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Forum Review

Vitamin K Epoxide Reductase Complex Subunit 1 (VKORC1): The Key Protein of the Vitamin K Cycle

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ABSTRACT

Vitamin K epoxide, a by-product of the carboxylation of blood coagulation factors, is reduced to vitamin K by an enzymatic system possessing vitamin K epoxide reductase (VKOR) activity. This system is the target of coumarin-derived drugs widely used in thrombosis therapy and prophylaxis. Recently, the key protein of the VKOR system has been identified. The human *VKORC1* gene maps to chromosome 16 and consists of 3 exons encoding a 163-amino acid integral ER membrane protein with three or four predicted transmembrane α-helices. Expression of human VKORC1 in *Spodoptera frugiperda* (Sf9) cells and in *Pichia pastoris* results in enhanced VKOR activity over low endogenous constitutive levels. Sequence based search methods reveal that human *VKORC1* belongs to a large family of homologous genes found in vertebrates, insects, plants, protists, archea, and bacteria. All orthologs share five completely conserved amino acids, including two cysteines found in a tetrapeptide motif presumably required for redox function. The recent discovery of the *VKORC1* gene has initiated renewed interest in understanding VKOR activity. Analysis of VKORC1 protein structure and function will be crucial in understanding the VKOR catalytic mechanism, how anticoagulant drugs modulate VKOR activity, and the role of VKORC1 in downstream physiological and pathological pathways. *Antioxid. Redox Signal.* 8, 347–353.

INTRODUCTION

In 1929, Henrik Dam published the first detailed observations of vitamin K deficit symptoms as a result of studying cholesterol metabolism in chickens fed fat-extracted diets (10). Although he was not yet aware of what caused the symptoms, including subcutaneous and internal hemorrhage, development of crooked bones, markedly prolonged coagulation times, and early death, he insightfully suggested that poor development in affected animals was likely due to a deficiency of fat-soluble vitamins. Having ruled out scurvy and calcium deficiency as the cause of the symptoms induced by the fat-extracted diet (11), Dam turned his research focus to identifying the causative factors of the new dietary deficiency disease (13). In 1935, he published a milestone report in which, after having excluded the absence of any known fat-soluble vitamins (A, D, and E) as possible cause of the disease, he concluded that the substance whose absence in the diet was responsible for the coagulation and bone growth pathologies should be a new fat-soluble vitamin he named vitamin K (from the Danish and German words *Koagulation*) (12). For the discovery of vitamin K, Dam was awarded the Nobel Prize in Physiology or Medicine in 1943, together with Edward Doisy for the purification, characterization, and synthesis of the vitamin. After that, quite some time passed until the first light was shed on the biochemical pathways requir-

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ing vitamin K and, ultimately, the target proteins upon which it acts.

Eventually, it was found that a number of clotting factors present in blood plasma require vitamin K for their activation (reviewed in Ref. 45). The enzyme system responsible for activating the clotting factors effects a post-translational γ-carboxylation of glutamate residues (18), while abstracting electrons from vitamin K to produce the oxidized form, vitamin K epoxide.

Earlier, the oxidized epoxide form of vitamin K had been discovered in metabolic labelling experiments in rats (31) and elucidating its role in γ-carboxylation was pursued. It was also noted that administration of warfarin, an anticoagulant drug developed around 1943, caused an increase in the level of vitamin K epoxide by blocking enzymatic vitamin K epoxide reductase (VKOR) activity in the liver (2, 3). Hepatic coagulation factors II, VII, IX, and X, as well as protein C, protein S, and protein Z were shown to be γ-carboxylated at several amino terminal glutamyl residues in a vitamin K dependent process (18). In 1978, chick osteocalcin (also referred to as bone Gla protein) was found to be the first vitamin K dependent γ-carboxylated protein not involved in blood coagulation (22). Since then, several other γ-carboxylated proteins have been discovered in key positions of physiological processes. Examples include matrix Gla protein (bone metabolism) (37), growth arrest protein gas6 (cell growth and survival) (30), various receptor tyrosine kinase ligand proteins (apoptosis and cell survival) (43), connexin26 and connexin32 (members of the gap junction family of proteins that facilitate direct intercellular signaling and homeostasis) (28), and four putative transmembrane proteins of unknown function including PRGP1, PRGP2 (24), TmG3, and TmG4 (25).

