Conserved Loop Cysteines of Vitamin K Epoxide Reductase Complex Subunit 1-like 1 (VKORC1L1) Are Involved in Its Active Site Regeneration*

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Background: The structure and the physiological function(s) of vitamin K epoxide reductase complex subunit 1-like 1 (VKORC1L1) are unknown.
Results: VKORC1L1 has four transmembrane domains and employs an intra-molecular electron transfer pathway for active site regeneration.
Conclusion: The different structure and reaction mechanism of VKORC1L1, as compared with VKORC1, suggest that VKORC1L1 has different physiological function(s).
Significance: Four conserved cysteines in VKORC1L1 function in concert for active site reduction.

Vitamin K epoxide reductase complex subunit 1 (VKORC1) reduces vitamin K epoxide in the vitamin K cycle for post-translational modification of proteins that are involved in a variety of biological functions. However, the physiological function of VKORC1-like 1 (VKORC1L1), a paralogous enzyme sharing about 50% protein identity with VKORC1, is unknown. Here we determined the structural and functional differences of these two enzymes using fluorescence protease protection (FPP) assay and an *in vivo* cell-based activity assay. We show that *in vivo* VKORC1L1 reduces vitamin K epoxide to support vitamin K-dependent carboxylation as efficiently as does VKORC1. However, FPP assays show that unlike VKORC1, VKORC1L1 is a four-transmembrane domain protein with both its termini located in the cytoplasm. Moreover, the conserved loop cysteines, which are not required for VKORC1 activity, are essential for VKORC1L1's active site regeneration. Results from domain exchanges between VKORC1L1 and VKORC1 suggest that it is VKORC1L1's overall structure that uniquely allows for active site regeneration by the conserved loop cysteines. Intermediate disulfide trapping results confirmed an intra-molecular electron transfer pathway for VKORC1L1's active site reduction. Our results allow us to propose a concerted action of the four conserved cysteines of VKORC1L1 for active site regeneration; the second loop cysteine, Cys-58, attacks the active site disulfide, forming an intermediate disulfide with Cys-139; the first loop cysteine, Cys-50, attacks the intermediate disulfide resulting in active site reduction. The different membrane topologies and reaction mechanisms between VKORC1L1 and VKORC1 suggest that these two proteins might have different physiological functions.

Vitamin K epoxide reductase complex subunit 1 (VKORC1)[2] is a polytopic membrane protein of the endoplasmic reticulum (ER)

(1, 2). It is responsible for the reduction of vitamin K 2,3-epoxide (KO, a quinone epoxide) to vitamin K (a quinone), and can also reduce vitamin K to vitamin K hydroquinone (KH_2). This series of quinone reductase reactions is an essential part of a redox cycle known as the vitamin K cycle (3). The active form of the vitamin in the cycle, KH_2, is a cofactor for γ-glutamyl carboxylase, which catalyzes post-translational modification of vitamin K-dependent proteins essential for blood coagulation, bone homeostasis, vascular mineralization, signal transduction, and cell proliferation (4–7).

VKORC1 is the target of warfarin, the most commonly prescribed oral anticoagulant used for prevention and treatment of thrombosis (8). Warfarin exerts its effect by inhibition of VKORC1 activity, which reduces the availability of KH_2 and impairs the synthesis of functional clotting factors. Finding the appropriate dosage of warfarin required to achieve stable anticoagulation has been a challenge, because of its narrow therapeutic index and broad individual variability (9). Since the identification of the *VKORC1* gene (1, 2), single nucleotide polymorphisms have been found in the coding as well as non-coding regions of VKORC1 in warfarin-resistant/sensitive patients. These genetic variations have been successfully used as a marker for managing warfarin maintenance dosage (10, 11).

Site-directed mutagenesis studies show that VKORC1 uses a C*XX*C redox motif as its active site (12–14). Membrane topology studies suggest that VKORC1's active site is located at the N-terminal of the last transmembrane domain (TMD) facing the ER lumen (15, 16). During the reduction of KO or vitamin K, VKORC1's active site cysteines (Cys-132 and Cys-135) are oxidized to a disulfide bond that must be reduced to free cysteines to reactivate the enzyme. Thioredoxin was proposed as the physiological reductant for VKORC1's active site regeneration

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(17–20); however, this hypothesis has been questioned (21). Currently, the physiological reductant of VKORC1 is unknown.

VKORC1 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 only in strains lacking DsbB, a cytoplasmic membrane protein that is important for protein oxidative folding in the periplasm (23). Bacterial VKORHs can functionally complement *Escherichia coli* strains that lacking DsbB to restore protein-disulfide formation (23, 24). It has been shown that the conserved loop cysteines in bacterial VKORHs shuttle electrons to the disulfide-bonded C*XX*C active site through an intra-molecular electron transfer pathway, similar to that of DsbB (25, 26). This mechanism has been extended to human VKORC1 (26–28). However, our recent study shows that the intra-molecular electron transfer pathway for active site regeneration does not apply to human VKORC1 (15).

When the *VKORC1* gene was identified, homologous searches with VKORC1 detected a paralogous protein, VKORC1L1 (1). Although the structure-function relationships of VKORC1 have been extensively studied (29), functional studies of VKORC1L1 have been reported only recently (30, 31). Unlike VKORC1, single-nucleotide polymorphisms of the *VKORC1L1* gene appear not to be directly associated with the warfarin maintenance dosage (32). In addition, results from an *in vitro* study suggest that VKORC1L1 functions as a housekeeping protein that helps to prevent intracellular oxidative damage (31). Therefore, it has been proposed that VKORC1L1 is not involved in the vitamin K cycle (31). On the other hand, a more recent study suggests that VKORC1L1 might rescue VKORC1 activity in extrahepatic tissues during anticoagulation therapy (30). Moreover, our results suggest that VKORC1L1 can support vitamin K-dependent carboxylation as efficiently as does VKORC1 (33). However, it appears unlikely that VKORC1L1 plays a key role in the vitamin K cycle under physiological conditions (33, 34). If it were, the murine knock-out of VKORC1 would be viable (34), and no significant decrease of KO reductase activity in the VKORC1 knock-out cells would have been observed (33). Therefore, despite the high homology of VKORC1L1 and VKORC1, the physiological function of VKORC1L1 is still unclear.

To better understand the structure-function relationships that define VKORC1L1 activity, we report here: 1) the membrane topology of VKORC1L1, determined using a fluorescence protease protection (FPP) assay in live mammalian cells; 2) the role of VKORC1L1's conserved loop cysteines in KO reduction, using our recently established cell-based activity assay (35); 3) a possible mechanism for VKORC1L1's active site regeneration by the conserved loop cysteines. Our results show that VKORC1L1 has a different membrane topology and reaction mechanism than that of VKORC1.

