The Inhibitory Effect of Calumenin on the Vitamin K-dependent γ-Carboxylation System

CHARACTERIZATION OF THE SYSTEM IN NORMAL AND WARFARIN-RESISTANT RATS*

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The vitamin K-dependent γ-carboxylation system is responsible for post-translational modification of vitamin K-dependent proteins, converting them to Gla-containing proteins. The system consists of integral membrane proteins located in the endoplasmic reticulum membrane and includes the γ-carboxylase and the warfarin-sensitive enzyme vitamin K 2,3-epoxide reductase (VKOR), which provides γ-carboxylase with reduced vitamin K, cofactor. In this work, an in vitro γ-carboxylation system was designed and used to understand how VKOR and γ-carboxylase work together as a system and to identify factors that can regulate the activity of the system. Results are presented that demonstrate that the endoplasmic reticulum chaperone protein calumenin is associated with γ-carboxylase and inhibits its activity. Silencing of the calumenin gene with siRNA resulted in a 5-fold increase in γ-carboxylase activity. The results provide the first identification of a protein that can regulate the activity of the γ-carboxylation system. The prepropeptides of vitamin K-dependent proteins stimulate γ-carboxylase activity. Here we show that the factor X and prothrombin propeptides do not increase reduced vitamin K, cofactor production by VKOR in the system where VKOR is the rate-limiting step for γ-carboxylation (Wallin, R., Sane, D. C., and Hutson, S. M. (2002) Thromb. Res. 108, 221–226). These findings put calumenin in a central position concerning regulation of γ-carboxylation of vitamin K-dependent proteins. Reduced vitamin K, cofactor transfer between VKOR and γ-carboxylase is shown to be significantly impaired in the in vitro γ-carboxylation system prepared from warfarin-resistant rats. Furthermore, the sequence of the 18-kDa subunit 1 of the VKOR enzyme complex (Rost, S., Fregin, A., Ivaskevicius, V., Conzelmann, E., Hortnagel, K., Pelz, H-J., Lappegard K., Seifried, E., Scharer, I., Tuddenham, E. G. D., Muller, C. R., Storm, T. M., and Olden burg, J. (2004) Nature 427, 337–341) was found to be identical in the two rat strains. This finding supports the notion that different forms of genetic warfarin resistance exist.

The vitamin K-dependent γ-carboxylation system is a multicomponent system of integral membrane proteins and lipids located in the endoplasmic reticulum (ER) which modifies vitamin K-dependent proteins post-translationally by adding an extra carbonyl group onto the -carbon of specific Glu residues located normally in the N-terminal part of the proteins. This modification converts Glu residues to Gla residues, which bind calcium. The calcium binding is essential for allowing the proteins to fulfill their physiological functions. In the blood coagulation system, seven proteins produced by the liver (prothrombin, factors VII, IX, and X, protein S, protein C, and protein Z) are dependent on the modification. Vitamin K-dependent proteins are also made outside the liver. They include the bone proteins osteocalcin and matrix Gla protein, the Axl ligand Gas6, and four putative membrane proteins PRGP1, PRGP2, TmG3, and TmG4, some of which are located in the brain.

Activity measurements have shown that the vitamin K-dependent γ-carboxylation system is present in most cells and tissues isolated from an eukaryotic organism. This indicates that vitamin K-dependent proteins play vital roles in a variety of physiological processes. One example is the functioning of matrix Gla protein as a binding protein for bone morphogenetic protein-2 (11), a mechanism that may be involved in regulation of the growth factor activity of bone morphogenetic protein-2. Certain bleeding disorders (12) and pathological calcification of the arterial wall (13) have been associated with impaired vitamin K-dependent γ-carboxylation of proteins (14).

