The Membrane Topology of Vitamin K Epoxide Reductase is conserved between Human Isoforms and the Bacterial Enzyme.

Zhenbo Cao[1], Marcel van Lith[1], Lorna J. Mitchell[1], Marie Anne Pringle[1], Kenji Inaba[2] and Neil J. Bulleid[1]

[1]The Institute of Molecular, Cell and Systems Biology, CMVLS, University of Glasgow, Glasgow, G12 8QQ

[2]Institute of Multidisciplinary Research for Advanced Materials, Tohoku University Katahira 2-1-1, Aoba-ku, Sendai 980-8577, Japan

To whom correspondence may be addressed: Neil J Bulleid, Institute of Molecular, Cell and Systems Biology, CMVLS, University of Glasgow, Glasgow, G12 8QQ. Tel +44 141 330 3870; fax +44 141 330 5481; e-mail: neil.bulleid@glasgow.ac.uk

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ABSTRACT

The membrane topology of vitamin K epoxide hydrolase (VKOR) is controversial with data supporting both a three transmembrane and a four transmembrane model. The positioning of the transmembrane domains and the loops between these domains is critical if we are to understand the mechanism of vitamin K oxidation and it's recycling by members of the thioredoxin family of proteins and the mechanism of action of warfarin, an inhibitor of VKOR. Here we show that both mammalian VKOR isoforms adopt the same topology, with the large loop between transmembrane one and two facing the lumen of the endoplasmic reticulum (ER). We used a redox sensitive GFP fused to the N- or C- terminus to show that these regions face the cytosol, and introduction of glycosylation sites along with mixed disulfide formation with thioredoxin like transmembrane protein (TMX) to demonstrate ER localisation of the major loop. The topology is identical to the bacterial homologue from *Synechococcus* sp., for which the structure and mechanism of recycling has been characterized. Our results provide a resolution to the membrane topology controversy and support previous results suggesting a role for members of the ER protein disulfide isomerase family in recycling VKOR.

Keywords: vitamin K epoxide reductase, redox-sensitive GFP, TMX, vitamin K, membrane protein, endoplasmic reticulum.

Summary statement: Vitamin K epoxide reductase ensures the efficient recycling of vitamin K. Our understanding of enzyme mechanism relies upon resolving controversy surrounding its membrane orientation. Here we show that the mammalian enzyme adopts a 4-transmembrane organisation similar to its bacterial homologue.

INTRODUCTION

Mammalian VKOR is responsible for the recycling of vitamin K during the γ-carboxylation of glutamic acid side chains in proteins involved in blood clotting [1]. The active site of the enzyme contains a CXXC motif within one of the transmembrane domains that becomes oxidised during the reduction of vitamin K epoxide and needs to be recycled to maintain activity [2]. This CXXC motif is highly conserved amongst VKOR homologues from plants, bacteria, archaea and mammals [3]. There are also two additional cysteines that are present within one of the loops between transmembrane domains which are conserved between homologues. The

structure of the bacterial enzyme has been solved and, in combination with structure-function studies, the mechanism of active site recycling has been elucidated [4, 5]. Hence the loop cysteines have been shown to shuttle elections between the active site CXXC motif and a thioredoxin-like domain. The bacterial enzyme differs from the other homologues in that it contains a thioredoxin domain as an extension at the carboxy-terminus. Such a relay of disulfide exchange would ultimately need to end with an electron donor. While this ultimate donor has not been identified in bacteria, it likely involves the transfer of elections from the periplasm to the cytosol via DsbD [6] and coupling to NADPH via thioredoxin reductase. Alternatively, it is possible that proteins that undergo oxidative folding donate electrons required for the catalysis of bacterial VKOR, as is the case with the DsbA-DsbB disulfide bond formation system in *E. coli* [7].

The mechanism of disulfide relay and particularly the involvement of the loop cysteines in the recycling of the CXXC active site within the mammalian enzyme has been the focus of sustained controversy over the last few years [8-12]. Essentially two conflicting models for the membrane topology of VKORC1 exist predicting either 3 or 4 transmembrane domains (TM) (Fig. 1) [13]. The 4TM model is compatible with the known structure of the bacterial VKOR and its functional homologue DsbB in *E.coli* [14-16] and places the amino- and the carboxy-termini within the cytosol, whilst the loop cysteines as well as the active site on the side of the membrane facing the ER. Evidence has been presented that supports the role of the loop cysteines in the active site recycling and for the involvement of thioredoxin-like proteins, present within the ER, in disulfide exchange [8, 9]. The 3TM model places the amino-terminus and the CXXC active site on the ER side of the membrane and the carboxy-terminus and the loop cysteines within the cytosol. In this orientation the loop cysteines would not play a role in recycling as they are on the opposite side of the membrane to the active site. Evidence has been presented for the suggested topology as well as data indicating that the loop cysteines are not required to recycle the active site [10, 12]. Several of the warfarin-resistant mutations in mammalian VKORC1 are located within the cysteine containing loop suggesting this sequence has an important role to play in the inhibition of activity by warfarin [17, 18]. Hence there is a need to resolve the membrane topology both to understand more fully the mechanism of enzyme recycling and inhibition by warfarin.