EXPERIMENTAL PROCEDURES

Materials—Vitamin K1, warfarin, digitonin, and trypsin (bovine pancreas) were obtained from Sigma-Aldrich. Vitamin K epoxide was prepared as described previously (36). Vitamin K1 (10 mg/ml) for cell culture was from Abbott Laboratories (Lake Forest, IL). Methoxy-polyethylene glycol maleimide, molecular weight 5000 (mPEG-MAL-5000), was purchased from Nektar (Huntsville, AL). Slide-A-Lyzer MINI dialysis unites (2K MWCO) were from Pierce Biotechnology, Inc. Complete protease inhibitor mixture tablets were from Roche (Indianapolis, IN). Xfect transfection reagent was from Clontech Laboratories, Inc. (Mountain View, CA). Mammalian expression vector pBudCE4.1, baculovirus expression vector pFastBac I, and all cell culture mediums and oligonucleotide primers were from Invitrogen Corp. (San Diego, CA). HEK293 cell line was from ATCC (Manassas, VA). The Sf9 (*Spodoptera frugiperda*) cells were obtained from the Lineberger Cancer Center at the University of North Carolina-Chapel Hill. Quick-Change site-directed mutagenesis kits were from Agilent Technologies (Santa Clara, CA). Mouse anti-carboxylated FIX gla domain monoclonal antibody was a gift from GlaxoSmithKline (Philadelphia, PA) and Green Mountain Antibodies (Burlington, VT). Anti-HA monoclonal antibody was from Covance Inc. (Princeton, NJ). Horseradish peroxidase conjugated sheep anti-human Protein C IgG and goat anti-mouse IgG were from Affinity Biologicals, Inc. (Ancaster, ON, Canada) and Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

DNA Manipulations and Plasmid Constructions—Mammalian expression vector pBudCE4.1 that independently expresses two genes from a single vector was used for expressing VKORC1, VKORC1L1, or their variants in HEK293 cells. *Metridia* luciferase cDNA was cloned into one of the multi-cloning sites of pBudCE4.1 as an internal control for the cell-based activity assay; VKORC1 or VKORC1L1 constructs were cloned into the other multi-cloning site as described previously (15). All the VKORC1 or VKORC1L1 constructs contain a warfarin-resistant mutation of Y139F or Y146F, respectively. Site-directed mutagenesis was performed by a QuickChange mutagenesis kit to create cysteine mutations in VKORC1L1 according to the manufacturer's instructions. Replacement of VKORC1L1's conserved cysteine loop region or active site sequences with that of VKORC1 was performed by overlap PCR. For Western blot detection, an HA tag (YPYDVPDYA) was introduced at the C terminus of the VKORC1 or VKORC1L1. GFP fusions of the VKORC1 or VKORC1L1 used in the FPP assays were constructed as described previously (15). For the *in vitro* activity assay, VKORC1L1 and its cysteine mutants were cloned into pFastBac I for expression in insect cells. The nucleotide sequences of all the constructs were verified by sequencing at Eton Bioscience Inc. (RTP, NC).

FPP Assay—The FPP assay was performed as described previously (15). Briefly, HEK293 cells were subcultured into chambered coverglass and transiently transfected with pBudCE4.1 plasmid DNA containing the GFP-tagged VKORC1 or VKORC1L1. Forty-eight hours post-transfection, cells were washed three times with KHM buffer (110 mM potassium acetate, 2 mM MgCl$_2$, and 20 mM HEPES pH 7.2) and 200 μl of KHM buffer was added to each chamber before placing 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 40×/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 emissions 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. Trypsin was added to the chamber 2 min after the addition of digitonin at a final concentration of 2 μM to elicit protease digestion of GFP tag in the cytoplasm.

In Vivo and in Vitro VKORC1 and VKORC1L1 Activity Assays—The *in vivo* activity assay for VKORC1, VKORC1L1, and their variants used in this study were performed according to our recently established cell-based activity assay (35). It is based on the ability of VKORC1 (Y139F warfarin-resistant mutant) or VKORC1L1 (Y146F warfarin-resistant mutant) to convert sufficient KO to vitamin K in the native milieu (HEK293 cells) to support the carboxylation of a chimera reporter protein FIXgla-PC. Endogenous KO reductase activity in HEK293 cells was inhibited by warfarin. Therefore, wild-type VKORC1 and VKORC1L1 activity in this study refers to the activity of warfarin-resistant mutants of VKORC1-Y139F and VKORC1L1-Y146F, respectively. VKORC1L1, VKORC1, and their variants were transiently expressed in FIXgla-PC/HEK293 cells. *Metridia* luciferase was coexpressed as the internal control from the same expression vector, pBudCE4.1. Transfected cells were cultured in complete medium containing 5 μM KO with 2 μM warfarin. Forty-eight hours post-transfection, carboxylated FIXgla-PC in the cell culture medium was measured by ELISA. Typically, cells transfected with VKORC1L1 produce ~150 ng/ml carboxylated FIXgla-PC in the culture medium. Mock-transfected cells produce ~5 ng/ml carboxylated reporter protein. Luciferase activity was determined by injecting 100 μl of coelenterazine solution (1 μM in PBS with 300 mM NaCl) to 10 μl of cell culture medium. Luminescence emission (relative light units (RLU)) from the mixture was recorded at 480 nm with a delay of 6 s and integration time of 1 s. VKORC1L1 activity was expressed as normalized carboxylated FIXgla-PC (ng/ml/RLU). Wild-type VKORC1L1 activity was normalized to 100%.

For the *in vitro* activity assay, VKORC1L1 or its cysteine mutants were expressed in Sf9 cells. The conversion of KO to vitamin K was determined by the traditional DTT-driven activity assay as described previously (2, 35).

Differential Modification of the Cysteines of VKORC1L1—Differential modification of the cysteines of VKORC1L1 by NEM and mPEG-MAL-5000 was performed as described previously (15, 37) with additional modifications. HA-tagged VKORC1L1, or their cysteine mutants were transiently expressed in FIXgla-PC/HEK293 cells in a 24-well plate. Cells were cultured in complete medium containing 5 μM KO to facilitate the formation of the possible intermediate intra-molecular disulfide during KO reduction. 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 resuspended in a lysis buffer (1% Triton X-100, 2 mM PMSF, and 1× protease inhibitor mixture in PBS) for cysteine modification. For mPEG-MAL-5000 modification of all the cysteines, cell pellets were resuspended in the lysis buffer with 5 mM DTT. Samples were incubated on ice for 10 min, then freshly prepared mPEG-MAL-5000 solution was added to a final concentration of 20 mM. The reactions were incubated on ice for 1 h and stopped by adding DTT to a final concentration of 50 mM.

For mPEG-MAL-5000 modification of the intra-molecular disulfide, cell pellets were resuspended in a lysis buffer containing 50 mM NEM. Samples were incubated on ice for 1 h followed by centrifuging at 14,000 rpm for 5 min. Supernatants were transferred to Slide-A-Lyzer MINI dialysis units and dialyzed against the lysis buffer to remove excess NEM. Intra-molecular disulfide bonds were then reduced by adding freshly prepared DTT to a final concentration of 5 mM, and the reaction mixture was incubated on ice for 10 min. Freshly prepared mPEG-MAL-5000 solution was added to the sample to a final concentration of 20 mM and incubated on ice for 1 h. 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 as described previously (15).

RESULTS

Determination of the Membrane Topology of VKORC1L1 in HEK293 Cells—The membrane topology of VKORC1L1 was predicted using ten different topology prediction programs (38). Seven out of the ten programs predict four TMDs in VKORC1L1. One program (SPOCTOPUS) predicts three TMDs with a signal peptide. Two programs (PolyPhobius and Phobius) predict three TMDs in VKORC1L1. All these programs predict that the C terminus of VKORC1L1 is located in the cytoplasm, which is significant, since the reliability of topology predictions is greatly increased if different prediction methods give the same prediction (39). The prediction program TOPCONS, which is based on five different topology prediction methods, performs better than other programs (40); like most of the programs, TOPCONS predicts that VKORC1L1 is a 4-TMD membrane protein with both the N terminus and the C terminus located in the cytoplasm. The predicted membrane topology of VKORC1L1 agrees with that of VKORC1 in the orientation of the last two TMDs, but differs with regard to the location of the N terminus with respect to the ER membrane (15, 16).

To experimentally determine the membrane topology of VKORC1L1, we fused green fluorescence protein (GFP) to the N or C terminus of VKORC1L1. We used the FPP assay to probe the location of the two termini of VKORC1L1 molecules in a single live HEK293 cell by fluorescence confocal microscopy. As a control, we fused GFP to the lumenal (N-) or the cytoplasmic (C-) terminus of VKORC1 (Fig. 1*A*). These fusion 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 located in the ER lumen of the control protein (GFP-VKORC1), it is protected from trypsin digestion in digitonin-permeabilized cells (Fig. 1*B*). However, the cytoplasmic terminal GFP fusion of the control protein (VKORC1-GFP) is susceptible to trypsin digestion.

