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Citation

Begley, Gail S., Barbara C. Furie, Eva Czerwiec, Kevin L. Taylor, Gregg L. Furie, Ludmila Bronstein, Johan Stenflo, and Bruce Furie. 2000. "A Conserved Motif within the Vitamin K-Dependent Carboxylase Gene Is Widely Distributed across Animal Phyla." *Journal of Biological Chemistry* 275 (46): 36245–49. <https://doi.org/10.1074/jbc.m003944200>.

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A Conserved Motif within the Vitamin K-dependent Carboxylase Gene Is Widely Distributed across Animal Phyla*

Received for publication, May 9, 2000, and in revised form, June 26, 2000
Published, JBC Papers in Press, July 12, 2000, DOI 10.1074/jbc.M003944200

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The vitamin K-dependent γ -glutamyl carboxylase catalyzes the posttranslational conversion of glutamic acid to γ -carboxyglutamic acid, an amino acid critical to the function of the vitamin K-dependent blood coagulation proteins. Given the functional similarity of mammalian vitamin K-dependent carboxylases and the vitamin K-dependent carboxylase from *Conus textile*, a marine invertebrate, we hypothesized that structurally conserved regions would identify sequences critical to this common functionality. Furthermore, we examined the diversity of animal species that maintain vitamin K-dependent carboxylation to generate γ -carboxyglutamic acid. We have cloned carboxylase homologs in full-length or partial form from the beluga whale (*Delphinapterus leucas*), toadfish (*Opsanus tau*), chicken (*Gallus gallus*), hagfish (*Myxine glutinosa*), horseshoe crab (*Limulus polyphemus*), and cone snail (*Conus textile*) to compare these structures to the known bovine, human, rat, and mouse cDNA sequences. Comparison of the predicted amino acid sequences identified a nearly perfectly conserved 38-amino acid residue region in all of these putative carboxylases. In addition, this amino acid motif is also present in the *Drosophila* genome and identified a *Drosophila* homolog of the γ -carboxylase. Assay of hagfish liver demonstrated vitamin K-dependent carboxylase activity in this hemichordate. These results demonstrate the broad distribution of the vitamin K-dependent carboxylase gene, including a highly conserved motif that is likely critical for enzyme function. The vitamin K-dependent biosynthesis of γ -carboxyglutamic acid appears to be a highly conserved function in the animal kingdom.

γ -Carboxyglutamic acid is a unique amino acid synthesized via the posttranslational modification of specific glutamic acid residues. This reaction is catalyzed by the vitamin K-dependent γ -glutamyl carboxylase in a reaction that requires reduced vitamin K, molecular oxygen, carbon dioxide, and a glutamate-containing peptide substrate. Vitamin K is a required human dietary nutrient, and its sole function is to support the biosyn-

thesis of γ -carboxyglutamic acid. The full-length cDNAs encoding four mammalian vitamin K-dependent carboxylases, human (1), bovine (2), rat (3) and mouse,¹ have been cloned, and comparison of the predicted amino acid sequences has revealed a very high degree of conservation, with sequence identity ranging from 88 to 95%.

Since the discovery in 1974 of γ -carboxyglutamic acid in prothrombin, a vitamin K-dependent blood coagulation protein (5, 6), many laboratories have sought to find this unique amino acid in a broad variety of organisms. Although initial reports identified γ -carboxyglutamic acid convincingly in a number of mammalian proteins that included all of the vitamin K-dependent blood coagulation proteins (7), osteocalcin (8, 9) and matrix gla protein (10) from mineralized tissue, the complexity of the chemical assay of γ -carboxyglutamic acid in crude tissue led to unconfirmed reports of γ -carboxyglutamic acid in *Escherichia coli* and wheat germ ribosomes (11) and in *Limulus* (12). The definitive finding of γ -carboxyglutamic acid in the conotoxins of the marine cone snail demonstrated for the first time the presence of γ -carboxyglutamic acid in invertebrates (13).

To assess the role of γ -carboxyglutamic acid and the importance of vitamin K in animal phyla, we have studied vitamin K-dependent carboxylation in the cone snail and compared this process to γ -carboxylation in mammalian systems. We and others have established numerous common features between mammalian carboxylation and cone snail carboxylation: 1) both have absolute requirements for vitamin K or a vitamin K-like cofactor (14, 15); 2) the propeptide on Glu-containing substrates directs carboxylation by greatly reducing the K_m of the carboxylase reaction (16–19); 3) the vitamin K-dependent carboxylases are membrane proteins (15, 20). The carboxylation recognition sites of carboxylase substrates direct carboxylation with carboxylase from both the bovine and cone snail species, although optimal carboxylation is observed in allotypic systems. The apparent functional similarity of the bovine and *Conus* vitamin K-dependent carboxylase led us to hypothesize that regions important to unique function would be highly conserved. To identify such highly conserved regions and establish useful molecular probes for the presence of the carboxylase gene in diverse animal species, we have determined the complete or partial carboxylase cDNA sequences from a variety of vertebrate and invertebrate species. Here, we describe the full-length clones of the beluga whale (*Delphinapterus leucas*) and toadfish (*Opsanus tau*) carboxylases. Using probes based upon the regions of sequence identity between the toadfish carboxylase sequence and the sequences of the mammalian

* This work was supported by National Institutes of Health Grants HL38216 and HL42443. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF278713 and AF278714.

