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On a Potential Global Role for Vitamin K-dependent γ-Carboxylation in Animal Systems

EVIDENCE FOR A γ-GLUTAMYL CARBOXYLASE IN *DROSOPHILA**

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The vitamin K-dependent γ-carboxylation of glutamate to γ-carboxyglutamate was originally well characterized in the mammalian blood clotting cascade. γ-Carboxyglutamate has also been found in a number of other mammalian proteins and in neuropeptides from the venoms of marine snails belonging to the genus *Conus*, suggesting wider prevalence of γ-carboxylation. We demonstrate that an open reading frame from a *Drosophila melanogaster* cDNA clone encodes a protein with vitamin K-dependent γ-carboxylase activity. The open reading frame, 670 amino acids in length, is truncated at the C-terminal end compared with mammalian γ-carboxylase, which is 758 amino acids. The mammalian gene has 14 introns; in *Drosophila* there are two much shorter introns but in positions precisely homologous to two of the mammalian introns. In addition, a deletion of 6 nucleotides is observed when cDNA and genomic sequences are compared. *In situ* hybridization to fixed embryos indicated ubiquitous presence of carboxylase mRNA throughout embryogenesis. Northern blot analysis revealed increased mRNA levels in 12–24-h embryos. The continued presence of carboxylase mRNA suggests that it plays an important role during embryogenesis. Although the model substrate FLEEL is carboxylated by the enzyme, a substrate containing the propeptide of a *Conus* carboxylase substrate, conantokin G, is poorly carboxylated. Its occurrence in vertebrates, molluscan systems (*i.e. Conus*), and *Drosophila* and the apparently strong homology between the three systems suggest that this is a highly conserved and widely distributed post-translational modification in biological systems.

The functions of proteins are coordinated physiologically by post-translational modification. For example, phosphorylation-dephosphorylation cascades integrate the biochemistry of individual proteins into cellular physiology. In addition to post-translational modifications that occur primarily within cells, post-translational modifications also occur on extracellular proteins. The most familiar of these are *N*-glycosylation of asparagine residues and *O*-glycosylation of serine and threonine residues.

One of the most distinctive of the extracellular post-translational modifications is the vitamin K-dependent γ-carboxylation of glutamate residues to give γ-carboxyglutamate (1). When it was first characterized, γ-carboxylation was thought to be a biochemical specialization of the mammalian blood-clotting cascade. However, several bone proteins (2, 3) as well as an extracellular ligand, gas6 (4), were subsequently identified as having the post-translational modification, although in the latter cases the precise mechanistic role of γ-carboxylation for proper protein function has not been established definitively. In addition, two novel proline-rich γ-carboxyglutamic acid-containing proteins, PRGP1 and PRGP2, of unknown function have been identified (5).

Long after its characterization in blood-clotting factors, vitamin K-dependent γ-carboxylation of glutamate residues was discovered in a phylogenetically distant system: the neuropeptides made in the venom duct of the predatory cone snails *Conus* (6, 7). The venoms of these snails have ~100 different peptides; ~5% of these are believed to be γ-carboxylated (8). This post-translational modification has been found in a number of diverse *Conus* peptides but has been studied most intensively in an unusual *Conus* neuropeptide family, the conantokins, which are NMDA receptor antagonists.

In the conantokins, the significance of the post-translational modification can readily be demonstrated: these peptides are inactive in analogs without γ-carboxylation of glutamate residues. Incomplete γ-carboxylation of blood-clotting factors results in poor coagulation. It has been postulated that γ-carboxylation of both the conantokins and of factors of the blood-clotting cascade induces a helical conformation in the post-translationally modified regions. This postulated role of γ-carboxylation in determining conantokin structure has been generally supported by a number of subsequent structural studies on various conantokins (9–12). γ-Carboxyglutamic acid confers the property of Ca^{2+} binding to the modified protein. In the case of the blood-clotting factors, the binding to Ca^{2+} results in a conformational change exposing hydrophobic residues for interaction with membranes (13–17).

The enzymatic reaction in the invertebrate system has recently been shown to have many striking similarities (*e.g.* a requirement for reduced vitamin K and the presence of a γ-carboxylation recognition site on the substrate) to that of the γ-carboxylation of factors involved in the mammalian blood-clotting cascade (18, 19). Despite the clear functional importance of γ-carboxylation in these two disparate phylogenetic systems, γ-carboxylation of glutamate residues has been re-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF170280.