In order to understand the underlying pathology caused by impaired γ-carboxylation, it is necessary to understand the γ-carboxylation system at the molecular level. The vitamin K-dependent γ-carboxylation system consists of 1) the vitamin K-dependent γ-carboxylase, which requires the reduced hydroquinone form of vitamin K 1 (vit.K 1H 2) as cofactor, and 2) the warfarin-sensitive enzyme vitamin K 2,3-epoxide reductase (VKOR), which produces the cofactor (15). Concomitant with γ-carboxylation, the hydroquinone is converted to the metabolite vitamin K 2,3-epoxide, which is reduced back to the vit.K 1H 2 cofactor by VKOR (15). This interconversion of vitamin K metabolites is known as the vitamin K cycle (16). Of all of the components that constitute the γ-carboxylation system, only the γ-carboxylase has been purified, cloned, and characterized extensively (17). The γ-carboxylase is a 94-kDa protein with five putative transmembrane domains spanning the ER membrane (18). VKOR, on the other hand, appears to be an enzyme complex embedded in the ER membrane, where more than one protein is needed for expression of warfarin-resistant proteins.

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sensitive vitamin K$_{1}$ 2,3-epoxide reducing activity (2). Recently, an 18-kDa hydrophobic ER membrane protein has been identified that appears to be a subunit of the VKOR protein-lipid membrane complex (19).

In our efforts to understand the VKOR enzyme complex and warfarin resistance, we compared kinetically the complex in normal rats and warfarin-resistant rats and used 4-azido-3$_{1}$H-warfarin alcohol for labeling and identification of the warfarin binding component of VKOR (1). From experiments with partially purified preparations of VKOR from normal and warfarin-resistant rats, we demonstrated that the ER chaperone protein calumenin became labeled radioactively (1). Additional experiments showed that 1) calumenin was overexpressed in the liver of rats from our warfarin-resistant colony, 2) the protein sequence of calumenin in resistant rats and normal rats were identical, and 3) the recombinant protein inhibited VKOR activity and at the same time made VKOR more resistant to inhibition by warfarin (1). We concluded from these experiments that overexpression of calumenin in liver may represent one form of genetic warfarin resistance seen in the rat.

Most biochemical data on VKOR have been derived from experiments with crude liver microsomes. Consistent findings from these experiments have been 1) genetic warfarin resistance, attributed to VKOR, is associated with low VKOR activity in liver, and 2) resistant rats require high intake of vitamin K to avoid bleeding (20–22). Collectively, these observations have led to the consensus that warfarin resistance is linked to a genetically altered VKOR enzyme (16). Recently, this has been verified in humans and rats that are genetically resistant to warfarin (19). However, work with our colony of warfarin-resistant rats questions this model as the only mechanism for resistance, since we have been able to produce γ-carboxylation systems from normal and warfarin-resistant rats that are kinetically indistinguishable. Thus, the mechanism(s) responsible for the poorly functioning γ-carboxylation system in our colony of warfarin-resistant rats could not be the result of dysfunctional VKOR and γ-carboxylase enzymes. In order to explain this mechanism(s) and also to expand our understanding of the γ-carboxylation system, we have undertaken further studies on the γ-carboxylation system in normal and warfarin-resistant rats. Here we demonstrate that calumenin is an inhibitory protein of γ-carboxylation and is associated with γ-carboxylase in the ER. In addition, we demonstrate that transfer of vit.K$_{1}$H$_{2}$ cofactor from VKOR to γ-carboxylase is impaired in our colony of warfarin-resistant rats, which may be a contributing factor to the poorly functioning γ-carboxylation systems normally observed in warfarin-resistant rats. Furthermore, we found the sequence of the 18-kDa VKOR subunit 1 (19) to be identical in normal and warfarin-resistant rats.

**EXPERIMENTAL PROCEDURES**

**Materials**

The γ-carboxylase peptide substrate FLEEL was from Sigma. The factor X propeptide ESLFIRREQANNILARVTRA and the prothrombinase sequence (23) was synthesized by the Protein Core Laboratory at Wake Forest University School of Medicine (Winston-Salem, NC). All peptides were more than 99.5% pure. CHAPS, cyclophilin B and α-tubulin were from Affinity Bioreagents (Golden, CO) and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), respectively.

**γ-carboxylase activity**. Cells were prepared for experiment with the calumenin-pcDNA3.1/Zeo expression vector was carried out with the FuGene 6 transfection system from Roche Applied Science. Forty-eight h after transfection, cells were harvested in phosphate-buffered saline and suspended in 250 mM phosphate, 0.5 M KCl, 20% glycerol, 0.75% CHAPS, pH 7.85 (buffer D) containing 10 μl of the Sigma protease inhibitor mixture per ml. Final protein concentration was 2 mg/ml.