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carboxylases, we have isolated partial clones of the vitamin K-dependent carboxylase genes from the cone snail (*Conus textile*), the hagfish (*Myxine glutinosa*), chicken (*Gallus gallus*), and horseshoe crab (*Limulus polyphemus*) and established a 38-residue region of the carboxylase that is nearly perfectly conserved. These studies indicate that the vitamin K-dependent biosynthesis of γ -carboxyglutamic acid is a highly conserved function in the animal kingdom.

EXPERIMENTAL PROCEDURES

Materials—mRNA from Beluga whale liver, toadfish liver, hagfish liver, and horseshoe crab hepatopancreas were the generous gift of Dr. N. Cornell at the Marine Biological Laboratory (Woods Hole, MA). Horseshoe crab blood was provided by Dr. N. Wainwright (Marine Biological Laboratory). The chicken liver cDNA library was purchased from Stratagene (La Jolla, CA). Live cone snails were obtained from Fiji and imported into the United States under the jurisdiction of the United States Fish and Wildlife Service. TRIzol reagent, Superscript reverse transcriptase, and synthetic oligonucleotide primers were purchased from Life Technologies, Inc. RACE² kits and Advantage cDNA polymerase mix were obtained from CLONTECH (Palo Alto, CA). TOPO TA cloning and FastTrack mRNA purification systems were purchased from Invitrogen (Carlsbad, CA). Reagents for DNA purification were from Qiagen (Santa Clarita, CA). Amplitaq Gold polymerase was purchased from PE Applied Biosystems (Foster City, CA). All other reagents were of highest grade available.

RNA Extraction and Generation of cDNA—RNA was extracted from frozen cone snail (*C. textile*) venom ducts with TRIzol reagent, and poly(A)⁺ RNA was isolated with oligo(dT) cellulose (FastTrack) according to the manufacturer's recommended protocols. Horseshoe crab blood was collected and immediately centrifuged to pellet cells. RNA was extracted and poly(A)⁺ RNA purified using the FastTrack system. First strand cDNA synthesis was performed with Superscript reverse transcriptase according to the manufacturer's protocol.

Degenerate Primer PCR Amplification—cDNA was amplified with Amplitaq Gold DNA polymerase in Geneamp buffer (PE Biosystems) using 1 μ M degenerate primers (Table I) in a PerkinElmer Life Sciences 9700 thermocycler. Cycling conditions were as follows: 95 °C, 9-min preactivation, five cycles each at annealing temperatures of 60, 55, and 50 °C and 25 cycles at an annealing temperature of 45 °C with extension times at 72 °C increasing 2 s/cycle starting from 1 min 30 s.

Nested Primer PCR—cDNAs that produced no amplification product following PCR with degenerate primers were reamplified using nondegenerate nested primers based upon the toadfish DNA sequence (Table I, fish gene-specific primers). For the nested reaction, 2 μ l of the first PCR reaction was used as template with 0.2 μ M nondegenerate primers. Cycling conditions were identical to those used in the first reaction.

RACE—SMART RACE technology (CLONTECH) was used to obtain full-length cDNA sequence. First strand cDNA was synthesized with Superscript reverse transcriptase, and adaptor sequences were incorporated via oligonucleotide primers during this step. The resulting cDNA preparations were used as templates in PCR reactions containing 0.2 μ M gene-specific primer (see Table I) and universal primer mix (CLONTECH). cDNA was amplified with Advantage cDNA polymerase mix in a PerkinElmer Life Sciences thermocycler according to the manufacturer's recommended protocols.

Cloning and Sequencing—Single-stranded overhangs generated by the polymerase during PCR were used to clone PCR products into vector pCR2.1-TOPO. The ligation reactions were used to transform competent TOP10 *E. coli* by heat shock. Transformants were selected on LB agar containing 50 mg/ml kanamycin and containing 5-bromo-4-chloro-3-indolyl β -D-galactopyranoside for blue/white screening. White colonies were analyzed by PCR and/or restriction enzyme digest for inserts. Plasmid DNA was extracted from positive clones by alkaline lysis column minipreps (Qiagen). DNA was sequenced on an Applied Biosystems 373 DNA sequencer.

In Vitro Assay of Carboxylase Activity—Microsomes were prepared from hagfish liver using standard techniques (17). Carboxylase activity was assayed as the incorporation of ¹⁴CO₂ into the synthetic peptide FLEEL, as described previously (17).

TABLE I
Oligonucleotide primers used in PCR amplification

Primer	Sequence
Carboxylase degenerate primer 1 ^a	ACICARGGITAYAAAYATGG
Carboxylase degenerate primer 2 ^a	CATRTCIGCRTGRCTCYTTCCA
Whale gene-specific primer 1	GGTCTTCCAACGCCGGCTCTGTG
Whale gene-specific primer 2	AGGGGTACAACAACCTGGACAAACGG
Fish gene-specific primer 1	GGTCTTCCAACGACGGCTTTGTG
Fish gene-specific primer 2	AAGGGTACAACAACCTGGACCAACGGC

^a Code for degenerate nucleotide positions: R = A + G, Y = C + T; I = deoxyinosine.

RESULTS

Molecular Cloning of Vitamin K-dependent Carboxylase cDNAs from Toadfish and Beluga Whale—Nondegenerate primers based on bovine and rat carboxylase nucleotide sequences and degenerate primers derived from predicted amino acid sequences of the most highly conserved regions of the published mammalian carboxylases were used in PCR reactions with cDNA templates derived from toadfish liver and whale liver mRNA. Only a single set of degenerate primers yielded a specific amplification product of the predicted size from both species (Table I). The 180-base pair fragments from both species were cloned into pCR2.1-TOPO and the insert sequenced. The predicted amino acid sequences were 94 and 100% identical to the bovine sequence for toadfish and whale clones, respectively.