Results in Fig. 1*C* shows that GFP fusions of either the N- or C-terminal of VKORC1L1 are susceptible to protease digestion indicating that both the N and C terminus of VKORC1L1 are located in the cytoplasm. This result suggests that VKORC1L1 has an even number of TMD, which agrees with the above four-TMD topology prediction. However, this result also supports a two-TMD topology model for VKORC1L1. Because the pre-
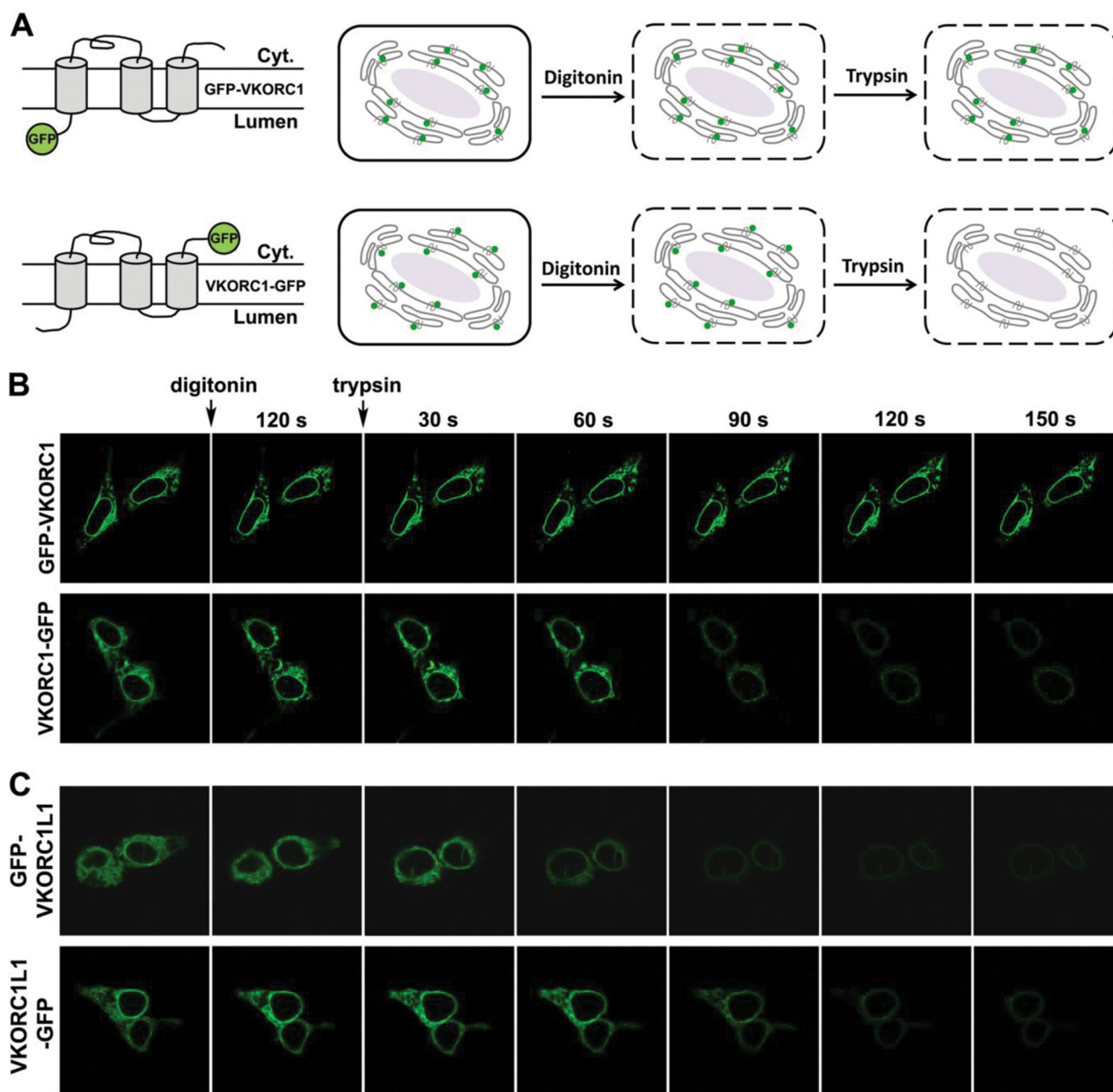
FIGURE 1. **Localization of the N and C termini of VKORC1L1 in HEK293 cells by FPP assay.** *A*, scheme of the FPP assay of GFP-tagged VKORC1 in a single cell before and after digitonin and trypsin treatment. Lumenal (*top*) or cytoplasmic (*bottom*) terminus GFP-tagged VKORC1 was expressed in HEK293 cells. Digitonin treatment permeabilizes the cell membrane while leaving the ER membrane intact. Only the cytoplasmic terminus-tagged GFP fusion is susceptible to protease (trypsin) treatment (*bottom*). *B*, FPP assay of VKORC1 in HEK293 cells. GFP-tagged VKORC1 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. *C*, FPP assay of VKORC1L1 in HEK 293 cells as described above.

dicted TMD regions of VKORC1L1 and VKORC1 share high sequence homology, and all four predicted TMDs in VKORC1 can function as authentic TMDs in both the *in vitro* (16) and the *in vivo* (15) assay, we prefer the four-TMD rather than the two-TMD topology model for VKORC1L1.

To confirm that the GFP fusions of VKORC1L1 used in the FPP assay are properly folded and active, we used our recently established cell-based activity assay to examine their ability to convert KO to vitamin K to support vitamin K-dependent carboxylation (Fig. 2*A*) (35). We transiently expressed GFP fusions of VKORC1L1 in HEK293 cells stably expressing the reporter

protein FIXgla-PC. The transfected cells were cultured with KO and warfarin. Fig. 2*B* shows that VKORC1L1 fusions with GFP, which is approximately twice the size of VKORC1L1, retain 50~70% of their activity compared with the wild-type enzyme. This result demonstrates that the GFP-tagged VKORC1L1 used in the FPP assay for the membrane topology study has passed ER quality control and is enzymatically active.