Within mammalian cells there is a paralogue of the enzyme called VKORC1 like protein 1 or VKORC1L1 which may have arisen by gene duplication [19]. While both paralogues can catalyse the same reaction there are differences in their pattern of tissue expression and VKORC1L1 does not complement for the absence of VKORC1 given the severity of the phenotype of the VKORC1 knock out mouse [20]. VKORC1L1 has been shown to protect cells from oxidative stress suggesting a distinct role for this enzyme [19]. While controversy over VKORC1 membrane topology remains it is agreed that VKORC1L1 has a 4TM topology similar to the bacterial enzyme [11]. Finally it should be noted that there is evidence to suggest that VKORC1 can form multimeric structures though there is no evidence for a heterooligomeric complexes between VKORC1 and VKORC1L1 [21, 22].

To address the membrane topology of both human paralogues we combined the use of a redox-sensitive GFP (roGFP) tag with an evaluation of glycosylation of the loop sequence and the ability of the loop cysteines to form mixed disulfides with a member of the protein disulfide isomerase (PDI) family - thioredoxin-like membrane protein (TMX) [9]. The use of roGFP to determine topology of membrane proteins has the advantage that it can be carried out in live cells

eliminating any artefacts due to cell permeablization or fractionation [23]. The technique relies upon the different redox environments of the ER and the cytosol which can be sensed by changes to the fluorescent properties of roGFP. These changes are ratiometric allowing analysis which is independent of protein concentration and unambiguously determines the environment sensed by the reporter. Our results conclusively support the 4TM model for both human VKOR paralogues.

EXPERIMENTAL

Reagents and antibodies: All reagents including mouse monoclonal anti-FLAG antibody and rabbit polyclonal anti-HA antibody were purchased from Sigma-Aldrich (Dorset, UK) unless stated otherwise. The fluorescent-conjugated anti-mouse and anti-rabbit IgG 680 and 800 secondary antibodies were purchased from either Li-Cor IRDye or Thermo-Scientific DyeLight.

DNA constructs and site-directed mutagenesis: DNA constructs containing the FLAG tag at the C termini and roGFP1-iE at the N or C termini of VKORC1 or C1L1 were all custom made by GenScript (NJ, USA). For all the roGFP constructs, a 30 amino acids flexible linker $(GGSGG)_6$ was included between VKOR and roGFP as described previously [10, 24]. Mutagenesis was performed as described [25] using FLAG tagged pcDNA3.1 VKORC1 or VKORC1L1 as template. The TMX substrate trapping mutant was a gift from Maurizio Molinari (Bellinzona, Switzerland) [26]. Mutant constructs were expressed for 24 h in HT1080 cells following polyethyleneimine-mediated transfection [27].

Live cell microscopy: HT1080 cells transfected with VKOR-roGFP constructs were seeded on poly-D-lysine coated coverslips. After washing with HEPES buffer (20 mM HEPES, pH 7.4 containing 130 mM NaCl, 5 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 10 mM D-glucose), the coverslip was mounted in a perfusion chamber (POCmini-2, Zeiss). The cells were imaged on a Zeiss Axia Observer A1 inverted microscope equipped with a 40x oil immersion Fluor objective by sequential excitation using 385 and 470 nm LEDs. Emission was detected through a 495 nm long pass emission filter (Chroma) by an Axiocam MRm camera (Zeiss) with 2x2 binning. The 385/470 nm ratio at each time point was calculated after background subtraction using Axiovision software (Zeiss).

EndoH deglycosylation: Cells were harvested, washed and lysed in buffer A (50 mM Tris-HCl buffer, pH 8.0, containing 150 mM NaCl, 5 mM EDTA, 1% (w/v) Triton X100). Lysates were cleared by centrifugation (15,000 xg for 10 min at 4°C) and reaction set up following the manufacture's protocol (NEB). The samples were digested overnight at 37°C and using 500 units of either EndoH, and separated on a 15% SDS-PAGE gel.

Immunoisolation: Cells were washed with PBS, before being lysed in IP buffer (50mM Tris buffer, pH 7.5 containing 1% Triton X-100, 150mM NaCl, 2mM EDTA, 0.5mM PMSF, 0.02% sodium azide). Cell debris was removed by centrifugation (20,000 xg for 3 min at 4°C). The lysate was pre-cleared by adding protein A Sepharose (PAS, Generon) and incubated for 30 min at 4°C. Samples were immunoisolated by using ANTI-FLAG® M2 Affinity Gel or Protein A Sepharose and anti-HA antibody at 4 °C overnight on a roller mixer. The beads were pelleted by centrifugation at 3000 xg for 1 min and washed three times with 1 ml of IP buffer. SDS sample buffer (final concentration 60 mM Tris/HCL, pH 6.8, 2% SDS, 10% glycerol and 0.01% bromophenol blue) was added and the samples were boiled for 10 min before being separated by SDS-PAGE.

