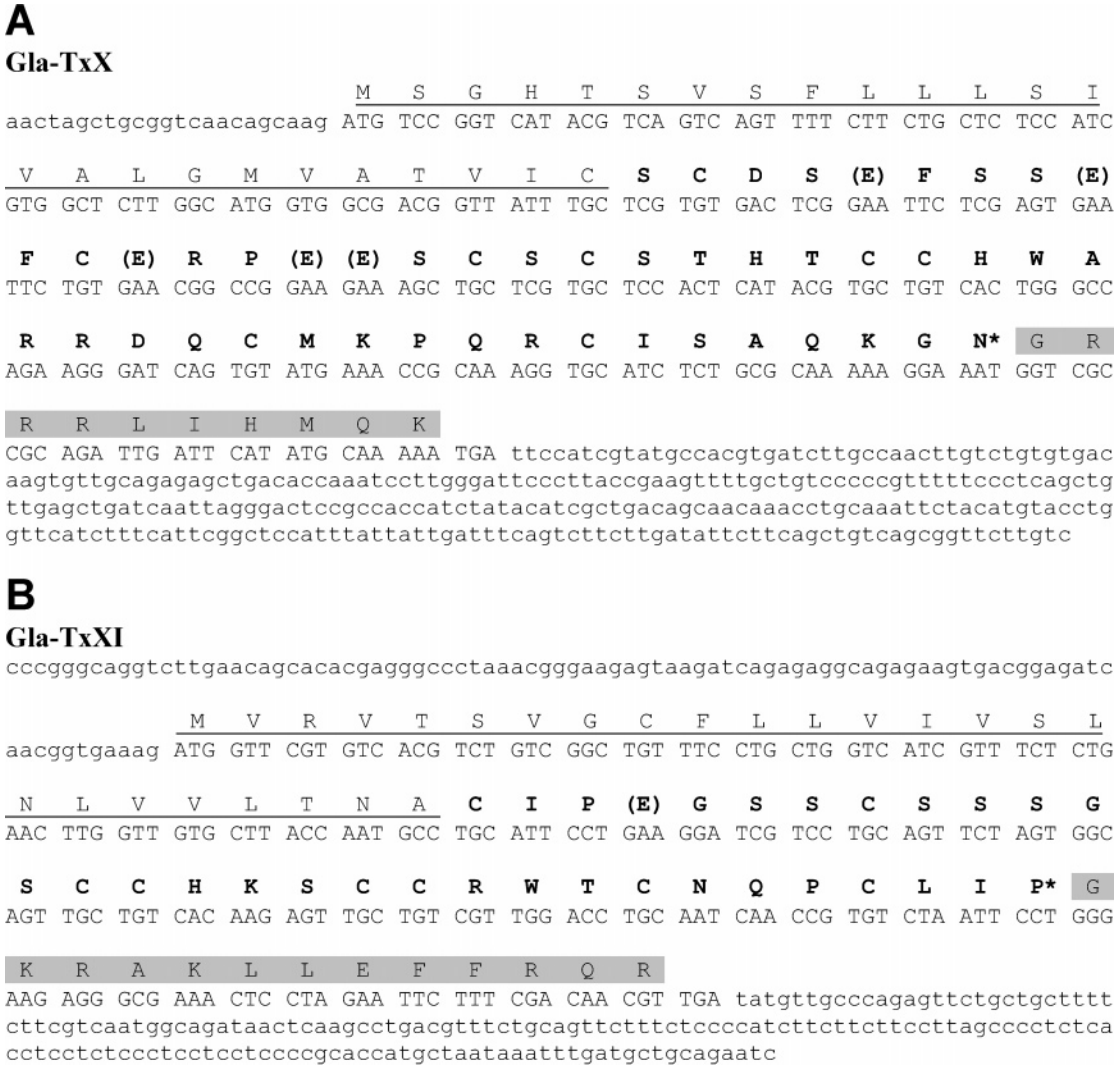

FIGURE 4: The cDNA and deduced amino acid sequences of Gla-TxX (A) and Gla-TxXI (B). The open-reading-frames of the cDNA sequences are shown in uppercase typeface and untranslated regions in lowercase. The amino acid sequences of the mature conotoxins, as determined by Edman degradation and mass spectrometry, are shown in bold typeface, and Glu residues that are post-translationally modified to Gla are shown in parentheses. In both cases, the mature peptides have an amidated C-terminal residue (indicated by an asterisk). The signal peptide is underlined and the postpeptide, which contains the γ-CRS, is shaded.