We next compared VKORC1L1's ability to reduce KO to vitamin K with that of VKORC1 by transiently expressing the two proteins in FIXgla-PC/HEK293 cells. Fig. 2*C* shows that, like VKORC1, VKORC1L1 efficiently supports reporter-pro-
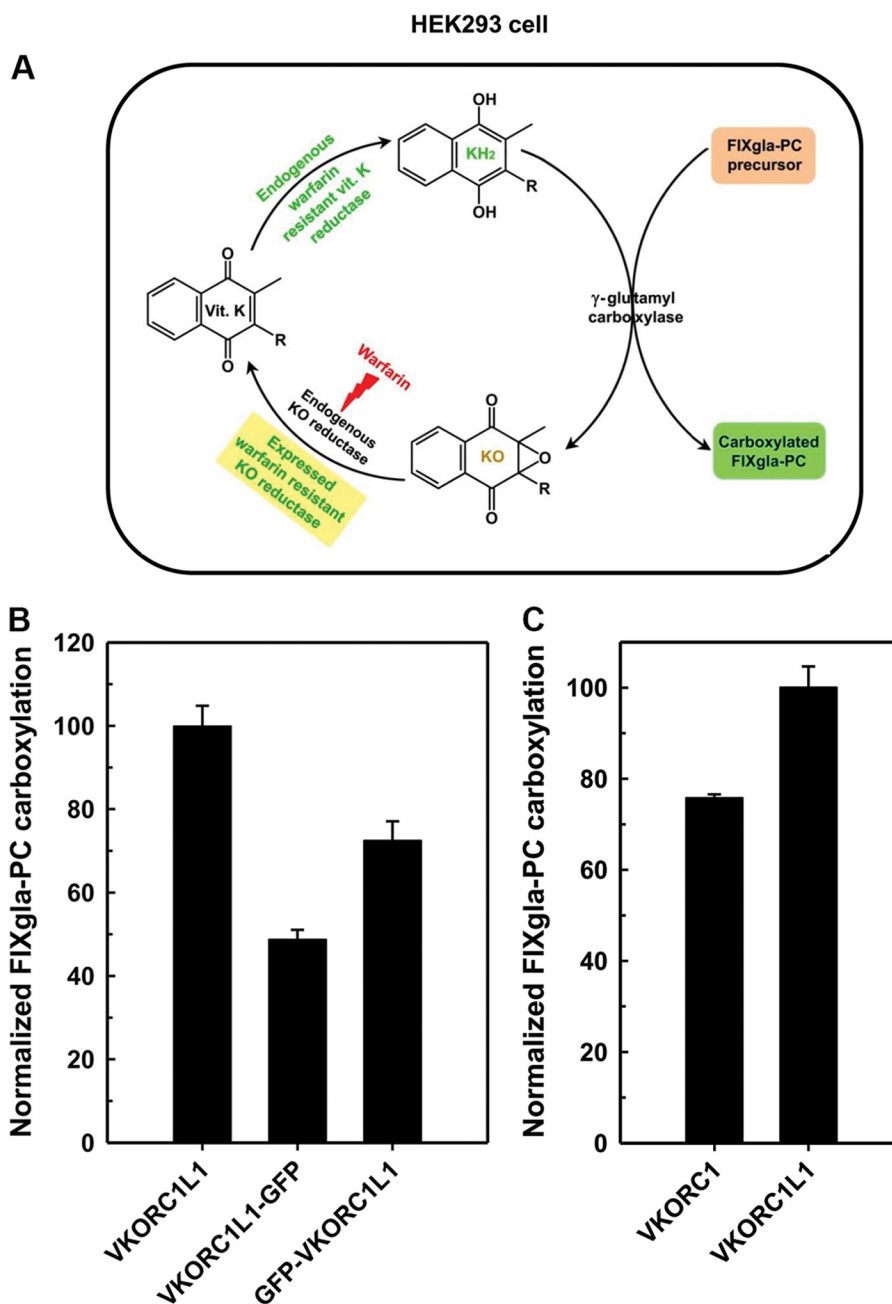
FIGURE 2. Cell-based *in vivo* KO reductase activity assay. *A*, scheme of the cell-based *in vivo* KO reductase activity assay. HEK293 cells stably expressing the chimeric reporter protein FIXgla-PC were used for *in vivo* activity assay. Warfarin was used to inhibit the endogenous KO reductase activity. Exogenously expressed warfarin-resistant VKORC1 or VKORC1L1 converts KO to vitamin K. Vitamin K is further reduced to KH_2 by the endogenous warfarin-resistant vitamin K reductase (antidotal enzyme) for reporter protein carboxylation. Carboxylated FIXgla-PC secreted in the cell culture medium was measured by ELISA to evaluate the functionality of the exogenously expressed VKORC1 or VKORC1L1. *B*, cell-based *in vivo* KO reductase activity of VKORC1L1 and its GFP fusions used in the FPP assay. Warfarin-resistant VKORC1L1 (Y146F) and its GFP fusions were transiently expressed in FIXgla-PC/HEK293 cells. Transfected cells were cultured in complete medium containing 5 μM KO and 2 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 under "Experimental Procedures." *C*, KO reductase activity of VKORC1L1 and VKORC1. Warfarin-resistant VKORC1 (Y139F) or VKORC1L1 (Y146F) was transiently expressed in FIXgla-PC/HEK293 cells, and the enzymatic activity was determined as described above. Data are presented as mean ± S.D. (*n* = 3).

tein carboxylation using KO as the substrate, which agrees with our recent study (33). This result suggests that, *in vivo*, both VKORC1L1 and VKORC1 are able to efficiently convert KO to vitamin K to support vitamin K-dependent carboxylation.

The Conserved Loop Cysteines in VKORC1L1 Are Essential for Its Function—We previously reported that the conserved loop cysteines (Cys-43 and Cys-51) in VKORC1 are located in the cytoplasm and are not required for VKORC1 function (12,

15, 41). In contrast to VKORC1, the conserved loop cysteines of VKORC1L1 (Cys-50 and Cys-58) are located in the ER lumen, the same side of the ER membrane as the C*XX*C active site. To examine whether the loop cysteines in VKORC1L1 are essential for its function, we mutated these two loop cysteines to serine either individually (C50S or C58S) or together (C50S/C58S). We then measured their activities using our cell-based assay. Our results show that mutating the loop cysteines, either indi-
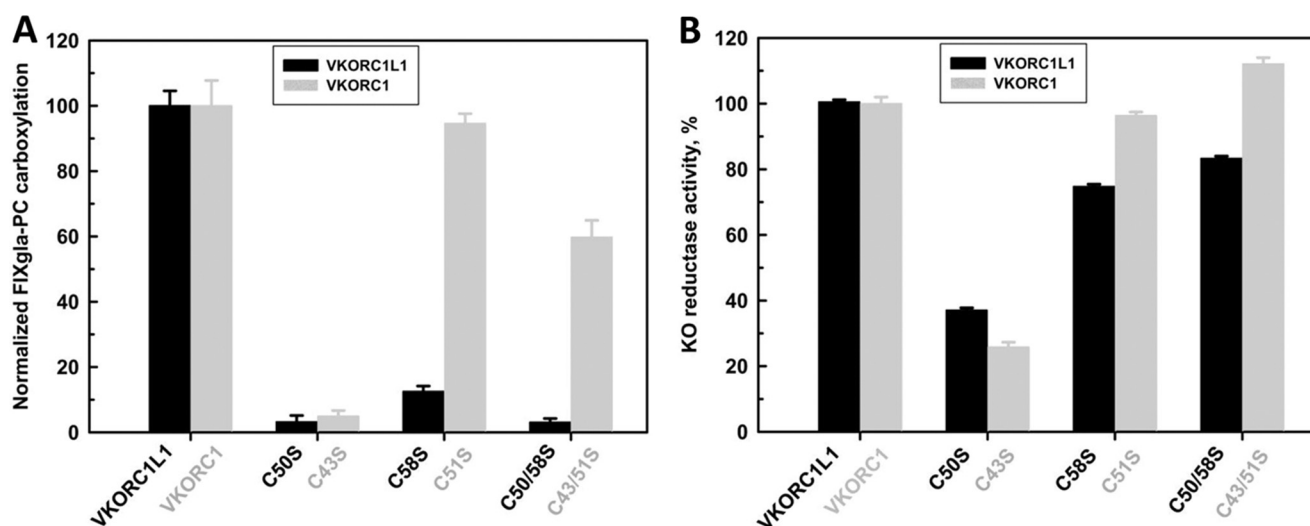
FIGURE 3. *In vivo (**A**)* **and** *in vitro (**B**)* **activity assays of the loop cysteine mutants of VKORC1 and VKORC1L1.** *A*, cell-based *in vivo* KO reductase activity assay. Loop cysteine mutants of VKORC1L1 (*black bars*) or VKORC1 (*gray bars*) were transiently expressed in FIXgla-PC/HEK293 cells, and the enzymatic activity was determined as described in the legend for Fig. 2. *B*, DTT-driven *in vitro* KO reductase activity assay. VKORC1L1 and its loop cysteine mutants were expressed in insect cells (Sf9). The enzymatic activity of converting KO to vitamin K of microsomes containing these mutants was measured by DTT-driven *in vitro* activity assay and normalized by the total microsomal protein concentration (*black bars*). The *gray bars* are the *in vitro* activities of VKORC1 and its loop cysteine mutants from our previous study (12). Data are presented as mean ± S.D. (*n* = 3).

vidually or together, abolished VKORC1L1 activity (Fig. 3*A*). This suggests that, unlike VKORC1, the conserved loop cysteines (Cys-50 and Cys-58) are essential for VKORC1L1 activity.

