Human vitamin K epoxide reductase and its bacterial homologue have different membrane topologies and reaction mechanisms*

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Running title: Membrane topology and reaction mechanism of VKOR

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Background: The membrane topology and the role of certain cysteines in human vitamin K epoxide reductase (VKOR) are disputed.

Results: VKOR has three transmembrane domains and only the active site cysteines are required for function.

Conclusion: Despite similarities, VKOR and its bacterial homologues (VKORHs) have different topologies and reaction mechanisms.

Significance: The VKOR does not employ the intra-molecular electron transfer pathway proposed for VKORH.

SUMMARY

Vitamin K epoxide reductase (VKOR) is essential for the production of reduced vitamin K that is required for modification of vitamin K-dependent proteins. Three- and four-transmembrane domain (TMD) topology models have been proposed for VKOR. They are based on in vitro glycosylation mapping of the human enzyme and the crystal structure of a bacterial (Synechococcus) homologue respectively. These two models place the functionally disputed conserved loop cysteines, C43 and C51 on different sides of the endoplasmic reticulum (ER) membrane. In this study, we fused green fluorescent protein to the amino- or carboxyl-terminus of human VKOR, expressed these fusions in HEK293 cells and examined their topologies by fluorescence protease protection assays. Our results show that the N-terminus of VKOR resides in the ER lumen while its C-terminus is in the cytoplasm. Selective modification of cysteines by polyethylene glycol maleimide confirms the cytoplasmic location of the conserved loop cysteines. Both results support a three-TMD model of VKOR. Interestingly, human VKOR can be changed to a four-TMD molecule by mutating the charged residues flanking the first TMD. Cell-based activity assays show that this four-TMD molecule is fully active. Furthermore, the conserved loop cysteines, which are essential for intra-molecular electron transfer in the bacterial VKOR homologue, are not required for human VKOR whether they are located in the cytoplasm (three-TMD molecule) or the ER lumen (four-TMD molecule). Our results confirm that human VKOR is a three-TMD protein. Moreover, the conserved loop cysteines apparently play different roles in human VKOR and in its bacterial homologues.

Vitamin K epoxide reductase (VKOR) is a 163-amino acid polytopic membrane protein of the endoplasmic reticulum (ER) (1,2). It is responsible for the conversion of vitamin K 2,3-
epoxide (KO) into vitamin K and is highly sensitive to inhibition by coumarin drugs, such as warfarin, the most commonly prescribed oral anticoagulant (3). Warfarin inhibition of VKOR reduces the availability of vitamin K hydroquinone (KH₂), which is a cofactor for gamma-glutamyl carboxylase (GGCX). GGCX catalyzes the functionally critical post-translational modification of a family of vitamin K-dependent proteins involved in blood coagulation, bone homeostasis, signal transduction, and cell proliferation (4,5). Deficiency of KH₂ causes partial carboxylation or non-carboxylation of vitamin K-dependent proteins resulting in defects primarily of blood coagulation (4,6,7). Concomitant with carboxylation, KH₂ is oxidized to KO which must be converted back to KH₂ by VKOR and an as yet unidentified vitamin K reductase for the carboxylation reaction to continue.

Based on a chemical model study, a reaction mechanism for converting KO to vitamin K by VKOR was proposed by Silverman (8). A quantum chemical study of the reaction mechanism of KO reduction by VKOR supports and extends the Silverman mechanism (9). This study predicts that once a key disulfide bond of VKOR is broken, the reaction is energetically favorable. Experimental data supporting the proposed chemical model comes from a study of VKOR activity with a variety of thiol compounds (10). It has been proposed that thioredoxin (Trx) family proteins serve as the physiological reductant for VKOR (11-14); however, this hypothesis has been questioned (15). Currently, the physiological reductant of VKOR remains unknown.

With the identification of the gene encoding human VKOR (1,2), it became possible to study its structure-function relationship at the molecular level. Site-directed mutagenesis studies confirmed that two conserved cysteines, C132 and C135, comprise the active site redox center (CXXC) in VKOR (16-18). While identity of the active site residues is clear, reports of the function of the other pair of conserved cysteines, C43 and C51 (the loop cysteines) do not allow a consistent interpretation for their role. Results with Trx/Trx reductase suggest that a redox protein transfers electrons to the loop cysteines, which in turn reduces the membrane-embedded active site C132-C135 disulfide bond to activate VKOR (19). On the other hand, a VKOR mutant with both loop cysteines changed to alanine has wild-type enzyme activity with dithiothreitol (DTT) as reductant, suggesting that the loop cysteines are not essential for VKOR activity (16,18). We extended this observation to an in vivo activity assay system where VKOR uses the endogenous reductant in the native milieu rather than DTT (20). Our in vivo results support the conclusion that the loop cysteines are not required for VKOR activity.

To better understand the structure-function relationships of VKOR, we have determined the membrane topology of human VKOR by in vitro translation/co-translocation (21). Our results suggest that VKOR is a three-transmembrane domain (TMD) protein with its N-terminus located in the ER lumen and C-terminus in the cytoplasm (Figure 1A). In this model, the active site cysteines (C132 and C135) are located at the N-terminal of the third TMD facing the ER lumen. However, this topology places the conserved loop cysteines C43 and C51 in the cytoplasm, the opposite side of ER membrane from the active site cysteines. Our three-TMD model is consistent with the other results suggesting that the loop cysteines are not directly involved in VKOR activity (16,18,20).