**Immunoprecipitations** — Cells were transfected and harvested as described above and extracted with the modified RIPA buffer 50 mM Tris, 1% Nonidet P-40, 0.25% deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, pH 7.4, containing 1 μg/ml each of the protease inhibitors aprotinin, leupeptin, and pepstatin as recommended by Upstate Biotechnology, Inc. (Lake Placid, NY).

**Agarose beads** with affinity-purified antibodies to the calumenin gene were used to analyze the calumenin mRNA sequence, and four Smart-selected siRNA duplexes were synthesized and provided in a single pool for use in experiments aimed at silencing the calumenin gene. A human cyclophilin B siRNA duplex was provided as the positive control. The negative control consisted of four pooled nonspecific siRNA duplexes with an average GC content of 33%.

**Cellular Transfection with siRNA**

HEK293 cells were cultured in 10-cm dishes in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. siRNAs were combined with Lipofectin (Invitrogen) and transfected according to the protocol provided by Dharmaco, Inc. (Lafayette, CO) using the Dharmaco Smart technology. A sophisticated algorithm was used to analyze the calumenin mRNA sequence, and four Smart-selected siRNA duplexes were synthesized and provided in a single pool for use in experiments aimed at silencing the calumenin gene. A human cyclophilin B siRNA duplex was provided as the positive control. The negative control consisted of four pooled nonspecific siRNA duplexes with an average GC content of 33%.
Experiments were done as approved by the Animal Care and Use Committee at Wake Forest University School of Medicine. Liver microsomes and partially purified preparations of VKOR were prepared according to procedures published by our laboratory (1, 26).

**Extraction of Microsomes**

For removal of microsomal luminal and peripherally bound membrane proteins, microsomes obtained from 4 g of rat liver were suspended in 8 ml of 100 mM Na2CO3, 1.2 M KCl, 0.025% deoxycholate, pH 11.5, with a Potter Elvehjem glass homogenizer and centrifuged at 100,000 x g for 45 min (27). The pellet was resuspended in 8 ml of 50 mM Tris base with the same homogenizer and centrifuged a second time at 100,000 x g for 45 min. Pellets (extracted microsomes) were stored at -85 °C until used for experiments.

**SDS-PAGE and Western Blotting**

Prior to SDS-PAGE, proteins in buffer D-solubilized microsomes were precipitated with cold acetone (−20 °C) by mixing 1 part of a protein solution with 5 parts of acetone. The mixture was left overnight at −20 °C, and precipitated protein was harvested by centrifugation. The precipitated protein was washed consecutively with 5 ml of cold 10% trichloroacetic acid (4 °C) and 5 ml of cold ether/ethanol (1:1, v/v) (−20 °C). The protein pellet from the ether/ethanol wash was dried by N2 aspiration and dissolved in SDS-PAGE running buffer containing 2% mercaptoethanol. Cell proteins in RIPA buffer were mixed with SDS-PAGE running buffer prior to electrophoresis. Western blotting of proteins transferred to polyvinylidene difluoride membranes was carried out as described (1).

**Reverse Transcriptase-PCR and DNA Sequencing**

Five μg of total RNA from livers of normal and warfarin-resistant rat was used for cDNA synthesis using oligo(dT) according to the manufacturer’s recommendation (Invitrogen). The following primers were used to produce a cDNA corresponding to the ortholog on chromosome 1 in the rat representing the human gene on chromosome 16 identified by Root et al. (19) to express the 18-kDa warfarin-sensitive subunit 1 of the VKOR enzyme complex. Primer design was based on the rat mRNA sequence with accession number XM 219366. The primer for the sense strand was 5ʹ-TGT CGA CAT GGG CAC CAC CTG GAG-3ʹ, which covered positions 14-37 bp in the published sequence. The antisense primer was 5ʹ-ATG AGG TGC CAC CTC AGG CCT TTT TG-3ʹ, which covered positions 494-519 bp. Thirty-five cycles of PCR were performed with the following conditions: denaturation at 94 °C for 30 s, annealing for 1 min at 60 °C, and then extension for 2 min at 72 °C. At the end of 35 cycles, the reaction was extended for 7 min at 72 °C. The PCR products were purified using the Qiagen PCR purification kit and separated on a 1.2% EtBr-stained agarose gel. The purified PCR products were then ligated into the TA cloning vector pCR 2.1 (Invitrogen). Positive clones were identified by EcoRI digestion and sequenced on both strands using M13 reverse and forward primers.