In bacterial VKORHs, the conserved loop cysteines are involved in active site regeneration by an intra-molecular electron transfer pathway. Mutating either of the loop cysteines diminished enzyme activity in an *E. coli* cell-based assay (25); however, the same cysteine mutants are active in an *in vitro* assay where the active site is directly reduced with DTT (26). Since VKORC1L1 and bacterial VKORHs have similar membrane topologies (25), one would expect that they might have a similar mechanism of function. To test this hypothesis, we expressed VKORC1L1 and its loop cysteine mutants in insect cells (Sf9). The enzymatic activity of reducing KO to vitamin K was measured by an *in vitro* assay using DTT as the reductant. Our results show that the VKORC1L1 C50S mutant has ~40% activity compared with the wild-type enzyme (Fig. 3*B*). Importantly, C58S and C50S/C58S mutants retain ~80% activity of the wild-type VKORC1L1. These results are similar to that of VKORH in bacteria (26), suggesting that the intra-molecular electron transfer pathway for active site regeneration by the loop cysteines applies to VKORC1L1.

Capture of the Intermediate Disulfide Bond between the Loop and the Active Site Cysteines of VKORC1L1—To further confirm the above conclusion and to identify the intra-molecular disulfide linkage between the loop and the active site cysteine, we used differential chemical modification of free cysteines and disulfide-linked cysteines in VKORC1L1. For detection purposes, we fused a HA tag to the C terminus of VKORC1L1 and its cysteine mutants. We transiently expressed HA-tagged VKORC1L1 or its variants in FIXgla-PC/HEK293 cells and cultured the cells with KO to facilitate the formation of the possible intermediate disulfide during KO reduction. Transfected cells were lysed and free cysteines were blocked by *N*-ethylma-

leimide (NEM) modification to trap the intermediate disulfide. Protein-disulfide bonds were then reduced by DTT and labeled by mPEG-MAL-5000. The procedure is illustrated in the scheme shown in Fig. 4*A*. Modification of each cysteine by mPEG-MAL-5000 is expected to increase the apparent molecular mass of the parent protein by ≥10 kDa (37), making it easily separated by SDS-PAGE and visualized by Western blot analysis.

Fig. 4*B* shows the results of differential modification of VKORC1L1 and its cysteine mutants. As the control, we reduced all the possible disulfide linkages in VKORC1L1 and labeled all the free cysteines directly by mPEG-MAL-5000 without NEM blocking. Our result shows that five PEG-labeled protein bands with higher molecular weight are observed, indicating that all five cysteines in VKORC1L1 can be modified by PEG (Fig. 4*B*, *lane 2*). The most populated forms of VKORC1L1 have five and one cysteines modified by PEG. It should be noted that except for the 5-PEG modified protein band, all other bands represent the number of cysteines modified by PEG; it does not necessarily mean modification of the same cysteines. When the free cysteines of VKORC1L1 are blocked by NEM before DTT reduction and PEG modification, the most dominant population of VKORC1L1 has two cysteines labeled by PEG (Fig. 4*B*, *lane 3*) indicating that two cysteines are protected from NEM blocking and can be reduced by DTT and labeled by PEG. This result suggests the existence of a disulfide bond within VKORC1L1. In addition, different from the control sample (Fig. 4*B*, *lane 2*), a non-PEG-labeled protein band was observed due to NEM blocking of the free cysteines. Unexpectedly, we also observed the 3- and 4-PEG modified protein bands. This result suggests that either there are more than one disulfide bonds in VKORC1L1 during KO reduction or NEM did not efficiently block all the free cysteines before PEG modification.
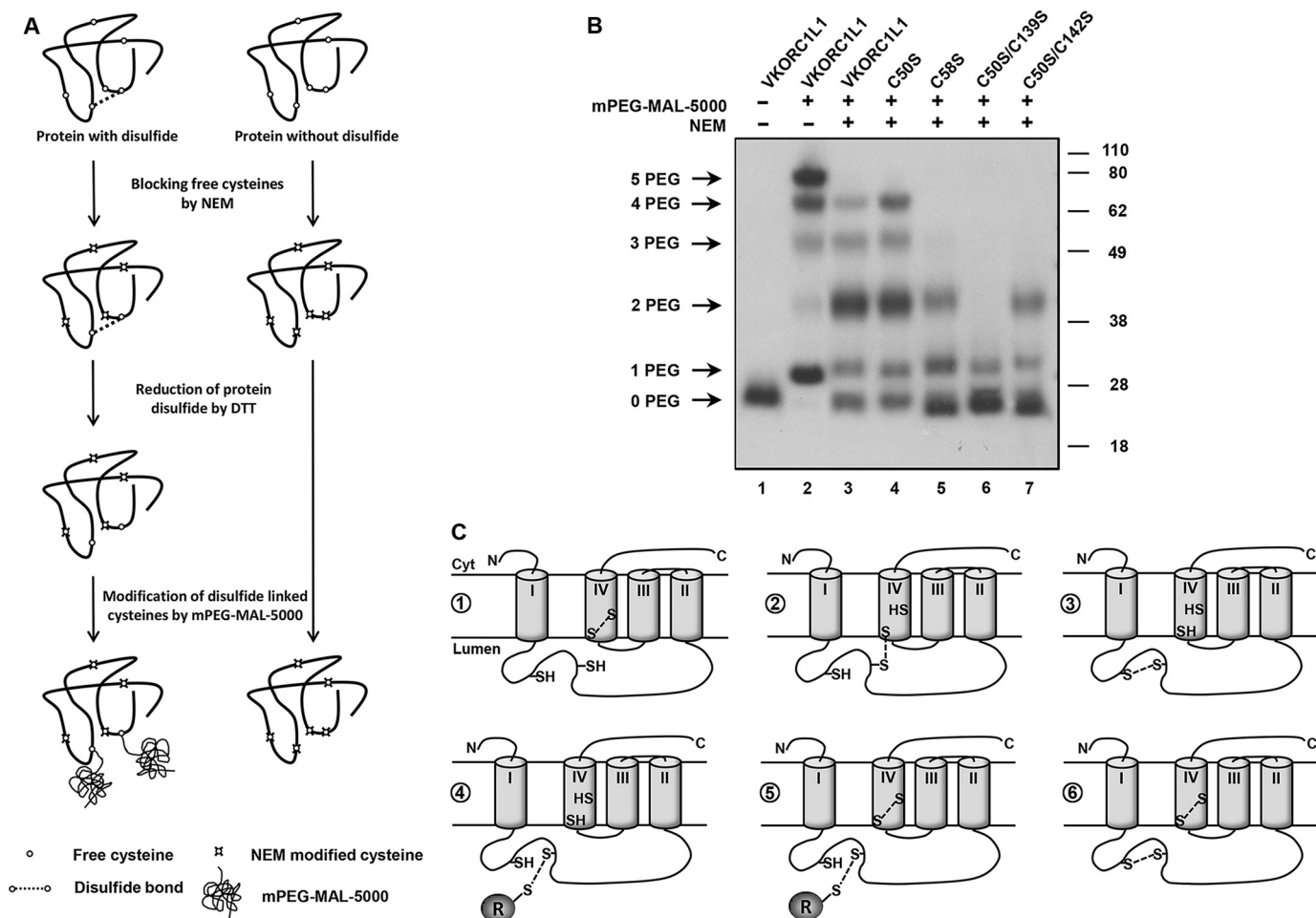
FIGURE 4. **Identification of the intermediate disulfide linkage between the loop cysteine and the active site cysteine in VKORC1L1.** *A*, scheme of differential modification of protein free cysteines and intra-molecular disulfide. Free cysteines in the protein are blocked by NEM modification. Intra-molecular disulfide is reduced by DTT and then labeled by mPEG-MAL-5000. Modification of each cysteine by PEG-MAL-5000 increases the apparent molecular weight of the parent protein by $\geq$10 kDa, which can be separated by SDS-PAGE. *B*, capture of the intermediate-disulfide of VKORC1L1. HA-tagged VKORC1L1 or its cysteine mutants was transiently expressed in FIXgla-PC/HEK293 cells. Forty-eight hours post-transfection, cells were lysed with Triton X-100 before NEM or mPEG-MAL modification. As the control, cell lysate that express wild-type VKORC1L1 was reduced by DTT and directly labeled with mPEG-MAL-5000 (*lane 1*). All other samples were first modified by NEM and excess NEM was removed by dialysis. Intra-molecular disulfide(s) was reduced by DTT and then labeled with mPEG-MAL-5000. *C*, scheme of the possible disulfide linkage in VKORC1L1. The disulfide bond is indicated by a *dashed line*. *Scheme 1*, disulfide-linked active site cysteines; *scheme 2*, intermediate disulfide between the loop cysteine and the active site cysteine; *scheme 3*, disulfide-linked loop cysteines with reduced active site; *scheme 4*, partially reduced loop disulfide with reduced active site (VKORC1L1's reductant is depicted as a *gray circle with the letter R*); *scheme 5*, partially reduced loop disulfide with oxidized active site; *scheme 6*, disulfide-linked loop cysteines and active site cysteines.

