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Expression of bovine vitamin K-dependent carboxylase activity in baculovirus-infected insect cells

(γ-carboxyglutamic acid/coagulation/*Spodoptera frugiperda* cells/*Autographa californica* nuclear polyhedrosis virus)

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ABSTRACT **A vitamin K-dependent carboxylase has recently been purified from bovine liver microsomes and candidate cDNA clones have been isolated. Definitive identification of the carboxylase remains circumstantial since expression of candidate carboxylase cDNAs in mammalian cells is confounded by the presence of endogenous carboxylase activity. To overcome this problem, a recombinant strain of baculovirus (*Autographa californica* nuclear polyhedrosis virus, AcMNPV) encoding a putative carboxylase (vbCbx/AcMNPV) was used to infect Sf9 insect cells, which we demonstrate have no endogenous carboxylase activity. Infection with vbCbx/AcMNPV conferred vitamin K-dependent carboxylase activity to Sf9 insect cells. Carboxylase activity was demonstrated to peak 2–3 days after infection with vbCbx/AcMNPV. Metabolic radiolabeling with L-[^{35}S]methionine revealed that the 90-kDa recombinant protein is the major protein synthesized at the time of peak activity after infection. An anti-peptide antibody directed against residues 86–99 reacted with bovine liver carboxylase on Western blot analysis and immunoprecipitated recombinant carboxylase from infected Sf9 microsomal protein preparations. Since Sf9 insect cells lack endogenous vitamin K-dependent carboxylase activity, expression of carboxylase activity in Sf9 insect cells with recombinant baculovirus demonstrates that the protein encoded by this cDNA is a vitamin K-dependent γ-glutamyl carboxylase.**

Vitamin K-dependent γ-carboxylation is a physiologically important post-translational modification of blood clotting proteins prothrombin, factor VII, factor IX, and factor X, regulatory proteins of blood coagulation, protein C and protein S, and two proteins of bone, osteocalcin and matrix γ-carboxyglutamic acid (Gla) protein (1). In what is the only known enzymatic reaction dependent on vitamin K, specific glutamic acid residues are carboxylated at the γ-carbon position to form Gla (2–4). There are 10–12 Gla residues in the blood clotting proteins located near the N termini. The Gla residues are critical components of a structural domain, the Gla domain, responsible for promoting calcium ion-dependent interactions of these proteins with membrane surfaces. The membrane binding properties of the blood clotting proteins are essential for their biological activity.

A microsomal γ-glutamyl carboxylase activity that is vitamin K-dependent catalyzes this post-translational modification (5). The carboxylase is an integral membrane protein that resides in the endoplasmic reticulum (6). Carboxylation of substrate proteins requires vitamin K hydroquinone, molecular oxygen, and carbon dioxide (7). The propeptides of the vitamin K-dependent proteins have highly conserved sequences (8) that contain a γ-carboxylase recognition site necessary for binding of carboxylase to protein substrates (9).

While a considerable amount of information concerning carboxylase function has been generated with crude preparations of the enzyme, detailed mechanistic analyses of this unique vitamin K-dependent enzyme have been impeded by its low abundance, its instability, and difficulty in solubilization and purification of sufficient quantities of the enzyme. By using an affinity-purification strategy in which the propeptide is used to bind to the carboxylase (10), the propeptide of factor IX was used to affinity purify bovine liver carboxylase (11). The cDNAs encoding the entire protein sequence of the 94-kDa human liver carboxylase (12) and bovine liver carboxylase (13) have been isolated. Expression of these cDNAs in mammalian cells, which already contain vitamin K-dependent carboxylase, led to an increase in carboxylase activity above the endogenous level. Nonetheless, there remains controversy whether these cDNAs encode a carboxylase and whether another purified protein might represent the carboxylase (14).