around 35%; more than 3 times the average occurrence in proteins (*34*). An alignment of the propeptide and postpeptide sequences from Gla-containing conotoxins revealed a high degree of variability, though a possible consensus involving one hydrophobic and two basic residues was noted (Lys/Arg-*X*-*X*-*J*-*X*-*X*-*X*-*X*-Lys/Arg, where *J* is a hydrophobic residue, usually Leu, and *X* is any amino acid; see Table 2). This consensus also occurs in the propeptide of the vertebrate γ-carboxylase substrate prothrombin.

We investigated whether the consensus sequence might play a role in substrate recognition by making amino acid substitutions at each of the three pertinent positions in the Gla-TxXI postpeptide and testing whether γ-carboxylation of the Gla-TxXI precursor was affected. Consistent with such a role, the substitutions Arg34 → Ala, Leu37 → Ser, or Arg42 → Ala and the triple substitution Arg34 → Ala/Leu37 → Ser/Arg42 → Ala, all interfered with the enzyme−substrate interaction. This was reflected by 3- to 7-fold increases in the K_m for the reaction, as compared to the wild-type postpeptide (Table 1). The substitutions would not be expected to markedly alter the secondary structure of the postpeptide, at least as predicted by the Jpred2 algorithm (*35*).

We also tested the combined effect of substituting two of the Arg residues for Ala or Gly and shuffling the positions of most of the other amino acids in the postpeptide upon γ-carboxylation of various substrates. In all cases, the shuffled sequence (i.e., GLAGEFKKLQAFR) was less effective at directing γ-carboxylation of an adjacent substrate than the wild-type postpeptide sequence. However, the magnitude of the effect depended upon the substrate. Thus, while a 5.5-fold increase in the apparent K_m (to 42.3 ± 4.7 μM) was observed when the shuffled postpeptide was placed at the C-terminus of Gla-TxXI, a 22-fold increase (to 848 ± 133 μM) was observed when it was placed at the C-terminus of conantokin-G.

DISCUSSION

The vitamin K-dependent proteins contain γ-carboxyglutamate, an amino acid that is generated from glutamyl residues during protein synthesis. This process has been variously assumed to be either co-translational, post-

Table 1: Sequences of the Synthetic Peptide Substrates and Their Apparent Michaelis Constants

Peptide	Amino acid sequence [a]	K_m (μM) [b]	Fold difference [c]
TxXI-Pp	CIPEGSSCSSSGSCCHKSCCRWTCNQPCLIPGKRAKLLEFFRQR	7.8 ± 1.7	1.0
TxXI-Pp(R42A)	CIPEGSSCSSSGSCCHKSCCRWTCNQPCLIPGKRAKLLEFFAQR	22.5 ± 3.0	2.9
TxXI-Pp(R34A)	CIPEGSSCSSSGSCCHKSCCRWTCNQPCLIPGKAAKLLEFFRQR	45.8 ± 3.4	5.9
TxXI-Pp(L37S)	CIPEGSSCSSSGSCCHKSCCRWTCNQPCLIPGKRAKSLEFFRQR	54.4 ± 5.6	7.0
TxXI-Pp(R34A/L37S/R42A)	CIPEGSSCSSSGSCCHKSCCRWTCNQPCLIPGKAAKSLEFFAQR	59.5 ± 6.0	7.6
Pp-TxXI [d]	GKRAKLLEFFRQRCIPEGSSCSSSGSCCHKSCCRWTCNQPCLIP-NH_2	7.5 ± 1.7	1.0
ConG-Pp	GEEELQENQELIREKSNGKRAKLLEFFRQR	39.1 ± 2.9	1.0
ConG	GEEELQENQELIREKSN	1495 ± 100	38
Pp-ConG	GKRAKLLEFFRQRGEEELQENQELIREKSN	3.4 ± 0.9	0.1
FLEEL-Pp	FLEELGKRAKLLEFFRQR	67.9 ± 0.4	1.0
FLEEL	FLEEL	406 ± 15	6.0