In humans and animals, vitamin K epoxide, resulting from the γ-carboxylation of vitamin K dependent proteins, is reduced to vitamin K by the vitamin K epoxide reductase (VKOR) system. The reduced vitamin K serves as a cofactor in the γ-glutamyl carboxylase (GGCX)-mediated carboxylation reaction. The carboxylation process begins with the abstraction of hydrogen from glutamyl residues (Glu). This generates a carbanion that can incorporate CO_2, resulting in γ-carboxylated Glu (abbreviated as Gla). Simultaneously, vitamin K is oxidized to vitamin K 2,3- epoxide (17, 35). Due to the limited availability of vitamin K in tissues *in vivo*, the epoxide must be rapidly reduced again to vitamin K in what is known as the vitamin K cycle (6) (Fig. 1). It was also recognized that administration of anticoagulant drugs including warfarin and phenprocoumon led to a significant accumulation of vitamin K epoxide. Although it was initially believed that the epoxide was merely a degradation product of vitamin K, it was soon hypothesized that the epoxide is reconverted to vitamin K by a warfarin-sensitive enzymatic reaction (31). The action of coumarin derivatives gained worldwide recognition through use as the first oral anticoagulant drug for the treatment of human thromboembolic disorders as well as for use as a potent rodenticide. In humans, problematic and sometimes fatal bleeding represents a serious complication during oral anticoagulant therapy (33, 48). Among wild rodent populations where coumarin derivatives have been used for pest control, at least eight independent natural point mutations in VKORC1 have resulted in anticoagulant resistance (34).