The presence of vitamin K-dependent γ-glutamyl carboxylase activity in mammalian cells confounds expression experiments in these cells to prove that the cDNAs cloned encode the carboxylase. The possibility exists that the cDNA encodes a protein that stimulates the endogenous carboxylation reaction. Since the vitamin K-dependent blood clotting mechanism as defined in vertebrates does not occur in insects (15), we explored whether insect cell lines suitable for protein expression with recombinant baculovirus contained endogenous vitamin K-dependent carboxylase activity. Unlike mammalian cells tested, we demonstrated that Sf9 insect cells lack endogenous vitamin K-dependent carboxylase activity, giving these cells an advantage over mammalian cells for expression and analysis of the protein encoded by this cDNA. In addition, baculovirus expression systems are characterized by very high levels of protein expression (16, 17) and might provide a means of isolating abundant quantities of the enzyme for mechanistic and structure–function studies. We therefore chose to express the cDNA encoding bovine liver carboxylase in insect cells. In the present study, we demonstrate that infection with recombinant baculovirus containing the cDNA encoding bovine liver carboxylase confers vitamin K-dependent γ-glutamyl carboxylase activity to Sf9 insect cells, which lack endogenous activity. This provides strong evidence that the protein encoded by this cDNA is a vitamin K-dependent γ-glutamyl carboxylase. A preliminary report of this work has been presented (18).

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Abbreviations: Gla, γ-carboxyglutamic acid; FLEEL, Phe-Leu-Glu-Glu-Leu; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; AcMNPV, *Autographa californica* nuclear polyhedrosis virus.

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MATERIALS AND METHODS

Plasmid Construction. The cDNA encoding bovine liver carboxylase (13) was subcloned into the *Nhe* I site of the baculovirus transfer vector pBLII (Invitrogen) to generate pBLII/bCbx by standard methods (19). The cDNA was digested with *Nco* I. A 3.5-kb fragment containing the entire coding region was blunt-ended with the large fragment of DNA polymerase I and ligated into pBLII, which had been linearized with *Nhe* I and blunt-ended with the large fragment of DNA polymerase I. Recombinant plasmid pBLII/bCbx was isolated by CsCl gradient ultracentrifugation and the proper orientation of the cDNA insert with respect to the polyhedron promoter in pBLII was confirmed by restriction enzyme analysis and DNA sequence analysis.

Cell Culture. *Spodoptera frugiperda* (fall armyworm) cell line Sf9 cells were grown in complete TNM-FH [supplemented Grace's insect medium (Invitrogen)/10% (vol/vol) fetal bovine serum (Hyclone)/gentamicin (50 μg/ml) (GIBCO)] at 27°C in a humidified incubator (20). Suspension cultures >100 ml were adjusted to 0.1% pluronic surfactant F-68 (JRH Biosciences, Lenexa, KS) to protect the cells from hydrodynamic stress observed in large suspension cultures (21).

Production of Recombinant Baculovirus. Cotransfection of 2.0×10^6 Sf9 cells was performed with 1 μg of linearized wild-type *Autographa californica* nuclear polyhedrosis virus (AcMNPV) baculovirus DNA (Invitrogen) and 3 μg of either pBLII/bCbx or pBLII using cationic liposomes (Invitrogen). Recombinant baculovirus generated with the pBLII transfer vector also contains the gene for β-galactosidase under control of the AcMNPV ETL promoter (22), such that viral clones of recombinant baculovirus generated with this transfer vector give rise to blue plaques in the presence of 5-bromo-4-chloro-3-indolyl β-D-galactoside that demonstrate an occlusion-negative phenotype. Viral clones of recombinant baculovirus (either vbCbx/AcMNPV, encoding carboxylase, or vBLII/AcMNPV, a control virus without carboxylase cDNA) were plaque-purified, propagated by passage in Sf9 cells infected at low multiplicity of infection (0.1–0.5 plaque-forming unit per cell), and titered by plaque assay (20).

