Engineering of a Recombinant Vitamin K-dependent γ-Carboxylation System with Enhanced γ-Carboxyglutamic Acid Forming Capacity

EVIDENCE FOR A FUNCTIONAL CXXC REDOX CENTER IN THE SYSTEM

The vitamin K-dependent γ-carboxylation system in the endoplasmic reticulum membrane responsible for γ-carboxyglutamic acid modification of vitamin K-dependent proteins includes γ-carboxylase and vitamin K 2,3-epoxide reductase (VKOR). An understanding of the mechanism by which this system works at the molecular level has been hampered by the difficulty of identifying VKOR involved in warfarin sensitive reduction of vitamin K 2,3-epoxide to reduced vitamin K1H2, the γ-carboxylase cofactor. Identification and cloning of VKORC1, a proposed subunit of a larger VKOR enzyme complex, have provided opportunities for new experimental approaches aimed at understanding the vitamin K-dependent γ-carboxylation system. In this work we have engineered stably transfected baby hamster kidney cells containing γ-carboxylase and VKORC1 cDNA constructs, respectively, and stably double transfected cells with the γ-carboxylase and the VKORC1 cDNA constructs in a bicistronic vector. All engineered cells showed increased activities of the enzymes encoded by the cDNAs. However increased activity of the γ-carboxylation system, where VKOR provides the reduced vitamin K1H2 cofactor, was measured only in cells transfected with VKORC1 and the double transfected cells. The results show that VKOR is the rate-limiting step in the γ-carboxylation system and demonstrate successful engineering of cells containing a recombinant vitamin K-dependent γ-carboxylation system with enhanced capacity for γ-carboxyglutamic acid modification. The proposed thioredoxin-like CXXC redox center in VKORC1 was tested by expressing the VKORC1 mutants Cys132/Ser and Cys135/Ser in BHK cells. Both of the expressed mutant proteins were inactive supporting the existence of a CXXC redox center in VKOR.

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The family of vitamin K-dependent proteins include the liver produced blood coagulation factors II, VII, IX, X, protein S, protein C, and protein Z (1) and several proteins made in extrahepatic tissues. These proteins are osteocalcin (2), matrix γ-carboxyglutamic acid (Gla) protein (2), protein S (3), growth arrest gene 6 (Gas6) (4), and four putative trans-membrane proteins, PRGP1, PRGP2, TmG3, and TmG4, some of which are located in the brain (5, 6). The vitamin K-dependent proteins are modified post-translationally to contain Gla, Ca2+ binding residues. The modification is carried out by γ-carboxylase, an integral protein of the endoplasmic reticulum (ER) membrane, which requires the reduced form of vitamin K (Vit.KH2) as cofactor (7). Concomitant with formation of Gla residues, Vit.KH2 is converted to vitamin K 2,3-epoxide (Vit.K2,3-O) (7). The epoxide can be reduced and recycled to the Vit.KH2 cofactor by the warfarin sensitive enzyme vitamin K 2,3-epoxide reductase (VKOR) (7), which also is an integral protein of the ER membrane (8). This redox cycling of vitamin K is known as that vitamin K cycle (9). The vitamin K-dependent γ-carboxylation system in the ER membrane consists of the vitamin K cycle enzymes plus additional proteins involved in regulation and formation of the Gla modification in vitamin K-dependent proteins (7).

