SUPPLEMENTAL DATA

METHODS

Construction of mutants in human VKORC1. VKORC1-Y139F and –Y139H with or without a FLAG epitope were constructed by overlap PCR. The FLAG-tagged versions encode a AAAGGSGGSGGSGGSDYKDDDDK peptide appended to the C-terminus. Y139F$_{flag}$ was generated by performing PCR with Expand Polymerase (Sigma-Aldrich) using pZEM228-VKORC1-linker-FLAG[1] as template and the primers A and B, or C and D (Table S1) to produce 341 bp and 164 bp fragments, respectively. Products were purified and combined in a PCR reaction for 10 cycles, followed by the addition of primers A and D and 25 additional cycles of PCR. The 586 bp fragment was purified, treated with Taq DNA polymerase and dATP and cloned into pCR2.1-Topo (Thermo Fisher Scientific). A BglII fragment with the correct sequence was isolated and cloned into pZEM229[2] or pBacPAK8 (Clontech). The VKORC1-Y139H$_{flag}$ mutant was cloned using the same method as above except with A and E or F and D. Untagged mutants were similarly constructed using the same template but replacing primers A and D with primers G and H to generate VKORC1-Y139F and VKORC1-Y139H, respectively. The PCR products produced by overlap PCR were purified and cloned into pCR2.1-TOPO for sequencing, and BglII fragments containing cDNAs with the correct sequence were cloned into pZEM229 or pBacPAK8 for expression in mammalian and insect cells, respectively.

Activity assays on VKORC1 mutants. Baculoviruses containing tagged or untagged Y139H and Y139F were generated as before[3] for expression in SF21 insect cells. Multiple clonal isolates were analyzed in a Western using anti-VKORC1 antibody and in an activity assay that measured KO to K reduction[1]. A representative isolate was then chosen for subsequent study.

KO to K reduction was determined in microsomes from SF21 cells infected with baculoviruses containing wild type or mutant VKORC1s. Both the microsomal preparation, which includes a washing step that removed peripheral membrane proteins, and activity assay have previously been described[3]. Full KO reduction to KH_2 was also measured, using a method that performs the entire procedure under oxygen-free conditions to prevent KH_2 oxidation[1]. Microsomes (200 μl, 4 mg/ml in 50 mM sodium phosphate pH 7.9, 25 mM KCl, 20% glycerol) were incubated with KO (65 μM) and thioredoxin reductant (25 μM thioredoxin, 12.5 μg/ml thioredoxin reductase and 1 mM NADPH, all from Sigma) for 1 hr. Reactions were quenched by the addition of 500 μl of a 1:1 mixture of ethanol and hexane, and a synthetic vitamin K (K25, GL Synthesis) was added to monitor subsequent vitamin K recovery. The samples were vortexed, centrifuged (750xg, 5 min) and the organic phase was evaporated to dryness. Vitamin K forms were separated by HPLC, using continuous helium sparging to block KH_2 oxidation, and detection was by fluorescence as before[1]. Vitamin K standards (KO, K, KH_2 and K25) were included to quantitate the vitamin K forms. The same assay was used to analyze purified $Y139F_{flag}$, which was prepared as previously described[1].

VKORC1 variants were also assayed for the ability to support carboxylation that requires KH_2. Washed microsomes were prepared from SF21 cells coinfected with VKORC1 variants and $caboxylase_{flag}$ at mois of 2 and 5, respectively. The microsomes (200 µl, 0.8 mg/ml) were incubated in reaction cocktail containing 0.8 M ammonium sulfate, 50 mM BES pH 6.9, 2.5 mM Phe-Leu-Glu-Glu-Leu (FLEEL, Anaspec), 10 µM factor X propeptide (Anaspec), 1.3 mM [^{14}C] sodium bicarbonate (Perkin Elmer), 0.16% CHAPS, 0.16% phosphatidyl choline (Sigma) and thioredoxin reductant (as above). Reactions were initiated by the addition of KO or K (both at 65 µM), and after 1 hr were quenched with 10% trichloroacetic acid (1 ml). The samples were centrifuged and the supernatant boiled to remove unfixed [^{14}C]-CO_2, and incorporation of [^{14}C]-CO_2 into FLEEL was quantitated by scintillation counting.