Expression of Recombinant Protein. Metabolic radiolabeling studies were performed in Sf9 cells grown as adherent cell monolayers in 24-well tissue culture plates. Sf9 cells (6×10^5 cells) were infected at high multiplicity of infection (10 plaque-forming units per cell) and were grown in complete TNM-FH for the specified time interval after infection. Medium was changed to methionine-deficient Grace's insect medium and incubated at room temperature for 1 h to deplete intracellular methionine pools, prior to exchange with fresh methionine-deficient Grace's insect medium supplemented with L-[^{35}S]methionine at 100 μCi/ml (1180 Ci/mmol; 1 Ci = 37 GBq; Du Pont/New England Nuclear). Cells were then incubated for 1 h at 27°C followed by chilling on ice. Cells were collected by centrifugation for 5 min at $1000 \times g$ and then washed three times by resuspension in ice-cold Grace's insect medium followed by centrifugation for 5 min at $1000 \times g$ prior to further analysis. Radiolabeled cells were resuspended in reducing SDS/PAGE loading buffer, sonicated in a water-bath sonicator for 2 min, and boiled for 5 min, and equivalent volumes were analyzed by reducing SDS/PAGE in 10% gels. Samples that were not radiolabeled were resuspended in 25 mM Mops, pH 7.0/0.5 M NaCl/5% (vol/vol) glycerol and sonicated with a microtip ultrasonic probe (Heat Systems-Ultrasonics, model W-220) prior to protein quantitation and carboxylase activity analysis. Samples not used immediately were stored at −80°C.

Large-scale protein expression was performed with Sf9 cells propagated and infected in suspension culture. Cells were infected at high multiplicity of infection (10 plaque-forming units per cell) and harvested 2–3 days after infection by centrifugation at $1000 \times g$ for 10 min at 4°C. All further processing was done with ice-cold buffers or at 4°C, unless otherwise stated. Cell pellets were washed once by resuspension in phosphate-buffered saline (137 mM NaCl/2.7 mM KCl/4.3 mM Na_2HPO_4/1.5 mM KH_2PO_4, pH 7.4), followed by a repeat centrifugation. Washed cell pellets were resuspended in a hypotonic lysis buffer (10 mM Mops, pH 7.0/10 mM KCl/1 mM $MgCl_2$) supplemented with protease inhibitors [phenylmethylsulfonyl fluoride (100 μg/ml)/leupeptin (0.5 μg/ml)/pepstatin A (1.0 μg/ml)/aprotinin (2.0 μg/ml)]. After a 5-min incubation, cells were lysed with 20 strokes in a Dounce homogenizer followed by eight 15-sec pulses with a Polytron (Brinkmann PT 3000) tissue homogenizer at a setting of 20. Nuclei were sedimented at $600 \times g$ for 5 min. Microsomes were collected from the postnuclear supernatant by centrifugation at $100,000 \times g$ for 60 min. Microsomal pellets were resuspended in 25 mM Mops, pH 7.0/0.5 M NaCl/5% glycerol/protease inhibitors (as above) and sonicated with a microtip ultrasonic probe prior to further analysis.

Antibody Purification. A 14-aa synthetic peptide corresponding to residues 86–99 in the bovine carboxylase sequence was synthesized, and the peptide was conjugated to ovalbumin and used to immunize New Zealand White rabbits (13). Anti-carboxylase-(86–99) antibodies were affinity-purified as described (13). Preimmune immunoglobulin was purified from rabbit antisera by affinity chromatography with protein A-Sepharose (Sigma).

Western Blot Analysis of Carboxylase. Western blot analysis employed the Enhanced Chemiluminescent detection system (Amersham) with biotinylated donkey anti-rabbit second antibody and streptavidin-horseradish peroxidase conjugate using the cyclic diacylhydrazide luminol as a substrate in the presence of hydrogen peroxide according to manufacturer's instructions.

