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In vitro and *in vivo* functional characterization of bovine vitamin K-dependent γ-carboxylase expressed in Chinese hamster ovary cells

(post-translational modification/γ-carboxyglutamic acid/factor IX/PACE/furin)

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ABSTRACT **Coagulation factor IX is a serine protease for which high-level expression of biologically active protein in heterologous cells is limited due to inefficient proteolytic removal of the propeptide as well as vitamin K-dependent carboxylation of multiple amino-terminal glutamic acid residues. We have overexpressed the vitamin K-dependent γ-carboxylase cDNA and monitored its ability to improve factor IX processing in Chinese hamster ovary (CHO) cells. From amino acid sequence analysis of bovine liver vitamin K-dependent γ-carboxylase, degenerate oligonucleotides were used to isolate a 3.5-kbp bovine cDNA that encoded a 758-residue open reading frame. Expression of the cDNA in COS-1 and CHO cells yielded 17- and 16-fold increases in the *in vitro* γ-carboxylase activity of microsomal preparations, respectively. Antiserum raised against a predicted peptide sequence reacted with a 94-kDa polypeptide in the partially purified bovine liver preparation as well as in stably transfected CHO cells. The amount of antibody reactivity correlated with the increased ability to carboxylate a peptide substrate *in vitro*. These results strongly support the conclusion that the cDNA encodes the vitamin K-dependent γ-carboxylase. Transient transfection of the γ-carboxylase expression vector into factor IX-expressing CHO cells did not improve the specific procoagulant activity of secreted factor IX. In contrast, transfection of an expression vector encoding the propeptide processing enzyme PACE (*p*aired basic *a*mino acid *c*leaving *e*nzyme) did improve the specific activity of secreted factor IX by 3-fold. These results demonstrate that the ability of CHO cells to modify glutamic acid residues to γ-carboxyglutamic acid in secreted factor IX is not limited by the expression of the vitamin K-dependent γ-carboxylase alone.**

The vitamin-K dependent plasma proteins contain γ-carboxyglutamic acid residues that are required for these proteins to attain a calcium-dependent conformation, which promotes interaction with phospholipid surfaces that is essential for their function (1, 2). Analysis of recombinant coagulation factor IX expressed in Chinese hamster ovary (CHO) cells revealed that the protein had a much lower specific activity compared to the natural human plasma-derived protein. The reduced specific activity was attributed to the limited ability of CHO cells to cleave the propeptide of factor IX as well to efficiently perform γ-carboxylation of glutamic acid residues (3, 4). To overcome these limitations, we have engineered CHO cells that are capable of efficient propeptide processing. Previously we identified a propeptide cleaving enzyme, PACE (*p*aired basic *a*mino acid *c*leaving *e*nzyme)/furin, that when overexpressed in CHO cells facilitates propeptide cleavage even when the recombinant protein is being expressed at very high levels (5, 6). The vitamin K-dependent γ-glutamylcarboxylase converts glutamate residues to γ-carboxyglutamyl (Gla) residues (7, 8). In an attempt to overcome the limited capacity of CHO cells to γ-carboxylate the vitamin K-dependent coagulation factors, we here describe the isolation of a cDNA for bovine vitamin K-dependent γ-carboxylase (hereafter referred to as γ-carboxylase), demonstrate expression and function of the recombinant protein, and access its effects in processing factor IX§.