The effect of warfarin on VKORC1-supported carboxylation versus VKORC1 reduction was compared using two different assays. The carboxylation assay was performed as above but using a range of warfarin concentrations (indicated in the Results). The reductase reaction was performed using the same reaction cocktail as in the carboxylation assay, except that unlabeled sodium carbonate replaced the radioactive form. The reaction was performed in sealed vials gassed with oxygen-free nitrogen to prevent carboxylase recycling of KH_2 to KO. After 1 hr, the reactions were quenched with 500 µl of a 1:1 mixture of ethanol and hexane, K25 (2 nmol) was added, and vitamin K isolation and HPLC were performed as described above.

The effect of warfarin on the K to KH_2 reaction was also tested, using the K concentrations that result from warfarin inhibition of KO to K reduction. Microsomes coinfected with the carboxylase and either wild type VKORC1 or the

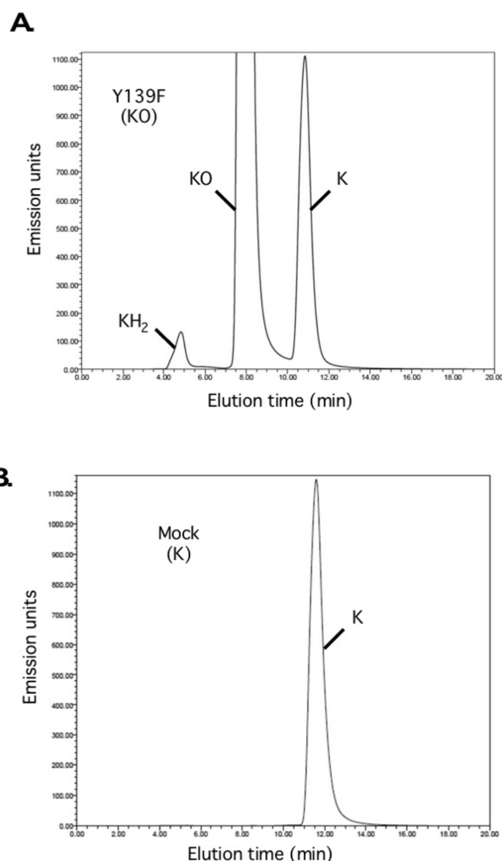
Y139F mutant were incubated with KO (65 μM) in the presence or absence of warfarin (1 μM (wild type VKORC1) or 10 μM (Y139F)) and then analyzed for K reduction as described above. Warfarin resulted in K concentrations of 0.2 μM and 0.3 μM for wild type VKORC1 and Y139F, respectively. The microsomes were incubated with 0.2 μM K and 1 μM warfarin (wild type VKORC1) or with 0.3 μM K and 10 μM warfarin (Y139F). Carboxylation was then monitored using a previously developed method that increases the sensitivity of Gla detection[4]. The reactions were terminated by dilution into water (2 ml) and immediate boiling to a volume of ~200 μl. Samples were precipitated with 800 μl acetone (4°C, 16 hr) and then centrifuged (18,000 x g, 4°C, 15 min). Supernatants were evaporated to 200 μl, and carboxylated peptide was purified by chromatography on a P-2 column (Biorad), followed by scintillation counting.

Supplemental References

1. Rishavy MA, Hallgren KW, Wilson LA, Usubalieva A, Runge KW, Berkner KL. The vitamin K oxidoreductase is a multimer that efficiently reduces vitamin K epoxide to hydroquinone to allow vitamin K-dependent protein carboxylation. *J Biol Chem*. 2013;288(44):31556-31566.
2. Hallgren KW, Qian W, Yakubenko AV, Runge KW, Berkner KL. r-VKORC1 expression in factor IX BHK cells increases the extent of factor IX carboxylation but is limited by saturation of another carboxylation component or by a shift in the rate-limiting step. *Biochemistry*. 2006;45(17):5587-5598.
3. Rishavy MA, Usubalieva A, Hallgren KW, Berkner KL. Novel insight into the mechanism of the vitamin K oxidoreductase (VKOR): electron relay through Cys43 and Cys51 reduces VKOR to allow vitamin K reduction and facilitation of vitamin K-dependent protein carboxylation. *J Biol Chem*. 2011;286(9):7267-7278.
4. Rishavy MA, Hallgren KW, Berkner KL. The vitamin K-dependent carboxylase generates gamma-carboxylated glutamates by using CO2 to facilitate glutamate deprotonation in a concerted mechanism that drives catalysis. *J Biol Chem*. 2011;286(52):44821-44832.
5. Grinnell BW, Walls JD, Gerlitz B. Glycosylation of human protein C affects its secretion, processing, functional activities, and activation by thrombin. *J Biol Chem*. 1991;266(15):9778-9785.