**Enzyme Assays**

Warfarin-sensitive VKOR activity was measured as described (28) by estimating the percentage conversion of vitamin K1 2,3-epoxide to vitamin K. The vitamin and the epoxide were separated on a reversed phase C18 column in 100% methanol and quantified against external standards. γ-Carboxylase activity was assayed as described (29) as 14CO2 incorporation into the synthetic peptide FLEEL. The reaction was either triggered by adding chemically reduced vitamin K1H2 to the assay mixture or triggered by VKOR-produced reduced vitamin in a reaction mixture containing 40 μM vitamin K1 2,3-epoxide and 8 mM dithiothreitol. Both assays were carried out as described (29) with saturating FLEEL concentration for the reactions.

**RESULTS**

**Inhibition of γ-Carboxylase Activity by Calumenin**—In a previous publication (1), we showed that calumenin inhibits VKOR and VKOR supported γ-carboxylase activity. The observed inhibition of VKOR-supported γ-carboxylase activity could have reflected a reduction in delivery of vit.K1H2 cofactor to γ-carboxylase, but similar results could also have been obtained if calumenin inhibits γ-carboxylase activity. To test this hypothesis, we expressed calumenin in COS-1 cells and measured γ-carboxylase activity in transiently transfected cells. As shown in Fig. 1A, specific γ-carboxylase activity was reduced 53% in transfected cells (Transfect.) compared with the control (Cont.). Fig. 1B shows a Western blot of cell proteins present in the test system. Our affinity-purified calumenin antibodies recognized calumenin in control cells and transfected cells. However, a significantly higher concentration of calumenin was present in transiently transfected cells, supporting the hypothesis that calumenin does interfere with γ-carboxylase and inhibits its enzymatic activity. Additional evidence supporting this hypothesis was obtained from experiments with rat liver microsomes where we physically removed calumenin by carrying crude microsomes through an extraction procedure. Fig. 2, C and D, shows that calumenin was removed from microsomal vesicles when microsomes from warfarin-resistant rats were extracted sequentially with 1) the deoxycholate-containing carbonate buffer, pH 11.5 (Carb.-Doc-pH11.5 ext.) and 2) the 50 mM Tris base solution (Tris base ext.) (see “Experimental Procedures”). The proteins remaining in the microsomal vesicles after extraction are shown in Fig. 2C, lane 4. These proteins represent integral proteins of the ER membrane (27). Fig. 2D shows a Western blot of the proteins shown in Fig. 2C. Calumenin antibodies recognized calumenin in the deoxycholate-containing carbonate buffer, pH 11.5, and the 50 mM Tris base extracts (lanes 2 and 3) but failed to recognize calumenin among the proteins present in the extracted vesicles (lane 4). The extraction procedure did not remove γ-carboxylase and VKOR from the microsomal membrane, since no activity of these enzymes could be detected in the two extracts (data not shown).

As shown in Fig. 2, A and B, both VKOR and γ-carboxylase activities are significantly lower in microsomes (Mic.) from warfarin-resistant rats (R) compared with microsomes from normal rats (N). Fig. 2A also shows that VKOR in microsomes from warfarin-resistant rats is less sensitive to warfarin (W) inhibition (compare Mic.; R and R + W) than VKOR in microsomes from normal (N) rats (compare Mic.; N and N + W). However, when extracted microsomes (Ext.-Mic.) were tested for VKOR and γ-carboxylase activities, both specific activities...
were found to be not significantly different in warfarin-resistant and normal rats (see A and B, Ext.-Mic.). The extraction procedure also produced microsomal vesicles from resistant and normal rats with VKOR activities that were equally sensitive to inhibition by warfarin (A, Ext.-Mic.). In summary, these results demonstrate that we could prepare γ-carboxylation systems from warfarin-resistant and normal rats with indistinguishable VKOR and γ-carboxylase activities and that the observed increase in these activities coincided with the removal of calumenin from the test system.