MATERIALS AND METHODS

Cloning of the Bovine γ-Carboxylase cDNA. The bovine liver γ-carboxylase protein was partially purified essentially as described (9) with modifications (10). Fractions containing the greatest amount of γ-carboxylase activity (specific activity, 1.9×10^9 cpm/mg per hr) were concentrated by ultrafiltration, electrophoresed on an SDS/10% polyacrylamide gel, and electroblotted onto nitrocellulose membranes in the presence of 0.05% SDS/20% methanol (11). The 94-kDa band obtained from preparations described here and by Kuliopulos *et al.* (10) was digested *in situ* with trypsin and the sequences of three tryptic peptides were determined. The sequences obtained were TGELGYLNPGVFTQSR, VYTVSSSPSCYMYIYV–T, and NLAFGRPSLEQLAQEVTYANLRPFEPAGEPS–VN. An additional peptide sequence, FLFGLMMVLDIPQE, was similarly obtained from a preparation of the 94-kDa protein generously provided by D. Stafford (University of North Carolina). A degenerate oligonucleotide (ATGATGGTICTIGAYATICC) based on the peptide sequence MMVLDIP was used to isolate a 330-bp fragment from a bovine liver cDNA library (Stratagene). The clone contained an open reading frame that spanned residues 1–136 of the human γ-carboxylase not present in the partial bovine sequence described by Wu *et al.* (12). To isolate a full-length coding sequence, the 330-bp fragment was used to screen a second bovine liver cDNA library (Clontech). Three overlapping clones were obtained, which encoded an open reading frame of 758 residues and contained all of the peptide sequences obtained from our partially purified band of 94 kDa. Using restriction fragments from two of the clones, a full-length coding sequence was constructed and inserted into the *Xba* I site of the dicistronic mRNA expression vector pED (13) to generate the plasmid pEDγ.

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Abbreviations: CHO, Chinese hamster ovary; MTX, methotrexate; PACE, *p*aired basic *a*mino acid *c*leaving *e*nzyme.

§The sequence reported in this paper has been deposited in the GenBank data base (accession no. L09726).

Expression of Vitamin K-Dependent γ-Carboxylase. Plasmid DNA was transfected into COS-1 cells by the DEAE-dextran procedure as described (16). At 36–40 hr after transfection cells were harvested and microsomes were prepared as described (15). Transient expression in CHO cells was performed by DEAE-dextran-mediated transfection with modifications (16). At 36–40 hr after transfection, transcription of the cDNA was induced by the treatment of the cells with 2 mM sodium butyrate (Sigma) in the presence of serum-free medium containing vitamin K_3 at 200 ng/ml (Menadione, Sigma). After 24 hr conditioned medium was harvested for factor IX analysis.

CHO cell lines stably expressing γ-carboxylase were obtained by electroporation of pEDγ into dihydrofolate reductase-deficient CHO cells (DUKX-B11) and selection for growth in the absence of nucleosides. Subsequently cells were selected for amplification of γ-carboxylase expression by growth in increasing concentrations of methotrexate (MTX) as described (14). Expression of γ-carboxylase in the various cell lines was monitored by Northern blot hybridization analysis as described (17). Microsomal fractions of cell lines expressing γ-carboxylase mRNA were prepared from two 100-mm^2 plates and the pellets were resuspended in 100 μl of resuspension buffer (25 mM Mops, pH 7.0/0.5 M NaCl/5% glycerol/2 mM dithiothreitol/2 mM EDTA/0.5 μg of leupeptin per ml/0.7 μg of pepstatin A per ml/2 μg of aprotinin per ml/1 mM phenylmethylsulfonyl fluoride/1 mM benzamidine) (10). The γ-carboxylase assay was done as described (18).

Production of Anti-Peptide Antibodies. The peptide SSLDRRYLDGLEVC was synthesized on a polystyrene resin with *p*-butoxycarbonyl protecting groups on an Applied Biosystems synthesizer (model 430) as recommended by the manufacturer. The peptide was purified by HPLC using a C_4 column. Purified peptide was conjugated to chicken egg albumin (Calbiochem) using the SMBP protocol (19), and 200 μg of the conjugated peptide was injected subcutaneously into female New Zealand White rabbits with Freund's complete adjuvant and subsequently with Freund's incomplete adjuvant at 4-week intervals.