Western blotting: After separation by SDS-PAGE, proteins were transferred on to nitrocellulose membranes (GE Healthcare). Nonspecific binding was blocked using 5%(w/v) non-fat dried skimmed milk in Tween/Tris-buffered saline (TTBS) (50 mM Tris buffer pH7.5 containing 150mM NaCl and 0.1% (v/v) Tween 20). Membranes were incubated with primary antibody for 16 h at 4 °C in TTBS. The secondary antibody was diluted 1:5000 in TTBS and incubation was performed in a light-shielded box for 45 min at room temperature. Western blots were visualized on an Odyssey® SA infrared scanner.

RESULTS

Membrane topology as judged by the localisation of roGFP

The current controversy over the orientation of VKORC1 or VKORC1L1 revolves around a 3 vs 4 TM model as depicted (Fig. 1). Given that these two models can be distinguished by the location of the N-terminus we sought to determine its location in live cells by creating fusion proteins with roGFP1-iE at the N-terminus. For completion we also fused roGFP1-iE to the C-terminus though this should be present in the cytosol in both models (Fig. 2A). We created the constructs in the same way as carried out previously [10]. It has been demonstrated that such GFP-fusion proteins are enzymatically active [10], the presumption being that they form the same membrane topology as the wild-type protein. The version of roGFP used (roGFP1-iE) will be partially reduced if present within the ER but fully reduced if present within the cytosol [28, 29]. The redox status of roGFP1-iE can be determined in live cells by measuring the ratio of emission at two excitation wavelengths providing a non-invasive approach to determine subcellular location [23].

As a prelude to determining the fluorescent properties we first considered the expression of the various constructs transfected into a human fibrosarcoma cell line (HT1080). All constructs were efficiently expressed as judged by western blotting (Fig. 2B). When roGFP1-iE was expressed it revealed a diffuse pattern of fluorescence as judged by live cell imaging characteristic of a cytosolic location (Fig. 2C, top left panel). In contrast, a roGFP1-iE construct containing an amino-terminal signal and an ER retrieval sequence (roGFP1-iE-KDEL) localised to the ER as judged by a perinuclear reticular pattern of live cell fluorescence (Fig. 2C, top right panel). All the VKOR-roGFP fusion proteins gave a very similar fluorescence pattern to the roGFP1-iE-KDEL, indicating that they localised to the ER membrane (Fig. 2, middle and bottom panels). Given the previous work carried out on VKOR constructs tagged with GFP [10, 11] and our own results, we can conclude that the addition of the tag does not affect activity of the enzyme or prevent membrane integration and localisation to the ER.

To ascertain the subcellular location of the various roGFP domains we determined the ratio of fluorescence emission following excitation at 385 and 470 nm (385/470). Ratios were recorded in live cells and in real time at steady state and after the addition of an oxidant (diamide) followed by a reductant (DTT). The addition of diamide to cells fully oxidises the roGFP (increases ratio) whereas the addition of DTT fully reduces the roGFP (decreases the ratio). For roGFP1-iE, the steady state ratio corresponds to the fully reduced probe as would be expected for its localisation to the cytosol (Fig. 3, left top panel). In contrast, for roGFP-iE-KDEL the steady state ratio lies between the fully oxidised and fully reduced ratio demonstrating that it is localised to the ER (Fig. 3, right top panel). The steady state ratio of all the VKOR constructs was fully reduced, indicating that the roGFP1-iE tag is localised to the cytosol. Hence, the C- and N-

termini of VKORC1 and VKORC1L1 are located on the cytosolic side of the membrane consistent with the 4-TM model (Fig 2A).

Determining the location of the loop cysteines within VKORC1 and VKORC1L1

The live cell imaging experiments with roGFP provide compelling evidence that these constructs adopt a 4 TM topology. To eliminate the possibility that these constructs are in some way different than the unmodified proteins, we used alternative approaches to evaluate the position of the loop between TM1 and TM2 (Fig. 1). If the 3TM model is correct, this loop would be located on the cytosolic side of the membrane whereas if the 4TM model is correct, we would expect the loop to be on the ER side of the membrane. We introduced potential acceptor sites for N-linked glycosylation within this loop by creating a F55N mutation in VKORC1 or K57N in VKORC1L1 (Fig. 4A). These mutations create either a NSS or a NCS acceptor site respectively. When these mutants were expressed in HT1080 cells, they were both glycosylated as judged by the appearance of a slower migrating protein which was sensitive to digestion with endoglycosidase H (Fig. 4B). For glycosylation to occur this loop must be exposed to the machinery for glycosylation within the ER lumen, therefore, our results demonstrate that the loop between TM1 and TM2 is located in the ER lumen for both VKORC1 and VKORC1L1.