VKOR appears to be a member of a large family of homologues (VKORHs) widely distributed among vertebrates, invertebrates, plants, bacteria, and archaea (22). In bacteria, VKORHs are present in strains lacking the cytoplasmic membrane protein DsbB which is important for protein oxidative folding in the periplasm (23). The functional similarity between bacterial VKORHs and DsbB is shown by the observation that bacterial VKORHs can complement DsbB to restore DsbA-dependent disulfide bond formation of secreted proteins in E. coli (23-25). Moreover, the crystal structure of a bacterial VKORH from Synechococcus reveals a four-TMD structure of the VKOR domain which is similar to DsbB (26). Based on this structure, a four-TMD topology model for human VKOR has been proposed (Figure 1B). In addition, an intramolecular electron transfer pathway between the
two pairs of conserved cysteines, similar to that of DsbB, has been proposed for the bacterial VKORHs as well as for mammalian VKOR (26,27). These results raise the question as to whether the bacterial enzymes and the mammalian ones have the same membrane topology and whether they employ different mechanisms to regenerate the active site cysteines.

To further clarify the membrane topology and the reaction mechanism of human VKOR, we employed independent biochemical techniques, fluorescence protease protection (FPP) assay (28) and selective modification of the VKOR endogenous cysteines by polyethylene glycol maleimide (PEG-MAL) (29) to probe the membrane topology of the intact VKOR molecule in mammalian cells. We used our recently established cell-based activity assay to examine the role of the conserved loop cysteines in VKOR. Our results support the three-TMD model of human VKOR and suggest that intra-molecular electron transfer between the loop cysteines and the active site cysteines of VKOR is not necessary for its function.

EXPERIMENTAL PROCEDURES

Materials  Vitamin K₁, warfarin, digitonin and trypsin (bovine pancreas) were obtained from Sigma-Aldrich (St. Louis, MO). Methoxy-polyethylene glycol maleimide, molecular weight 5000 (mPEG-MAL-5000), was purchased from Nektar (Huntsville, AL) and Laysan Bio. Inc. (Arab, AL). Luciferase substrate coelenterazine was from NanoLight Technology (Pinetop, AZ). Complete protease inhibitor cocktail tablets and ABTS solution for ELISA were purchased from Roche Molecular Biochemicals (Indianapolis, IN). Immobilon-P PVDF membrane was from Millipore Corp. (Bedford, MA). ECL Western blotting detection reagents were from GE Healthcare (Piscataway, NJ). Mammalian expression vector pIRESpuro3, Xfect transfection reagent and green fluorescence protein (GFP) containing vector pEGFP-N1 were from Clontech Laboratories, Inc. (Mountain View, CA). Cytochrome B5 cDNA was from Open Biosystems (Huntsville, AL). The gene encoding asialoglycoprotein receptor (ASGPR) subunit H₁ was kindly provided by Dr. Martin Spiess (University of Basel, Switzerland) (30). Restriction enzymes and PNGase F were from New England Biolabs (Ipswich, MA). QuickChange site-directed mutagenesis kit was from Stratagene (La Jolla, CA). HEK293 cell line was from ATCC (Manassas, VA). Mammalian expression vector pBudCE4.1, all cell culture mediums and oligonucleotide primers were from Life Technologies Corp. (San Diego, CA). Mouse anti-carboxylated FIX gla domain monoclonal antibody was a gift from GlaxoSmithKline (Philadelphia, PA) and Green Mountain Antibodies (Burlington, VT) (31,32). Horseradish peroxidase conjugated affinity purified sheep anti-human Protein C IgG was from Affinity Biologicals, Inc. (Ancaster, ON, Canada). Anti-HPC4 monoclonal antibody was from Dr. Charles T. Esmon (Oklahoma Medical Research Foundation, Oklahoma City, OK). Lab-Tek II chambered cover glass (4 well) were from Nalge Nunc International (Rochester, NY). 96-well EIA/RIA high binding flat bottom plates were purchased from Corning Inc. (Corning, NY).

DNA manipulations and plasmid constructions

Mammalian expression vector pIRESpuro3 was used as the basic cloning and expression vector. For the FPP assay, GFP was fused to the N- or C-terminal of the target protein with a 30-amino acid flexible linker (GGSGG)₆ between the two entities as described previously (33). The linker was generated by the annealing of four oligonucleotides with a BamHI site and a Bgl II site flanking the 5’ and the 3’ end respectively. The annealed fragment was cloned into the BamHI site of the pIRESpuro3 vector. The Bgl II site at the 3’ end of the linker introduces an arginine residue which is susceptible to trypsin cleavage in the FPP assay. For the N-terminal GFP fusion, the gene encoding GFP was amplified by PCR using a 5’ primer containing the Kozak sequence flanked by an EcoRI site and a 3’ primer flanked by a BamHI site without the stop codon. The PCR product was cloned into the pIRESpuro3 vector with the (GGSGG)₆ linker by EcoRI/BamHI to yield pIRESpuro3-GFP-N. The gene encoding the target protein was amplified by PCR using primers.
flanked with a Not I site at both ends and sub-cloned into pIRESpuro3-GFP-N at its Not I site. For the C-terminal GFP fusion, the gene encoding GFP was amplified by PCR using primers flanked with Not I sites at both ends. The PCR product was cloned into the Not I site of the pIRESpuro3 vector with the (GGSGG)$_6$ linker to yield pIRESpuro3-GFP-C. The gene encoding the target protein was amplified by PCR using a 5’ primer flanked with an EcoR I site with the Kozak sequence and a 3’ primer flanked with a BamH I site (Bgl II for VKOR) without the stop codon. The PCR product was sub-cloned into pIRESpuro3-GFP-C by EcoR I/BamH I.