The serum collected from the immunized rabbits was precleared over a Sepharose 4B column followed by a column of chicken egg albumin that was conjugated to CNBR-activated Sepharose 4B (Pharmacia) as recommended by the manufacturer. Unbound protein from the above two columns was then applied to a peptide/agarose column. The column was washed extensively with 15 mM Tris, pH 7.4/1 M NaCl/0.02% Tween 20 followed by equilibration in 20 mM Tris, pH 7.4/150 mM NaCl (TBS) after which bound antibody was eluted with 4 M guanidine hydrochloride and the eluate was dialyzed in TBS immediately.

Western Blot Analysis. Samples to be analyzed were resolved by SDS/PAGE and transferred to a nitrocellulose membrane using a Bio-Rad electroblotter as recommended by the manufacturer. The membrane was probed with the purified anti-peptide antibody at a concentration of 1–5 μg/ml followed by an anti-rabbit antibody coupled to horseradish peroxidase. Chemiluminescence was used for detection as recommended by the manufacturer (Amersham).

Factor IX Activity and Antigen Assay. Factor IX activity in conditioned medium was quantitated by a two-stage clotting assay using factor IX-deficient plasma (George B. King Biomedical, St. Louis). Normal pooled human plasma was used as a standard. Total factor IX antigen levels were quantitated by a monoclonal antibody sandwich ELISA using anti-human factor IX antibody (Haematologic Technologies, Burlington, VT) as capture and bESN3 antibody (American Diagnostica, Greenwich, CT) as detector. One unit of factor IX activity represents the amount of factor IX in 1 ml of normal pooled human plasma (5 μg).

RESULTS

Isolation of the Coding Sequence for Bovine γ-Glutamylcarboxylase. Four independent clones were isolated from two bovine liver cDNA libraries as described above. A contiguous open reading frame of 758 amino acids was constructed from these clones that predicted all of the peptides sequenced from the 94-kDa γ-carboxylase protein. Analysis of the protein sequence revealed the absence of a signal peptide and the presence of 50 hydrophilic residues at the amino terminus followed by a hydrophobic segment of 250 residues. Analysis using the program of Klein *et al.* (20) identified the presence of five (residues 61–80, 114–135, 136–153, 200–216, and 252–271) transmembrane domains within the hydrophobic segment. In contrast, similar analysis using the algorithm of Eisenberg *et al.* (21) predicted three (residues 109–130, 249–271, and 277–298) transmembrane domains. Comparison of the bovine sequence described here with the human sequence (12) showed 94% homology at the protein level (Fig. 1) and 88% homology at the DNA level. In addition, the sequence described here was identical to the partial bovine sequence described (12).

Functional Analysis of Recombinant Vitamin K-Dependent γ-Carboxylase. An expression vector containing the cDNA for γ-carboxylase (pEDγ) was transfected into COS-1 cells. After 40 hr, microsomes were prepared from the transfected cells and tested for γ-carboxylase activity. Compared to microsomes prepared from COS-1 cells transfected with the original pED vector, microsomes prepared from cells transfected with pEDγ incorporated 17-fold greater amounts of $^{14}CO_2$ into the synthetic pentapeptide substrate FLEEL (Table 1). Incorporation of radiolabeled $^{14}CO_2$ into FLEEL was dependent on the presence of reduced vitamin K.

CHO cell lines were engineered by amplification of the γ-carboxylase expression vector using MTX selection. Two cell lines (Eγ0.1 and Eγ1.0) selected for resistance to 0.1 and 1.0 μM MTX, respectively, were further analyzed for functional activity. Microsomes prepared from Eγ0.1 and Eγ1.0 cells incorporated 14- and 16-fold, respectively, greater amounts of $^{14}CO_2$ compared to control CHO cells (Table 1). This indicated that stable integration of the pEDγ vector resulted in increased γ-carboxylase activity due to expression of the inserted cDNA.