Calumenin siRNA Inhibits Synthesis of Calumenin and Stimulates γ-Carboxylase Activity—In this experiment, we used the highly specific technology of siRNA to directly demonstrate an effect of calumenin on γ-carboxylase activity. Fig. 3A shows that knockdown of the positive control protein cyclophilin B was lower at 72 h than at 48 h. Scanning of the immunoreactive protein bands showed integrated band intensities that were 49% (48 h) and 30% (72 h) of the control (100%) (see Fig. 3C). No significant change in α-tubulin cell concentration was measured, indicating that the cells were viable for 72 h. Based on these data, we elected to transfect the cells with calumenin siRNA for 72 h. As shown in Fig. 3, B and D, after 72 h calumenin was reduced 75% in cells transfected with calumenin siRNA (Calumenin siRNA) compared with the control (Control siRNA). Thus, the calumenin Smart siRNA pool had silenced the calumenin gene significantly, and its effect on γ-carboxylase activity could be determined. These results are shown in Fig. 4. Compared with the control (Control siRNA), specific γ-carboxylase activity triggered with chemically reduced vitamin K$_2$H$_2$ had increased 5-fold in cells transfected with calumenin siRNA (Calumenin siRNA). This result added strong support to the hypothesis that calumenin is an inhibitor of the γ-carboxylation system.

Calumenin Is Physically Associated with γ-Carboxylase in the ER—Calumenin is a chaperone for ER proteins and has been shown by electron microscopy to be associated with ER membrane proteins (30). Since we could demonstrate effects of calumenin on γ-carboxylase activity, we designed experiments to find out whether a physical association could be demonstrated between calumenin and γ-carboxylase. Affinity-purified anti-calumenin antibodies covalently linked to agarose beads were used for immunoprecipitation of calumenin and proteins associated with calumenin in the RIPA buffer extract obtained from COS-1 cells transfected transiently with the calumenin cDNA construct. γ-Carboxylase activity could be measured in these cell extracts (data not shown), which showed that the modified RIPA buffer (see “Experimental Procedures”) used for extraction released γ-carboxylase from the ER membrane. Fig. 5A shows Western blots with anti-calumenin and anti-γ-carboxylase antibodies of proteins present in the wash and the three consecutively collected protein fractions eluted from the beads with the pH 2.8 elution buffer. Calumenin antibodies identified calumenin in the wash and in the second and third eluted fractions (Fig. 5A, Calumenin abs.). γ-Carboxylase was identified in the wash and the third eluted fraction (A, anti-γ-carboxylase abs.), suggesting that γ-carboxylase was attached to calumenin that was bound to the beads via the immobilized anti-calumenin antibodies. Control beads with rabbit IgG retained neither calumenin nor γ-carboxylase (data not shown). We also carried out immunofluorescence chromatography of the RIPA buffer extract using an open column packed with the beads. Fig. 5B shows an SDS-PAGE image of Coomasie Blue-stained proteins (Coomassie stain) retained by the column resin and eluted from the column with 4 M urea, 0.5 M NaCl, pH 4.0, and Western blots of these proteins with anti-calumenin (Calumenin abs.) and γ-carboxylase antibodies (γ-carboxylase abs.), respectively. The antibodies identified calumenin and γ-carboxylase in the retained fraction. When this experiment was carried out with a column packed with beads attached to rabbit IgG, the column retained neither calumenin nor γ-carboxylase (data not shown). Several other proteins were also retained by the anti-calumenin affinity column (see Fig. 5B, Coomassie stain). Some of these proteins are likely to represent additional ER proteins to which calumenin binds as a chaperone but could also represent additional proteins needed for assembly of the γ-carboxylation system as a supramolecular protein complex.