Gene-specific primers (Table I) were designed for each species and used to amplify 5'- and 3'-flanking regions by RACE PCR. RACE products were cloned and sequenced, and full-length cDNA sequences assembled. The whale cDNA contains an open reading frame of 2274 nucleotides (GenBankTM accession number AF278713) encoding a predicted protein of 758 amino acids (Fig. 1). The predicted full-length amino acid sequence is 92% identical to the human sequence and 94% identical to the bovine sequence. This marked similarity among these mammalian vitamin K-dependent carboxylases precluded identification of regions of functional importance, since there were few sequence differences among the five carboxylases. The toadfish cDNA contains an open reading frame of 2286 nucleotides (GenBankTM accession number AF278714) encoding a predicted protein of 762 amino acids (Fig. 1). The deduced toadfish translation product has five insertions relative to the mammalian sequences, one of 7 amino acids, three of 2 amino acids and one of 1 amino acid, one deletion of 2 amino acids and a 9 amino acid truncation, relative to the mammalian sequences.

Analysis of alignments of the predicted protein products of these full-length cDNAs and the published carboxylase sequences revealed extremely high conservation of the central region of the protein. One-hundred twenty-four of the 131 amino acids between residues 365 and 496 (bovine numbering) are identical or highly conserved in all the mammalian and the single fish species. Indeed, residues 365–418 are conserved, and residues 384–415 are identical (Fig. 1). Although the mammalian carboxylase sequences are near perfectly conserved, there are 105 residues of 758 where the toadfish amino acid disrupts conserved residues at the same position for bovine, human, rat, mouse, and whale carboxylases. Given the extremely high conservation of the mammalian carboxylases, we relied heavily on the fish carboxylase to indicate regions that might be conserved across animal phyla and focused on the central region of sequence identity as a strategy for exploring nonvertebrate carboxylases.

² The abbreviations used are: RACE, rapid amplification of cDNA ends; PCR, polymerase chain reaction.