Characterization of Cell Lines that Overexpress γ-Carboxylase. The Eγ0.1 and Eγ1.0 lines were further analyzed by Northern blot hybridization analysis to detect the presence of γ-carboxylase mRNA. Total RNA was prepared from Eγ0.1 and control CHO cells as well as from COS-1 cells and COS-1 cells transfected with the pEDγ construct. Hybridization to an 880-bp fragment from the 5′ end of the γ-carboxylase cDNA detected a 5.5-kb band in transiently transfected COS-1 cells (Fig. 2, lane 2) and stably transfected CHO cells (Fig. 2, lane 4) that was not detected in either nontransfected COS-1 cells or CHO cells (Fig. 2, lanes 1 and 5, respectively). Since the same sized mRNA was detected in stably transfected CHO cells and transiently transfected COS-1 cells, it is unlikely that the expression vector rearranged upon integration into the CHO cell genome. The 5.5-kb mRNA detected is the expected size derived from the expression vector containing a 3.5-kbp insert since the transcriptional unit contains 2 kb of additional 5′ and 3′ sequences (22). Interestingly, low levels of a 4-kb mRNA were detected in RNA prepared from the human hepatoma cell line HepG2, indicating that the γ-carboxylase mRNA is 4 kb in size. Thus, the 3.5-kb cDNA we have obtained may lack 3′ and/or 5′ noncoding sequences or may represent a difference between the human and bovine mRNA structures.

An anti-peptide antibody generated against the predicted amino acid sequence from residues 86–99 was used to analyze microsomal fractions prepared from Eγ0.1, Eγ1.0, and

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MAVSARPARAPRGSDKVKKDKAAQTSGPRQGSRMGKLLGFEWTDVSSWERLVTLLNRPTD  60
     GS  TSPS    Q    ELI     D  I          L   R
PAGLAVFRFLFGLMMVLDIPQERGLSSLDRRYLDGLEVCRFPLLDALQPLPLDWMYLIYT  120
  S         FL                K     D          R         V
IMFLGALGMMLGLCYRISCVLFLLPYWYVFLLDKTSWNNHSYLYGLLAFQLTFVDAHHYW  180
                                                      M  N
SVDGLLRARKRNAHVPLWNYAVLRGQIFIVYFIAGIKKLDADWVEGYSMEYLSRHWLFSP  240
      N HR                          V
FKLVLSEEMTSLLVVHWCGLLLDLSAGFLLFFDASRPIGFVFVSYFHCMNSQLFSIGMFP  300
   L    L        G               V  S  LF                  S
YVMLASSPLFCSPEWPRKLVAHCPKKLQELLPLRTAPQPSTSCMYKRSRARGSQKPGLRH  360
                    SY  QR  Q    KA     V  V     GKSG
KLSTAFTLLYLLEQLFLPYSHFLTQGYNNWTNGLYGYSWDMMVHSRSHQHVKITYRDGRT  420
Q GA
GELGYLNPGVFTQSRRWKDHADMLKQYATCLSRLLPKYNVTEPQIYFDIWVSINDRFQQR  480

IFDPRVDIVQAAWSPFQRTPWLQPLLMDLSPWRTKLQEIKSSLDNHTEVVFIADFPGLHL  540
                    S V           A
ENFVSEDLGNTSIQLLQGEVTVELVAEQKNQTLQEGEKMQLPAGEYHKVYTVSSSPSCYM  600
                                  R                 T P
YIYVNTTEVALEQDLAYLQELKEKVENGSETGPLPPELQPLLEGEVKGGPEPTPLVQTFL  660
 V      L
RRQQRLQEIERRRNAPFHERLVRFLLRKLFIFRRSFLMTCISLRNLAFGRPSLEQLAQEV  720
              T     FF       YV               IL
TYANLRPFEPAGEPSPVNTDSSNPNPPEPDSHPVHSEF                        758
        AV  LN S     HS    SNPD
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FIG. 1. Comparison of the bovine (top) and human (bottom) γ-carboxylase protein sequences. Residues of the human sequence are only shown when they diverge from the bovine sequence. The bovine sequence was deduced from the cDNA described here and the human sequence was obtained from ref. 12.