Fig. 4*C* elucidates the possible disulfide linkage in VKORC1L1 during KO reduction. When KO is reduced to vitamin K, the active site cysteines are oxidized to a disulfide (Fig. 4*C, scheme 1*). Partial reduction of the active site disulfide by a loop cysteine results in an intermediate disulfide between the loop and the active site cysteines (Fig. 4*C, scheme 2*). Full reduction of the active site results in the oxidation of the loop cysteines to a disulfide (Fig. 4*C, scheme 3*). When the loop disulfide is partially reduced by the reductant of VKORC1L1, it forms an intermediate disulfide between the loop cysteine and the cysteine of the reductant (Fig. 4*C,* scheme 4). The active site cysteines of VKORC1L1 can form a disulfide by KO reduction before the loop disulfide is fully reduced (Fig. 4*C, scheme 5*). If the rate of reduction for the loop disulfide is significantly slower than that of oxidation of the active site cysteines, both the loop and active site cysteines may be disulfide linked (Fig. 4*C, scheme 6*). Of all these possible disulfide linkages, most of them contain one intra-molecular disulfide. Differential modification of any

of these intermediates would result in a 2-PEG modification of VKORC1L1, which agrees with the most intense 2-PEG-labeled protein band (Fig. 4*B, lane 3*). In the case in which VKORC1L1's active site cysteines are oxidized and its loop cysteines are partially reduced (Fig. 4*C, scheme 5*), three cysteines would be protected from NEM blocking. This species may be represented by the 3-PEG-labeled protein band observed in Fig. 4*B, lane 3*. However, the source of VKORC1L1 with 4-PEG modification is not clear (Fig. 4*B, lane 3*). It seems unlikely that this is due to the PEG labeling of four cysteines protected by two disulfide bonds (Fig. 4*C, scheme 6*). If this were the case, VKORC1L1 would not be an efficient enzyme for KO reduction due to the slow rate of active site regeneration which disagrees with the result of the activity assay (Fig. 2*C*). In addition, it also seems unlikely that the 4-PEG-labeled protein band is due to the inefficient blocking of free cysteine by NEM, as we observed a similar result when we increased NEM concentration to 100 mM in the blocking reaction (data not shown). Moreover, if a cysteine residue is

not accessible to NEM modification at a higher concentration (50 mM), theoretically, it should not be accessible to a maleimide derivative with a bulky side chain (PEG) at a low concentration (20 mM).

To identify the disulfide linkage between the loop and the active site cysteines, we first mutated the loop cysteines, Cys-50 and Cys-58, individually to serine and repeated the above differential modification experiment. PEG modification of VKORC1L1 C50S mutant shows a pattern similar to that of the wild-type enzyme (Fig. 4*B*, *lane 4*), suggesting that mutating Cys-50 does not affect the number of cysteines protected from NEM blocking by disulfide bond. However, mutating Cys-58 abolishes PEG modification of two cysteines in VKORC1L1, as evidenced by the disappearance of two PEG-labeled protein bands (Fig. 4*B*, *lane 5*). Moreover, compared with the wild-type enzyme (Fig. 4*B*, *lane 3*), the C58S mutation significantly decreased the population of the 2-PEG-labeled protein band with a dramatic increase in the non-PEG-labeled protein band due to NEM blocking of free cysteines. This result suggests that mutating Cys-58 decreased the amount of disulfide formation and increased the free cysteines in VKORC1L1. The 2-PEG-labeled protein band in the C58S mutant could result from PEG modification of the disulfide-linked active site cysteines (Fig. 4*C*, *scheme 1*). These results together suggest that Cys-58 is the catalytic cysteine that forms a disulfide with the active site cysteine, and that Cys-50 serves as the canonical resolving cysteine that reduces the intermediate disulfide to release the active site cysteines as the fully reduced form (42).

To further identify the active site cysteine that forms disulfide with Cys-58, we mutated Cys-139 and Cys-142 individually and repeated the NEM and mPEG-MAL-5000 differential modification experiment. To stabilize the intermediate disulfide bond between the loop and the active site cysteines (Fig. 4*C*, *scheme 2*), we also mutated the resolving cysteine, Cys-50, to serine in the C139S and C142S VKORC1L1 mutants. Therefore, the only possible disulfide linkage of the electron transfer chain in these two mutants is either between the loop (Cys-58) and the active site cysteines (Fig. 4*C*, *scheme 2*) or the loop and VKORC1L1's reductant cysteines (Fig. 4*C*, *scheme 4*), with a maximum number of two cysteines being protected by NEM and labeled by PEG. Our result shows that mutating Cys-139 (C50S/C139S) abolishes three PEG-labeled protein bands (Fig. 4*B*, *lane 6*) compared with the C50S mutant (Fig. 4*B*, *lane 4*). In addition, the intensity of the non-PEG-labeled protein band increased significantly. This result suggests that Cys-139 is involved in disulfide formation with the loop cysteine, Cys-58. This result is further supported by the result of differential modification of the C50S/C142S mutant where two cysteines can be labeled by PEG (Fig. 4*B*, *lane 7*), and the only possible disulfide linkage that protects two cysteines from NEM blocking in C50S/C142S mutant is the disulfide between Cys-58 and Cys-139 (Fig. 4*C*, *scheme 2*).