Immunoprecipitation of Carboxylase. Immunoprecipitation of recombinant carboxylase was carried out at 4°C. Microsomal protein prepared from vbCbx/AcMNPV-infected Sf9 cells was adjusted to 1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid (CHAPS)/1 M NaCl and mixed vigorously for 30 min at 4°C to solubilize carboxylase activity. The sample was subjected to centrifugation at $100,000 \times g$ for 60 min at 4°C, and the supernatant containing solubilized carboxylase was concentrated by precipitation with 55% saturated ammonium sulfate at 4°C for 60 min followed by centrifugation at $10,000 \times g$ for 10 min at 4°C. The ammonium sulfate-precipitated pellet was resuspended in TBS (20 mM Tris·HCl, pH 7.4/140 mM NaCl)/0.1% CHAPS and extensively dialyzed against TBS/0.1% CHAPS at 4°C. The dialysate was precleared of aggregates by centrifugation at $16,000 \times g$ for 30 sec at 4°C. Anti-carboxylase-(86–99) antibody or preimmune immunoglobulin was added to 15 units of carboxylase activity (*vide infra*) in a final reaction volume of 100 μl of TBS/0.1% CHAPS and incubated with intermittent mixing for 4 h at 4°C. After this incubation, 25 μl of 50% (vol/vol) protein A-Sepharose in TBS/0.1% CHAPS was added and incubated for 1 h with intermittent mixing at 4°C. Samples were centrifuged at $16,000 \times g$ for 30 sec to separate the microsomal protein suspension from the resin pellet. Duplicate samples of microsomal protein suspensions were each assayed in duplicate for residual carboxylase activity.

Bovine Liver Carboxylase Purification. Isolation of microsomes from fresh bovine liver was performed as described (10). Carboxylase was solubilized with CHAPS and concentrated by precipitation with ammonium sulfate. The ammonium sulfate-precipitated enzyme was resuspended, sonicated, and affinity-purified essentially as described (11) using FIX59Q/S as an immobilized affinity ligand. FIX59Q/S was

synthesized using solid-phase 9-fluorenylmethyloxycarbonyl chemistry on an Applied Biosystems model 430A peptide synthesizer as recommended by the manufacturer. The cleavage reactions were performed in thioanisole/ethyl methyl sulfide/water/trifluoroacetic acid, 5:2.5:5:87.5 (vol/vol), for 3 h at 25°C. Proteins were analyzed by electrophoresis in SDS/10% polyacrylamide gels under reducing conditions and silver stained using the Daiichi silver stain kit (ISS, Natick, MA) as recommended by the manufacturer.

***In Vitro* Carboxylase Activity.** $^{14}CO_2$ incorporated into the pentapeptide substrate FLEEL (Phe-Leu-Glu-Glu-Leu) (23) was measured in 125-μl reaction mixtures of 0.88 mM vitamin K hydroquinone (Merck Sharp & Dohme)/10 μCi of $NaH^{14}CO_3$ (52 mCi/mmol, Amersham)/3.6 mM FLEEL (Sigma)/25 mM Mops, pH 7.0/0.5 M NaCl/0.16% CHAPS/0.16% phosphatidylcholine/8 mM dithiothreitol/16 μM pro-FIX18/0.8 M ammonium sulfate for 30 min as described (24). Carboxylase activity is defined as 1000 cpm of $^{14}CO_2$ incorporated into FLEEL in 30 min equals 1 unit. Protein concentrations were determined by the modified method of Lowry using a bovine serum albumin standard (25).

RESULTS

We have determined that unlike mammalian cells, Sf9 insect cells lack endogenous vitamin K-dependent carboxylase activity. This cell line thus provides a γ-carboxylase-free expression system in which it can be further verified that the candidate bovine γ-glutamyl carboxylase clone encodes a vitamin K-dependent carboxylase.

The cDNA encoding bovine liver carboxylase was subcloned into the baculovirus transfer vector pBLII to generate pBLII/bCbx. Cotransfection of Sf9 cells with linearized wild-type AcMNPV baculovirus DNA and either pBLII/bCbx or pBLII resulted in the production of both wild-type baculovirus and recombinant baculovirus. Homologous recombination between the transfer vector (pBLII/bCbx or pBLII) and AcMNPV DNA occurred with high frequency such that recombinant blue plaques with the occlusion-negative phenotype made up 30–35% of the plaques generated. Viral clones of recombinant baculovirus (either vbCbx/AcMNPV or vBLII /AcMNPV) were plaque-purified and used for recombinant protein expression.