bovine	MAVS--ARPARAPRGS ^{DRVKKDKAAQ} --TSGPRQGR ^{MRGKLLGFEWT} DVSSWRLVTL ^{LLN}
human	MAVS--AGSARTSPSS ^{SDKVQDKKAE} --ISGPRQDSR ^{IGKLLGFEWT} DLSSWRRVLT ^{LLN}
rat	MAVH--RGSARAAPAS ^{DKVQKNKPAQ} --TSGLEQGR ^{MARI FGFWEADLSSWQS} VVTL ^{LLN}
mouse	MAVH--RGSALVAPAS ^{DKVQKNKSAQ} --TSGLKQGR ^{MRMKEILGFEWT} DLSSWQS ^{VVTL} LLN
whale	MAVS--ARSARSP ^{SDKVQDKKAGQ} --TSGRRQGR ^{MRGKLLGFEWT} DVSSW ^{GKLVTL} LLN
fish	MEARDATAGAFV ^{SSDSEKESR} KQADARK ^{DTLPAKSRMEQLGFEW} KKEDLTS ^{SRVLV} ALLN
bovine	RPTDPAGLAVFR ^{FLFGLMMVLDI} POERGLSS ^{LDRYLDGLDVC} RFP ^{LLDALQ} PLP ^{LDW} MY
human	RPTDPASLAVFR ^{FLFGLMVLDI} POERGLSS ^{LDRYLDGLDVC} RFP ^{LLDALR} PLP ^{LDW} MY
rat	RPTDPANLAVFR ^{FLFAFLMLLDI} POERGLSS ^{LDRYLDGLDVC} RFP ^{LLDALR} PLP ^{LDW} MY
mouse	KPTDPANLAVFR ^{FLFAFLMLLDI} POERGLSS ^{LDRYLDGLDVC} RFP ^{LLDALR} PLP ^{LDW} MY
whale	RPTDPASLAVFR ^{FLFGLMMVLDI} POERGLSS ^{LDRYLDGLDVC} RFP ^{LLDALQ} PLP ^{LDW} MY
fish	RPTDPASL ^{GLFR} FLFGLM ^{AIDITQ} ERGLS ^{HLDYKLDGSL} VCRF ^{PLFNF} LO ^{PL} LDW ^{MY}
bovine	LYITIMFLGALG ^{MMLG} LCYRISCV ^{LFL} LYWYV ^{FLLDKT} SN ^{NHNSY} LYGLLAF ^{QIT} FMDA
human	LVYITIMFLGALG ^{MMLG} LCYRISCV ^{LFL} LYWYV ^{FLLDKT} SN ^{NHNSY} LYGLLAF ^{QIT} FMDA
rat	LVYITIMFLGALG ^{MMLG} LCYRISCV ^{LFL} LYWYV ^{FLLDKAS} SN ^{NHNSY} LYGLLAF ^{QIT} FMDA
mouse	LVYITIMFLGALG ^{MMLG} LCYRISCV ^{LFL} LYWYV ^{FLLDKT} SN ^{NHNSY} LYGLLAF ^{QIT} FMDA
whale	LVYITIMFLGALG ^{MMLG} LCYRISCV ^{LFL} LYWYV ^{FLLDKT} SN ^{NHNSY} LYGLLAF ^{QIT} FMDA
fish	MVYV ^{VMFV} GALG ^{IMLGC} FYR ^{LSCL} MF ^{ISSY} WYV ^{IF} FLDK ^{TAN} NHNSY ^{LYGL} IG ^{FGI} AL ^{MDG}
bovine	HHYWSVDGLLR ^{ARKRNAH} VPLW ^{NYAVLRG} QIFIVY ^{FIAGV} KKL ^{DADW} VEGYS ^{MEYLS} RHW
human	NHYWSVDGLLN ^{AHRRNAH} VPLW ^{NYAVLRG} QIFIVY ^{FIAGV} KKL ^{DADW} VEGYS ^{MEYLS} RHW
rat	NHYWSVDGLLSA ^{QKRNAH} VPLW ^{NYAVLRG} QIFIVY ^{FIAGV} KKL ^{DADW} VEGYS ^{MEHLS} RHW
mouse	NHYWSVDGLLN ^{ARKRNAH} VPLW ^{NYAVLRG} QIFIVY ^{FIAGV} KKL ^{DADW} VEGYS ^{MEHLS} RHW
whale	NRYWSVDGLLSA ^{RKRNAH} VPLW ^{NYAVLRG} QIFIVY ^{FIAGV} KKL ^{DADW} VEGYS ^{MEYLS} RHW
fish	NRYW ^{SLDGLR} PVIR ^{NAH} VPLW ^{NYAVLRG} QIFIVY ^{FIAGV} KKL ^{DADW} VEGYS ^{MSYLA} RHW
bovine	LFSPFKLVSEEM ^{TSLLVVH} CG ^{LLLDL} SAG ^{FLLFFD} ASR ^{PIGFV} VS ^{YFHC} MNS ^{QLFS} I
human	LFSPFKLLSEEM ^{TSLLVVH} CG ^{LLLDL} SAG ^{FLLFFD} VSR ^{IGLFF} VS ^{YFHC} MNS ^{QLFS} I
rat	LFSPFKLVSEEM ^{TSLLVVH} CG ^{LLLDL} SAG ^{FLLFFD} ASR ^{PIGLF} VS ^{YFHC} MNS ^{QLFS} I
mouse	LFSPFKLVSEEM ^{TSLLVVH} CG ^{LLLDL} SAG ^{FLLFFD} ASR ^{PVGLF} VS ^{YFHC} MNS ^{QLFS} I
whale	LFSPFKLVSEEM ^{TSLLVVH} CG ^{LLLDL} SAG ^{FLLFFD} ASR ^{IGLFF} VS ^{YFHC} MNS ^{QLFS} I
fish	LF ^{DPFR} VIL ^{PVEL} VS ^{LLVVH} CG ^{LLLDL} TAG ^{FLFFD} ATR ^{PYAF} FFV ^{TY} FHC ^{MNS} QLFS ^I
bovine	GMFPYV ^{MLASS} PL ^{FCS} PEW ^{PRKLV} AHC ^{PKK} LQ ^{ELLE} PL ^R TA--PQ ^{PST} SC ^{MY} -----KRS