It has previously been shown that the conserved cysteines within this loop can form mixed disulfides with members of the PDI family of proteins, particularly ERp18, TMX and TMX4 [9]. We also evaluated the ability of our constructs to form mixed disulfides with TMX. For these experiments we co-expressed our FLAG-tagged VKOR constructs with a HA-tagged TMX mutant which has the second cysteine in its active site mutated to alanine. Such a mutant can form mixed disulfides with its substrate, but the absence of the second cysteine stabilises this disulfide allowing enzyme-substrate complexes to be identified [30]. The exogenously expressed VKORC1 or VKORC1L1 was first immunoisolated from cell lysates using a FLAG antibody and any TMX co-isolated was identified by subsequent western blotting using a HA antibody (Fig. 5). Non-covalent and covalent complexes between TMX and both VKOR paralogues were identified, indicating that TMX associates with VKOR and forms a mixed disulfide. To determine whether the mixed disulfide was between TMX and one of the loop cysteines, we mutated both conserved cysteines within the TM1 to TM2 loop in VKORC1 and VKORC1L1. For completion we also included a mutant that had the two loop cysteines as well as the active site cysteines mutated. Both the double cysteine mutant (DM) and the quadruple mutant (QM) were still able to associate with TMX but crucially DM did not form a mixed disulfide complex. This clearly demonstrates that the loop cysteines are able to form a mixed disulfide with TMX. As the active site of TMX is localised to the ER lumen [31], the formation of a mixed disulfide via the loop cysteines strongly suggests that the TM1-TM2 loop is located on the ER face of the membrane.

Discussion

The main conclusion from the work present here is that the membrane topology of both mammalian VKOR paralogues is similar to the bacterial homologue. This topology places the conserved cysteines on the same side of the membrane as the active site. The consequence for such an arrangement is that the mechanism of recycling is most likely to involve disulfide exchange between the active site cysteine pair and the cysteine pair within the TM1 to TM2 loop which acts as a disulfide shuttle finally exchanging a disulfide with a thioredoxin like protein [8]. For the bacterial enzyme this thioredoxin domain is part of the enzyme itself [4], for the

mammalian enzymes this role is fulfilled by a member of the PDI family of oxidoreductases [9]. The formation of a disulfide within the active site PDI could then contribute to disulfide formation in newly synthesised proteins as has been suggested previously [32]. This orientation also places the majority of the mutations that cause warfarin resistance on the ER side of the membrane, suggesting that warfarin binding could interfere with the recycling of VKOR and thereby inhibit its activity [13].

While the evidence for a 4TM topology for VKOR seems compelling, it does contradict previous evidence which supports a 3TM model for VKORC1 [10, 12]. The evidence for a 3TM topology is based upon a fluorescent protease protection (FPP) assay, the glycosylation of VKOR constructs and enzymatic activity of mutants lacking the TM1 to TM2 loop cysteine residues. We created the same GFP-tagged construct in our work to this previous study using identical linker sequences. The difference between the approaches is that our assay is carried out in live cells with the roGFP acting as a sensitive reporter of its environment while the FPP assay depends upon the relative susceptibility of GFP to digestion with trypsin following permeablization of the cells with digitonin. Crucially a lack of digestion of GFP by trypsin is taken to indicate an ER localisation of the tag. It may well be that the differences in the way the location of the GFP is evaluated forms the basis for the contradictory results. A previous study on glycosylation of an N-terminally tagged VKOR demonstrated that this region could become glycosylated when expressed in an *in vitro* translation/translocation system [12]. This suggests that the N-terminus is in the ER lumen which would support the 3-TM model. However, the efficiency of glycosylation was poor and it is likely that the N-terminus would enter the ER lumen prior to the reorientation of the TM domain as is the case with proteins containing TM domains with a N_{cyt}/C_{exo} orientation [33]. Hence, it would be exposed to the glycosylation machinery briefly during its synthesis. In our experiments the glycosylation site on the TM1-TM2 loop is efficiently glycosylated following expression of the proteins in cells. Hence, the differences in results could reflect both the positioning of the potential glycosylation site and the mechanism of insertion of TM domains acting as signal anchors.

The formation of mixed disulfides between the two mammalian VKOR paralogues and TMX which was dependent on the presence of the loop cysteines, providing further evidence for the TM1 to TM2 loop being present within the ER. Intriguingly we also noted a non-covalent association of VKOR with TMX which was not dependent upon the presence of the loop or the active site cysteines. The co-isolation of the two proteins might indicate their close association in cells, potentially forming a complex to facilitate efficient enzyme recycling. TMX is a single spanning transmembrane protein that has been shown to localise to the mitochondria-associated membrane (MAM) following palmitoylation of the cytosolic tail [34]. There is no evidence that VKOR itself becomes palmitoylated but it will be of interest to determine whether VKOR is associated with the MAM via association with TMX given the role of vitamin K in mitochondrial electron transport [35].