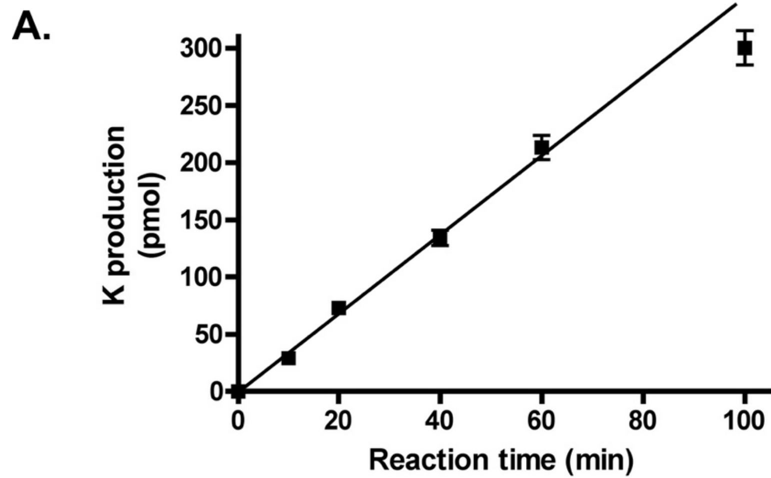
Table S1. Oligonucleotides used in this study.

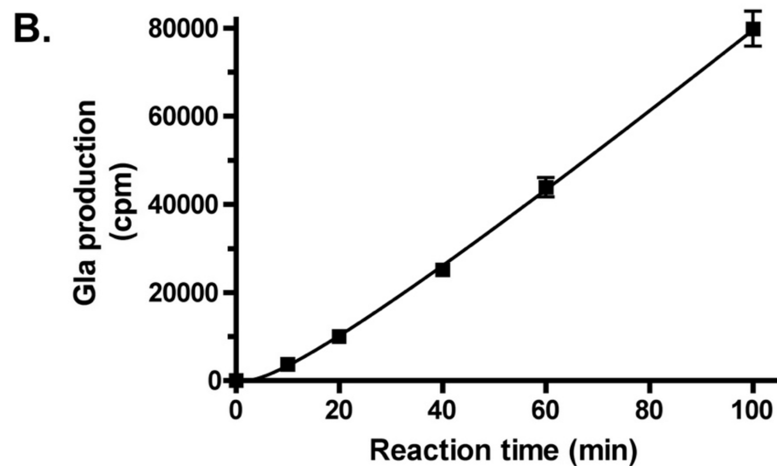
Name	sequence
A	CACCCAGATCTACCATGGGCAG
B	CGTTGATCGCGAAGGTGGTGATACAAACAATGCAG
C	CACCACCTTCGCGATCAACGTGAGCCTGATGTG
D	ATGTCTGAGATCTTCACTTGTC
E	CGTTGATAGCATGCGTGGTGATACAAACAATGCAG
F	CACCACGCATGCTATCAACGTGAGCCTGATG
G	CCCGGGAGATCTACCATGGGCAGCACC
H	AGCTCTAGATCTTCAGTGCCTCTTAGCC



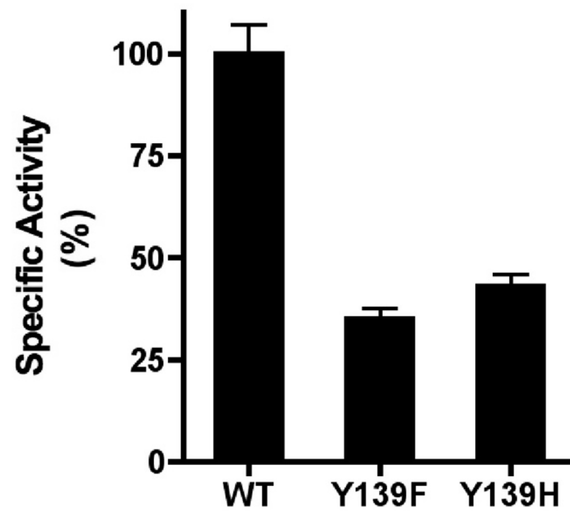
Supplemental Figure S1. The warfarin resistant Y139F VKORC1 mutant fully reduces KO to KH_2. A. Microsomes containing Y139F were assayed for conversion of KO to KH_2 by incubation with KO and the thioredoxin system, followed by vitamin K isolation and analysis by HPLC. All steps were performed under nitrogen to allow KH_2 detection. We previously showed that this thioredoxin system does not reduce either KO or K in these assays[3]. B. To assess the possibility that insect cells have a quinone reductase that might account for K to KH_2 reduction, mock microsomes

lacking Y139F were incubated with K. KH_2 production was not observed, indicating that Y139F was responsible for the full reduction of KO to KH_2 in part A.