For the FPP control assays, we used the single-TMD protein ASGPR and the tail-anchored protein CYB5 as model proteins. Both proteins are type II integral membrane proteins with the N terminus located in the cytoplasm and C-terminus in the ER lumen. We replaced the cytoplasmic N-terminal heme-binding domain (residues 1-90) of CYB5 (34) and the luminal C-terminal carbohydrate recognition domain of ASGPR (residues 147-290) (35), respectively, with GFP (Figure 2B).

Site-directed mutagenesis was performed by a QuickChange kit to create cysteine mutations or change the charged residues in VKOR according to the manufacturer’s instructions (Stratagene). For Western blot detection, a HPC4 tag (EDQVDPRLIDGK) was introduced at the carboxyl terminus of the VKOR with its ER retention sequence (KAKRH). As an internal control for our cell-based VKOR activity assay, Metridia luciferase cDNA was cloned into one of the multi-cloning sites of the dual mammalian expression vector pBudCE4.1 to yield pBudCE4.1-Met.Luc. All the VKOR constructs were subcloned into a different multi-cloning site of pBudCE4.1-Met.Luc. All the VKOR constructs for the in vivo cell-based activity assay have a warfarin resistant mutation of Y139F. The nucleotide sequences of all the constructs were verified by sequencing at Eton Bioscience Inc. (RTP, NC).

**FPP assay**  FPP assay was performed as described previously (28) with minor modifications. HEK293 cells were sub-cultured into chambered cover glass with complete growth medium to yield ~80% confluency at the time for the FPP assay. Cells were transiently transfected with pIRESpuro3 plasmid DNA containing the GFP tagged target protein using transfection reagent Xfect according to the manufacturer's protocol. Forty-eight hours post-transfection, cells were washed three times for 1 min each, with KHM buffer (110 mM potassium acetate, 2 mM MgCl$_2$ and 20 mM HEPES pH 7.2) at room temperature. After washing, 200 µl of KHM buffer was added to each chamber before placing the chambered cover glass on the microscope stage. Confocal microscopy was performed on a Zeiss LSM710 confocal laser scanning microscope (Carl Zeiss Microimaging, Thornwood, NY). Images were collected using a 40X/1.2NA C-Apochromat objective lens. Visualization of the GFP was achieved by use of a 488 nm Argon laser line for excitation and the detector was set to collect emission at 493–530 nm. We collected a Time Series of images of the chosen cell using ZEN software. Successive images were collected at an interval of 30 s. After the fourth image was taken, 200 µl of KHM buffer containing 0.2 mM digitonin was added to the chamber (final concentration is 0.1 mM) to selectively permeabilize the plasma membrane. Two minutes after the addition of digitonin (i.e. after the 8th image was taken), 400 µl of KHM buffer containing 2 µM trypsin was gradually added to the chamber to elicit protease digestion of GFP in the cytoplasm. The essential factor that affects the FPP assay is the concentration of digitonin. To determine the effective digitonin concentration for permeabilizing the plasma membrane, increasing concentration of digitonin in KHM buffer was added to HEK293 cells transiently expressing GFP. The lowest digitonin concentration (0.1 mM) resulting in the disappearance of the GFP signal within 60 s was chosen (28). In order to compare changes in fluorescence intensity post treatment, instrument software was used to define and mark areas of interest within cells. Mean pixel intensity within the area of interest was used as a marker for comparison.

Selective modification of the endogenous VKOR cysteines by mPEG-MAL  Selective modification
of the cysteines of VKOR by mPEG-MAL was performed as described previously (29) with additional modifications. VKOR or VKOR C43/51A mutant with a C-terminal HPC4 tag was transiently expressed in HEK293 cells in a 6-well plate. Forty-eight hours post-transfection, cells were washed twice in situ with PBS (pH 6.8) and detached by pipetting. Cells were harvested by centrifugation and cell pellets were re-suspended in PBS. Samples were aliquoted into three tubes; one was a control with no detergent, while the other two were incubated with 0.1 mM digitonin or 1% Triton X-100 in the presence of protease inhibitor cocktail on ice for 10 min. The digitonin concentration used for selectively permeabilizing the plasma membrane was as described above with the GFP test in the FPP assay. Freshly prepared mPEG-MAL-5000 solution was added to each sample to a final concentration of 20 mM and incubated on ice for 30 min. The reaction was stopped by adding DTT to a final concentration of 50 mM, followed by SDS loading buffer for SDS-PAGE and Western blot detection.