Cofactor Transfer between VKOR and γ-Carboxylase in Normal and Warfarin-resistant Rats—As shown in Fig. 2, extraction of liver microsomes produced γ-carboxylation systems from normal and warfarin-resistant rats that were indistinguishable when characterized by their individual VKOR and γ-carboxylase activities. If the systems in the two rat strains behaved identically, we expected VKOR-supported γ-carboxylase activity to also be the same in the extracted microsomes from the two rat strains. Fig. 6 shows FLEEL carboxylation by γ-carboxylase when the vitamin K$_2$H$_2$ cofactor was provided by VKOR as a result of vitamin K$_1$ 2,3-epoxide reduction. As shown, the specific activity in the lipid-protein-detergent vesicles from normal rats was 2.5-fold higher than this activity measured in the vesicles from warfarin-resistant rats at saturating concentrations of FLEEL for the reaction. This result was reproduced in three separate experiments with three dif-
Fig. 3. Calumenin siRNA reduces calumenin protein in HEK293 cells. HEK293 cells were cultured and transfected with siRNAs as described under “Experimental Procedures.” A, a control experiment with siRNA specific for cyclophilin B. Cells were harvested at times 0, 48, and 72 h, and cellular proteins were processed for SDS-PAGE and Western blotting (see “Experimental Procedures”). Lanes labeled Control and Control siRNA have proteins from cells transfected with Lipofectin only and nonspecific siRNA, respectively. Lanes labeled Cyclophilin B have proteins from cells transfected with cyclophilin B siRNA. Each lane was adjusted to contain equal amounts of protein (25 μg) and probed with antibodies against cyclophilin B and α-tubulin, respectively. B, a 72-h transfection experiment with control siRNA (Control siRNA) and the Smart pool of calumenin siRNA (Calumenin siRNA). Each lane contains 25 μg of protein and was probed with anti-calumenin and α-tubulin antibodies, respectively. C and D, integrated band intensities of the cyclophilin B and anti-calumenin immunoreactive bands as a percentage of controls (100%).

Fig. 4. Calumenin siRNA enhances γ-carboxylase activity in HEK293 cells. Cells transfected with control siRNA (Control siRNA) and the calumenin Smart pool of siRNA (Calumenin siRNA) were harvested after 72 h and prepared for measurements of γ-carboxylase activity triggered with chemically reduced vitamin K₃H₂ as described under “Experimental Procedures.” Specific γ-carboxylase activities in control and calumenin siRNA-transfected cells are shown. Each measurement is the average of three parallel incubations, and S.D. values are shown.

Different preparations of extracted microsomes from normal and warfarin-resistant rats and revealed a significant difference in the γ-carboxylation systems present in normal rats and our warfarin-resistant rats at the level of cofactor transfer in the two systems.

Communication between VKOR and γ-Carboxylase within the γ-Carboxylation System—We have shown previously that VKOR is the rate-limiting step in the γ-carboxylation system (29). Thus, in order to cope with a demand for increased output of γ-carboxylated proteins by the system, increased cofactor production by VKOR is needed. Since the propeptides of vitamin K-dependent proteins stimulate γ-carboxylase activity (31), we asked whether the propeptides could produce a signal within the γ-carboxylation system that would result in increased cofactor production by VKOR. For these experiments, we elected to use the factor X propeptide, since this propeptide has been shown to have the greatest affinity for γ-carboxylase (31). We also investigated the effect of the prothrombin propeptide. We used extracted microsomes from normal rat livers as
did not affect VKOR activity. However, when present together, the propeptides, when present in the test system, showed that these propeptides, when present in the test system, had the same \( \gamma \)-carboxylase activity when triggered with chemically reduced vitamin \( \mathrm{K}_1 \) (22,500 \( \pm \) 990 cpm/mg, \( n = 3 \)). VKOR activities were also insignificantly different in the two preparations (2.5 \( \pm \) 0.1 nmol/mg, \( n = 3 \)). Shown is \( \gamma \)-carboxylase activity measured with increasing FLEEL concentration present in the test system. Activity was triggered with vitamin \( \mathrm{K}_1 \) produced by VKOR at saturating dithiothreitol and vitamin K \( 1 \), 2,3-epoxide concentrations for the reaction (see “Experimental Procedures”). Each data point is the average of three parallel incubations differing by <5%. Filled circles, vesicles from normal rats. Filled triangles, vesicles from warfarin-resistant rats.