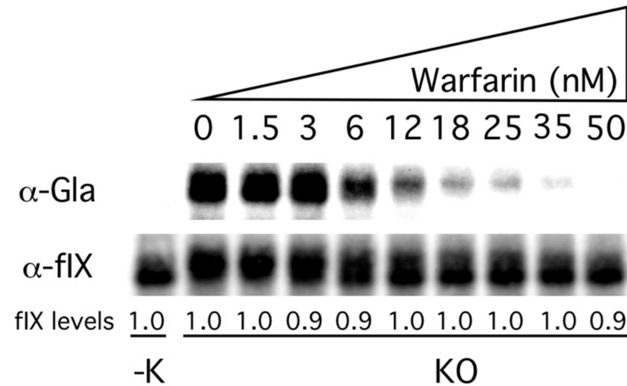




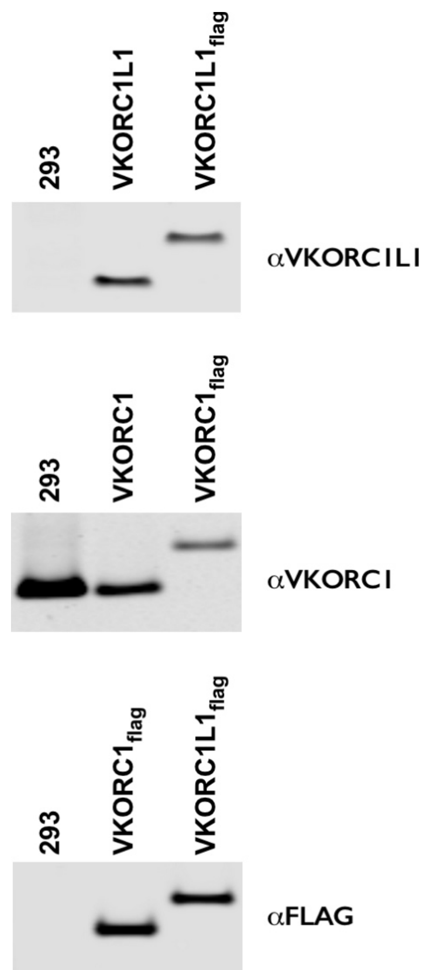
Supplemental Figure S2. The timepoints (60-90 min) used to analyze KO reduction and Gla production in these studies represent steady-state conditions. KO reduction (A) and Gla production (B) were monitored over time, using the reaction conditions described in the Supplemental Methods.



Supplemental Figure S3. VKORC1 mutants substituted in residue 139 have impaired activity. A. Microsomes from wild type (WT) or mutant $VKORC1_{flag}$ variants expressed in insect cells were assayed for KO reduction to K. $VKORC1_{flag}$ was quantitated using anti-FLAG antibody (0.4 μg/ml) and secondary antibody conjugated to IR dye 800 CW (0.2 μg/ml).



Supplemental Figure 4. An assay to monitor warfarin inhibition of carboxylation in cells. r-wt VKORC1/r-fIX BHK cells cultured with varying concentrations of warfarin were monitored for carboxylated fIX or total fIX, using Western analysis with anti-Gla or anti-fIX antibodies, respectively. Warfarin caused small differences in the molecular weight of fIX, consistent with previous studies showing that carboxylation impacts glycosylation[5]. Nonetheless, LiCor quantitation indicated that the fIX levels were similar at all warfarin concentrations, indicating that the decrease in anti-Gla signal was due to loss of fIX carboxylation.



Supplemental Figure S5. VKORC1 but not VKORC1L1 is detectable in 293 cells. Cell extracts from 293 cells were analyzed in Westerns with antibodies against VKORC1[1] and VKORC1L1 (Aviva), along with controls from insect cells expressing untagged or FLAG-tagged VKORC1 and VKORC1L1. The FLAG-tagged variants were quantitated in a Western with anti-FLAG antibody and a FLAG-BAP (Sigma) control (not shown). This quantitation and comparison to the untagged controls and 293 lysates showed that virtually all KO-supported activity in 293 cells is due to VKORC1.