Purification of γ-carboxylase from the ER membrane and cloning of the enzyme (10) has resulted in a broad understanding of this membrane enzyme and the mechanism by which the enzyme carries out vitamin K-dependent modification of proteins (11). On the other hand, very little is known about the enzymology of VKOR and the mechanism by which the vitamin K-dependent γ-carboxylation system operates in the ER membrane. This lack of insight into the mechanism of operation of the system stems from the difficulty of obtaining purified preparations of VKOR (12) for identification of the protein(s) responsible for VKOR activity. In our attempts to identify protein components of VKOR by photo affinity labeling with azido derivatives of warfarin, we identified a regulatory protein of the vitamin K-dependent γ-carboxylation system (12). The protein was shown to be calumenin, a known ER chaperone belonging to the calcium binding, EF hand motif containing family of proteins (7, 8). The azido derivative of warfarin did not label the catalytic protein responsible for warfarin sensitive VKOR activity (12). Recently the location of genetic warfarin resistance in man was mapped to the 16p12-q21 region on chromosome 16, and the effector gene was identified (13). Rost et al. (14) and Li et al. (15) showed that the gene encodes an 18-kDa transmembrane protein that exhibits warfarin sensitive VKOR activity when expressed in cell lines. The protein is believed to be a subunit (VKORC1) of a larger VKOR lipid-enzyme complex in the ER membrane that we have shown is

† The abbreviations used are: ER, endoplasmic reticulum; Vit.KH2, chemically reduced vitamin K1H2; Vit.K2,3-O, vitamin K 2,3-epoxide; BHK, baby hamster kidney; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.
Cloning of Rat \( \gamma \)-Carboxylase and Creation of an Expression Vector—

The plasmid pCR 2.1 containing full-length rat \( \gamma \)-carboxylase was a kind gift from Dr. David A. Roth, Division of Hemostasis & Thrombosis, Department of Medicine, Harvard Medical School. NotI and XhoI sites were generated at the 5’ and 3’ ends to clone the cDNA into the pBUDCE4.1 mammalian expression vector. A Kozak sequence was also generated at the 5’ end. The modified cDNA for rat \( \gamma \)-carboxylase was then cloned into the NotI and XhoI sites on plasmid pBUDCE4.1 under the control of the EF-1 \( \alpha \)-promoter. Recombinant plasmid with cDNA for rat \( \gamma \)-carboxylase was sequenced on both the strands to eliminate any PCR errors. The new plasmid with rat \( \gamma \)-carboxylase cDNA is named pBUDCE4.1-\( \gamma \)-Carb.

Construction of Plasmid Containing VKORC1 and \( \gamma \)-Carboxylase cDNAs—Plasmid pBUDCE4.1 is a dual promoter vector capable of expressing two independent recombinant proteins. It has two multiple cloning sites each under the control of two independent promoters, primers, virus and EF-1. The first, \( \gamma \)-Carb, was used to transfected BHK cells obtained from the QuikChange mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions. For the Cys\textsuperscript{122}/Ser mutant protein the following primers were used: sense primer, 5’-GTT GAT GTT TGC ACC ACC-3’; antisense primer, 5’-GGT GGT GAT GAT GTA AAC AAT GGA ATC ATG GAG AAA G-3’. (Bold and italic base was changed.) For the Cys\textsuperscript{122}/Ser mutant protein the following primers were used: sense primer, 5’-GAT GCC ACC TAC GCT ACC-3’; antisense primer, 5’-GGT GAC ACC TAC ACC TGC-3’. (Bold and italic base was changed.) After mutagenesis the modified rat VKOR cDNAs were cloned into the pBUDCE4.1 vector as described above for cloning of the wild type VKORC1 cDNA into the vector.

Stable Cell Lines Expressing Rat VKOR—Plasmid pBUDCE4.1-VKOR was used to transfect BHK 21 cells (ATCC number CCL-10) (American Tissue Culture Collection, Manassas, VA). Cells were transfected using the FuGENE 6 transfection reagent (Roche Applied Science) according to the manufacturer’s recommendations. After 24 h, cells were passaged at 1:20 dilution into fresh growth medium (Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum) with Zeocin antibiotic (InviVoGen, San Diego, CA) for selective pressure. After selecting 400 \( \mu \)g/ml of Zeocin, cells were maintained in 200 \( \mu \)g/ml of Zeocin in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. Frozen stocks were made.