Author contribution

Zhenbo Cao, Kenji Inaba and Neil Bulleid conceived and planned the research. Zhenbo Cao, Marcel van Lith, Lorna Mitchell and Marie Anne Pringle performed the experiments. Zhenbo Cao, Marcel Van Lith and Neil Bulleid analysed the data. Zhenbo Cao and Neil Bulleid wrote the paper.

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Abbreviations

The abbreviations used are: ER, endoplasmic reticulum; PDI, protein disulfide isomerase; DTT, dithiothrietol; TTBS, Tris-Tween buffered saline; VKOR; vitamin K epoxide reductase; GFP, green fluorescent protein; TMX, thioredoxin; TM, transmembrane; endoH, endoglycosidase H; PNGase, peptide N-glycosidase F; PMSF, phenylmethylsulfonyl fluoride; DM, double cysteine mutant; QM, quadruple cysteine mutant.

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Figure legends

FIGURE 1: Proposed topological model for VKORC1 and VKORC1L1

Cartoon depicting the potential membrane topology of the human VKOR paralogues. The Roman numerals represent the order of the transmembrane domain. The loop cysteines and the active site cysteines are coloured in yellow. Human VKORC1 4TM model was based on data from Uniprot database with both amino- and carboxy termini located in the cytosol. VKORC1 3TM model adds the TM2 in the 4TM model to the loop region in the cytosol. The amino-termini and CXXC active side are on the ER side of the membrane. The human VKORC1L1 4TM model was based on the VKORC1 4TM model by using sequence alignment.

FIGURE 2: VKOR roGFP expression and localization in HT1080 cells

(A) Schematic illustrating the cellular location of the C- or N- terminal roGFP1-iE domain if the 3 or 4TM model is correct. (B) HT1080 cells were transiently transfected with different VKOR-roGFP constructs and western blotted with anti-GFP antibody. Note VKORC1L1 is larger than

VKORC1. (C) transfected HT1080 cells were seeded on coverslips and imaged by live-cell fluorescence microscopy. The perinuclear reticular pattern suggests that the VKOR-roGFP constructs are localised to the ER membrane.

FIGURE 3: Measuring the steady state redox states of roGFP in different VKOR constructs

Transfected cells were excited by using 385 and 470 nm LEDs and the 385/470 emission ratio was calculated at each time point. Error bars represent mean ± S.D. from at least 3 cells in each field of view. Cells were then treated with 1mM diamide and 10mM DTT sequentially as indicated to demonstrate the fully oxidized (highest ratio) and fully reduced states (lowest ratio) of roGFP1-iE. The experiments were repeated with similar results.

FIGURE 4: determining TM1 to TM2 loop location by introducing a glycosylation site

(A) The location of the potential glycosylation site (star) introduced by mutagenesis in proposed 3 or 4TM model. (B) VKORC1 and VKORC1L1 mutants migrate slower compared to wild type (WT) demonstrated by western blot with anti-FLAG antibody. Endoglycosidase H (endoH) treatment resulted in the mutants migrating similar to wild type.

FIGURE 5: TMX forms a mixed disulfide with VKOR via the TM1 to TM2 loop cysteines

Cells were co-transfected with a HA-tagged substrate-trapping mutant of TMX and either FLAG-tagged wild type (WT) VKORC1, VKORC1L1 or mutants in which the TM1 to TM2 loop cysteines were mutated to alanine alone (DM) or in combination with the active site cysteines (QM). Cell lysates were immunoisolated with FLAG antibodies followed by immunoblotting with HA antibody. Samples were separated by SDS-PAGE run under non-reducing (left) or reducing (right) conditions.