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garded as a highly specialized post-translational modification. In this report, we provide evidence that is strongly consistent with vitamin K-dependent γ-carboxylation in fact being a much more widely distributed biological phenomenon. We demonstrate by molecular techniques the presence of a vitamin K-dependent γ-carboxylase-related protein that is expressed in the fruit fly *Drosophila melanogaster*, which has a high degree of sequence identity with the mammalian enzyme. Similar observations have recently been reported by Li *et al.* (20). Although the role of γ-carboxylation in *Drosophila* remains unknown, this post-translational modification is present in arthropods, suggesting that it is generally distributed in animal systems. The strong conservation in sequence of the γ-glutamyl carboxylase in *Drosophila* and in mammals suggests an important functional role for the enzyme, resulting in strong selection for sequence conservation.

EXPERIMENTAL PROCEDURES

Materials—*Conus* textile venom ducts were obtained from Dr. L. J. Cruz (University of the Philippines). Vitamin K (phytonadione) was from Abbott Laboratories, and $NaH^{14}CO_3$ (55 mCi/mmol) was from PerkinElmer Life Sciences. Enzymes were purchased from Life Technologies, Inc. PCR[1] reactions were performed in an Air Thermo-Cycler (Idaho Techology). Oligonucleotides were synthesized at the peptide sequencing facility at the University of Utah.

Preparation of mRNA—Adult *Drosophila* (Oregon) were frozen in liquid nitrogen and ground to a fine powder, and total RNA was isolated (21). Poly(A)$^+$ RNA was isolated using a Qiagen Oligotex mRNA kit according to the vendor's instructions. Molecular biology experiments were done according to methods described by Sambrook *et al.* (22).

Sequence Analysis—The cDNA sequence of *Drosophila* γ-carboxylase was assembled from sequences of PCR products obtained by amplification of oligo(dT)- or Q_T-primed cDNA using primers shown in Table I. The primer combinations used in the PCRs are shown in Table I. Primers 1 and 2 correspond to amino acid sequences conserved in human, bovine, and rat. Primers 5 and 6 were selected from the *Drosophila* genomic sequence (Berkeley *Drosophila* Genome Project, accession number AC005557). 3′-Sequences of the carboxylase mRNA were determined by the technique of rapid amplification of cDNA ends described by Frohman (23). The PCR product was cloned into the TA cloning vector (Invitrogen), and the nucleic acid sequence was determined. DNA sequencing was performed using ABI Prism BigDye terminators and cycle sequencing with *Taq* FS DNA polymerase (Life Technologies). The DNA sequence was collected and analyzed on an ABI Prism 377 automated DNA sequencer (Applied Biosystems, Foster City, CA). To obtain the 5′ end of the coding sequences, we selected primer 6 from the genomic sequence. This allowed us to characterize a transcript with an open reading frame from nucleotides 52 (ATG) to 2198 (TGA). The sequences have been submitted to GenBank; the accession number is AF170280.

Analyses of amino acid homology between human (24), bovine (25), rat (26), and *Drosophila* γ-glutamyl carboxylases were carried out using Gap and PileUp programs version 4.0, 1998 (Genetics Computer Group).

Northern Blot Analysis—Total RNA was isolated from *Drosophila* at various stages of development. Embryos at 0–2, 2–4, 4–8, 12–16, and 16–24 h of development, larval stages 1–3, pupae, and adult flies were used in the experiment. Northern blot analysis was performed using reagents provided in the Northern Max kit (Ambion). Nine μg of total RNA from each sample were electrophoresed in a 1.5% denaturing agarose gel, transferred to a Gene Screen Plus membrane (PerkinElmer Life Sciences), and hybridized to [α-^{32}P]UTP-labeled antisense *Drosophila* γ-carboxylase RNA. As a control for loading, a membrane containing identical samples was hybridized to [α-^{32}P]dCTP-labeled *Drosophila* rp49. RNA molecular weight standards (RNA Millennium) were purchased from Ambion. Membranes were exposed to Molecular Dynamics (Sunnyvale, CA) Phosphor Screen and scanned. Images were analyzed using the NIH Image processing program.