Carboxylase activity of uninfected and infected Sf9 cells was measured in an *in vitro* assay performed in the presence or absence of reduced vitamin K (Table 1). Microsomes were prepared from uninfected Sf9 cells as well as vBLII/AcMNPV control virus- and vbCbx/AcMNPV carboxylase virus-infected Sf9 cells. Total microsomal protein (200 μg) was assayed *in vitro* for carboxylase activity. Microsomal protein from vbCbx/AcMNPV-infected Sf9 cells had 185 units of carboxylase activity. In the absence of reduced vitamin K, no carboxylase activity was observed. Thus, the carboxylation of FLEEL in this assay was vitamin K-dependent. Sf9 cells had no endogenous carboxylase activity as microsome resuspension buffer and microsomal protein from uninfected or vBLII/AcMNPV control virus-infected Sf9 cells had background radioactivity (200–400 cpm) in the *in vitro* assay. The vitamin K-dependent carboxylase activity of the vbCbx/AcMNPV-infected Sf9 microsomes was stimulated ≈20-fold in the *in vitro* assay system by the inclusion of an 18-residue peptide based upon residues −18 to −1 of human profactor IX. This stimulation parallels that observed with rat liver vitamin K-dependent γ-glutamyl carboxylase using the propeptide of factor X (26).

Aliquots of Sf9 cells were taken at various times after infection with carboxylase-encoding virus (vbCbx/AcMNPV). The cells were lysed and the lysate was assayed *in vitro* for carboxylase activity using FLEEL as a substrate. Uninfected cells exhibited no vitamin K-dependent carboxylase activity. After infection with vbCbx/AcMNPV, there was a time-dependent increase in carboxylase activity, with the specific activity maximal at 2–3 days after infection. The time course of appearance of carboxylase activity after infection correlated with the expected kinetics of a protein expressed under polyhedron viral promoter control (27) and further demonstrates the acquisition of vitamin K-dependent carboxylase activity by Sf9 cells after infection with vbCbx/AcMNPV (Fig. 1). No carboxylase activity was generated in Sf9 cells infected with either wild-type virus (vAcMNPV) or control recombinant virus (vBLII /AcMNPV), generated with the pBLII transfer vector lacking carboxylase cDNA.

Metabolic radiolabeling of vbCbx/AcMNPV-infected Sf9 cells with L-[^{35}S]methionine revealed a radiolabeled protein of ≈90 kDa whose appearance correlated with the peak expression of vitamin K-dependent carboxylase activity (Fig. 2, lane 4, arrow). This molecular mass is similar in size to the expected 94 kDa of purified bovine carboxylase (11) or recombinant carboxylase expressed in mammalian cells (13). Lysates of wild-type AcMNPV-infected cells revealed the predominant 29-kDa polyhedron protein (Fig. 2, lane 2). Lysates of control virus-infected cells were consistent with the disruption of the polyhedron gene in that the polyhedron protein was not observed (Fig. 2, lane 3).

Table 1. Vitamin K dependence of *in vitro* carboxylase activity of Sf9 microsomal protein

Virus	Carboxylase activity, units	
	+ vitamin K	− vitamin K
None	0	0
vBLII/AcMNPV	0	0
vbCbx/AcMNPV	185 ± 4.6	0

Sf9 microsomal protein (200 μg) from uninfected, vBLII /AcMNPV (control virus)-infected or vbCbx/AcMNPV (carboxylase virus)-infected cells was assayed. Assays were done in triplicate and results are presented as units of activity (mean ± SD).