Figure 1

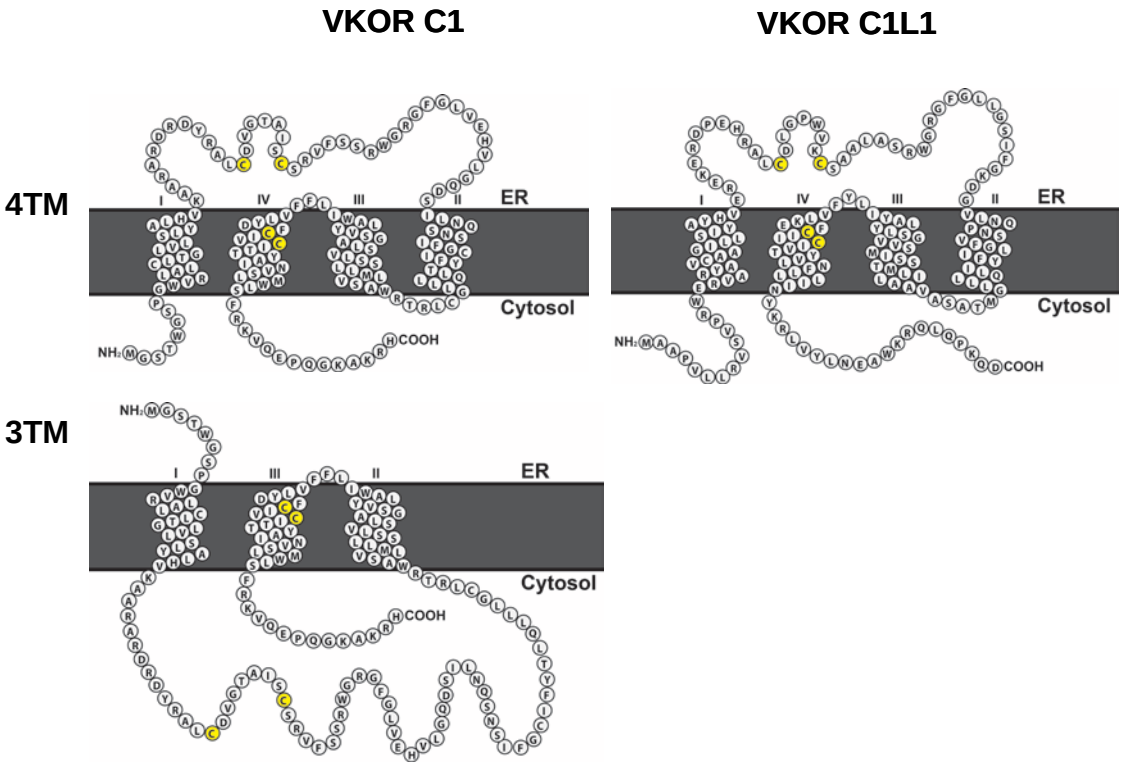
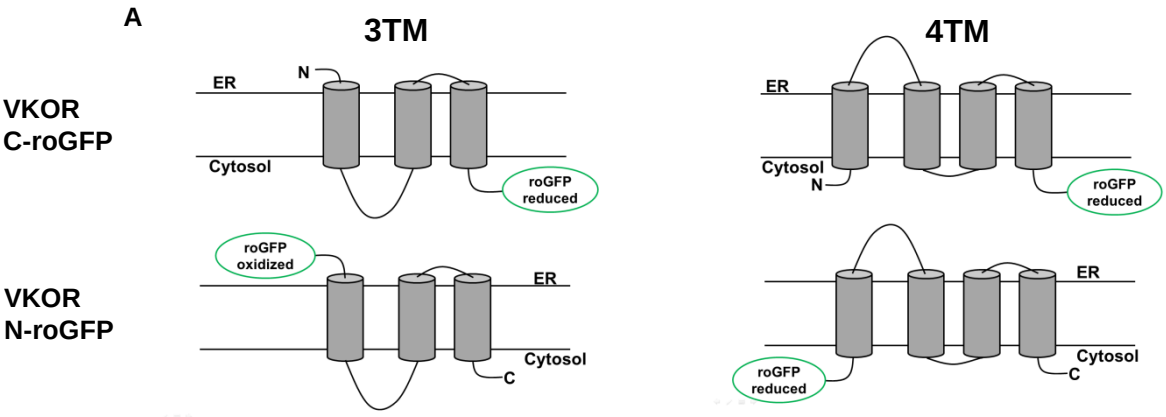
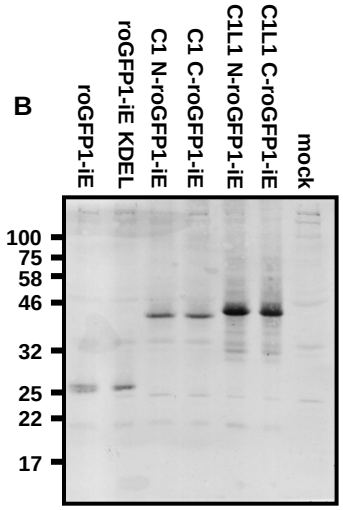


Figure 2


A
3TM
4TM
VKOR
C-roGFP
VKOR
N-roGFP
ER
Cytosol
N
C
roGFP
reduced
roGFP
oxidized


B
roGFP1-iE
roGFP1-iE KDEL
C1 N-roGFP1-iE
C1 C-roGFP1-iE
C1L1 N-roGFP1-iE
C1L1 C-roGFP1-iE
mock
100
75
58
46
32
25
22
17