[a] The postpeptide sequence is shaded and amino acid substitutions are underlined. [b] K_m values were calculated by the Lineweaver−Burk method and are given as mean ± 1 SD ($n \geq 3$). [c] Compared to K_m for relevant substrate with wild-type postpeptide at the C-terminus. [d] Peptide synthesized with an amidated C-terminus.

Table 2: Comparison of Postpeptide and Propeptide Amino Acid Sequences

Conotoxin	Amino acid sequence[a]	Position	pI	Ref.
	* * *			
Gla-TxX	GRRRLIHMQK	+48/+57	12.81	–
Gla-TxXI	GKRAKLLEFFRQR	+32/+44	12.24	–
κ-BtX	GKRSKLQEFFRQR	+32/+44	12.24	(33)
PnVIIA	QQAKINFLSKRKPSAERWRR	−1/−20	12.52	(10)
TxVIIA	RKAEINFSETRKLARNKQKR	−1/−20	12.12	(8)
ε-TxIX	PLSSLRDNLKRTIRTRLNIR	−1/−20	12.68	(27)
Tx9.1	DNRRNLQSKWKPVSLYMSRR	−1/−20	12.11	(12)
ConG	GKDRLTQMKRILKQRGNKAR	−1/−20	12.53	(26)
GlaCon-M	GRDNPGRARRKRMKVL	−1/−16	12.69	(40)
Human II	HVFLAPQQARSLLQRVRR	−1/−18	12.98	(49)

[a] Amino acids forming the consensus sequence are boxed and their positions highlighted by an asterisk. Basic amino acids are shown in bold. Shaded residues are those predicted to form an α-helix by the program Nnpredict (http://www.cmpharm.ucsf.edu/~nomi/nnpredict.html). The γ-CRS identified in the propeptide of human prothrombin (factor II) is underlined.

translational, or both, with no experimental data to support either mechanism. In our continuing study of the synthesis and function of γ-carboxyglutamate, we have discovered two novel Gla-containing conotoxins, designated Gla-TxX and Gla-TxXI, that must be γ-carboxylated via a post-translational process.

The precursor polypeptides encoded by the cloned Gla-TxX and Gla-TxXI cDNAs are unusual among proteins that undergo γ-carboxylation in that the signal peptide is followed immediately by the mature toxin sequence. Thus, they lack the intervening propeptide typically found in precursors of vitamin K-dependent proteins that contains the γ-CRS required to target and tether the protein to the γ-carboxylase (*21*). Instead, the precursors to both of these novel conotoxins possess a C-terminal extension, or "postpeptide", which bears some resemblance in its amino acid sequence to the propeptides of other conotoxins known to undergo γ-carboxylation. As is characteristic of propeptides, which are excised in the late-Golgi after γ-carboxylation is complete (*36*), the encoded postpeptides are cleaved to generate the mature Gla-TxX and Gla-TxXI conotoxins. We therefore hypothesized that despite their unusual location at the C-terminus, the postpeptides would contain a γ-CRS.