Cell-based VKOR activity assay The in vivo activity assay for VKOR and its variants used in this study were performed according to our recently established cell-based assay (36) with minor modification. It is based on the ability of VKOR or its homologue to convert KO to vitamin K in mammalian cells to support the carboxylation of a reporter protein. The reporter chimera protein FIXgla-PC, that has the gla domain of factor IX replacing the gla domain of protein C, was stably expressed in HEK293 cells (FIXgla-PC/HEK293). Plasmid DNA of pBudCE4.1-Met.Luc containing the cDNA of wild-type or mutant VKOR was transiently expressed in FIXgla-PC/HEK293 cells in a 24-well plate using Xfect transfection reagent according to the manufacturer’s instructions (Clontech). Five hours post-transfection, the transfection medium was replaced by a complete cell culture medium containing 5 µM KO with 4 µM warfarin. The cell culture medium was collected after a 48-hour incubation and directly used for ELISA to determine the level of carboxylated reporter protein (FIXgla-PC), and for the luciferase activity assay to normalize for the transfection efficiency (20). Since endogenous VKOR in HEK293 cells can efficiently carboxylate the reporter protein (250 ~ 300 ng/ml carboxylated FIXgla-PC in the cell culture medium), 4 µM warfarin was used in all assays to inhibit the endogenous VKOR activity. Warfarin reduced carboxylation of reporter protein to extremely low background levels (5-10 ng/ml carboxylated FIXgla-PC). For this reason, all the VKOR molecules used in the cell-based activity assay have the Y139F warfarin resistant mutation. Therefore, wild-type VKOR activity in this study refers to the activity of VKOR-Y139F warfarin resistant mutant.

RESULTS

Localization of the N- and C- termini of human VKOR in HEK293 cells by FPP assay The two proposed membrane topology models for human VKOR agree with each other in terms of the location of the C-terminus, but are in conflict as to the location of the N-terminus and the conserved loop cysteines (C43 and C51) (Figure 1). To clarify this disagreement, we fused GFP to either the N- or C-terminus of VKOR and then used the FPP assay to probe the location of the two termini in intact VKOR molecules in mammalian cells HEK293 by confocal microscopy. These chimeric proteins were transiently expressed in HEK293 cells and the plasma membranes were selectively disrupted by digitonin followed by the addition of trypsin. Results show that when GFP is fused to the N-terminus of VKOR (GFP-VKOR), it is protected from trypsin digestion (Figure 2A). However, the C-terminal GFP fusion of VKOR (VKOR-GFP) is susceptible to protease digestion in digitonin permeabilized cells. This result suggests that the N-terminus of VKOR is located in the ER lumen and the C-terminus is located in the cytoplasm. Control experiments were performed by fusing GFP to either the cytoplasmic or luminal terminus of the single TMD model proteins ASGPR and CYB5 (Figure2B & 2C). These results agree with our previous in vitro results suggest that VKOR is a three-TMD protein (21).

To examine the enzymatic activity of the GFP tagged VKOR molecules, we used our
recently established cell-based activity assay (36). Figure 3 shows that the fusion of GFP, which is approximately twice the size of VKOR, to VKOR decreased its activity to about 50% compared with the wild-type enzyme. This result demonstrates that the GFP tagged VKOR used in the FPP assay for the membrane topology study has passed ER quality control and is enzymatically active.

Selective modification of the endogenous cysteine residues in VKOR by mPEG-MAL

Another difference between the three- and four-TMD models for VKOR is the location of the cysteine residues in terms of the ER membrane (Figure 1). In the three-TMD model, the conserved loop cysteines C43 and C51 along with C85 and C96 are located in the cytoplasm. In contrast, none of the cysteine residues are located in the cytoplasm in the four-TMD model. To clarify these two topology models, we used a membrane impermeable sulfhydryl modification reagent mPEG-MAL-5000 to selectively modify the cysteines of VKOR in HEK293 cells. Modification of each cysteine by mPEG-MAL-5000 is expected to increase the apparent molecular weight of VKOR by ≥10kDa (29), which can be easily separated by SDS-PAGE and visualized by Western blot analysis. In this experiment, we transiently expressed HPC4 tagged VKOR in HEK293 cells. The cells were again treated with digitonin which preferentially permeabilizes the cell membrane over the ER membrane. Thus, the modification reagent should react only with the cysteine residues exposed to the cytoplasm. If VKOR is a four-TMD protein, none of the 7 endogenous cysteines should be available to react with mPEG-MAL because they would be found within the protected ER membrane or lumen.

Figure 4A shows that when cells expressing wild-type VKOR are selectively permeabilized by digitonin and modified by mPEG-MAL-5000, two PEG labeled protein bands with higher molecular weight are observed (Figure 4A, lane 2). This result suggests that at least two cysteine residues of VKOR are located in the cytoplasm. When both the plasma and ER membranes are disrupted with Triton X-100, at least five PEG modified protein bands are observed and most of the VKOR molecules have two or three mPEG-MAL modifications (Figure 4A, lane 3). Since PEG molecule is large, when two cysteines are close to each other (for example cysteine 132 and 135), they may not be modified efficiently. In addition, when more cysteines are modified, it may decrease the transfer efficiency of the modified protein from the gel to the membrane. Our result suggests that at least five of the seven cysteines in VKOR can be modified simultaneously by mPEG-MAL when the plasma and ER membrane were permeabilized. These results agree with the above FFP assay and again, are consistent with VKOR being a three-TMD protein.