Stable Cell Lines Expressing \( \gamma \)-Carboxylase—Plasmid pBUDCE4.1-\( \gamma \)-Carb was used to transfected BHK 21 cells. Cells were transfected and stably transfected clones of cells selected as described above. Frozen stocks were made.

Stably Transfected Cells Expressing VKORC1 Mutant Proteins—Plasmid pBUDCE4.1 containing the Cys\textsuperscript{122}/Ser and the Cys\textsuperscript{135}/Ser mutations, respectively, were used to transfected BHK 21 cells. Cells were transfected and stably transfected clones of cells selected as described above. Frozen stocks were made.

Cellular in Vitro System for Assaying Enzyme Activities Associated with the Vitamin K-dependent \( \gamma \)-Carboxylation System—Cells were washed and harvested in phosphate-buffered saline and suspended in a Potter Elvehjem homogenizer in 250 \( \mu \)l phosphate, 0.5 \( \times \) KCl, 20% glycerol, 0.75% CHAPS, pH 7.85 (buffer D) containing 10 \( \mu \)g/ml of the Sigma protease inhibitor mixture per ml. Final protein concentration was 2.0 mg/ml.

Immunoprecipitation of VKORC1 from Solubilized Extracted Liver Membranes—Extracted liver membrane vesicles were suspended in a Potter Elvehjem homogenizer in 50 mM Hepes, 125 mM KCl, 0.75% CHAPS, pH 7.85 containing 10 \( \mu \)g/ml of the Sigma protease inhibitor mixture. The supernatant was adsorbed with Sepharose-protein A beads before anti-peptide 1 VKORC1 affinity purified antibodies were added. The mixture was rotated over night at 4 °C. An identically prepared sample without the peptide 1 antibodies served as control. Sepharose-protein A particles were again added to the samples, and immune A beads before anti-peptide 1 VKORC1 affinity purified antibodies were added. The mixture was rotated over night at 4 °C.An identically prepared sample without the peptide 1 antibodies served as control. Sepharose-protein A particles were again added to the samples, and immune
complexes were removed by centrifugation. VKOR activities in the anti-peptide 1 immunoabsorbed, and the control samples were measured.

Two-dimensional SDS-PAGE, Mass Spectrometry, and Western Blotting of Proteins in Extracted Liver Microsomal Membranes—Extracted microsomal membranes were solubilized in 9.5 M urea containing sample buffer for isoelectric focusing as described by O’Farrell (23) and modified by our laboratory (24). Insoluble material was removed by centrifugation before the sample was applied to the focusing gel. Isoelectric focusing was carried out overnight and SDS-PAGE in the second dimension run in 10–15% gradient gels as described (12). Gels were stained with Coomassie Brilliant Blue R-250. Selected stained protein spots were excised from the gel and subjected to tandem mass spectrometry analysis for protein identification. The analysis was performed at the Harvard Microchemistry Facility by micro capillary reverse-phase HPLC nanoelectrospray tandem mass spectrometry on a Finnigan LCQ DECA XP Plus quadrupole ion trap mass spectrometer. For Western blotting, proteins were transferred to polyvinylidene difluoride membranes and developed with antibodies to peptide 1 and 2 as described (18) using the ECL plus detection system from Amersham Biosciences.

SDS-PAGE and Western Blotting of Proteins in Cultured Cells—Cells were washed and harvested in phosphate-buffered saline and extracted with the modified radioimmune precipitation assay buffer 50 mM Tris, 1% Nonidet P-40, 0.25% deoxycholate, 150 mM NaCl, 1 mM EDTA, pH 7.4 containing 10 μg/ml of the Sigma protease inhibitor mixture. Cell proteins in radioimmune precipitation assay buffer were mixed with SDS-PAGE running buffer prior to electrophoresis. Western blotting of cell proteins transferred to polyvinylidene difluoride membranes was carried out as described above.