control CHO cells as well as partially purified fractions of bovine γ-carboxylase. The antibody detected a 94-kDa species as well as a higher molecular mass species (Fig. 3, lane 4) in partially purified bovine γ-carboxylase prepared as described (10). A band comigrating with this 94-kDa species was detected in microsomes from cell lines Eγ0.1 and Eγ1.0 but was not detected in the nontransfected CHO cells (Fig. 3). The antibody also detected lower molecular mass species in nontransfected CHO cells as well as Eγ0.1 and Eγ1.0 cells, indicating that they were due to nonspecific reactivity of the antibody. A species migrating at 200 kDa was present in partially purified preparations of bovine γ-carboxylase and also detected in the stable cell lines Eγ0.1 and Eγ1.0 when large quantities of microsomal protein were analyzed (data not shown).

Table 1. COS-1 cells or CHO cells assayed for endogenous γ-carboxylase activity or activity after transient (COS-1) or stable (CHO) transfection with pEDγ

DNA	cpm/μg per 30 min	Fold increase
COS-1		
pED	268	—
pEDγ	4470	17
CHO		
CHO cells	65	—
CHO Eγ0.1	915	14
CHO Eγ1.0	1060	16

Microsomes were prepared from cells and assayed as described in the text. Microsome preparations were assayed for protein concentration and the γ-carboxylase activity in each sample was expressed as cpm of $^{14}CO_2$ incorporated per μg of protein in a 30-min assay. Samples pED and pEDγ represent microsome preparations prepared from COS-1 cells transfected with the vector alone or the vector containing the γ-carboxylase cDNA, respectively. CHO cells were stably transfected with pEDγ and selected for resistance to MTX at 0.1 μM (CHO Eγ0.1) or 1.0 μM (CHO Eγ1.0).

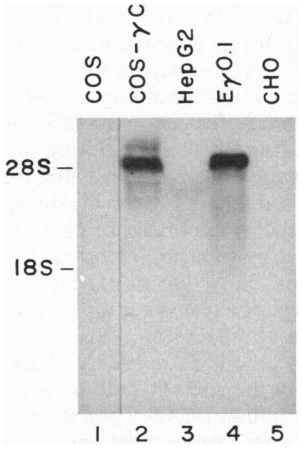

FIG. 2. Analysis of γ-carboxylase mRNA expression. Northern blot analysis was performed using total RNA prepared from nontransfected CHO cells (lane 5), Eγ0.1 (lane 4), COS-1 monkey kidney cells (lane 1), COS-1 cells transfected with pEDγ (lane 2), and HepG2 cells (lane 3). Total RNA was loaded for HepG2 (10 μg) cells and 1 μg was loaded for the rest.

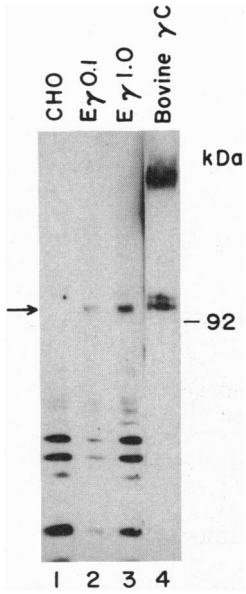

FIG. 3. Analysis of γ-carboxylase protein expression. Western blot analysis was performed using partially purified γ-carboxylase from bovine liver (lane 4) and microsomes prepared from CHO cells (lane 1) or CHO Eγ0.1 (lane 2) and CHO Eγ1.0 (lane 3). Approximately 25 μg of microsomal protein prepared as described or partially pure carboxylase (described above) was resolved on 8% SDS/PAGE gel and transferred to nitrocellulose. The filters were probed as described. The arrow indicates a unique 94-kDa band present in CHO cells that contain pEDγ as well as partially purified γ-carboxylase from bovine liver but not untransfected CHO cells.