Indeed, compelling evidence to support this notion was obtained from kinetic experiments performed in vitro with the *Conus* γ-carboxylase. Small peptides such as FLEEL, which lack a γ-CRS, can diffuse into the active site of the γ-carboxylase but are high-K_m substrates per se (*37*). Conjugation of the 13-residue Gla-TxXI postpeptide to the C-terminus of FLEEL or to the C-terminus of another high-K_m substrate, acarboxy conantokin-G, converted both peptides to low-K_m substrates for the γ-carboxylase, in accordance with the postulated role of the postpeptide as a recognition element for the enzyme. A similar effect was observed when the postpeptide was conjugated to the N-terminus of acarboxy conantokin-G to mimic a conven-

tional γ-carboxylase substrate structure. In addition, the Gla-TxXI precursor exhibited a low and almost identical K_m regardless of whether the postpeptide was located at the N- or C-terminus. Together, these experiments demonstrate that the Gla-TxXI postpeptide is sufficient to effectively direct γ-carboxylation of an adjacent substrate. This ability is a general property of this postpeptide and not limited to its natural substrate partner or natural location at the C-terminus of the substrate. Thus, the postpeptide contains a bona fide γ-CRS, enabling it to function in a manner analogous to a typical γ-CRS-containing propeptide. The recognition element may be specified by as few as 10 amino acids, given the length of the Gla-TxX postpeptide and the critical size of the 12-residue sequence that delineates the γ-CRS within the propeptide of ϵ-TxIX (*27*).

The structural organization of the Gla-TxX and Gla-TxXI precursors (signal peptide-mature toxin-postpeptide) is exceptional among vertebrate and invertebrate vitamin K-dependent proteins, having been reported previously only for the precursor to the γ-carboxylated conotoxin κ-BtX (*33*). On the basis of the location we have established for the γ-CRS in the Gla-TxX and Gla-TxXI precursors, synthesis of γ-carboxyglutamate must occur by post-translational processing. Current evidence indicates that the active site of the γ-carboxylase resides in the lumen of the endoplasmic reticulum. In previously known vitamin K-dependent proteins, the propeptide is located near the N-terminus of the nascent polypeptide and the precursors in many cases are long enough to span a membrane. It therefore had been possible to speculate that γ-carboxylation could be co-translational: with translocation occurring during chain elongation, the propeptide could bind to the γ-carboxylase and the substrate region could contact the active site of the enzyme. Such a mechanism is not plausible for substrates such as Gla-TxX and Gla-TxXI, in which the γ-CRS is encoded at the extreme 3′-end of the open-reading-frame of the messenger RNA transcript. For these peptides, the γ-CRS is available for binding to the γ-carboxylase active site only after translocation of the peptide into the endoplasmic reticulum is complete. Hence, this type of gene organization argues for post-translational γ-carboxylation, occurring after the polypeptide chain of the precursor has been synthesized, released from the ribosome, and translocated to the lumen of the endoplasmic reticulum. This type of gene organization also has spatial consequences in that the enzyme must tether and process substrates with the γ-CRS located on either side of the targeted glutamyl residues. The ability of the γ-carboxylase to modify Glu residues located N-terminal to the γ-CRS may not be limited to the invertebrate enzyme. In the vertebrate vitamin K-dependent protein matrix Gla protein, a putative Gla residue resides N-terminal to an internal region that likely constitutes the γ-CRS, but this Gla residue has not been directly identified (*23, 38*).

Our attempt to define a potential consensus sequence within the γ-CRS in the Gla-TxXI postpeptide was inconclusive. Three positions in the postpeptide (Arg+34, Leu+37, and Arg+42) display some relationship to propeptide sequences from other γ-carboxylated conotoxins. Amino acid substitutions at these positions increased the K_m for the reaction with the Gla-TxXI precursor severalfold, which suggests they may have some involvement in substrate recognition. A γ-CRS has also proven difficult to define in propeptide sequences, though the functional importance of certain residues has been demonstrated by mutational studies (*19*−*22, 25, 39*). Notably, the propeptides and postpeptides from γ-carboxylated conotoxins display a marked preference for basic amino acids, while at the same time the occurrence of acidic amino acids is low. Thus the estimated pIs of these regions are in the range of 12−13, and this appears to be a distinguishing feature of the propeptides of γ-carboxylated conotoxins and not those of their non-carboxylated counterparts. For example, the prepro-region is highly conserved among all contryphan family members with one exception: glacontryphan-M, the only member known to be γ-carboxylated (*40*). Whereas the signal peptide of glacontryphan-M is similar to other family members, it stands apart in having a propeptide region that contains 55% (6/11) basic amino acids.