In Situ Hybridization—The spatial distribution of γ-glutamyl carboxylase RNA was probed by hybridization to whole-mount embryos *in situ* (27). The cDNA was cloned into a dual-promoter (T7 and Sp6) vector. Both sense and antisense RNAs were synthesized using the appropriate RNA polymerase in the presence of digoxigenin-labeled uridine triphosphate. The digoxigenin-labeled RNA was used as probe in hybridizations to fixed embryos. The hybridized digoxigenin-labeled RNA was detected by incubating the embryos with alkaline phosphatase-conjugated anti-digoxigenin antibody and developed using nitroblue tetrzolium and 5-bromo-4-chloro-3-indolyl phosphate.

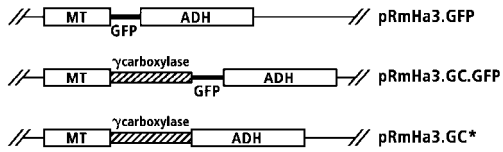

FIG. 1. **Vectors used for the expression of GFP, γ-carboxylase.GFP, and γ-carboxylase in S2 cells.**

Transient Expression of γ-Glutamyl Carboxylase Activity—DNA containing the coding sequence of *Drosophila* γ-glutamyl carboxylase was obtained by PCR amplification of the genomic sequences. The 3′ PCR primer was designed such that the γ-carboxylase (GC) coding sequences would be in frame with coding sequences of green fluorescent protein (GFP) in the expression plasmid pRmHa-3.GFP (Fig. 1). The γ-glutamyl carboxylase encoding sequences include the two introns in the genomic sequence. pRmHa-3.GFP was constructed by introducing the coding sequence of GFP from the pEGFP vector (CLONTECH) into pRmHa-3 (28). Expression in this plasmid is under the control of the inducible metallothionein promoter and carries the alcohol dehydrogenase poly(A) addition signal. *Drosophila* Schneider 2 (S2) cells were transfected with pRmHa-3.GC.GFP DNA using CellFECTIN (Life Technologies). 24 h after transfection cells were induced with 0.7 mM $CuSO_4$. 48 h after induction 50% of the cells expressed GFP as judged by fluorescent microscopy. The results also indicated that the introns were properly processed, and a continuous reading frame was present in the cloned GC. pRmHa-3.GC.GFP was modified to introduce a stop codon at the end of the GC coding sequences and to delete the GFP coding sequences. The modified plasmid pRmHa-3.GC* was transfected into S2 cells. The cells were induced with 0.7 mM $CuSO_4$ and harvested 48 h after induction. Cells were washed twice with phosphate-buffered saline and resuspended in buffer containing 25 mM 4-morpholinepropanesulfonic acid, pH 7.0, 0.5 M NaCl, 0.2% 3-[(3-cholamidopropyl)dimenthylammonio]-1-propanesulfonic acid/phosphatidyl choline, 2 mM EDTA, 2 mM dithiothreitol, 0.2 μg/ml leupeptin, 0.8 μg/ml pepstatin, and 0.04 mg/ml phenylmethylsulfonyl fluoride. The cell suspension was briefly sonicated using a Branson 450 sonifier and incubated in ice for 20 min. The lysate was assayed for carboxylase activity.

Enzyme Assays—γ-Glutamyl carboxylase assays were performed as described by Stanley *et al.* (18). Reactions were done in a total volume of 125 μl containing cell lysate and a final concentration of reagents as follows: 25 mM 4-morpholinepropanesulfonic acid, pH 7.4, 0.5 M NaCl, 0.2% 3-[(3-cholamidopropyl)dimenthylammonio]-1-propanesulfonic acid, 0.2% phosphatidyl choline, 0.8 M ammonium sulfate, 5 μCi of $NaH^{14}CO_3$′, 6 mM dithiothreitol, 222 μM reduced vitamin K, and 1.2 mM of a model substrate, the pentapeptide FLEEL (29). Reaction mixtures were incubated at 25 °C for 120 min and were quenched by addition of 75 μl of 1 N NaOH. The quenched reaction mixture (160 μl) was transferred to 1 ml of 5% trichloroacetic acid and boiled to remove unincorporated $^{14}CO_2$. After cooling, 5 ml of Ecolite (PerkinElmer Life Sciences) was added, and the $^{14}CO_2$ incorporated was determined in a Beckman LS 9800 counter.