Enzyme Assays—Warfarin sensitive VKOR activity was measured as described (25) by estimating the percent conversion of Vit.K$_{1}$O to vitamin K$_{1}$. The vitamin and the epoxide was separated on a reversed phase C18 column in 100% methanol and quantified against external standards. γ-Carboxylase activity was assayed as described (22) as $^{14}$CO$_{2}$ incorporation into the synthetic peptide FLEEL. The reaction was either triggered by adding chemically reduced vitamin K$_{1}$H$_{2}$ (Vit.KH$_{2}$) (100 μg/ml) to the assay mixture or triggered by VKOR-produced reduced Vit.KH$_{2}$ in a reaction mixture containing 40 μM Vit.K$\rightarrow$O and 8 mM dithiothreitol. Both assays were carried out as described (22) with saturating FLEEL concentration for the reactions. The factor X propeptide was added to the incubations to give a final concentration of 10 μM.

RESULTS

Preparation of Peptide Antibodies against VKOR1—Fig. 1 shows a Kyte-Doolittle hydropathy plot of the predicted amino acid sequence of rat VKOR1 (gi:45827740). The plot was carried out with a window setting at 9. The plot above the heavy horizontal line indicates a plot with window setting at 19, which identifies three potential transmembrane domains labeled 1, 2, and 3. The horizontal axis is scaled to include only those amino acids for which a windowed hydropathy score is computed. The positions of peptide 1 and 2 in the VKOR1 sequence used for peptide antibody productions are indicated, and their sequences shown. Peptide 2 has an extra C-terminal Cys (C) residue used for conjugation. The position of the predicted CXXC redox center in trans-membrane domain 3 is also indicated.

![Kyte-Doolittle hydropathy plot of the predicted amino acid sequence of rat VKOR1 (gi:45827740).](image-url)

**Fig. 1.** Kyte-Doolittle hydropathy plot of the predicted amino acid sequence of rat VKOR1 (gi:45827740). The plot was carried out with a window setting at 9. The plot above the heavy horizontal line indicates a plot with window setting at 19, which identifies three potential transmembrane domains labeled 1, 2, and 3. The horizontal axis is scaled to include only those amino acids for which a windowed hydropathy score is computed. The positions of peptide 1 and 2 in the VKOR1 sequence used for peptide antibody productions are indicated, and their sequences shown. Peptide 2 has an extra C-terminal Cys (C) residue used for conjugation. The position of the predicted CXXC redox center in trans-membrane domain 3 is also indicated.
Blue stained two-dimensional SDS-PAGE gel of proteins present in extracted microsomal membranes. C shows the protein spots on the two-dimensional SDS-PAGE gel identified by the peptide 1 and the peptide 2 antibodies, respectively. As shown in C, two major immunoreactive proteins spots, a (26 kDa) and b (32 kDa) were identified with the C-terminal peptide 2 antibodies, whereas the peptide 1 antibodies only recognized the basic 26-kDa protein in spot a. The acidic 32-kDa protein in spot b was subjected to tandem mass spectrometry and identified as membrane-associated progesterone receptor component 1 (gi:6647578) (26). A homology search revealed no sequence homology between the receptor and VKORC1. Thus we conclude that the peptide 2 antibodies recognized an epitope on the progesterone receptor that mimicked peptide 2. Based on these findings we focused on the peptide 1 antibodies as specific antibodies for VKORC1 which runs as a 26-kDa protein in SDS-PAGE and not as an 18-kDa protein predicted by its amino acid composition. To confirm that the peptide 1 antibodies recognized VKORC1, we tested the ability of these antibodies to remove VKOR from solubilized extracted microsomal membranes. Immune complexes were trapped with Sepharose-protein A beads. The supernatant remaining after removal of the Sepharose-protein A beads by centrifugation had 55% lower VKOR activity than the control, which was treated identically except that it did not contain the peptide 1 antibodies. Although these peptide antibodies gave excellent Western blots of VKORC1, the antibodies were unable to remove all enzymes exhibiting VKOR activity in a soluble preparation of extracted microsomal membranes.