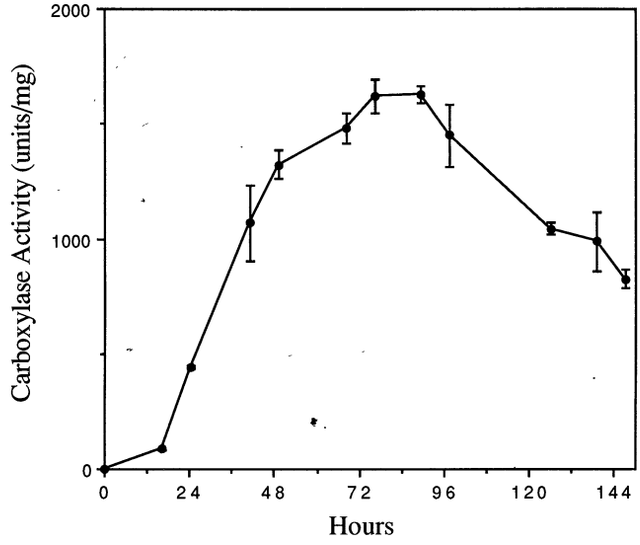

FIG. 1. Kinetics of expression of carboxylase activity in vbCbx/AcMNPV-infected Sf9 cells. Cells were infected with vbCbx/AcMNPV as described for protein expression studies. Cells were harvested at the indicated times after infection. Total cell lysates were assayed for carboxylase activity *in vitro*. The experiment was done twice and samples were assayed in duplicate. The results are expressed as specific carboxylase activity (units of activity per mg of protein assayed; mean ± SD). No carboxylase activity was generated in Sf9 cells infected with either wild-type virus (vAcMNPV) or control recombinant virus (vBLII/AcMNPV).

To demonstrate that the carboxylase activity in the vbCbx/AcMNPV-infected Sf9 cells was conferred by the newly synthesized recombinant protein, antibodies were generated against a 14-aa synthetic peptide corresponding to an N-terminal hydrophilic region of the carboxylase [SSLDRRYLDGLEVC, residues 86–99; carboxylase-(86–99)]. The sequence of the synthetic peptide was based upon the cDNA sequence of the bovine carboxylase. Purified anti-carboxylase-(86–99) antibodies were used in Western blot analysis of bovine liver microsomes estimated to be 100-fold enriched for carboxylase. After analysis by SDS/PAGE and silver staining, the protein pattern revealed the presence of many bovine liver microsomal proteins (Fig. 3, lane 1). None of these bands were reactive with preimmune immunoglobulin (Fig. 3, lane 2). However, purified anti-carboxylase-(86–99) antibody reacted with the 94-kDa protein band, consistent with the size of the purified protein (11) (Fig. 3, lane 3). This antibody has previously been shown to react with recombinant γ-carboxylase expressed from the same cDNA in Chinese hamster ovary (CHO) cells (13), and it specifically reacts with recombinant γ-carboxylase when expressed in baculovirus-infected Sf9 insect cells (data not shown). When larger quantities of recombinant protein from CHO cells (13) or insect cells were analyzed on Western blots, a higher molecular mass band of ≈200 kDa could also be detected. This band was observed when metabolically radiolabeled protein from vbCbx/AcMNPV-infected Sf9 cells was analyzed (Fig. 2, lane 4) and when purified bovine liver carboxylase was affinity labeled with *N*-bromoacetyl-FLEELY (28).

Reactivity of the anti-carboxylase-(86–99) antibody with recombinant carboxylase from vbCbx/AcMNPV-infected Sf9 cells was evaluated. Microsomal protein was prepared from vbCbx/AcMNPV-infected Sf9 cells and recombinant carboxylase was solubilized with CHAPS, ammonium sulfate-precipitated, and dialyzed extensively against TBS/0.1% CHAPS. The dialysate was precleared of insoluble aggregates by centrifugation and the activity of the preparation was determined prior to incubation with antibodies. Equal quantities of recombinant carboxylase activity (15 units) were incubated with various quantities of either preimmune immunoglobulin or purified anti-carboxylase-(86–99) antibody as indicated in Fig. 4. Neither antibody inhibited carboxylase activity when tested in the *in vitro* carboxylase assay. After removal of the immunoprecipitate with protein A-Sepharose by centrifugation, the microsomal protein suspension supernatants revealed depletion of carboxylase activity in samples treated with anti-carboxylase-(86–99) antibody. Increasing amounts of anti-carboxylase-(86–99) antibodies led to depletion of increasing amounts of carboxylase activity. In contrast, preimmune immunoglobulin failed to immunoprecipitate activity.