![Figure 6](https://example.com/fig6.png)

**Fig. 6.** VKOR-supported \( \gamma \)-carboxylase activity in normal and warfarin-resistant rats. Microsomal vesicles extracted with the carbonate-deoxycholate, pH 11.5, buffer and 50 mM Tris base (see “Experimental Procedures”) were suspended in buffer D and used for \( \gamma \)-carboxylase assays. Proteins were adjusted to the same concentration (5.4 mg/ml) in both preparations, which had the same \( \gamma \)-carboxylase activity when triggered with chemically reduced vitamin \( \mathrm{K}_1 \) \( \pm \) 990 cpm/mg, \( n = 3 \)). VKOR activities were also insignificantly different in the two preparations (2.5 \( \pm \) 0.1 nmol/mg, \( n = 3 \)). Shown is \( \gamma \)-carboxylase activity measured with increasing FLEEL concentration present in the test system. Activity was triggered with vitamin \( \mathrm{K}_1 \) produced by VKOR at saturating dithiothreitol and vitamin K \( 1 \), 2,3-epoxide concentrations for the reaction (see “Experimental Procedures”). Each data point is the average of three parallel incubations differing by <5%. Filled circles, vesicles from normal rats. Filled triangles, vesicles from warfarin-resistant rats.

![Figure 7](https://example.com/fig7.png)

**Fig. 7.** The propeptides of factor X and prothrombin do not stimulate vitamin \( \mathrm{K}_1 \) \( \gamma \)-carboxylase activity in an \( \text{in vitro} \) \( \gamma \)-carboxylation system. Extracted microsomal vesicles from normal rats were prepared as described under “Experimental Procedures” and suspended in buffer D for \( \gamma \)-carboxylase and VKOR activity measurements. A, \( \gamma \)-carboxylase activity triggered with chemically reduced vitamin \( \mathrm{K}_1 \), in the absence (FLEEL) and presence of 10 \( \mu \)M of the prothrombin (FLEEL + Pro.II) and the factor X propeptides (FLEEL + Pro.X), respectively. Activities are shown as percentage of the control (FLEEL, 100%), which contained no propeptide. Activities were assayed at 37°C. B, VKOR activity in the presence of 10 \( \mu \)M of the prothrombin propeptide (Pro.II), 10 \( \mu \)M of the factor X propeptide (Pro.X), and 10 \( \mu \)M FLEEL (FLEEL), 10 \( \mu \)M prothrombin propeptide plus 40 \( \mu \)M FLEEL (FLEEL + Pro.II) and 10 \( \mu \)M factor X propeptide plus 40 \( \mu \)M FLEEL (FLEEL + Pro.X), respectively. VKOR activity is shown as the percentage of the control (100%) that contained no propeptide and FLEEL (Cont.). C, VKOR activity in a partially purified preparation of VKOR in the absence (Cont.) and presence of 40 \( \mu \)M FLEEL (FLEEL). All activities are the average of three parallel incubations. S.D. values are indicated on the bars.

In this work, we have identified, for the first time, a protein that can regulate the activity of the \( \gamma \)-carboxylation system. This conclusion is based on data that include 1) inhibition of \( \gamma \)-carboxylase activity by transient transfection with a calumenin cDNA construct, 2) silencing of the calumenin gene by Smart siRNA, and 3) a proteomic approach that demonstrates the existence of protein-protein interactions between \( \gamma \)-carboxylase and calumenin. In a previous paper (1), we showed that calumenin inhibits VKOR activity and protects the enzyme from warfarin inhibition. Together, these data indicate that calumenin plays a role in regulation and performance of the \( \gamma \)-carboxylation system and thus biosynthesis of functional vitamin K-dependent \( \gamma \)-carboxylase.

**Discussions**

In this work, we have identified, for the first time, a protein that can regulate the activity of the \( \gamma \)-carboxylation system. This conclusion is based on data that include 1) inhibition of \( \gamma \)-carboxylase activity by transient transfection with a calumenin cDNA construct, 2) silencing of the calumenin gene by Smart siRNA, and 3) a proteomic approach that demonstrates the existence of protein-protein interactions between \( \gamma \)-carboxylase and calumenin. In a previous paper (1), we showed that calumenin inhibits VKOR activity and protects the enzyme from warfarin inhibition. Together, these data indicate that calumenin plays a role in regulation and performance of the \( \gamma \)-carboxylation system and thus biosynthesis of functional vitamin K-dependent proteins.