RESULTS

Characterization of a Putative Drosophila γ-Carboxylase mRNA Sequence—Poly(A)$^+$ RNA from adult *Drosophila* was used as template for the reverse transcription of cDNA using an oligo(dT) primer. An initial segment of cDNA was amplified and sequenced using γ-carboxylase primers encoding amino acid sequences highly conserved between *Drosophila* sequences from the Berkeley *Drosophila* Genome Project (BDGP) and all mammalian enzymes; the 5′-oligonucleotide primer (primer 1; Table I) corresponded to amino acids 395–402, YGYSWDMM, and the 3′-primer (primer 2; Table I) corresponded to amino acids 465–471, IYFDIWC (the amino acid positions correspond to human γ-glutamyl carboxylase sequence). The PCR product was cloned, and the nucleic acid sequence was determined; the

[1] The abbreviations used are: PCR, polymerase chain reaction; GC, γ-carboxylase; BDGP, Berkeley *Drosophila* Genome Project; γ-CRS, γ-carboxylation recognition signal sequence; GFP, green fluorescent protein; S2, Schneider 2.

TABLE I
Primers used in PCR to identify Drosophila γ-glutamyl carboxylase

The primer pairs used in PCR amplification (see Fig. 2) were 1 and 2, 1 and Q_o, 3 and Q_o, 5 and 4, 5 and 7, 6 and 7. RACE, rapid amplification of cDNA ends.

Primer	Nucleotides	Sequence
1	1361–1384	TAYGGITAYTCATGGGAYATGATG
2	1599–1581	CACCAGATGTCGAAGTAGA
3	1513–1532	GCAGTAYGCCAGGTGCATCG
4	1532–1513	CGATGCACCTGGCRTACTGC
5	567–587	GTGGAATAACCACAGCTATCT
6	2–22	TGCAAAGGACGTGTTCTTTCG
7	2510–2419	ATCAGTTTTATCACCCATAC
8	Q_T (3′-RACE primer)	CCAGTGAGCAGAGTGACGAGGACTCGAGCTCAAGCTTTTTTTTTTTTTTT
9	Q_0	CCAGTGAGCAGAGTGACG

sequence obtained was identical to a DNA sequence in the *Drosophila* genome (BDGP, accession number AC005557).

We carried out further PCR analysis of the cDNA using the primers indicated schematically in Table I and Fig. 2*A*. The 3′-end of the transcript to the poly(A) addition site was determined using 3′-rapid amplification of cDNA ends. Polyadenylation takes place 20 bases downstream of a consensus Poly(A) signal, AATAAA. The remainder of the cDNA was characterized by PCR amplification using primers shown in Table I; PCR products were cloned, and overlapping sequences were combined to yield the cDNA shown schematically in Fig. 2*A*.

The cDNA sequence encodes an open reading frame of 670 amino acids. Examination of the genomic sequence revealed two notable differences from the cDNA. First, there are two short introns. A schematic comparison of the genomic and cDNA, shown in Fig. 2*B*, illustrates the position of these introns. The nucleic acid sequences at the splice junction are shown in Fig. 2*C*. Second, an as yet uncharacterized processing event removes 6 nucleotides from the cDNA (Fig. 2*D*).

The locations of the two introns are conserved between *Drosophila* and mammals. As is generally found when comparing *Drosophila* with mammalian introns (30), the *Drosophila* introns are significantly shorter (for intron I, 58 *versus* 2204 nucleotides; for intron II, 72 *versus* 646 nucleotides). Comparison of the amino acid sequences flanking *Drosophila* intron II and human intron VII is shown in Fig. 2*E*. There are a total of 14 introns in both the rat (26) and the human γ-carboxylase genes (31); therefore, *Drosophila* has both fewer and shorter introns compared with the mammalian gene. Fig. 3*A* shows a schematic of amino acid homology between human and *Drosophila* γ-carboxylase.

The surprising finding from the sequencing described above is that an uncharacterized mechanism of RNA processing results in the deletion of 6 nucleotides that would have been present if the DNA were faithfully transcribed. The deletion does not change the amino acid homology to the mammalian enzyme at this site. This region of cDNA was sequenced at least three times as parts of PCR amplification products synthesized by different primer pairs. To confirm the genomic sequence of our strain, we directly sequenced the genomic DNA from the *Drosophila* strain that was the source of our cDNA. It was identical to that from BDGP, which confirmed that the deletion of 6 nucleotides was real and not an artifact of strain differences or cloning or sequencing errors.