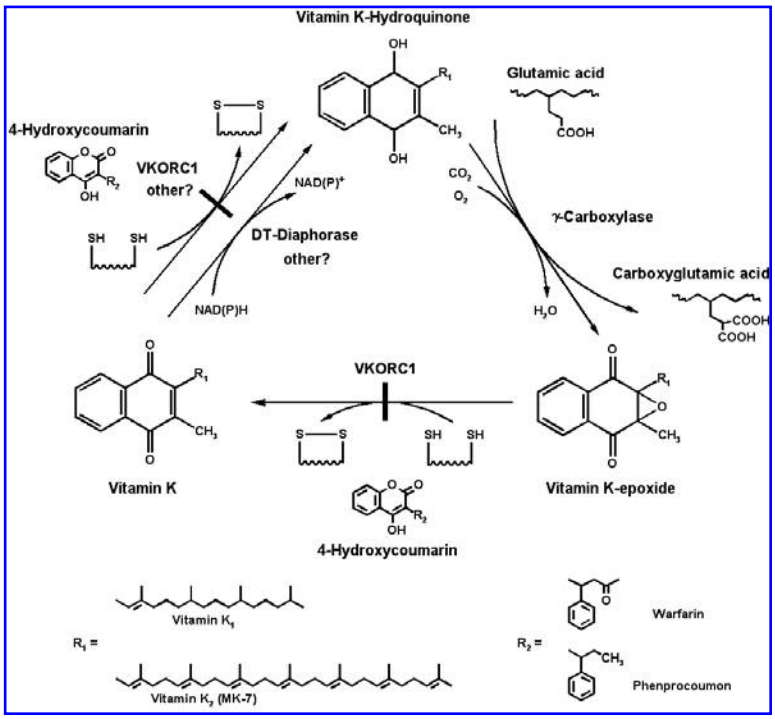

FIG. 1. The vitamin K cycle. Vitamin K-dependent γ-carboxylase uses reduced vitamin K hydroquinone (KH_2), carbon dioxide, and oxygen as cofactors to γ-carboxylate glutamic acid side chains in various vitamin K-dependent proteins. Vitamin K epoxide (KO) is generated as a by-product and, due to the overall scarcity of vitamin K in cells, must be rapidly recycled to KH_2 to sustain further γ-carboxylation reactions. Coumarin-derived anticoagulants such as warfarin or phenprocoumon block recycling of KO by inhibiting the two dithiol-dependent steps putatively performed by VKORC1. NADPH-dependent vitamin K reductase, formerly known as DT-diaphorase, can also reduce vitamin K quinone to the fully reduced KH_2, but is not capable of reducing KO. Thus, VKORC1 is the only enzyme that has been shown to catalyze KO deepoxidation.

IDENTIFICATION OF THE VITAMIN K EPOXIDE REDUCTASE (VKOR) GENE

Since the first direct evidence of VKOR activity was published in 1970 (2), all attempts to purify the membrane associated enzyme system have failed (1, 7, 26, 32, 52). The repeated failure in purifying proteins exhibiting VKOR activity led to the assumption that the putative VKOR enzyme complex was very labile and sensitive to detergent treatment. Additionally, some experiments provided evidence that the VKOR system may be a multiprotein complex consisting of at least a member of the glutathione-*S*-transferase family and a microsomal epoxide hydrolase that is closely associated with γ-glutamyl carboxylase (7). Furthermore, calumenin, an endoplasmic reticulum-localized chaperone, had been shown to partially inhibit VKOR activity, suggesting a direct interaction with the VKOR complex (53).

More recently, two independent genetic approaches have both identified a single protein that forms an essential component of the putative VKOR complex. Our group chose the classical approach of positional cloning in order to identify candidate proteins that display warfarin-sensitive VKOR activity. First, we mapped the locus for a rare human disorder, combined vitamin K-dependent clotting factor deficiency type 2 (VKCFD2, OMIM #607473), to a 20 Mb region on chromosome 16p12-q21 (16). This region is orthologous to a previously mapped locus for warfarin resistance in the rat genome on chromosome 1 and on chromosome 7 in mice (21, 23, 51). Pursuing the hypothesis that allelic mutations in the orthologous gene could cause warfarin resistance in rodents and man (OMIM #122700), as well as cause VKCFD2, we narrowed the search to a 4 Mb region on the short arm of human chromosome 16.

The annotated human genome available in the Ensembl database (http://www.ebi.ac.uk/ensembl/) revealed some 129 putative genes distributed across approximately 1100 exons mapped to this region. Using DNA from two diagnosed VKCFD2 patients and from four warfarin resistant probands, we systematically screened genes in this region and found putative mutations in a gene of unknown function. This gene extended over 5126 base pairs and comprised three exons encoding a protein of 163 amino acids with a calculated relative molecular mass of about 18 kDa ([41]). We named this gene vitamin K epoxide reductase complex subunit 1(VKORC1), as recombinant overexpression of the respective cDNA in HEK293 cells (human embryonic kidney cells with little endogenous VKOR activity, an immortalized cell line created by transformation with adenovirus) resulted in robust VKOR activity as assessed by a standard *in vitro* VKOR activity assay (54). The activity of the overexpressed wild type protein was inhibited by warfarin.

Furthermore, recombinant expression of warfarin-resistant rat VKORC1 mutants in HEK293 cells resulted in reduced VKOR activity, relative to wild-type rat VKORC1, along with resistance to warfarin inhibition. Expression of VKORC1 mutant proteins identical to those found in patients suffering from VKCFD2 almost completely abolished VKOR activity. This proved the expected functional correlation of VKOR activity, warfarin resistance, and VKCFD2 disease phenotype with VKORC1 expression. Additionally, Northern blot analysis revealed highest levels of VKORC1 expression in fetal and adult liver, with lesser amounts expressed in fetal heart, kidney, lung, and in adult heart and pancreas.