In the three-TMD model, of the four cysteines located in the cytoplasm, C43 and C51 are located in a hydrophilic region while C85 and C96 reside in a hydrophobic region of VKOR (Figure 4B). Therefore, we suspected that the two cysteines modified under ER intact conditions (Figure 4A, lane 2) are the cytoplasmic hydrophilic region loop cysteines C43 and C51. To test this hypothesis, we mutated both cysteines to alanine and repeated the mPEG-MAL modification. When C43 and C51 were mutated, under ER intact conditions, the two PEG protein adducts were significantly reduced in the mutant enzyme as compared to the wild-type enzyme (Figure 4A, lane 5). In addition, the four- and five-fold PEG-modified forms also dramatically decreased when the cells were treated with Triton X-100 (Figure 4A, lane 6). We demonstrated previously that the VKOR mutant C43/51A has almost wild-type enzyme activity, indicating it folds correctly and functions normally (16,20). Together our results suggest that the conserved loop cysteines C43 and C51 are located in the cytoplasm and again support the three-TMD model of VKOR.

VKOR topology can be changed by mutating the charged residues flanking TMD1

Our results thus far suggest that, consistent with our previous conclusion, VKOR is a three-TMD protein. The orientation of the first TMD in the three-TMD model agrees with the "positive inside rule" (37). A number of positively charged residues are found after the TMD1 that are located on the cytoplasmic
side of the ER membrane (inside). To test the contribution of the charged residues flanking TMD1 to VKOR topology, we removed four positively charged residues at the C-terminus of TMD1 by making the following mutations: K30L, R33G, R35G, and R37G. We also added three positive charged residues to the N-terminus of VKOR by making the mutations of G6R, S7R, and G9R. The topology prediction program TMHMM predicts three TMDs for wild-type VKOR, but four TMDs for the charge mutant VKOR-CM with both its N- and C-termini located in the cytoplasm.

To experimentally test that the membrane topology of this charged residue mutant protein, we repeated the FPP assay by fusing GFP to either the N- or C-terminus of the VKOR-CM mutant. Our result shows that GFP fused to either the N- or C-terminus of VKOR-CM is now sensitive to protease digestion when the cell membrane is disrupted with digitonin (Figure 5A). This result indicates that both ends of this mutated VKOR are located in the cytoplasm. Therefore, the membrane topology of the mutated protein has been changed to a four-TMD protein. The sequence 75-97, which we previously demonstrated could function as a stop-transfer sequence in vitro (21), now appears to span the membrane as a TMD. Therefore, instead of changing the topology of the whole protein, the C-terminal half of the molecule retains the orientation as in the wild-type enzyme. Compared with C-terminal tagged GFP, there is ~60 s delay in the disappearance of the fluorescence signal of the N-terminal GFP tagged VKOR-CM. But compared with wild-type VKOR, the accessibility of the N-terminal GFP tag to protease digestion is obvious (Figure 5B) indicating its cytoplasmic location.

To determine the enzymatic activities of the above mutant VKOR, we performed our in vivo cell-based activity assay. The results in Figure 5C show that VKOR-CM mutant has an even higher activity than the wild-type enzyme. This experimentally altered four-TMD human VKOR retains almost full activity when GFP is fused to the N-terminus. The C-terminal GFP fusion, however, shows significantly decreased activity. On the other hand, even this VKOR mutant fusion (VKOR-CM-GFP) has almost the same activity as the wild-type non-fusion enzyme.

Conserved loop cysteines C43 and C51 are not involved in intra-molecular electron transfer

The above results suggest that human VKOR normally has three TMDs. This places the conserved loop cysteines C43 and C51 on the opposite side of the ER membrane from the active site, CXXC. Changing the charged residues flanking TMD1 reverses the orientation of TMD1 and places the loop cysteines in the ER lumen. Furthermore, the charge mutated VKOR-CM with four TMDs appears to be more active than the wild-type enzyme. One possible reason for the higher activity of VKOR-CM could be that moving the loop cysteines to the same side (lumenal) of the ER membrane as the active site CXXC favors the electron transfer pathway proposed for bacterial VKORH and human VKOR (19,26,27). To test this possibility, we mutated the loop cysteines (both individually and together) of the VKOR-CM mutant and tested their activity in our cell-based in vivo activity assay. Mutation of C43 significantly decreased the VKOR activity as in the wild-type enzyme (Figure 6). However, C51A or the double cysteine mutant (C43/51A) had minor effect on VKOR activity. In addition, the activity of both the C51A and the double cysteine mutant (C43/51A) of the altered four-TMD form of VKOR are more active than the wild-type three-TMD enzyme. These results suggest that the increased activity in the charged residue mutant VKOR-CM is not due to the favorable location of the conserved loop cysteine for intra-molecular electron transfer.