With regard to the protein length, the alignment in Fig. 3*B* shows that although the *Drosophila* enzyme has 17 additional amino acids at the N terminus compared with the mammalian enzymes, it is significantly shorter at the C-terminal end. All of the mammalian enzymes are longer (758 *versus* 670 amino acids). A recent deletion analysis (32) of the bovine enzyme suggests that small deletions at the C terminus may be tolerated by the wild-type mammalian enzyme. Interestingly, a deletion that resulted in a bovine enzyme that was 676 amino acids in length had lower enzymatic activity (15-fold lower with respect to γ-carboxylation and 400-fold lower than for vitamin K epoxidation). It remains to be determined whether other *Drosophila* subunits are necessary to compensate for the shorter length of the *Drosophila* open reading frame.

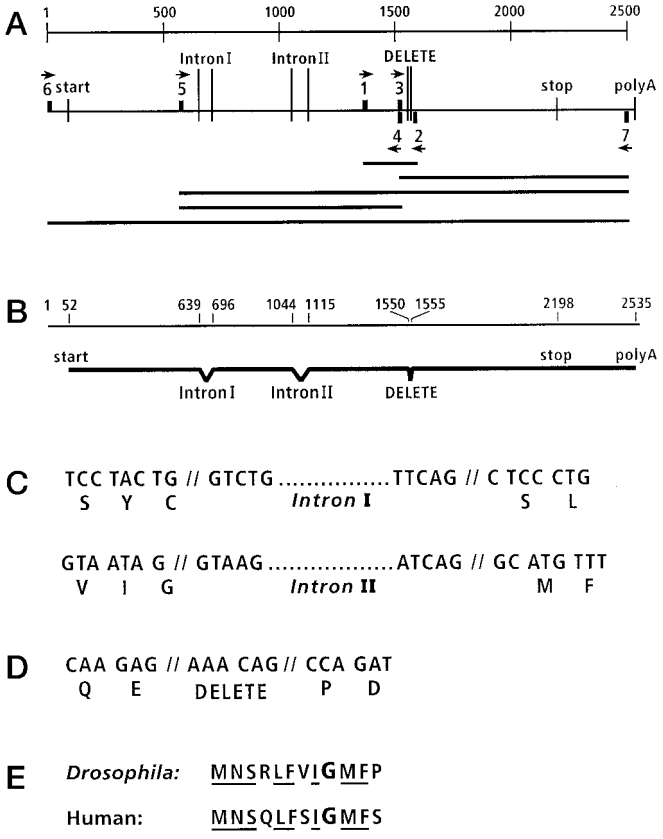

FIG. 2. *A*, schematic of PCR products. Coordinates refer to the genomic sequence. Positions of initiation and termination codons, introns, processing site (*DELETE*), and Poly(A) addition signals are shown. *B*, schematic comparison of genomic and cDNA maps of *Drosophila* γ-carboxylase. *C*, nucleic acid and amino acid sequences at the splice junctions of *Drosophila* introns I and II. *D*, cDNA sequences at the processing site (*DELETE*). *E*, comparison of amino acid sequences flanking *Drosophila* intron II and human intron VII. Amino acids at the splice junction are shown in *bold*.

Northern Blot Analysis—Fig. 4 shows the results of Northern blot analysis. *Drosophila* γ-glutamyl carboxylase mRNA is ~2.7 kb in size (Fig. 4*A*) and is predominantly expressed in 12–24-h embryos (Fig. 4*B*). (However, the more sensitive *in situ* hybridization experiments presented below reveal the presence of carboxylase mRNA throughout embryogenesis.) Ribosomal protein rp49 mRNA was also monitored in these experiments