Independently, the laboratory of Darrel W. Stafford identified the VKORC1 gene by an alternative approach (27). Starting from published data on VKCFD2 in human and warfarin resistance in rat, the same region of human chromosome 16 was analyzed. Considering that VKOR activity is a membrane-bound process, they identified just 13 candidate genes for membrane proteins of unknown function in the critical chromosome segment. The Stafford lab's strategy was to perform siRNA knockdown experiments for each of the 13 gene candidates in a cell system (A549 cultured cells) with high intrinsic VKOR activity in order to identify the gene. Several different interference RNAs were synthesized for each of the target genes. Knockdown of only one gene resulted in significant reduction of VKOR activity. The putative VKOR gene was cloned and heterologously overexpressed in insect cells to confirm VKOR function and warfarin sensitivity. This is the first time a mammalian gene has been identified using the siRNA gene knockdown approach (42).

Examining available sequences from the human, rat, and mouse genomes, highly conserved homologs and orthologs, as well as pseudogenes, were identified (27, 41). Orthologs have now been identified in *Xenopus*, *Drosophila*, *Anopheles*, bacteria, protists, and plants (20, 27, 39, 41). The unexpected high level of sequence identity across these species points toward an important, and likely ancient, conserved function. In vertebrates, VKOR activity is closely coupled to vitamin K recycling in γ-carboxylation. In protist and plant genomes, searches for the complementary carboxylase sequences, that would indicate the presence of vitamin K cycling, revealed no homologs. This finding suggests that VKORC1, and VKOR activity, likely arose very early in the development of cellular life and may fulfil another function not directly related to γ-carboxylation of proteins (39).

VKORC1 PROTEIN STRUCTURE AND FUNCTION

From biochemical studies it is well known that VKOR activity copurifies with the microsomal membrane fractions from liver and kidney (15, 52) and is located in the endoplasmic reticulum membrane of mammalian cells (7). These results have been confirmed by more recent studies using GFP- and Myc- tagged VKORC1-cDNA constructs and antibodies against the ER specific protein calnexin, demonstrating VKORC1 localization in the meshlike structures of the ER (41).

With a calculated isoelectric point of 10.73 and extremely high hydrophobicity (GRAVY hydrophobicity index = 0.546012, calculated using the SOSUI algorithm, GRAVY = grand average of hydropathicity; http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0E.html), VKORC1 may be exceptionally problematic to purify from native tissues. Various topol-

ogy prediction algorithms propose between one and five transmembrane domains (depending on the program used) (20, 47). Recent data strongly suggest a three-transmembrane topology from *in vitro* translation/cotranslocation experiments (47). The suggested structure can be described as follows: ER-lumenal N-terminus (~10 aa), 3 trans-membrane α-helices bracketing one large cytoplasmic loop (~69 aa) between the first and second transmembrane helices and a small ER-lumenal loop (~7 aa) between the second and third helices, and a cytoplasmic C-terminus (~16 aa) (Fig. 2).

Alignments of amino acid sequences of VKORC1 orthologs indicate several conserved amino acids and functional motives (20, 27, 41). Two absolutely conserved cysteine residues (Cys43 and Cys51 in human VKORC1) together with a conserved serine/threonine (Ser57) are located within the cytoplasmic loop (20, 47). Two additional conserved cysteines (Cys132 and Cys135) predicted to be partially buried in the ER membrane form a possible CIVC redox motif (20, 40) (Fig. 2). These five conserved polar residues have been proposed to form the active center of VKORC1 (20). None of the conserved residues are found to be naturally mutated in humans or rodents. *In vitro* mutagenesis of Cys132 or Cys135 to serine completely eliminates VKOR activity, confirming that the CIVC redox motif plays a crucial role in vitamin K epoxide reduction (50). Conserved throughout all species investigated, cysteine residues 43 and 51 and the redox motive confirm previous observations that VKOR activity is dependent on external reducing agents (46). Interestingly, in some plant VKORC1 orthologs as well as in those of cyanobacteria and bacteria, C- or N-terminal fusion with thioredoxin-like (Trx) protein domains or with thiol-disulfide interchange protein-like domains (dsbA) is observed. These fusion proteins suggest the possible involvement of Trx-like proteins as biological reducing partners for VKORC1. As cytoplasmic Trx seems not to directly provide reducing equivalents for VKORC1 (36), protein disulfide isomerases appear to be candidates for providing reducing equivalents that sustain VKOR activity (44). As both warfarin and vitamin K are known to partition into the lipid bilayer of the cell membrane, the predicted location of the redox motif and the warfarin binding motif (TYA motif, Fig. 2) within a hydrophobic transmembrane domain is consistent with the intramembrane distribution of both the enzyme's substrate and inhibitor, respectively (55).