In some conotoxin superfamilies encoding Gla-containing conotoxins, the signal peptide, propeptide, and mature region are each specified by different exons separated by large introns (*41, 42*). It has been postulated that this may allow mutational rates to differ among exons, leading to the hyperdivergence observed among sequences encoding the mature toxins (*41, 43*). Such a gene organization could conceivably facilitate shuffling of the propeptide-encoding exon to a downstream location, giving rise to a new modular sequence in the precursor polypeptide (signal peptide-mature toxin-postpeptide). In contrast, the vertebrate propeptide-Gla domain region is encoded by a single exon (*44*), thereby ensuring that the γ-CRS is located N-terminal to the Gla domain.

Besides post-translational γ-carboxylation, as directed by the γ-CRS in the postpeptide, these conotoxins undergo additional processing, including the formation of disulfide bonds and C-terminal amidation. The amidation of the C-termini on mature Gla-TxX and Gla-TxXI indicates that removal of the postpeptides is most likely the result of at least two separate cleavage events. The first cleavage(s) would expose the glycinyl residue located immediately after the mature peptide region. This corresponds to Gly+48 in Gla-TxX and to Gly+32 in Gla-TxXI. Such glycine-extended precursors are the requisite substrates for C-terminal amidation (*45*). The canonical signal for α-amidation (-*Xxx*-Gly-Lys/Arg-Lys/Arg-) is present in both conotoxin precursors, and exposure of the Gly residue is likely to occur in the typical manner: initial cleavage by a subtilisin-like endoprotease on the C-terminal side of the basic dipeptide (or tripeptide in Gla-TxX), with subsequent trimming by a carboxypeptidase. The Gly residue is probably cleaved during amidation by the sequential action of two enzymes, peptidylglycine-α-hydroxylating monooxygenase and peptidyl-α-hydroxyglycine α-amidating lyase, which together catalyze the oxidative cleavage of the N−Cα bond in the Gly residue to produce an amidated peptide (*45*).

Gla-TxXI has eight Cys residues in the pattern C-C-CC-CC-C-C, known as framework 11/XI in conotoxin nomenclature. This framework is characteristic of the recently identified I-superfamily of conotoxins (*46*). The disulfide framework of a conotoxin is a major determinant of its three-dimensional structure and, hence, molecular target (*5*), and where known, I-superfamily members have been found to be specific modulators of K^+ channels (*6, 33, 47*). The spacing of the Cys residues in Gla-TxXI ($CX_6CX_5CCX_3$-

$CCX_3CX_3CX_3$, where *X* denotes a residue other than Cys) matches that observed so far in only one other conotoxin, κ-BtX from *C. betulinus* (*33*). Gla-TxX exhibits an unusual arrangement of eight Cys residues in the pattern (CX_8CX_6CX-$CX_4CCX_7CX_5C$). This framework (C-C-C-C-CC-C-C) is shared with conotoxin Gla-MrII from *C. marmoreus* (*48*), though the spacing of the Cys residues differs. In keeping with the nomenclature used for designating the Cys-framework of conotoxins (*42*), we propose that the new framework be assigned the Roman numeral XII.

The vitamin K-dependent synthesis of γ-carboxyglutamate appears to be a critical metabolic process in animal phyla given the marked conservation of the vertebrate and invertebrate γ-carboxylase gene (*16*, *17*). Our fortuitous observation that γ-carboxylase substrates, in this case certain Gla-containing conotoxins, have the γ-CRS downstream of the targeted glutamyl residues formally demonstrates that γ-carboxylation is a post-translational rather than co-translational process.

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SUPPORTING INFORMATION AVAILABLE

Peptide synthesis procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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