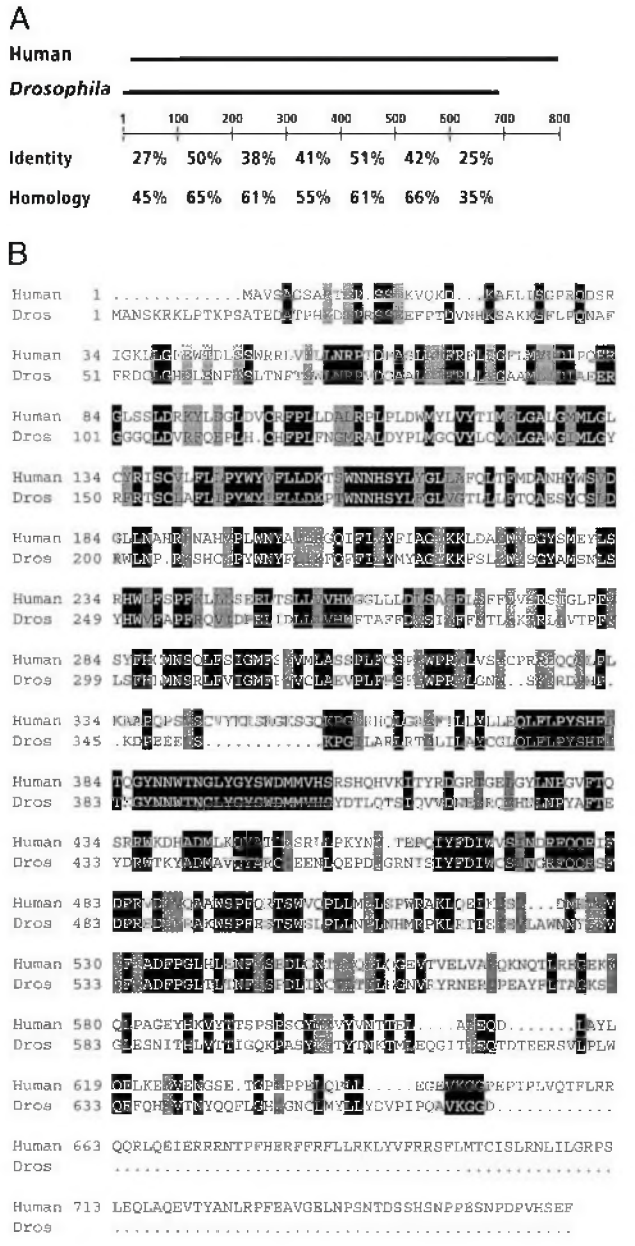

FIG. 3. *A*, schematic of amino acid homology between human and the *Drosophila* γ-glutamyl carboxylase. The two proteins are strongly homologous over the entire length of the predicted *Drosophila* open reading frame. *B*, protein sequence homology between human and *Drosophila* vitamin K-dependent carboxylases. Sequences in *black boxes* indicate identical residues; those in *gray boxes* are homologous residues. *Dots* indicate gaps.

(Fig. 4*C*). Although similar amounts of rp49 mRNA were present in the samples from embryos at different developmental stages (0–24 h), there was less RNA in larvae, pupae, and adult. The reduced level of rp49 mRNA observed at later stages is a reflection of reduced synthesis at these developmental stages (33, 34). From densitometric measurements using the NIH Image analysis program, we determined the amount of γ-carboxylase RNA present relative to rp49 RNA. Normalized to rp49 RNA, we estimate that there is at least three times as much γ-carboxylase RNA in late stage embryos (16–24 h) relative to that in adults.

Spatial Expression of γ-Carboxylase—When embryos were examined to identify the sites of expression of γ-glutamyl carboxylase, ubiquitous expression of γ-carboxylase RNA was observed throughout embryogenesis. Only antisense transcript probes cross-hybridized to embryonic mRNAs *in situ* (Fig. 5, *B* and *D*). No hybridization-positive embryos were observed when sense transcripts were used as probes (Fig. 5, *A* and *C*).

Assays for γ-Carboxylase Activity—Lysates of S2 cells transfected with DNA encoding the putative γ-glutamyl carboxylase were assayed for carboxylase activity. Table II demonstrates that S2 cells transfected with pRmHa-3.GC* expresses vitamin K-dependent γ-carboxylase activity that is not present in mock-transfected cells or cells transfected with pRmHa-3.GFP (data not shown).

Substrate Specificity of γ-Carboxylation—Endogenous substrates of both the mammalian (35) and *Conus* γ-glutamyl carboxylases contain a γ-carboxylation recognition signal (γ-CRS) in the propeptide region (19, 36). The presence of a γ-CRS at the amino terminus of a substrate greatly enhances the efficiency of carboxylation. We have previously identified the γ-CRS of a γ-carboxylated *Conus* peptide, conantokin-G (19). A peptide containing proconantokin-G sequences (−20 to +5, $^{-20}$GKDRLTQMKRILKQRGNKAR $^{-1}$GEEEL^{+5}Y) referred to as −20Y in the text below, is efficiently carboxylated by *Conus* carboxylase (19). We determined the carboxylation of both FLEEL and −20Y by *Conus* and *Drosophila* γ-glutamyl carboxylases. The ratio of $^{14}CO_2$ incorporated in the two substrates (−20Y/FLEEL) by the *Conus* enzyme was 4, whereas that for the *Drosophila* enzyme was 0.26.