Inhibition of VKOR activity by coumarin derivatives, including warfarin, is the most commonly used approach to anticoagulant therapy world-wide. Recently, the 18 kDa protein VKORC1 was identified as a warfarin-sensitive vitamin K epoxide reductase (27, 41). Yet, there are other known warfarin-sensitive reductases, such as quinone oxidoreductase (NQOR, previously referred to as DT-diaphorase; 14), that are capable of reducing oxidized vitamin K quinone to the reduced hydroquinone form required as a cofactor for γ-carboxylation of clotting factors. However, none of these other warfarin-sensitive enzymes is capable of converting vitamin K epoxide to the more reduced forms (quinone or hydroquinone) of vitamin K. Furthermore, VKORC1 possesses a hydrophobic sequence motif, Thr-Tyr-Ala (TYA), identified by photoaffinity labelling and site-directed mutagenesis experiments as essential for dicoumarol binding in rat liver NQOR (29). Recently, Pelz et al. (34) demonstrated that several different mutations in rat VKORC1 at residue Y139 produce warfarin-resistant phenotypes, suggesting that the TYA motif forms at least part of the binding site for coumarins in VKORC1. Interestingly, this sequence is found just adjacent to the CIVC redox motif. The physical adjacency of structural elements important for VKORC1 warfarin binding and vitamin K epoxide reduction suggests possible mechanisms for warfarin inhibition of VKOR activity. High resolution structural data will likely be required to discern the actual mecha-