human	GMFSYV ^{MLASS} PL ^{FCS} PEW ^{PRKLV} SY ^{CP} R ^{LQ} ELL ^{PL} KAA--PQ ^{PSV} SC ^{VY} -----KRS
rat	GMFPYV ^{MLASS} PL ^{FCS} SAE ^{WPRKLV} ARC ^{PKR} LQ ^{ELL} PAKAA--PR ^{FAS} CV ^Y -----KRA
mouse	GMFPYV ^{MLASS} PL ^{FCS} SAE ^{WPRKLV} ARC ^{PKR} LQ ^{ELL} TKAA--PR ^{FAS} CV ^Y -----KRS
whale	GMFPYV ^{MLASS} PL ^{FCS} PEW ^{PRKLV} AHC ^{PKK} LQ ^{ELLE} PL ^R TA--PQ ^{PAS} CV ^Y -----KRS
fish	GMFPY ^{AMLAT} S ^{PLFC} Y ^{PW} RR ^{RF} SH ^{FA} PL ^{VALE} LS ^{TP} D ^{OR} ST ^{SC} LY ^{HE} LQ ^{NT} K ^R HE
bovine	RARGSQ ^{KPGLR} HK ^{LST} AFT ^{LLY} LE ^{QL} FL ^{PY} SH ^{FT} Q ^{GY} NN ^{WT} NG ^{LY} GY ^{SD} MM ^{VH} SR ^{SH}
human	RAGSGQ ^{KPGLR} HQ ^{LGA} AFT ^{LLY} LE ^{QL} FL ^{PY} SH ^{FT} Q ^{GY} NN ^{WT} NG ^{LY} GY ^{SD} MM ^{VH} SR ^{SH}
rat	RKAGQ ^{KPGLR} HL ^{GT} VFT ^{LLY} LE ^{QL} FL ^{PY} SH ^{FT} Q ^{GY} NN ^{WT} NG ^{LY} GY ^{SD} MM ^{VH} SR ^{SH}
mouse	RKAGQ ^{KPGLR} HQ ^{LGA} I ^{FT} LLY ^{LE} QL ^{FL} PY ^{SH} FT ^Q GY ^{NN} WT ^{NG} LY ^{GY} SD ^{MM} VH ^{SR} SH
whale	RKAGSQ ^{KPGLR} HL ^{GAA} AFT ^{LLY} LE ^{QL} FL ^{PY} SH ^{FT} Q ^{GY} NN ^{WT} NG ^{LY} GY ^{SD} MM ^{VH} SR ^{SH}
fish	TVAKAS K ^{PKL} HK ^{LAA} I ^{FT} I ^F Y ^{IME} Q ^L FM ^{PY} SH ^{FT} Q ^{GY} NN ^{WT} NG ^{LY} GY ^{SD} MM ^{VH} SR ^{SH}
bovine	QHVKI ^{TYR} DGR ^{TG} EL ^{GY} LN ^{PG} VFT ^Q SR ^{RWK} D ^{HAD} ML ^{KQ} YAT ^{CLS} RL ^L PK ^{YN} VT ^{EP} Q ^I Y ^{FD}
human	QHVKI ^{TYR} DGR ^{TG} EL ^{GY} LN ^{PG} VFT ^Q SR ^{RWK} D ^{HAD} ML ^{KQ} YAT ^{CLS} RL ^L PK ^{YN} VT ^{EP} Q ^I Y ^{FD}
rat	QHVKI ^{TYR} DGR ^{TG} EL ^{GY} LN ^{PG} VFT ^Q SR ^{RWK} D ^{HAD} ML ^{KQ} YAT ^{CLS} RL ^L PK ^{YN} VT ^{EP} Q ^I Y ^{FD}
mouse	QHVKI ^{TYR} DGR ^{TG} EL ^{GY} LN ^{PG} VFT ^Q SR ^{RWK} D ^{HAD} ML ^{KQ} YAT ^{CLS} RL ^L PK ^{YN} VT ^{EP} Q ^I Y ^{FD}
whale	QHVKI ^{TYR} DGR ^{TG} EL ^{GY} LN ^{PG} VFT ^Q SR ^{RWK} D ^{HAD} ML ^{KQ} YAT ^{CLS} RL ^L PK ^{YN} VT ^{EP} Q ^I Y ^{FD}
fish	QHVKI ^{TYR} DGR ^{TG} E ^{IG} Y ^{LN} PG ^{VFT} Q ^{SR} RWK ^{DH} G ^{DM} L ^{KQ} YAT ^{CLS} OLL ^{PR} Y ^{NS} D ^{EP} E ^I Y ^{FD}
bovine	IWVSIN ^{DR} FQ ^{OR} I ^{FD} PR ^{VDI} VQ ^A AW ^S PF ^{ORT} PW ^{LQ} PL ^{LD} LS ^{PW} RT ^{KLQ} E ^{IK} SS ^{LD} NH ^{TE}
human	IWVSIN ^{DR} FQ ^{OR} I ^{FD} PR ^{VDI} VQ ^A AW ^S PF ^{ORT} SW ^Q PL ^{LD} LS ^{PW} RA ^{KLQ} E ^{IK} SS ^{LD} NH ^{TE}
rat	IWVSIN ^{DR} FQ ^{OR} L ^{FD} PR ^{VDI} VQ ^A V ^S PF ^{ORT} PW ^Q PL ^{LD} LS ^{PW} RT ^{KLQ} D ^{IK} SS ^{LD} NH ^{TE}
mouse	IWVSIN ^{DR} FQ ^{OR} L ^{FD} PR ^{VDI} VQ ^A V ^S PF ^{ORT} PW ^Q PL ^{LD} LS ^{PW} RT ^{KLQ} D ^{IK} SS ^{LD} NH ^{TE}
whale	IWVSIN ^{DR} FQ ^{OR} I ^{FD} PR ^{VDI} VQ ^A T ^{WS} PF ^{ORT} PW ^{LQ} PL ^{LD} LS ^{PW} RT ^{KLQ} E ^{IK} SS ^{LD} NH ^{TE}
fish	IWVSIN ^{ER} FQ ^{OR} I ^{FN} Q ^{VDI} V ^K AD ^{WS} PF ^{RNT} W ^{IM} PL ^{LD} LS ^{PW} RT ^K FQ ^E I ST LD ^{NQ} TE
bovine	VVFIAD ^{FP} GL ^{HLEN} FV ^{SE} DL ^{GNT} SI ^{QL} LQ ^{GE} VT ^{VEL} V ^{AE} CK ^{NOT} LQ ^E G ^E K ^M Q ^L PAGE ^{YHK}