Overexpression of γ-Carboxylase Does Not Increase *in Vivo* γ-Carboxylation of Factor IX. Expression of functional factor IX at high levels in CHO cells is limited by the fact that CHO cells are not able to efficiently carboxylate as well as proteolytically process large amounts of factor IX (3, 4). To examine if cells that express recombinant γ-carboxylase were able to efficiently γ-carboxylate factor IX, CHO cells expressing factor IX at high levels (IC4) or at moderate levels (B.1) were transfected with the γ-carboxylase expression vector and/or a propeptidase (PACE) expression vector (23) or an influenza hemagglutinin expression vector as a control. The factor IX secreted from the transfected cells was analyzed by a factor IX coagulation assay and a factor IX antigen-specific ELISA to determine the specific activity of the expressed factor IX. B.1 cells transfected with the influenza hemagglutinin expression vector as a control secreted 61 milliunits of factor IX activity per ml and 4.2 μg of factor IX antigen per ml, yielding a specific activity of 15 milliunits/μg (Table 2). Transfection of a duplicate plate with an expression vector for the propeptide processing enzyme (PACE/furin) resulted in an almost 3-fold increase in specific activity to 40 milliunits/μg. However, the specific activity of factor IX expressed in the presence of PACE/furin was still significantly lower than plasma-derived factor IX (200 milliunits/μg) and was likely due to partial γ-carboxylation occurring at this level of expression. In contrast to transfection with PACE/furin, transfection with the γ-carboxylase expression vector resulted in a decrease in factor IX antigen as well as activity but the specific activity remained essentially unchanged at 13 milliunits/μg. Coexpression of PACE with γ-carboxylase did not improve the specific activity of factor IX above that observed with PACE alone (Table 2). This was also observed in the IC4 cell line, which expresses 8-fold more factor IX but of a much lower specific activity (Table 2). The greater reduction in specific activity of factor IX secreted from IC4 cells results primarily from inefficient γ-carboxylation when factor IX is expressed at a high level since expression of PACE resulted in a 2-fold increase in specific activity but the specific activity did not equal that obtained from B.1 cells. These results were corroborated by analysis of secreted factor IX with polyclonal antibodies specific for the metal-induced Gla-dependent conformation of factor IX (24, 25).

Table 2. Specific activity of factor IX secreted from stable cell lines transiently transfected with γ-carboxylase and/or PACE

DNA	Activity, milliunits/ml	Antigen, μg/ml	Specific activity, milliunits/μg
B.1			
HA	61	4.2	15
PACE	182	4.6	40
γ-Carboxylase	33	2.6	13
+ PACE	90	3.4	27
IC4			
HA	173	30	6
PACE	326	28	12
γ-Carboxylase	204	29	7
+ PACE	305	29	11

B.1 and IC4 are stable CHO cell lines that express factor IX. Duplicate plates were transfected with an expression vector for influenza hemagglutinin (HA; ref. 5) as a control or with an expression vector encoding PACE (5) or γ-carboxylase; 48 hr after transfection, cells were placed in fresh medium and 24 hr later the medium was assayed for factor IX activity and antigen.

DISCUSSION

We have isolated a 94-kDa protein from bovine liver microsomes that has a high specific activity for carboxylation of a synthetic peptide, FLEEL. Amino acid sequence data were used to isolate a full-length sequence encoding bovine γ-carboxylase. Transient expression of the cDNA in COS-1 cells yielded microsomes that contained 17-fold higher amounts of γ-carboxylase activity measured *in vitro*. This was also confirmed in stably transfected and amplified CHO cell lines that yielded a 14- to 16-fold increase in *in vitro* γ-carboxylase activity compared to untransfected CHO cells. The increase in γ-carboxylase activity correlated with the presence of an abundant mRNA transcript by Northern blot hybridization analysis and an increase in a 94-kDa polypeptide reacting with γ-carboxylase-specific antibodies as detected by Western blot analysis.