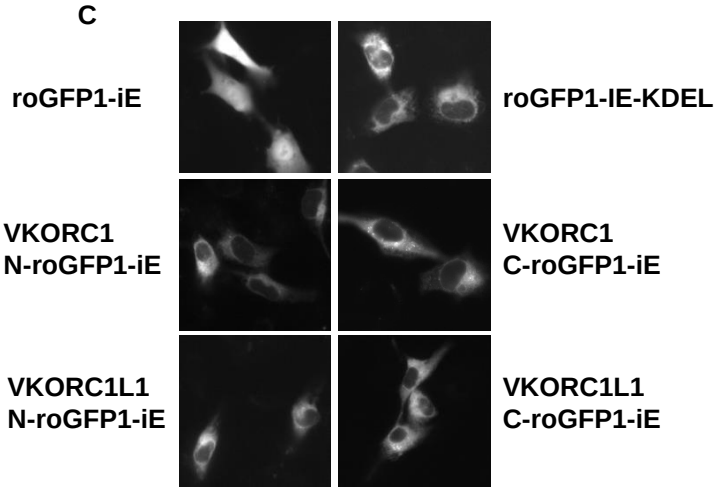

C
roGFP1-iE
roGFP1-IE-KDEL
VKORC1
N-roGFP1-iE
VKORC1
C-roGFP1-iE
VKORC1L1
N-roGFP1-iE
VKORC1L1
C-roGFP1-iE

Figure 3

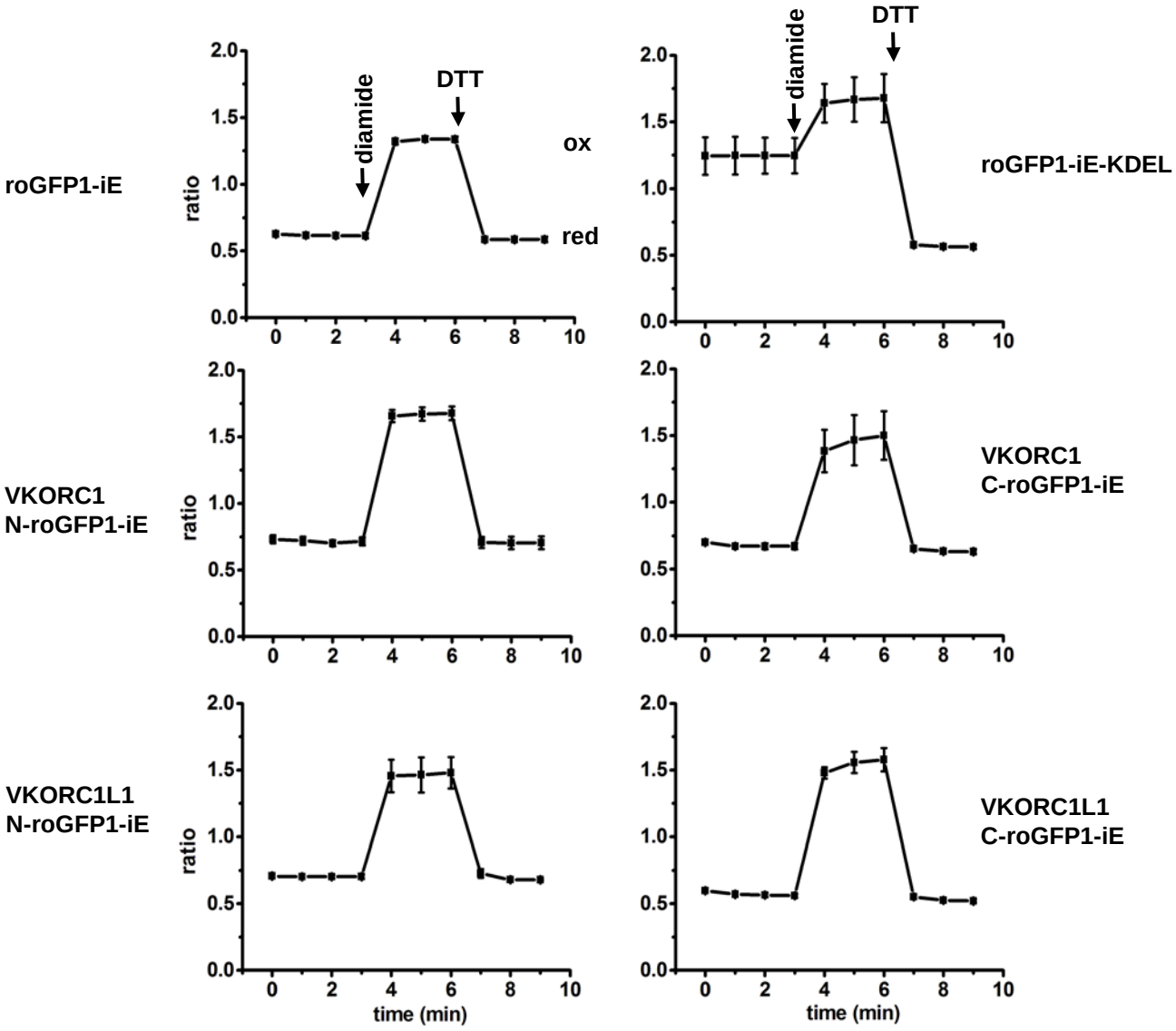
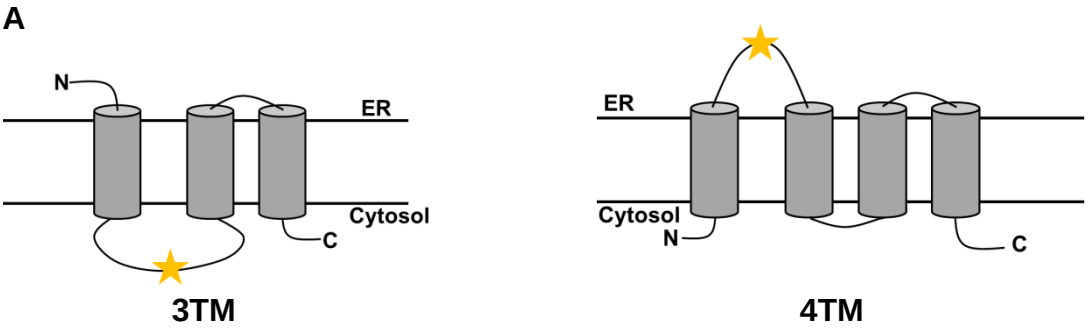