The sequences following the conserved loop cysteine appears not to be involved in the control mechanism for VKOR activity

The crystal structure of VKOR bacterial homologue revealed a 1/2-helix structure after the second conserved loop cysteine, C56 (26). According to the sequence homology, these authors proposed a similar 1/2-helix (residues S52 to S57) in human VKOR following the second conserved loop cysteine C51 that forms an amphipathic lid on the four-helix bundle. They suggested that “the 1/2-helix must move out of the way, perhaps by sliding parallel to the plane of the membrane, to allow C56 in the
loop to gain access to the C130 of the CXXC motif. In the resting state, the 1/2-helix would shield the active site, preventing it from non-selectively oxidizing proteins in the periplasm” (26). If this hypothesis holds for human VKOR, disruption of the 1/2-helix should leave the active site of VKOR open and susceptible to non-selective oxidation. In that case, VKOR (oxidized form) should be inactive. To test this hypothesis, we have mutated the conserved residue V54 which resides in the middle of the proposed 1/2-helix (26) to proline (Figure 7A), a residue that should break the alpha helix structure. Our in vivo cell-based activity assay shows that this mutant protein has similar activity as wild-type VKOR (Figure 7B). Therefore, the proposed 1/2-helix following the conserved loop cysteines of human VKOR appears not to function as a control mechanism as proposed for its bacterial homologue (26).

DISCUSSION

VKOR is an ER membrane protein that reduces KO to vitamin K and is the target of warfarin. Although the membrane orientation of the C-terminus of VKOR is not in doubt, the number of membrane-spanning domains and the orientation of the N-terminus of VKOR have been a source of contention. We presented a three-TMD model derived from in vitro glycosylation mapping indicating that the N-terminus of VKOR is located in the ER lumen (21). On the other hand, a four-TMD model based on the crystal structure of the bacterial VKOR homologue suggests that the N-terminus is located in the cytoplasm (26). These two topology models of VKOR place the conserved loop cysteines (C43 and C51) on different sides of the ER membrane. The location of these loop cysteines is critical for understanding the mechanism of VKOR. Our objective in the current study is to address the topology and reaction mechanism discrepancy of human VKOR.

To experimentally re-examine the membrane topology of human VKOR, we used two independent biochemical approaches to probe the intact VKOR molecule in living cells. One is a recently established FPP assay which has been successfully used for determining the membrane protein topology in single cells (38). Unlike in vitro translation studies, where ER-derived microsomes are used as the source of cellular membranes, the FPP assay surveys proteins that are delivered by the cellular machinery to their correct destination. In addition, all the VKOR fusions used in this study for the FPP assay are active as evidenced by results from the cell-based in vivo activity assay. The other approach is the widely used selective modification of cysteines in the membrane protein by PEG-MAL (29). We also extended this selective modification to mammalian cells that over express the functional human VKOR or its cysteine mutant. Our results from both the FPP assay and selective cysteine modification support our previous conclusion that human VKOR is a three-TMD protein (Figure 2 and Figure 4).

It is worth noting that selective modification of cysteine residues to define the membrane topology of human VKOR has also been used in a recent study by Schulman et al (27). However, these authors reported results that they interpreted as supporting the four-TMD model of VKOR. This is the only direct evidence to support the four-TMD model of human VKOR. These authors state, “Each cysteine modified by Mal-PEG is expected to increase the molecular weight of VKOR by ~5 kDa”. While explaining their results, they state that “With wild-type VKOR, little modification was observed. However, extensive modification was observed when the ER membrane was solubilized with Triton X-100, as expected from the presence of multiple endogenous cysteines located inside the ER membrane and in the ER lumen” (27). Based on the work of Lu et al. (29) and our results (Figure 4), one would expect modification of multiple cysteines in a protein to result in multiple gel bands with different sizes representing different extents of cysteine modification, rather than one band with different intensities (Figure 1B in reference (27)).

In addition, in that study, a cysteine residue was introduced either into the N-terminus (S3C) or the C-terminus (G158C) of VKOR (27). The N-terminal cysteine mutant VKOR (S3C)
modification shows an increased intensity of PEG modified VKOR band after Triton X-100 treatment compared with digitonin treatment (Figure 1B, lane 7 compared to lane 5 in reference (27)). With the C-terminal cysteine mutant VKOR (G158C) modification, however, the intensity of PEG modified VKOR band in the sample treated with digitonin is similar to that treated with Triton X-100 (Figure 1B, lane 9 compared to lane 11 in reference (27)). If the two cysteines were on the same side of the membrane as in the four-TMD model, one would expect the same degree of modification for either the N- or C-terminal cysteine mutant VKOR with digitonin treatment. On the other hand, the accessibility and the relative reactivity of the two added cysteines may be different at the N or C-terminus. For that reason, it appears that their results could be interpreted to support the three-TMD as well as the four TMD topology model for VKOR.