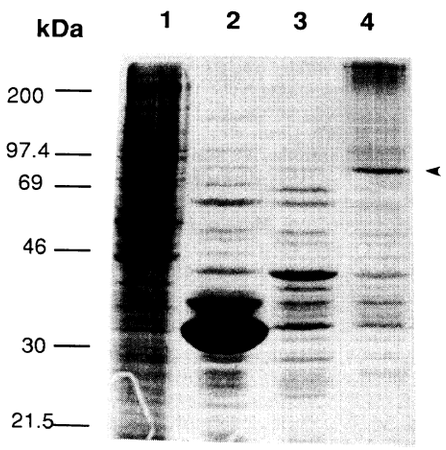

FIG. 2. Metabolic radiolabeling of Sf9 cells. Sf9 cells were infected as described for protein expression studies. At 48 h after infection, cells were radiolabeled with L-[^{35}S]methionine. Equivalent volumes of radiolabeled cell lysates were analyzed by electrophoresis in reducing SDS/10% polyacrylamide gels. The positions of molecular mass standards are indicated. Sf9 cells were infected as follows. Lanes: 1, uninfected; 2, wild-type vAcMNPV-infected; 3, control recombinant virus vBLII /AcMNPV-infected; 4, carboxylase virus vbCbx/AcMNPV-infected.

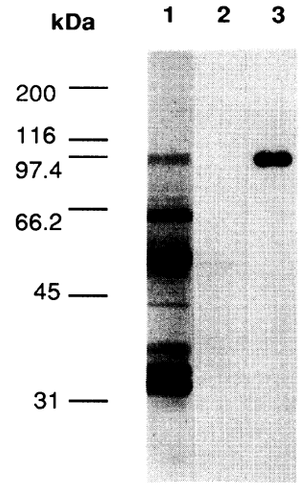

FIG. 3. Western blot analysis of bovine liver carboxylase. Partially purified bovine liver carboxylase was subjected to SDS/PAGE under reducing conditions in 10% gels (300 ng was applied in lane 1; 75 ng was applied in lanes 2 and 3). Lanes: 1, visualized with silver stain; 2 and 3, visualized by immunoblot analysis; 2, preimmune immunoglobulin; 3, anti-carboxylase-(86–99) antibodies.

DISCUSSION

γ-Glutamyl carboxylation is a critical posttranslational modification of the vitamin K-dependent blood coagulation and regulatory proteins in that the formation of γ-carboxyglutamic acid is required for protein function. The modification of specific glutamic acid residues to Gla residues promotes metal-ion-dependent interactions of the blood coagulation proteins with membrane surfaces enabling these proteins to express full biological activity. A recognition unit, termed the γ-carboxylation recognition site (9, 24, 29), resides adjacent to an amphipathic α-helix on the propeptide of the precursor forms of the vitamin K-dependent blood clotting proteins (30). This recognition element binds to the carboxylase and directs the vitamin K-dependent carboxylation of the specific glutamic acid residues. A detailed analysis of this unique vitamin K-dependent process has been hampered by the difficulty in obtaining stable highly purified carboxylase in suitable quantity.

Using a synthetic propeptide based upon the structure of proprothrombin, bovine liver carboxylase was significantly purified by affinity chromatography (10). However, a major protein contaminant, BiP (hsp78 or glucose-regulated protein), an abundant protein in the endoplasmic reticulum, copurified with carboxylase. Using a modification of this affinity-purification strategy, a recombinant peptide corresponding to the propeptide plus Gla domain of factor IX was used to purify a 94-kDa bovine liver carboxylase to near homogeneity (11). This preparation of purified enzyme exhibited high specific carboxylase activity. Expression of the human and bovine cDNAs corresponding to the 94-kDa bovine liver protein results in carboxylase activity above the endogenous activity level in transfected mammalian cells (12, 13). However, this does not rigorously distinguish between the expression of carboxylase activity encoded by the trans-