DISCUSSION

In this work, we have characterized a cDNA clone derived from adult *D. melanogaster* mRNA that encodes a protein with γ-glutamyl carboxylase activity. The open reading frame is homologous to the mammalian γ-glutamyl carboxylase throughout its length of 670 amino acids; the degree of sequence identity is strikingly high (39% identity and 55% homology) and comparable with the subset of enzymes highly conserved between *Drosophila* and mammals. One unexpected result when comparing the putative genomic DNA sequence with the cDNA is that there is an apparent small deletion in the cDNA sequence, suggesting that RNA processing of the *Drosophila* γ-glutamyl carboxylase mRNA occurs.

Northern blot analysis indicates that the putative *Drosophila* γ-glutamyl carboxylase mRNA is ~2.7 kb in size. This is ~350 nucleotides longer than the open reading frame and 3′-untranslated region previously characterized from cDNA. Because a single RNA isoform is observed, and the 3′-end of the RNA determined by cDNA analysis is unique, the additional 350 nucleotides represent the length of the poly(A) tail together with the 5′-untranslated region. *In situ* hybridization reveals the ubiquitous presence of carboxylase RNA throughout embryogenesis. By reverse transcription-PCR analysis, Li *et al.* (20) have also reported similar observations. In early embryos (0–2 h) it is probably of maternal origin, whereas in later embryos (2–12 h), there is considerable contribution from embryonic synthesis as suggested by the kinetics of RNA synthesis. In this regard, carboxylase mRNA expression has been demonstrated in neuronal and skeletal tissue of postimplantation rat embryos during early organogenesis (26).

The discovery that a γ-carboxylase is expressed in *Drosophila* opens the door to genetic analysis of vitamin K-dependent γ-carboxylation in this model system. The availability of the *Drosophila* γ-glutamyl carboxylase sequence already provides significant structure-function information. Some segments are clearly much more highly conserved when the *Drosophila* sequence is compared with that of mammalian enzymes; presumably, these are domains of the enzyme most critical for function. Among the most noteworthy, mammalian and *Drosophila* sequences corresponding to amino acids 385–404 in the *Drosophila* sequence (see Fig. 3) are identical. An extended con-

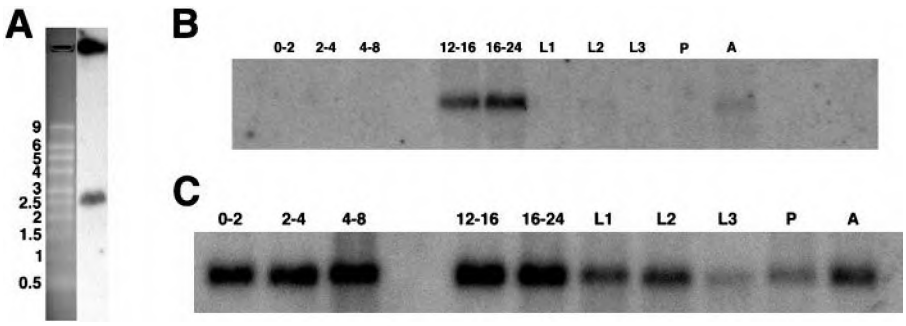

FIG. 4. **Northern blot analysis.** A, total RNA from *Drosophila* embryos (0–24 h) probed with ^{32}P-labeled antisense γ-carboxylase RNA. Molecular weight standards represent ethidium bromide-stained RNA Millennium markers (Ambion). *B* and *C*, analysis of developmental expression of γ-carboxylase (*B*) and rp49 (*C*) RNAs. *Lanes* containing RNA from embryos at 0–2, 2–4, 4–8, 12–16, and 16–24 h of development, larval stages 1–3, pupae, and adult flies are indicated (the *lane* between *4–8*-h and *12–16*-h embryo samples is blank).