Calumenin belongs to the CREC subfamily of \( \mathrm{Ca}^{2+} \)-binding proteins where an EF-hand motif binds the metal (32). The CREC proteins are found in the secretory pathway, and most of the proteins have been shown to have chaperone functions (32).
All proteins have C-terminal retrieval sequences (HDEL, HDEF, and HEEF) for retention either in the ER or the Golgi apparatus (32). In contrast to other EF-hand family members like calmodulin and parvalbumin (33), the CREC proteins show weak Ca\(^{2+}\) binding with association constants in the millimolar range (34). It is known that EF-hand proteins with strong affinity for Ca\(^{2+}\) expose hydrophobic amino acids upon metal binding, which is responsible for binding of these proteins to their targeted partner-proteins (38). Whether or not Ca\(^{2+}\) binding by the CREC proteins is important for their protein-protein interactions is an unsettled issue that needs to be addressed in order to understand the interactions between calumenin and VKOR and \(\gamma\)-carboxylase, respectively. EDTA stimulates VKOR activity when present in the test system.\(^2\) This finding may indicate that metals do play a role in binding.

Calumenin is a water-soluble acidic protein (1, 32). Consistent with electron microscopy studies of the ER membrane (30) and our previous demonstration that calumenin is strongly associated with lipid-detergent micelles derived from the ER membrane (1) is the notion that calumenin, despite being a hydrophilic protein, is associated with ER membrane proteins. As a chaperone, we propose that calumenin has an important regulatory function of the \(\gamma\)-carboxylation system.

Our previous (1) and current results suggest that calumenin targets both VKOR and \(\gamma\)-carboxylase of the \(\gamma\)-carboxylation system and is responsible for the genetic warfarin resistance in our colony of warfarin-resistant rats. Different forms of genetic warfarin resistance has been described for rats (36, 37). Our finding that the 18-kDa subunits 1 of the VKOR enzyme complex in normal and warfarin-resistant rats have identical sequences supports our previous hypothesis that overexpression of calumenin in liver represents one form of genetic warfarin resistance. Rost et al. (19) have identified mutations in the subunit that coincide with warfarin-resistant phenotypic humans and rats. However, the relationship between the mutations and the phenotypes is complex and will require more work to clarify the various types of resistance at the molecular level.

Extraction of microsomal vesicles allowed us to produce calumenin-deficient \(\gamma\)-carboxylation systems from warfarin-resistant and normal rats that were indistinguishable with respect to their individual VKOR and \(\gamma\)-carboxylase activities. On the other hand, the ability of VKOR to provide \(\gamma\)-carboxylase with reduced vit.K\(_1\)H\(_2\) cofactor was found to be significantly impaired in the system prepared from our colony of warfarin-resistant rats. We propose that the result reflects impaired transfer of reduced vit.K\(_1\)H\(_2\) cofactor from VKOR to \(\gamma\)-carboxylase within the \(\gamma\)-carboxylation system. The ER lumen harbors proteins in an oxidative environment (38). Since vit.K\(_1\)H\(_2\) is sensitive to oxidation, transfer of reduced cofactor between VKOR and \(\gamma\)-carboxylase in the ER membrane must be protected from oxidation. Therefore, as depicted by the putative model shown in Fig. 8, we propose that VKOR and \(\gamma\)-carboxylase reside close to each other in the ER membrane and constitute an enzyme complex that is structurally organized for maximum efficiency as a \(\gamma\)-carboxylation system. The lipid bilayer plays an essential role in this organization as we know from phospholipase A2 experiments that the complex will be destroyed by treatment with the lipase.\(^2\) If our results are representative for the system in vivo, the impaired vit.K\(_1\)H\(_2\) cofactor transfer in our resistant rats could explain the need for these rats to increase their intake of vitamin K in order to prevent internal bleeding. Our

\(^2\) N. Wajih, D. C. Sane, S. M. Hutson, and R. Wallin, unpublished data.
The Inhibitory Effect of Calumenin on the Vitamin K-dependent $\gamma$-Carboxylation System: CHARACTERIZATION OF THE SYSTEM IN NORMAL AND WARFARIN-RESISTANT RATS

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