The main difference between the two proposed topology models for VKOR is the orientation of the first TMD in the ER membrane (Figure 1). It has been shown that the orientation of the transmembrane helix is primarily determined by the charged residues flanking the hydrophobic core, the “positive-inside rule” that applies to both bacterial and mammalian membrane proteins (37,39). That is, positively charged residues are more abundant on the cytoplasmic side (inside) of membrane proteins as compared to the lumenal/periplasmic side. Since the four-TMD topology model of human VKOR derives from the crystal structure of the VKORH of Synchococcus (Syn-VKORH), we compared the charge distribution flanking TMD1 of human VKOR and Syn-VKORH. The net charge difference $\Delta (C-N)$ between the C- and N-terminal flanking regions of human TMD1 according to the rules by Hartmann et al. (39) is $+1.5$. By contrast, Syn-VKORH has a net charge difference of $-3.5$ between the C- and N-terminus of TMD1. Thus, these two proteins have a very different distribution of charged residues flanking TMD1. According to the “positive inside rule”, the N-terminus of VKOR should be located in the ER lumen, consistent with the three-TMD model. On the other hand, the N-terminus of Syn-VKORH should be located in the cytoplasm which agrees with the four-TMD topology model derived from its crystal structure. The overall charge distribution of these two proteins in their own topology model is consistent with the “positive inside rule” (Figure 8A). If VKOR had four TMDs as proposed from its bacterial homologue, the charged residue distribution flanking TMD1 and the putative TMD2 would violate the “positive inside rule” (Figure 8B). Therefore, deduction of the membrane topology of human VKOR from its bacterial VKORH may not be appropriate.

It has been shown that mutating the charged residues flanking a signal sequence can invert the orientation of the peptide in the membrane (40). To further test the contribution of the “positive inside rule” in VKOR topology, we made charged residue mutations around the first TMD of human VKOR (VKOR-CM). The topology prediction program TMHMM predicts that the membrane orientation of TMD1 has been inverted and this VKOR-CM mutant has been changed to a four-TMD molecule with the C-terminal half of the molecule staying the same as in the three-TMD form. FPP assays confirmed that VKOR-CM has both the N and C-terminus facing to the cytoplasm. Interestingly, this molecule is even more active than the wild-type enzyme. One possible explanation for the increased activity in the four-TMD human VKOR-CM is that changing the orientation of TMD1 may change the warfarin sensitivity of VKOR. Several residues responsible for warfarin resistance have been identified in the loop region following TMD1. These residues are located in the cytoplasm of the wild-type enzyme, but they are located in the ER lumen in the VKOR-CM mutant. This topological difference may alter warfarin sensitivity resulting in an apparent increase in VKOR activity since we include warfarin in the cell culture medium of our cell-based activity assay to inactivate the endogenous VKOR.

The other major difference in our data compared to other publications concerns the role of the loop cysteines. The loop cysteines are conserved essentially over all the known VKOR molecules. Thus there seems little doubt that they serve an important function. It is clear that the role of the conserved loop cysteines in bacterial
VKORH is to transfer electrons to the active site cysteines (24-26). Due to the homology between human and bacterial VKORs, it was reasonable to assume that the corresponding loop cysteines, C43 and C51 of human VKOR have a similar function as VKORH in bacteria.

On the other hand, biochemical data suggest that the two conserved loop cysteines are not required for human VKOR activity (16,18). These results agree with the three-TMD model since the loop cysteine and the active site are located in the opposite side of the ER membrane. These earlier studies were based on the in vitro VKOR activity assay that used DTT which could bypass the role of the endogenous reductant. Recently, experiments using a cell-based assay, where VKOR uses its endogenous reductant, showed that the loop cysteines are not required for VKOR activity (20). Even when the loop cysteines are located on the same side of the ER membrane as the active site cysteines (Figure 6, VKOR-CM mutant), these two cysteines appear not to be involved in VKOR activity. Interestingly, the Mycobacterium tuberculosis VKOR homologue has four TMDs in its VKOR domain with its loop cysteines located in the periplasm. These loop cysteines are clearly required for complementation of DsbB in E. coli (25), but are not essential for its function in reducing vitamin K or KO in mammalian cells (20). Therefore, the conserved loop cysteines in VKOR appear not to be required for the reduction of KO in the vitamin K cycle.

Based on the crystal structure of a bacterial VKORH, it has been proposed that in human VKOR there is a “1/2-segment” in the region of the conserved loop cysteines and a “1/2-helix” immediately after this segment (Figure 7A) (26). The “1/2-helix” is proposed to serve as a control mechanism to protect the enzyme active site. Results from the present study (Figure 7) and our previous study (16) show that deleting part of the proposed “1/2-segment” or disrupting the proposed “1/2-helix” has only a minor effect on VKOR activity, this suggests that human VKOR and its bacterial homologue have different reaction mechanisms.

In conclusion, we studied the membrane topology of human VKOR by two independent approaches in mammalian cells. Our results support the three-TMD topology model of VKOR which is consistent with the “positive inside rule”. We have shown that VKOR topology can be changed by changing the charged residue distribution flanking the first TMD. Cell-based in vivo activity assays show that the conserved loop cysteines are not essential to VKOR activity, whether they are located in the cytoplasm or the ER lumen. Together, our results indicate that human VKOR and its bacterial homologues have different membrane topologies and different mechanisms for electron transfer.