human	VVFIAD ^{FP} GL ^{HLEN} FV ^{SE} DL ^{GNT} SI ^{QL} LQ ^{GE} VT ^{VEL} V ^{AE} CK ^{NOT} LQ ^E G ^E K ^M Q ^L PAGE ^{YHK}
rat	VVFIAD ^{FP} GL ^{HLEN} FV ^{SE} DL ^{GNT} SI ^{QL} LQ ^{GE} VT ^{VEL} V ^{AE} CK ^{NOT} LQ ^E G ^E K ^M Q ^L PAGE ^{YHK}
mouse	VVFIAD ^{FP} GL ^{HLEN} FV ^{SE} DL ^{GNT} SI ^{QL} LQ ^{GE} VT ^{VEL} V ^{AE} CK ^{NOT} LQ ^E G ^E K ^M Q ^L PAGE ^{YHK}
whale	VVFIAD ^{FP} GL ^{HLEN} FV ^{SE} DL ^{GNT} SI ^{QL} LQ ^{GE} VT ^{VEL} V ^{AE} CK ^{NOT} LQ ^E G ^E K ^M Q ^L PAGE ^{YHK}
fish	LVFIAD ^{FP} GL ^{HLEN} FV ^{SE} DL ^{GNT} SV ^Q V ^{LQ} G ^{VN} VE ^{VV} DE ^K KN ^Y T ^{LQ} EP ^{EQ} M ^Q V ^{SS} GY ^{HK}
bovine	VYTVSS ^{SP} SC ^{MY} IY ^{VN} TE ^{VA} LQ ^{DL} AY ^{LQ} EL ^{KE} V ^{EN} GS ^{ET} GL ^{PP} EL ^Q PLI--EGEV
human	VYTVSS ^{SP} SC ^{MY} IY ^{VN} TE ^{VA} LQ ^{DL} AY ^{LQ} EL ^{KE} V ^{EN} GS ^{ET} GL ^{PP} EL ^Q PLI--EGEV
rat	VYTVSS ^{SP} SC ^{MY} IY ^{VN} TE ^{VA} LQ ^{DL} AY ^{LQ} EL ^{KE} V ^{EN} GS ^{ET} GL ^{PP} EL ^Q PLI--EGEV
mouse	VYTVSS ^{SP} SC ^{MY} IY ^{VN} TE ^{VA} LQ ^{DL} AY ^{LQ} EL ^{KE} V ^{EN} GS ^{ET} GL ^{PP} EL ^Q PLI--EGEV
whale	VYTVSS ^{SP} SC ^{MY} IY ^{VN} TE ^{VA} LQ ^{DL} AY ^{LQ} EL ^{KE} V ^{EN} GS ^{ET} GL ^{PP} EL ^Q PLI--EGEV
fish	VYTVSS ^{ED} SC ^{MY} IY ^{VN} TE ^{AA} LQ ^{DN} FT ^K L ^E I ^Q ER ^V R ^{NG} T ^E T ^E PL ^{PP} EL ^Q PLI ^{AA} D ^{EA}
bovine	KGGPE ^{PT} PL ^{VQ} T ^{FL} RR ^Q R ^{LQ} E ^I ERR ^N AP ^{PH} ER ^{IL} V ^{FL} LR ^K L ^F I ^F RR ^S FL ^{MT} TR ^{IS} LR ^{NL}
human	KGGPE ^{PT} PL ^{VQ} T ^{FL} RR ^Q R ^{LQ} E ^I ERR ^N T ^{PH} ER ^{FR} FL ^{LR} K ^L Y ^F RR ^S FL ^{MT} TR ^{IS} LR ^{NL}
rat	KGGPE ^{PT} PL ^{VQ} T ^{FL} RR ^Q R ^{LQ} E ^I ERR ^{NS} PL ^{HER} EL ^{FR} V ^{LR} K ^L Y ^F RR ^S FL ^{MT} TR ^{IS} LR ^{NL}
mouse	KGGPE ^{PT} PL ^{VQ} T ^{FL} RR ^Q R ^{LQ} E ^I ERR ^{NS} PL ^{HER} EL ^{FR} V ^{LR} K ^L Y ^F RR ^S FL ^{MT} TR ^{IS} LR ^{NL}
whale	KGGPE ^{PT} PL ^{VQ} T ^{FL} RR ^Q R ^{LQ} E ^I ERR ^N AP ^{PH} ER ^{IL} FL ^{LR} K ^L Y ^F RR ^S FL ^{MT} TR ^{IS} LR ^{NL}
fish	AEVN AT ^D PI ^{VRL} FL ^{KR} QR ^{RI} KE ^V K ^R KE ^{AG} V ^{LER} I ^{OR} F ^{AV} K ^Y TT ^{IR} RG ^{FL} MT ^{AT} AM ^{RN} I
bovine	AFGR ^{PS} LE ^Q LA ^Q EV ^{TY} AN ^{LR} PF ^E AV ^{GL} NP ^{SN} T ^D SS ^N PN ^{PF} EP ^D SH ^{PV} H ^{SE} F
human	ILGR ^{PS} LE ^Q LA ^Q EV ^{TY} AN ^{LR} PF ^E AV ^{GL} NP ^{SN} T ^D SS ^N PN ^{PF} ES ^N DP ^V H ^{SE} F
rat	LFGR ^{PS} LE ^Q LA ^Q EV ^{TY} AN ^{LR} PF ^E V ^D ESS ^{AS} N ^T D ^{SS} DP ^H SE ^P D ^{SE} H ^V H ^{SE} L
mouse	LLGR ^{PS} LE ^Q LA ^Q EV ^{TY} AN ^{LR} PF ^E V ^D ESS ^{AS} N ^T D ^{SS} N--HP ^{SE} PD ^{SE} H ^V H ^{SE} F
whale	VLGR ^{PS} LE ^Q LA ^Q EV ^{TY} AN ^{LR} PF ^E V ^{GE} PS ^{PN} T ^D SS ^N PN ^{PF} SE ^{PN} AD ^{AV} H ^{SE} F
fish	AVGL ^{PE} LQ ^L T ^{RE} V ^A FAN ^{MK} --E ^Q AD ^{AN} PD ^{ER} LK ^{DE} V ^{GH} EL-----