The γ-Carboxylation System in Stably Transfected BHK Cells—Fig. 3 shows the various enzyme activities of the γ-carboxylation system measured in BHK cells stably transfected with VKORC1 (VKOR) and γ-carboxylase (γ-Carb.) cDNA constructs, respectively, and BHK cells stably double transfected with both cDNA constructs (VKOR + γ-Carb.). VKOR activities measured in all cell lines were sensitive to warfarin inhibition (data not shown). As shown in Fig. 3A, γ-carboxylase activity triggered with Vit.K$_2$H$_2$ was not enhanced in cells transfected with the VKORC1 construct (VKOR) over the control (Cont.). On the other hand, Vit.KH$_2$ triggered γ-carboxylase activity was significantly higher in γ-carboxylase transfected (γ-Carb) and the double transfected cells (VKOR + γ-Carb.) than in the control. The results shown in A and C demonstrate that individual overexpression of VKORC1 and γ-carboxylase in BHK cells does not effect expression of its "partner" (VKOR or γ-carboxylase) in the γ-carboxylation system when judged on enzyme activity measurements. The results shown in B provide information about the γ-carboxylation system in the various stably transfected cell lines. In these experiments, which were carried out with the same samples used for the experiments shown in A and C,
VKOR is providing the reduced Vit.KH\textsubscript{2} cofactor for \(\gamma\)-carboxylase. In these experiments the double transfected BHK cells (VKOR/\(\gamma\)-Carb.) had the highest activity measured as \(^{14}\)CO\textsubscript{2} incorporation into the FLEEL-\(\gamma\)-carboxylase substrate. This activity was also enhanced in the VKORC1 transfected cells (VKOR). However, in the \(\gamma\)-carboxylase transfected cells (\(\gamma\)-Carb.) the activity was not significantly different from the control (Cont.). These results indicate that 1) overexpression of \(\gamma\)-carboxylase does not enhance post-translational \(\gamma\)-carboxylation of proteins, 2) VKOR is the rate-limiting step in the \(\gamma\)-carboxylation system, and 3) the double transfected BHK cells appear to be cells containing a recombinant vitamin K-dependent \(\gamma\)-carboxylation system capable of enhanced post-translational \(\gamma\)-carboxylation of proteins.

**Effect of Propeptide on the Recombinant \(\gamma\)-Carboxylation System in BHK Cells**—Newly synthesized precursors of vitamin K-dependent proteins are equipped with a propeptide that is the recognition element that docks the precursors to the \(\gamma\)-carboxylase for post-translational \(\gamma\)-carboxylation of the proteins (1). In addition, the propeptide activates the \(\gamma\)-carboxylase, which results in enhanced \(\gamma\)-carboxylation of proteins and synthetic peptides (27–29). To determine whether these propeptides would have an effect on the recombinant \(\gamma\)-carboxylation system in BHK cells, we added the factor X propeptide to the test samples prepared from the various stably transfected cell lines. The choice of the factor X propeptide was made upon the knowledge of its tight binding to \(\gamma\)-carboxylase (30). To test for propeptide activation of the \(\gamma\)-carboxylation system we used our test system, which requires VKOR to provide the \(\gamma\)-carboxylase with Vit.KH\textsubscript{2} cofactor. As shown in Fig. 4, 10 \(\mu\)M of the factor X propeptide activated the system 2-fold (Control panel). In \(\gamma\)-carboxylase transfected cells (\(\gamma\)-Carb. panel), 10 \(\mu\)M factor X propeptide activated the system 2.2-fold. It should be noted that the activity of the system in \(\gamma\)-carboxylase transfected cells was not significantly different from the activity measured in control cells, again reflecting that overexpression of \(\gamma\)-carboxylase does not result in enhanced \(\gamma\)-carboxylation of FLEEL. On the other hand, 10 \(\mu\)M factor X propeptide activated the system in VKORC1 transfected

![A Recombinant Vitamin K-dependent \(\gamma\)-Carboxylation System](image-url)
cells 7.6-fold (VKOR panel). In the double transfected cells, the system was activated 3.4-fold (VKOR + γ-Carb. panel). Although 10 μM factor X propeptide caused less activation of the system in the double transfected cells, the system in the double transfected cells had significantly higher activity than the system in the VKORC1 transfected cells.