Several lines of evidence suggest that the protein encoded for by the cDNA we have characterized encodes a γ-carboxylase. We have shown by Western blot analysis using an anti-peptide antibody made against a peptide predicted from the cDNA sequence that microsomal proteins prepared from CHO cell lines transfected with the γ-carboxylase expression vector contain a 94-kDa protein that is below detectable levels in control cells. The antibody also reacted with a comigrating species present in the most active fractions obtained from a bovine liver γ-carboxylase purification. In addition, reactive material was also detected migrating at ≈200 kDa in CHO recombinant-derived and bovine-derived microsomal preparations. The origin of the high molecular mass material is not known but one can speculate that it might represent a dimeric form of the protein that is resistant to dissociation under the gel conditions employed. Comparison of γ-carboxylase activity and the total amount of immunoreactive material seen on Western analysis (including the 94-kDa and the 200-kDa forms) suggests that the recombinant γ-carboxylase from CHO Eγ1.0 and that derived from bovine liver have similar specific activities. We have also recently demonstrated that the 94-kDa species isolated from bovine liver is specifically labeled using the affinity label *N*-bromoacetyl-FLEELY (10). The affinity peptide also labeled a

high molecular mass species similar in size to the high molecular mass immunoreactive material seen on Western blots, suggesting that indeed the high molecular mass material may represent γ-carboxylase and that it may result from dimerization of the 94-kDa form of γ-carboxylase. In addition, we have recently demonstrated that the above antipeptide antibody is able to deplete γ-carboxylase activity from microsomes prepared from cells expressing recombinant γ-carboxylase (26). Taken together, the biochemical, immunological, and functional evidence presented here identifies the protein encoded for by the above described cDNA as a γ-carboxylase.

Our results are consistent with those of Wu *et al.* (9) in describing isolation of a 94-kDa polypeptide having γ-carboxylase activity. These investigators cloned a human cDNA that demonstrated a 21-fold stimulation of *in vitro* activity in microsomes prepared from cells transfected with a cDNA expression vector (12). This cDNA has 88% homology to our bovine cDNA. The partial bovine cDNA described by Wu *et al.* (12) is identical to the cDNA described here. We further demonstrate here that expression of γ-carboxylase activity correlated with expression of the expected polypeptide. More recently, Berkner *et al.* (27) have described purification of a 98-kDa polypeptide that has 15-fold higher specific activity *in vitro* over that described by Wu *et al.* (9). Since different assay conditions were employed, it is difficult to directly compare specific activities of the two preparations. The relationship of the 98-kDa protein described by Berkner *et al.* and the 94-kDa protein described here as well as by Wu *et al.* remains to be determined. Cloning and functional analysis of the 98-kDa protein described by Berkner *et al.* will reveal if the protein is related to the one described here or if it represents a new protein.

Factor IX expression in CHO cells is limited due to inefficient propeptide processing as well as events leading to γ-carboxylation. Transient transfection of an expression vector encoding the propeptide processing enzyme PACE/furin improved the specific activity of factor IX secreted from CHO cells ≈3-fold. Recently, we have shown that increased PACE/furin expression increased the specific activity of secreted factor IX by improving processing of pro-factor IX at the correct cleavage site (5). Using this transient expression assay system, we tested whether increased expression of γ-carboxylase improves the specific activity of secreted factor IX. The results show that either in the presence or absence of PACE expression, overexpression of γ-carboxylase did not improve the specific activity of secreted factor IX nor did it improve reactivity of the factor IX with Gla-dependent metal conformation-specific anti-factor IX antibodies. In addition, increased γ-carboxylase expression did not increase the specific activity of factor IX when factor IX and γ-carboxylase were stably coexpressed in the same cell (data not shown). Thus one can conclude either that γ-carboxylase is apparently not the limiting factor in CHO cells or that additional activities may be required for efficient vitamin-K dependent γ-carboxylation of factor IX *in vivo*. Further investigation is necessary to determine whether failure to carboxylate factor IX is due to the fact that the recombinant γ-carboxylase when expressed in CHO cells is present in an inappropriate environment and hence is not able to γ-carboxylate factor IX.

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