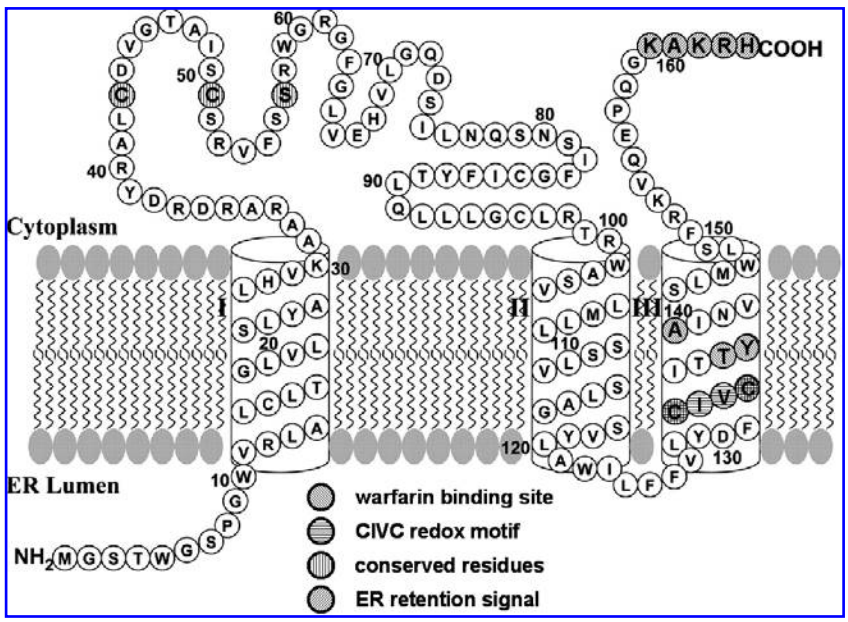

FIG. 2. Proposed membrane topology of VKORC1 according to Tie et al. (47). The model predicts three transmembrane α-helices. The amino terminus (10 aa) of the enzyme is located in the ER lumen, followed by α-helix 1 (aa 11–30), a large cytoplasmic loop comprising 69 aa, α-helix 2 (aa 101–120), a small ER lumenal loop of 7 amino acids, α-helix 3 (aa 128–147), and finally a 16 aa carboxy terminus located in the cytoplasm. *Various hatchings* are used to indicate amino acids belonging to five completely conserved residues (Cys 43, Cys51, Ser/Thr57, Cys132, Cys135), the proposed warfarin binding motif (TYA, aa 138–140), the proposed redox motif (CIVC, aa 132–135), and a proposed ER retention signal motif (aa 159–163).

nism from among the possibilities and such data can now be obtained through large-scale production of VKORC1 using heterologous expression systems (38).

VKOR-COMPLEX STRUCTURE

For a long time, it has been a generally accepted model that a membrane-bound multienzyme complex is required to perform the reaction steps of complete vitamin K epoxide reduction (7, 8). A microsomal epoxide hydrolase and a membrane-bound member of the glutathione-*S*-transferase family, as well as calumenin have been proposed to be members of this complex (7, 8, 49, 53). In contrast to these previous studies, VKORC1 appears not to require association with other proteins for its primary function. Recombinant expression of VKORC1 in *Spodoptera frugiperda* cells (Sf9), which possess undetectable constitutive VKOR activity, elevates vitamin K epoxide recycling to high levels (27). However, we cannot exclude the possibility that endogenous cofactors or coenzymes required for vitamin K recycling are present in SF9 cells and, together with heterologously expressed VKORC1, reconstitute a functional, albeit non-native, complex capable of reducing vitamin K epoxide.

Similarly, the observation that heterologous expression of VKORC1 in vitamin K recycling-deficient cells of the methylotrophic yeast *Pichia pastoris* results in measurable VKOR activity (our unpublished results) suggests that the presence of VKORC1 is necessary, if not sufficient, to reduce vitamin K epoxide.

In another recent study focusing on the γ-carboxylase, GGCX , Wajih *et al.* used an engineered recombinant expression system to demonstrate that VKORC1-dependent VKOR activity is the rate-limiting step for γ-carboxylation (50). Due to the oxidative environment in the ER lumen, VKORC1 and GGCX likely reside in close proximity in order to limit oxidation of nascent recycled vitamin K hydroquinone. However, it does not appear that VKORC1 and GGCX form a stable complex, as the two enzymes can be easily separated by mild chromatographic methods (7).

CONCLUSION

Cloning of VKORC1, the rate-limiting enzyme of vitamin K recycling, has elucidated the molecular basis of VKCFD2, a rare autosomal recessive bleeding disorder, and of warfarin resistance in man and rodents (34, 41). Further structural and functional characterization of the protein will be crucial for understanding the mechanism of the redox reaction in VKOR and its inhibition by warfarin. This may lead to the design of more potent and less harmful coumarin derivatives for use in anticoagulant therapy in humans.

Unexpectedly, recent reports have opened a much wider perspective on realizing near-term improvements in the therapeutic application of existing anticoagulants. Several independently conducted studies have explained the basis for interindividual and interethnic differences in therapeutic response to coumarin drugs based on common genetic variants that may modulate VKOR activity through altered levels of protein production (5, 9, 19, 48). Moreover, we are tempted to speculate that naturally occurring VKORC1 gene variants (i.e., variants encompassing mutations in nontranslated regions of the gene) may also have important influence on downstream function of vitamin K dependent proteins, including matrix Gla protein and osteocalcin, which have been suggested to play a role in the pathogenesis of atherosclerosis, myocardial infarction, and stroke (4).

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