VKORC1 Mutants with an Altered CXXC Redox Center—Bioinformatic analyses of VKORC1 from various species predicted a thioredoxin like CXXC redox center in all proteins analyzed (16). In the rat, the CXXC center is found at positions 132–135 (16). As shown in Fig. 1, the center is predicted to be located in the hydrophobic transmembrane domain 3. We prepared two mutant cDNA constructs of rat VKORC1 in which each of the two Cys residues in the CXXC center were replaced by Ser residues, respectively (mutant 1, 132SXXC135; mutant 2, 132–135SXXC135). Fig. 5 shows VKOR activities measured in BHK cells stably transfected with the mutant 1 and 2 cDNA constructs, respectively, and the wild type VKORC1 cDNA construct. VKOR activity measured in cells transfected with the wild type construct (VKOR cDNA) was enhanced 2.9-fold over the activity measured in control cells (Cont.). The activity measured in cells transfected with the mutant 1 construct (VKOR cDNA/Cys132Ser) was indistinguishable from the activity measured in control cells. The activity measured in cells transfected with the mutant 2 construct (VKOR cDNA/Cys135Ser) was 0.8-fold lower than the activity measured in control cells. Western blotting with the peptide 1 antibody confirmed overexpression of both the wild type protein and the mutant proteins (data not shown). The results support the hypothesis (16) that the 132CXXC135 center in VKORC1 functions as the redox center in VKOR.

**DISCUSSION**

This is the first paper to demonstrate engineering of stably transfected cells carrying a recombinant vitamin K-dependent γ-carboxylation system. These BHK cells are shown to have an enhanced capacity to modify the synthetic γ-carboxylase peptide substrate FLEEL to contain γ-carboxyglutamic acid residues (Gla). Enhanced γ-carboxylation of synthetic peptides by liver microsomal preparations has been shown to parallel enhanced γ-carboxylation of endogenous precursors of vitamin K-dependent proteins present in the microsomes (27–29). Thus the enhanced capacity of our engineered cells to γ-carboxylate FLEEL is likely to also reflect the ability of these cells to produce increased amounts of γ-carboxylated Glα containing proteins. Our finding could be of significant importance for production of recombinant vitamin K-dependent proteins used as pharmaceuticals in treatment of diseases associated with deficiency of such proteins. Hemophilia B is associated with deficiency of clotting factor IX (31). Treatment requires infusion of functional γ-carboxylated factor IX. Currently, recombinant factor IX produced by Chinese hamster ovary cells is used as a pharmaceutical (32). A major problem with production of recombinant vitamin K-dependent coagulation factors has been poor yield of functional γ-carboxylated proteins produced by and isolated from transfected cells (33). Following cloning of the γ-carboxylase (10), its cDNA was introduced into Chinese hamster ovary cells stably transfected with a factor IX cDNA construct with the hope of increasing production of γ-carboxylated factor IX by the cells (34). Increased production was not observed (34). This finding is in accordance with our results where we show that the VKOR-supported γ-carboxylation sys-