The Overall Structure Rather Than the Specific Loop Sequence Distinguishes the Mechanism of Action between VKORC1 and VKORC1L1—VKORC1L1 and VKORC1 share ~50% amino acid sequence identity, but results from this study show that these two proteins have different membrane topologies and reaction mechanisms. VKORC1L1 requires the con-

served loop cysteines to reduce the active site disulfide by an intra-molecular electron transfer pathway, which is not the case for VKORC1. To examine whether the specific loop sequence or the active site sequence of VKORC1L1 are unique for the intra-molecule electron transfer, we first replaced the active site sequence of VKORC1L1 with that of VKORC1 (*boxed residues* in Fig. 5*A*). The resulting chimeric protein was designated VKORC1L1′ (Fig. 5*B*). *In vivo* cell-based activity assays show that the chimeric protein of VKORC1L1′ that contains VKORC1's active site is fully active (~1.6-fold more active than wild-type VKORC1L1) (Fig. 5*C*). To explore whether VKORC1's active site in the chimeric protein VKORC1L1′ is regenerated by the loop cysteines of VKORC1L1 via the intra-molecular electron pathway or directly reduced by an unknown physiological reductant as in VKORC1 (15, 41), we mutated the loop cysteines of VKORC1L1′ and examined their effect on the enzymatic activity. Fig. 5*D* shows that mutating either or both of the loop cysteines abolished the enzymatic activity of VKORC1L1′. These results suggest that the loop cysteines of VKORC1L1 are required for the regeneration of VKORC1's active site in the chimeric protein.

Next, we examined whether the specific loop sequence contributes to the different reaction mechanisms between VKORC1L1 and VKORC1. Fig. 6*A* shows that amino acid residues around the second loop cysteine are substantially different between VKORC1 and VKORC1L1. We therefore replaced the loop region of VKORC1L1 with that of VKORC1 (*boxed residues* in Fig. 6*A*). The resulting chimeric protein was designated VKORC1L1″ (Fig. 6*B*). The results of our cell-based *in vivo* assay show that the activity of VKORC1L1″ is similar to the wild-type enzyme (Fig. 6*C*). Mutating the loop cysteines of this chimeric VKORC1L1″ results in the loss of enzymatic activity (Fig. 6*D*), suggesting that the loop cysteines of VKORC1 are essential for the regeneration of VKORC1L1's active site in the chimeric protein. Importantly, these loop cysteines are not required for VKORC1's active site regeneration (15). These results together suggest that the overall structure of VKORC1L1, rather than specific sequences within the loop region or the active site region, is responsible for the different reaction mechanisms of VKORC1L1 and VKORC1.

DISCUSSION

Characterization of the human VKORC1 and its bacterial homologues yielded important structure-function information about this family of enzymes (15, 24, 26). Unlike its bacterial homologues, human VKORC1 is a three-TMD membrane protein, with its N terminus located in the ER lumen and its C terminus in the cytoplasm (15). The biological function of VKORC1 is to reduce KO to vitamin K for vitamin K-dependent carboxylation, and its enzymatic activity is sensitive to warfarin inhibition (33, 35). VKORC1L1, a paralogous enzyme of VKORC1, shares ~50% protein sequence identity with VKORC1 (1). However, the physiological function and the structure of VKORC1L1 are not yet clear.

The results presented in this study (Fig. 2*C*), which agree with our previous results (33), indicate that VKORC1L1, like VKORC1, can efficiently reduce KO, as evidenced by its ability to support vitamin K-dependent carboxylation in HEK293
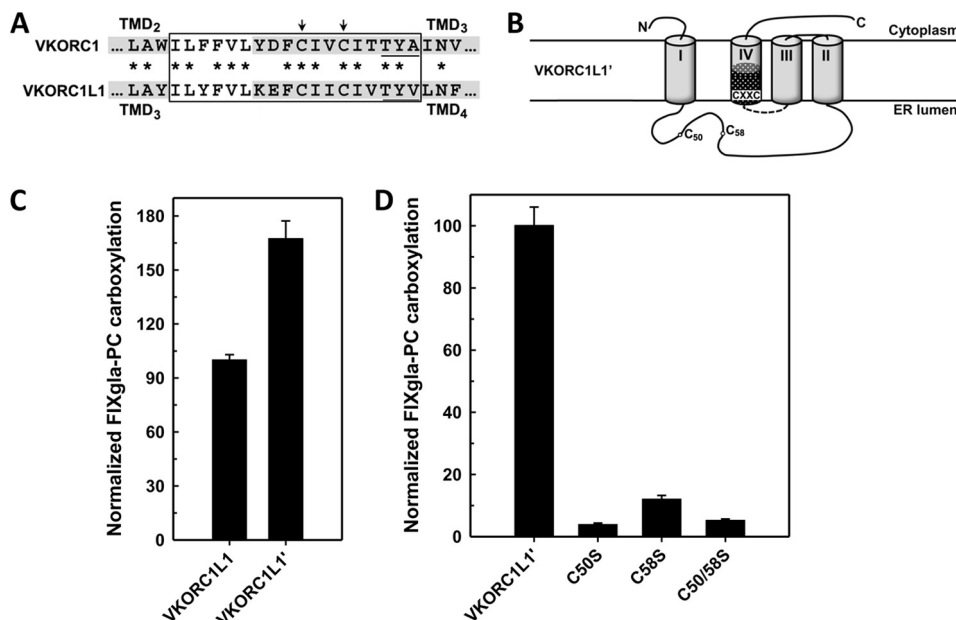
FIGURE 5. **VKORC1's activity site can be reduced by the loop cysteines of VKORC1L1.** *A*, sequence alignment of VKORC1 and VKORC1L1 near the active site region. Completely conserved residues are indicated by *asterisks*. Conserved active site cysteines are indicated by *arrows*. The warfarin binding motif (TYA/TYV) is *underlined*. Transmembrane regions are highlighted in *gray*. Active site sequence replacement is indicated by a *box*. *B*, scheme of VKORC1L1 molecule with its active site region replaced by that of VKORC1 (VKORC1L1'). *Dashed lines* and the *black cylinder* with *white dots* indicate sequences from VKORC1. *C*, cell-based *in vivo* activity assay of VKORC1L1 and VKORC1L1'. *D*, cell-based *in vivo* activity assay of loop cysteine mutants of VKORC1L1'. Enzymatic activity was determined as described in the legend for Fig. 2. Data are presented as mean ± S.D. (*n* = 3).
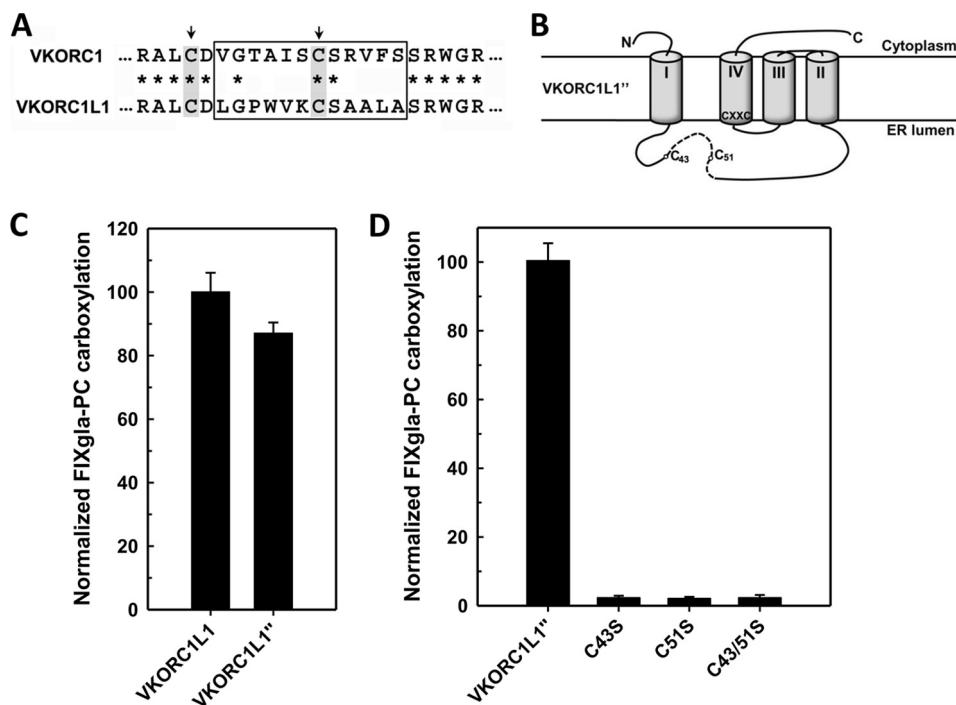