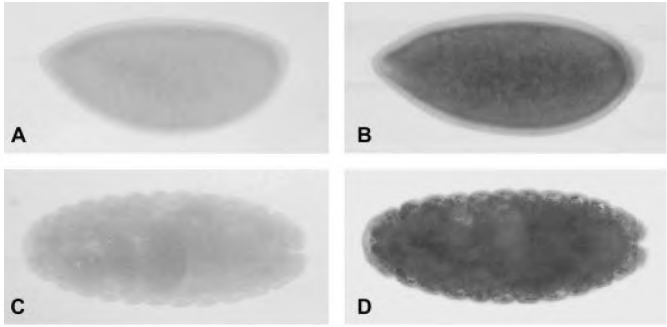

FIG. 5. **Ubiquitous transcription of γ-carboxylase in *Drosophila* embryos.** *In situ* hybridization to stage 5 (*A* and *B*) and 16 (*C* and *D*) whole-mount embryos with digoxigenin labeled strand-specific probes is shown. Ubiquitous gene expression is visible when the antisense strand is used as probe (*B* and *D*) but not when the sense strand is used (*A* and *C*).

TABLE II
Carboxylase activity in transfected Drosophila S2 cells

Enzyme[a]	$^{14}CO_2$ incorporated
	pmol
pRmHa-3.GC*-transfected S2 cell lysate	
Complete reaction	83
Minus vitamin K	4
Minus cell lysate	4
Mock-transfected S2 cell lysate	
Complete reaction	4

[a] Cell lysates from 2×10^5 cells were used in each reaction.

served motif in this region has been suggested by Begley *et al.* (37). However, nucleic acid sequences reported here, by Li *et al.* (20) and by the BDGP (AC005557), are not consistent with the suggestion. The sequences reported here, by Li *et al.* (20) and by the BDGP (AC005557), are ^{385}GYNNWTNGLYGYSWDMMVH^{404}SYDTLQTSIQVVD . . . , whereas the sequences at this site reported by Begley *et al.* (37) are ^{385}GYNNWTNGLYGYSWDMMVH^{404}SRSHQHVKITYRD.

A recently elucidated hereditary disease further emphasizes the functional significance of the total conservation of amino acid sequence in this region: mutation of residue 395 (Leu→Arg) in the human enzyme (38) results in a clinical syndrome characterized by a general deficiency of blood clotting. Interestingly, this clinical condition can be treated satisfactorily by an infusion of high doses of vitamin K, consistent with an enzymatic defect in the affinity of the enzyme for its substrate, reduced vitamin K. Thus, the comparison between *Drosophila* and mammalian enzymes may have helped define a conserved site involved in the binding of reduced vitamin K, consistent with more conventional biochemical studies.

Heterologous γ-CRS sequences are not or are poorly recognized by the γ-glutamyl carboxylases. γ-CRS containing *Conus* substrate, proconantokin G, is poorly carboxylated by the bovine enzyme, whereas a peptide, factor IX-18–41, which consists of the propeptide and all normally carboxylated residues of the vitamin K-dependent clotting protein factor IX, is not carboxylated by the *Conus* enzyme (18). The poor carboxylation of −20Y by the *Drosophila* enzyme further strengthens the suggestion that the enzymes have evolved to recognize their cognate γ-CRSs. This is also supported by the observation of Li *et al.* (20), who found that the propeptide of human blood coagulation factor IX did not stimulate carboxylation by the *Drosophila* enzyme. Because the *Drosophila* and human γ-glutamyl carboxylases share considerable sequence homology, it should be possible to identify substrate binding domains by studying carboxylation using chimeric enzymes.

Because γ-carboxylated molecules may serve as signals for growth and differentiation, differential regulation of γ-carboxylation may operate at multiple levels during development. Control may be at the level of synthesis of γ-carboxylase mRNA or its translation, or both. Although mRNA may be present, enzyme activity may not be obvious. Future experiments will be aimed at determining possible differences among levels of mRNA, expressed protein, and activity by immunological methods (for protein) and γ-carboxylase assay (for activity).

γ-Carboxyglutamate-containing proteins isolated to date are extracellular proteins. γ-Carboxyglutamate interacts with Ca^{2+}, induces a conformational change in the protein, and facilitates binding to membrane phospholipids. A number of γ-carboxyglutamate-containing vitamin K-dependent proteins (thrombin, factor Xa, protein S, and Gas6) are ligands for cell surface receptors. Interaction with the receptors induces cellular proliferative responses (39, 40). In *Drosophila*, high levels of γ-carboxylase RNA are detected in late stage embryos. During this period, a variety of developmental and morphogenetic events occur, among them cuticle deposition and central nervous system, peripheral nervous system, and gut differentiation. It is conceivable that some of the gene products signaling these events are γ-carboxylated and serve as ligands for corresponding receptors. The effects of γ-carboxylase knockout in flies will enable a systematic study of probable targets for this post-translational modification.

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