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**FIG. 4.** Effect of factor X propeptide on 14C-FLEEL γ-carboxylation triggered by VKOR-supported γ-carboxylase activity. The effect of the factor X propeptide on the γ-carboxylation systems in control BHK cells and the stably transfected cells described in the legend to Fig. 3 was investigated. The activity was measured as 14CO2 incorporated into the FLEEL γ-carboxylase substrate (cpm/mg of cell protein) triggered by Vit.KH2 produced by VKOR in a test system containing Vit.K-0 and dithiothreitol (see “Experimental Procedures”). The name of each panel refers to the cDNAs the cells were transfected with (see legend to Fig. 3). The factor X propeptide was added to each incubation to give a final concentration of 10 μM. Standard deviations are shown as vertical lines on top on the bars (n = 3). Filled bars show results from incubations containing the propeptide.
system in BHK cells stably transfected with a γ-carboxylase cDNA construct exhibits γ-carboxylation activity that is indistinguishable from the activity measured in control BHK cells. Our results clearly demonstrate that VKOR (and not γ-carboxylase) is the rate-limiting step in the system. This conclusion is consistent with earlier published data by our laboratory on the γ-carboxylation system in rat liver (22) and also with results published by others (35).

Only recently, after the successful cloning of the VKORC1 subunit of the putative VKOR lipid-protein enzyme complex, have new experimental approaches become available to understand how the vitamin K-dependent γ-carboxylation system works at the molecular level in the ER membrane. From earlier studies, it became clear that thiol groups are involved in the catalytic mechanism of Vit.K →O reduction by VKOR and that warfarin appears to bind to a thiol redox center in VKOR. The most plausible model for the mechanism of inhibition of VKOR by warfarin came from work by Fasce et al. (36). Their experimental data, which also were confirmed by us (37), led to the proposal that warfarin binds to an oxidized thiol redox center and prevents reduction of the center by an unknown electron donor. The identity of the physiological electron donor has not been established. Experiments with the recently discovered VKORC1 subunit of VKOR provide support for the earlier proposed thiol redox center model (36). As shown in Fig. 1, the predicted thioredoxin-like 132CXXC135 redox center in VKORC1 is located in the third α-helix trans-membrane domain. The center is embedded in a hydrophobic environment, which is consistent with our finding that hydrophobic but not hydrophilic thiol-reducing trialkylphosphines will reach the center and trigger VKOR activity (22). As shown by the inability of the 132SXXC135 and the 132CXXS135 mutants to trigger VKOR activity, our data strongly support that the putative 132CXXC135 redox center in VKORC1 is the redox center in VKOR.

A second problem that needs to be addressed is the biochemical composition of VKOR. Is the single 26-kDa VKORC1 protein by itself capable of reducing Vit.K →O via a warfarin-sensitive catalytic mechanism, or is the reaction dependent upon cooperation of additional proteins in the ER membrane? Kinetic analyses of Vit.K →O reduction by VKOR have demonstrated two different binding sites for Vit.K →O and warfarin (38). There are also strong data supporting the concept that warfarin binds to the thiol redox center in VKOR (36). Whether or not the two binding sites are located on the 26-kDa VKORC1 protein or are present on separate proteins constituting a VKOR enzyme complex remains to be established. The argument for the existence of a VKOR enzyme complex is supported by reconstitution experiments carried out by our laboratory (12) and by Begent et al. (39) where increased VKOR activity could be measured by combining protein fractions obtained from chromatography of ER proteins and by the finding that hydroxy-vitamin K1 can be produced enzymatically from Vit.K →O by an ER enzyme that is insensitive to warfarin (40).

Because of the oxidative environment in the ER (41, 42), VKOR and γ-carboxylase must reside close together in the ER membrane as a functional γ-carboxylation system (7). This is necessary to preserve the reduced Vit.KH2 cofactor produced by VKOR and used by γ-carboxylase (7). We know that the two enzymes cannot be linked tightly in the ER membrane because the two enzymes can be easily separated by chromatography (12). Overall these observations indicate the existence of a vitamin K-dependent γ-carboxylation system of significant complexity where ER membrane lipids also play an important part (12). In this work we have engineered a recombinant vitamin K-dependent γ-carboxylation system in BHK cells that will be used to further investigate the molecular mechanism by which this system carries out γ-carboxylation of vitamin K-dependent proteins.

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