FIGURE 6. **VKORC1's loop cysteines can transfer electrons to the active site of VKORC1L1.** *A*, sequence alignment of VKORC1 and VKORC1L1 near the conserved loop cysteine region. Completely conserved residues are indicated by *asterisks*. Conserved loop cysteines are highlighted in *gray* and indicated by *arrows*. Loop sequence replacement is indicated by a *box*. *B*, scheme of VKORC1L1 molecule with its conserved cysteine containing loop region replaced by that of VKORC1 (VKORC1L1"). *Dashed lines* indicate sequences from VKORC1. *C*, cell-based *in vivo* activity assay of VKORC1L1 and VKORC1L1". *D*, cell-based *in vivo* activity of loop cysteine mutants of VKORC1L1". Enzymatic activity was determined as described in the legend for Fig. 2. Data are presented as mean ± S.D. (*n* = 3).

cells. This contrasts with the results of Westhofen *et al.*, which indicated that VKORC1L1 reduces KO 18,054-fold slower than does VKORC1 (31). Based on their results, these authors hypothesized that the primary physiological function of VKORC1L1 is to reduce vitamin K to KH_2. It should be noted,

however, that the conclusion of Westhofen *et al.* is based on the comparison of the enzymatic activity of VKORC1L1 in crude cell lysate in the presence of detergent (31) to that of purified VKORC1 reconstituted in intact membranes (43). Additionally, without knowing the expression level of VKORC1L1, they

calculated their specific enzymatic activity of VKORC1L1 based upon total proteins in the whole-cell lysates (31). Inexplicably, they compared this activity with the specific activity of purified VKORC1 calculated from a published turnover number (43). On the other hand, results from the same study show that under the same conditions, VKORC1L1 and VKORC1 have a similar ability to reduce KO to vitamin K (31). Therefore, it is premature to conclude that the primary physiological function of VKORC1L1 is to reduce vitamin K to KH_2.

Recently, Hammed *et al.* reported that VKORC1L1 might rescue VKORC1 function in extrahepatic tissues during anticoagulation therapy (30). This hypothesis is based on their *in vitro* kinetic results, which suggest that VKORC1L1 is 50-fold more resistant to warfarin inhibition than VKORC1. Our *in vivo* results, however, show that VKORC1L1 and VKORC1 have similar warfarin resistance (33). The experiments of Hammed *et al.* were carefully done; however, there are at least two possibilities to explain the discrepancy. First, Hammed *et al.* used DTT-driven *in vitro* activity assay in their study, while we used our cell-based *in vivo* activity assay, in which the enzymes function in their native milieu. It has been reported that the DTT-driven *in vitro* assay is a misleading for the study of warfarin resistance (44). In addition, DTT directly reduces the active site disulfide in the *in vitro* assay, which bypasses the function of the loop cysteines; as shown in the present study, these cysteines are essential for the function of VKORC1L1 but not for that of VKORC1. Therefore, until all of the components required for VKORC1's and VKORC1L1's functions are known and can be reassembled into a stable functional membrane complex *in vitro*, it is problematic to infer too much functional significance from *in vitro* studies. Secondly, Hammed *et al.* expressed VKORC1L1 and VKORC1 in yeast (*Pichia pastoris*), while we expressed these enzymes in mammalian cells (HEK293). It has been reported that VKORC1L1 and VKORC1 expressed in HEK293 cells only exhibit a 1.8-fold difference in warfarin sensitivity (31) even in the *in vitro* activity assay, which agrees with our previous result (33). Thus, it seems unlikely that VKORC1L1 functions as the "antidotal" enzyme that allows carboxylation of proteins in the presence of warfarin in the native milieu. Therefore, the physiological function of VKORC1L1 remains unknown.

Our topology study shows that, unlike VKORC1, VKORC1L1 is a 4-TMD membrane protein with both termini located in the cytoplasm. The main topological difference between VKORC1L1 and VKORC1 is the orientation of the first TMD in the ER membrane. VKORC1's TMD1 has the orientation of N_{out}-C_{in}, while VKORC1L1's is N_{in}-C_{out}. The orientation of TMD is primarily determined by the charged residues flanking the hydrophobic core, the "positive-inside rule" (45, 46); that is, positively charged residues are more abundant on the cytoplasmic side (inside) than the lumenal/periplasmic side (outside) of the membrane. According to the rules laid out by Hartmann *et al.* (46), the net charge difference between the C- and N-terminal flanking the TMD1 of VKORC1 is $+1.5$. By contrast, the net charge difference of VKORC1L1's TMD1 is -3.0. The two proteins thus have a very different distribution of charged residues flanking TMD1; according to the "positive inside rule", the TMD1 of VKORC1 and that of VKORC1L1 should thus have different orientations. This agrees with the results from our experimentally-determined topology models.

Another topological difference between VKORC1L1 and VKORC1 is the location of the conserved loop cysteines in relation to the ER membrane. In VKORC1L1, the conserved loop cysteines (Cys-50 and Cys-58) are located in the ER lumen, while those of VKORC1 are located in the cytoplasm. We have shown previously, by both *in vitro* and *in vivo* studies, that VKORC1's loop cysteines, whether located in the cytoplasm or ER lumen, are not required for its function (12, 15, 41). Here, however, we find that VKORC1L1's loop cysteines are essential for its activity (Fig. 3). Results from replacement of VKORC1L1's active site sequence or loop sequence with that of VKORC1 (Figs. 5 and 6) suggest that it is VKORC1L1's overall structure that uniquely allows for active site regeneration by the loop cysteines.

Our results from intermediate disulfide-trapping experiments suggest the following intra-molecular electron transfer pathway for VKORC1L1's active site regeneration. The second loop cysteine (Cys-58) carries out a nucleophilic attack on the active-site disulfide bond; this leads to the reduction of Cys-142 and the formation of an intermediate disulfide bond between Cys-58 and Cys-139. Then, the first loop cysteine (Cys-50) serves as the canonical resolving cysteine that attacks the intermediate disulfide (42), resulting in the reduction of active-site cysteines and the oxidization of the loop cysteines (disulfide-bonded). These reactions transfer two electrons from the loop cysteines to the active site. The oxidized loop cysteines will then need to be reduced by an as-yet-unidentified reductant for the enzyme to be recycled. This electron flow in VKORC1L1, but not in VKORC1, is similar to that of DsbB and the bacterial VKORH (47).

Our studies suggest that the structure and the active site regeneration mechanism of human VKORC1L1 are similar to that of bacterial VKORHs but different from that of human VKORC1. This suggests that VKORC1L1 evolved earlier than VKORC1. Whether VKORC1 in the lower species functions as KO reductase or is involved in disulfide-bond formation is still an open question. However, it is clear that VKORC1L1 in higher species lost its function as KO reductase under physiological conditions (33, 34).

In conclusion, we studied the membrane topology and the function of VKORC1L1 in mammalian cells. Our results suggest that, unlike VKORC1, VKORC1L1 is a four-TMD protein with its conserved loop cysteines located in the ER lumen. Cell-based *in vivo* activity assay shows that VKORC1L1 can support vitamin K-dependent carboxylation as efficiently as VKORC1. The conserved loop cysteines of VKORC1L1, but not VKORC1, are involved in active site regeneration through an intra-molecular pathway. The different structures and reaction mechanisms of VKORC1L1 and VKORC1 may imply that these two enzymes have different physiological functions.

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