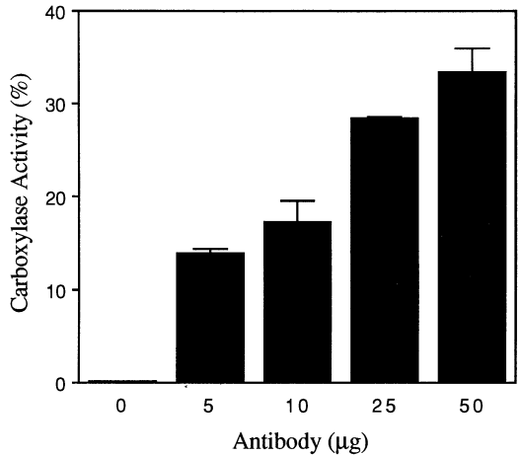

FIG. 4. Immunodepletion of recombinant carboxylase activity with anti-carboxylase-(86–99) antibodies. Solubilized recombinant carboxylase from vbCbx/AcMNPV-infected Sf9 microsomes was prepared. Equal quantities of carboxylase activity (15 units) were incubated with various quantities of anti-carboxylase-(86–99) antibodies as indicated. After removal of the immunoprecipitate with protein A-Sepharose followed by centrifugation, the microsomal protein suspension supernatants were assayed in duplicate for residual carboxylase activity *in vitro*. The carboxylase activity depleted from the supernatants is presented as a percentage of the starting activity. These data are the average (±SD) of duplicate samples. Preimmune immunoglobulin (50 μg) failed to immunodeplete recombinant carboxylase activity with the supernatant containing 103.8 ± 2.5% of the starting activity.

fected cDNA and a protein that augments endogenous carboxylase activity. For this reason, the evidence that the original cDNA encodes the putative carboxylase remains circumstantial. Furthermore, affinity purification of bovine liver carboxylase, exploiting the association of the enzyme with proprothrombin, has resulted in the purification of a 98-kDa protein thought to represent a vitamin K-dependent carboxylase with higher specific activity than the 94-kDa protein (14). The relationship of this 98-kDa protein to the bovine protein we have expressed is currently unknown. Thus, definitive identification of the vitamin K-dependent γ-glutamyl carboxylase is lacking.

The expression of vitamin K-dependent carboxylase activity in Sf9 insect cells, which lack endogenous activity, strongly supports the notion that the 94-kDa bovine liver protein encoded by this cDNA is a vitamin K-dependent γ-glutamyl carboxylase. Furthermore, anti-carboxylase antibodies, specific for bovine liver carboxylase on Western blots, recognize recombinant carboxylase on Western blots and immunoprecipitate recombinant carboxylase activity from vbCbx/AcMNPV-infected Sf9 cells. In addition, the purified 94-kDa protein from bovine liver, which is recognized by the anti-carboxylase antibody, was affinity-labeled with *N*-bromoacetyl-FLEELY, a substrate for carboxylase, and an irreversible time-dependent inactivator of the enzyme (28). Thus these data demonstrate that the cDNA corresponding to the 94-kDa protein represents one gene encoding a vitamin K-dependent γ-glutamyl carboxylase.

The estimated molecular mass of recombinant carboxylase expressed in insect cells (90 kDa) is less than the native protein (94 kDa) isolated from bovine liver or the recombinant carboxylase (94 kDa) expressed in CHO cells (13). Differences in higher-order glycosylation are known to exist between insect cells and mammalian cells (17). With eight potential N-linked glycosylation sites, differences in glycosylation are likely to account for the discrepancy in size. The vitamin K-dependent carboxylation of the pentapeptide substrate FLEEL and the stimulation of FLEEL carboxylation by addition of factor IX propeptide during the *in vitro* carboxylation reaction suggests that, despite putative differences in glycosylation, recombinant carboxylase synthesized in insect cells retains active-site reactivity toward glutamic acid and retains the ability to bind the γ-carboxylase recognition site present in the propeptide of substrate proteins.

The expression of the 94-kDa vitamin K-dependent carboxylase in baculovirus-infected insect cells will allow for the heterologous expression of mutant forms of carboxylase in the absence of contaminating endogenous wild-type activity and should permit detailed structure–function analysis of the enzyme. Further mechanistic analyses with purified enzyme should shed light on the role of vitamin K as a cofactor in this enzymatic reaction.

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