FIG. 1. Comparison of the amino acid sequence similarity predicted from the full-length nucleotide sequences of the vitamin K-dependent carboxylases of toadfish (*fish*) and beluga whale (*whale*) with the known amino acid sequences of bovine, human, rat, and mouse. Regions of near perfect homology in all six sequences are highlighted by shading. Residues in the predicted toadfish sequence that disrupt the sequence homology within the mammalian carboxylases are shown in bold. The highly conserved region 374–418 common to all of these carboxylases is shown in red.

Molecular Cloning of a Conserved Carboxylase Motif from Vertebrate and Invertebrate Species—We elected to evaluate hagfish as a primitive fish (*hemichordata*) that predates the chordates phylogenetically and to evaluate *Conus*, since we and others have studied γ -carboxyglutamic acid and the vitamin

K-dependent carboxylase in this invertebrate (14, 13, 15, 21, 22). Using the degenerate primers for the highly conserved amino acid motif (Table I), cDNA from chicken liver was PCR amplified. The sequence of the resulting DNA fragment predicted a translation product 92% identical to the corresponding

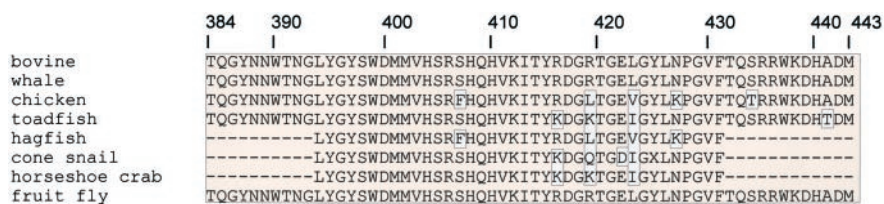


FIG. 2. **A conserved motif.** Multiple sequence alignment of amino acid sequences deduced from the cDNAs. Amino acids with sequence identity to the bovine vitamin K-dependent carboxylase are shaded. Residues that differ from the bovine sequence are boxed. Bovine, beluga whale (*Delphinapterus leucas*), toadfish (*Opsanus tau*), chicken (*Gallus gallus*), hagfish (*Myxine glutinosa*), horseshoe crab (*Limulus polyphemus*), and cone snail (*Conus textile*). In addition, a homologous sequence from fruit fly (*Drosophila melanogaster*) is shown. Amino acid numbering is based on the bovine vitamin K-dependent carboxylase sequence.

region of the bovine carboxylase. No product was observed after primary amplification with cDNA templates from hagfish liver, cone snail venom duct, and horseshoe crab hepatopancreas or blood using degenerate primers. However, a product of the correct predicted size was obtained for each species in a secondary PCR reaction using nested primers based on the toadfish DNA sequence (Table I; fish gene-specific primers) and the reaction mixture from the primary amplification. The alignment of these partial carboxylase sequences, from residues 384 to 443 using the bovine numbering, is shown in Fig. 2. This region is very highly conserved across these disparate vertebrate and invertebrate species. From residues 393 to 432, there are only 3 nonconservative substitutions among the bovine, chicken, toadfish, hagfish, cone snail, and horseshoe crab sequences. At residue 407, the chicken and hagfish carboxylases have a phenylalanine, while the other species studied have a serine. At residue 419, chicken and hagfish carboxylases have a leucine, the cone snail carboxylase has a glutamine, and other species have a basic amino acid, either lysine or arginine. At residue 427, the chicken and hagfish carboxylases have a lysine while the other species studied have an asparagine.

Demonstration of Carboxylase Activity in Hagfish—Carboxylase enzyme activity has been demonstrated in the chicken (23) and the cone snail (14, 15), and there is a single report of γ -carboxyglutamic acid in the horseshoe crab (12). To confirm the presence of carboxylase activity in hagfish, where it has not previously been observed, *in vitro* carboxylation assays were performed. Microsomes were prepared from hagfish liver and assayed for vitamin K-dependent incorporation of $^{14}\text{CO}_2$ into the synthetic carboxylase substrate Phe-Leu-Glu-Glu-Leu. In the presence of reduced vitamin K, the carboxylase in hagfish microsomes incorporated 2219 cpm of $^{14}\text{CO}_2$ into FLEEL. When proPT18, an 18-residue peptide based upon the sequence of the propeptide of prothrombin, is included in the reaction mixture, 10,064 cpm of $^{14}\text{CO}_2$ were incorporated into FLEEL—a stimulation of about 5-fold. ProPT18 also stimulates carboxylation of FLEEL by mammalian carboxylases (24). In the absence of vitamin K, no carboxylation was detected with the hagfish microsomes, indicating that the hagfish microsomes contain a carboxylase activity that is dependent upon the presence of vitamin K.

DISCUSSION

Although γ -carboxyglutamic acid has been found in vertebrates and a single invertebrate, the marine snail of the genus *Conus*, we have been unable to clone the *Conus* carboxylase cDNA using DNA probes derived from the bovine carboxylase or rat carboxylase cDNA. It would appear that the vitamin K-dependent carboxylase, albeit very well conserved in mammals, does not have sufficient DNA sequence similarity to allow identification of the carboxylase in tested invertebrate species. To explore structure-function relationships in an enzyme that has no significant homology to other proteins, we predicted that direct comparison of the amino acid sequences of functionally

homologous carboxylases derived from various species across animal phyla would allow identification of conserved amino acid sequences that are critical for the function of this enzyme. All vitamin K-dependent carboxylases should contain an active site for carboxylase activity, an active site for epoxidase activity, a vitamin K binding site, and a binding site for the carboxylation recognition site on the substrate (24). To this end, we obtained the full-length clones of the beluga whale carboxylase and the toadfish carboxylase to distinguish variable regions from well conserved regions, since the human, bovine, mouse, and rat carboxylase sequences are so similar. Comparison of the toadfish carboxylase sequence and the bovine carboxylase sequence revealed a single region, from 384 to 415 consisting of 32 amino acids that share the same sequence. We speculate that this may be a critical sequence for the function of this enzyme and that all vertebrate and invertebrate vitamin K-dependent carboxylases would share this sequence. Using probes based upon the toadfish cDNA, we were able to isolate cDNA encoding this highly conserved region in the carboxylases derived from chicken, hagfish, cone snail, and horseshoe crab. These results indicate that the vitamin K-dependent carboxylase is broadly distributed across animal phyla, and this motif is very highly conserved in this enzyme regardless of species of origin.