roGFP1-iE
diamide
DTT
ox
red
ratio
roGFP1-iE-KDEL
VKORC1
N-roGFP1-iE
VKORC1
C-roGFP1-iE
VKORC1L1
N-roGFP1-iE
VKORC1L1
C-roGFP1-iE
time (min)

Figure 4


A
N
ER
Cytosol
C
3TM
ER
Cytosol
N
C
4TM

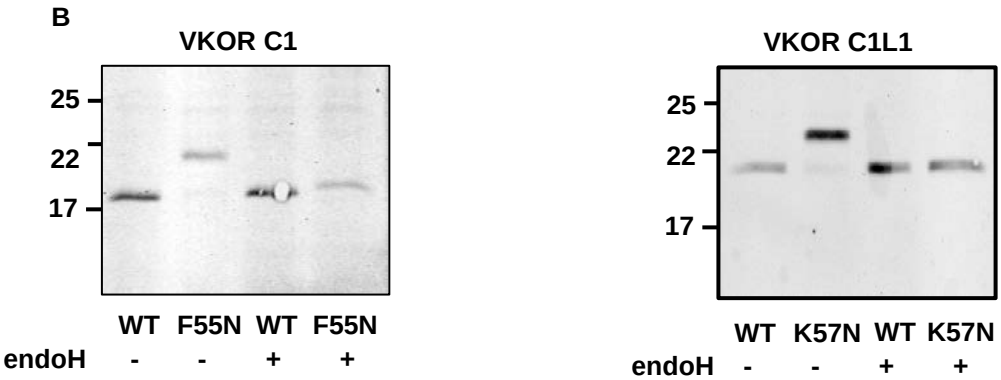
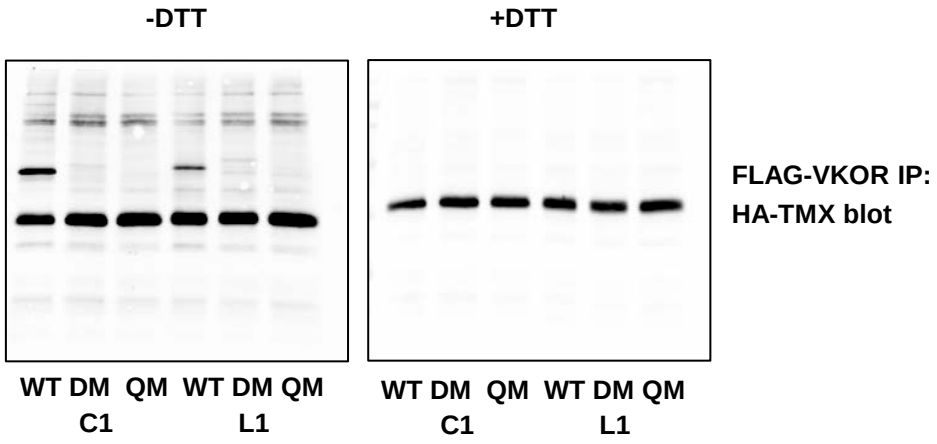

B
VKOR C1
25
22
17
WT F55N WT F55N
endoH - - + +
VKOR C1L1
25
22
17
WT K57N WT K57N
endoH - - + +

Figure 5


-DTT
+DTT
WT DM QM WT DM QM
C1 L1
WT DM QM WT DM QM
C1 L1
FLAG-VKOR IP:
HA-TMX blot