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**FOOTNOTES**

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3 The abbreviations used are: ER, endoplasmic reticulum; VKOR, vitamin K epoxide reductase; VKORH, vitamin K epoxide reductase homologue; VKOR-CM, charged residue mutated VKOR; KO, vitamin K 2,3-epoxide; KH₂, vitamin K hydroquinone; GGCX, gamma-glutamyl carboxylase; Trx, thioredoxin; FIXgla-PC, protein C with its gla domain replaced by the gla domain of FIX; FPP, fluorescence protease protection; mPEG-MAL, methoxy-polyethylene glycol maleimide; TMD, transmembrane domain; GFP, green fluorescence protein; CYB5, cytochrome B5; ASGPR, asialoglycoprotein receptor; Syn-VKORH, *Synechococcus* vitamin K epoxide reductase homologue
FIGURE LEGENDS

Figure 1. Proposed membrane topology models of human VKOR

(A) Three-TMD topology model of human VKOR based on in vitro translation/co-translocation (21). The N-terminus of VKOR is located in ER lumen and the C-terminus in the cytoplasm. Conserved active site and loop cysteines are indicated by gradient filling of the circle and they are located on the opposite side of ER membrane. The positively charged residues flanking TMD1 are shaded in gray. (B) Proposed four-TMD topology model of human VKOR based on the crystal structure of a bacterial VKOR homologue (26). In this model, both the N- and C-terminus are located in the cytoplasm. Conserved active site cysteines and the loop cysteines are located in the same side of the ER membrane.

Figure 2. Localization of the N- and C- termini of VKOR in HEK293 cells by fluorescence protease protection assay

(A) FPP assay of GFP fusions of human VKOR. GFP tagged VKOR fusions were transiently expressed in HEK293 cells. Forty-eight hours post-transfection, cells were selectively permeabilized with 0.1 mM digitonin. Then trypsin was added and images were collected at the indicated time intervals. (B) Schematic representation of the single TMD model proteins (ASGPR and CYB5) and their GFP fusions. The solid bars indicate the TMD, the gray bars indicate the luminal carbohydrate recognition domain (CMD) of ASGPR or the cytoplasmic heme-binding domain (HBD) of CYB5. Dark gray bars indicate the (GGSGG)₆ linker between GFP and the model proteins. (C) FPP assay of GFP fusion of the model proteins as described above.

Figure 3. In vivo cell-based activity assay of human VKOR and its GFP fusions used in FPP assay

Human VKOR and its GFP fusions were transiently expressed in FIXgla-PC/HEK293 cells. Transfected cells were cultured in complete medium containing 5 µM KO and 4 µM warfarin for 48 h. The concentration of carboxylated FIXgla-PC in the cell culture medium was measured by ELISA and normalized by the luciferase activity as described in Materials and Methods. Data are presented as mean±SD (n=3).

Figure 4. Selective modification of the endogenous cysteine residues of human VKOR and its C43/51A cysteine mutant by mPEG-MAL-5000

(A) C-terminal HPC4 tagged VKOR or its C43/51A mutant was transiently expressed in HEK293 cells. Forty-eight hours post-transfection, cells were harvested and permeabilized with digitonin or Triton X-100 before mPEG-MAL modification. Whole cell lysate were applied to SDS-PAGE and transferred to PVDF membrane, HPC4 tagged VKOR was analyzed by Western blot. (B) Hydrophobicity profile of VKOR and the location of the endogenous cysteine residues. Transmembrane regions determined by in vitro translation/co-translocation were marked as TMD1 to TMD3. The putative transmembrane region was marked as pTMD in gray.
Figure 5. FPP topology assay and *in vivo* cell-based activity assay of charged residue mutated VKOR

(A) FPP topology assay of charged residue mutated VKOR, VKOR-CM as described in the legend for Figure 2. (B) Time course of relative fluorescence intensity in FPP assay of GFP fusions of VKOR-CM compared with the N-terminal GFP fusion of wild-type VKOR. (C) Cell-based activity assay of charged residue mutated VKOR and its GFP fusions as described in the legend for Figure 3.

Figure 6. *In vivo* cell-based activity assay of the cysteine mutants of human VKOR and VKOR-CM

Cysteine mutants of VKOR or VKOR-CM were transiently expressed in FIXgla-PC/HEK293 cells and the enzymatic activity was determined as described in the legend for Figure 3.

Figure 7. *In vivo* cell-based activity assay of human VKOR and its V54P mutant

(A) Sequence alignment of VKORs from different species per reference (26). Sequences were truncated between TMD1 and TMD2 as in the four-TMD topology model. Conserved loop cysteines and conserved serine were highlighted in gray. The conserved valine in the middle of the 1/2-helix was boxed. The numbers on the top refer to the position of amino acid residues in human VKOR sequence. (B) Human VKOR and its V54P mutant were transiently expressed in FIXgla-PC/HEK293 cells and the enzymatic activity was determined as described in the legend for Figure 3.

Figure 8. Charge distribution of human VKOR and *Syn*-VKORH in the proposed topology models

(A) Net charge distribution in the three-TMD model of human VKOR and the four-TMD model of the VKOR domain of *Syn*-VKORH. Net charges of the loops and the termini are calculated according to the rules by Hartmann *et al.* (39) and indicated inside the circle. Arginine, lysine, and the N-terminal amino group were given a value of +1, histidine +0.5, and aspartate and glutamate -1 as described previously. (B) Net charge distribution in the proposed four-TMD model of human VKOR. The putative TMD that functions as an authentic TMD in the four-TMD variant is shown in light gray with dotted line.
Figure 1
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Figure 7
Figure 8
Human vitamin K epoxide reductase and its bacterial homologue have different membrane topologies and reaction mechanisms
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