Mapping of the vitamin K-dependent carboxylase to identify functional regions has revealed several general insights, some consistent with the identification of a functionally important motif within the central region of the enzyme. Roth (25) demonstrated that truncation of 46 amino acids at the C terminus of bovine carboxylase did not interfere with enzymatic function *in vitro*, whereas truncation of 82 residues from the C terminus interfered with epoxidation but not propeptide binding or glutamate binding. A naturally occurring carboxylase mutation in a patient with deficiency of the vitamin K-dependent carboxylase occurs at leucine 394 (26), within the most conserved region of the motif that we have identified. This residue is highly conserved, but it is not known how this mutation interferes with enzymatic activity. Modified scanning mutagenesis of bovine carboxylase included mutation of arginine 406 to alanine and histidine 408 to alanine (27). Expression of this double mutant in CHO cells was characterized by a carboxylase activity with a requirement for high propeptide concentrations for stimulation of carboxylation of FLEEL, suggesting defects in the propeptide binding site.

To confirm the presence of the vitamin K-dependent carboxylase in hemichordates, we assayed hagfish liver. Hagfish exhibited carboxylase activity that was dependent upon the presence of vitamin K. Because the propeptide of both low K_m mammalian carboxylase substrates and *Conus* carboxylase substrates are required for efficient carboxylation at low substrate concentration, and because the carboxylation recognition site in these propeptides differs (18, 19), we used FLEEL as a high K_m generic substrate for these assays. Bovine vitamin

K-dependent carboxylase has a K_m in the millimolar range with FLEEL. Microsomal preparations from hagfish demonstrate the carboxylation of FLEEL via the incorporation of CO_2 . However, the hepatopancreas preparation from horseshoe crab was highly atypical of microsomal preparations and contained large amounts of lipid. On this basis, the carboxylase assay of this material was considered unreliable. We have not evaluated any of these nonvertebrate carboxylases using mammalian substrates containing propeptides, but we speculate that these would not be low K_m substrates.

Using a consensus sequence derived from alignment of the cDNA sequences reported here, the nonredundant nucleotide data base was searched with the tBLASTn program. The only significant match obtained, other than the published mammalian carboxylase cDNAs, was a small stretch of *Drosophila melanogaster* genomic sequence (GenBankTM accession number AC005557). Genefinder analysis of this clone revealed a putative gene consisting of 3 exons and encoding a 629-amino acid-long sequence with 44% identity to the human carboxylase. Alignment analyses revealed that the most highly conserved region (corresponding to residues 384–443 of the bovine carboxylase) is the amino acid sequence that is completely conserved in all published mammalian sequences and almost completely conserved in all of the sequences obtained in this study (Fig. 2). Stafford and colleagues (28) have recently made a similar observation. They cloned this gene from *Drosophila*. Expression of this gene in insect cells resulted in the expression of vitamin K-dependent carboxylase activity, thus demonstrating that the product of this homologous gene has functional homology as well. Neither γ -carboxyglutamic acid nor γ -carboxyglutamic acid-containing proteins have been identified in *Drosophila*.

The marked homology of the mammalian carboxylases suggests the importance of vitamin K and the synthetic machinery to generate γ -carboxyglutamic acid. The evolutionary conservation of this complex enzymatic system requiring exogenous vitamin K or a vitamin K-like cofactor and multiple enzymes for vitamin K transport and metabolism suggests a critical role for γ -carboxyglutamic acid that has been retained throughout phylogeny. γ -Carboxyglutamic acid is important for calcium binding and plays a critical functional role in generating the membrane binding properties of the vitamin K-dependent blood clotting proteins (24). The role of γ -carboxyglutamic acid in other mammalian proteins such as osteocalcin and matrix Gla protein remains uncertain. The role of γ -carboxyglutamic acid in the conotoxins also remains speculative, despite the determination of the structures of some of these peptides (21, 22, 29). We suspect that γ -carboxyglutamic acid plays a broader role than the highly specialized activities associated with membrane interaction. Perhaps, γ -carboxyglutamic acid is critical for function of certain housekeeping proteins that are ubiquitous in animal cells, although it would appear that vitamin K-dependent carboxylation is not a required step until late in embryogenesis.¹ Isolation of novel γ -carboxyglutamic acid-containing proteins from these invertebrate species should provide insight into these functions.

The major implication of this work is that the vitamin K-dependent carboxylase is broadly distributed in animal phyla. Although γ -carboxyglutamic acid has not been directly identi-

fied in most of these phyla, it is highly likely that the preservation of the components of this complex enzymatic reaction for the biosynthesis of γ -carboxyglutamic acid throughout evolution in the animal kingdom indicates that γ -carboxyglutamic acid is present and functional in primitive and higher vertebrates as well as invertebrates. Although the function of γ -carboxyglutamic acid in the vitamin K-dependent blood coagulation proteins is understood within the context of conferring calcium and membrane binding properties (4, 30), it remains to be determined as to the functional role of γ -carboxyglutamic acid in other proteins and in lower organisms.

Acknowledgments—We are grateful to the late Dr. Neal Cornell and Dr. Norman Wainwright for providing biological samples and mRNA from various species.

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A Conserved Motif within the Vitamin K-dependent Carboxylase Gene Is Widely Distributed across Animal Phyla

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J. Biol. Chem. 2000, 275:36245-36249.

doi: 10.1074/jbc.M003944200 originally published online July 12, 2000

Access the most updated version of this article at doi: [10.1074/jbc.M003944200](https://doi.org/10.1074/jbc.M003944200)

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