Identification of VKORC1 interaction partners by split-ubiquitin system and coimmunoprecipitation

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Summary

Since the discovery of vitamin K epoxide reductase complex subunit 1 (VKORC1), the key enzyme for the regeneration of vitamin KH_2, numerous studies have addressed the role of VKORC1 in the posttranslational modification of vitamin K-dependent proteins. VKORC1 is also the target protein of anticoagulant drugs of the coumarin type (e.g. warfarin). Genetic variants in VKORC1 have recently been shown to significantly affect the coumarin dose and international normalised ratio level. In the present study, we have used the split-ubiquitin yeast two-hybrid system to identify potential interaction partners of VKORC1. With this system we could identify 90 candidates. Out of these, we focused on VKORC1 itself, its paralog VKORC1L1, emopamil binding protein (EBP) and stress-associated endoplasmic reticulum protein 1 (SERP1). By coimmunprecipitation and colocalisation experiments, we were able to demonstrate evidence for the interaction of these proteins. Mutations in the EBP gene cause X-linked dominant chondrodysplasia punctata (CDPX2) which can be considered as a phenocopy of warfarin embryopathy. The interaction could be a link between these phenotypes. SERP1 representing an oxidative stress-associated endoplasmatic reticulum protein with chaperon-like functions. Antioxidant capacities have been described for vitamin K hydroquinone, the substrate of VKORC1. Both VKORC1 and SERP1, might have a synergistic function in eliminating reactive oxygen species generated during the VKOR redox process. Further studies are needed to investigate the role of these proteins in the vitamin K pathway.

Keywords

VKOR, EBP, chondrodysplasia punctata, warfarin embryopathy, SERP1

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Introduction

Vitamin K epoxide reductase complex subunit 1 (VKORC1), the key enzyme of the vitamin K cycle was identified in 2004. The vitamin K cycle provides vitamin K hydroquinone ($Vit.K_1H_2$) as a cofactor for the γ-glutamyl-carboxylase (GGCX) which catalyses the posttranslational modification of vitamin K-dependent proteins (VKDP), including the blood coagulation factors II, VII, IX and X which require carboxylation to gain their full activity (1–4). Two other VKDPs, osteocalcin and matrix Gla protein, are key factors in bone metabolism (5). The VKOR reaction is the target for anticoagulant drugs of the coumarin type, e.g. warfarin, which inhibit blood clotting by repression of γ-carboxylation (6, 7). A wide spectrum of mutations scattered over the entire VKORC1 gene has been described to be causative for reduced warfarin response in patients (8) and warfarin-resistance in rodents (9). Mutations abolishing VKORC1 activity lead to a combined deficiency of vitamin K-dependent clotting factors (VKCFD2) with increased risk of bleeding (7, 10, 11). Homozygous VKORC1-deficient mice developed normally until birth but died due to extensive, predominantly intracerebral haemorrhage within 2–20 days after birth (12). Whereas polymorphisms in VKORC1 have no effect on vitamin K-dependent coagulation factor activity (13), allelic variation in the VKORC1 promotor region has recently been described as the most significant determinant of the dosage requirement of warfarin and other coumarins (14–16). Thus, VKORC1 has become a model for pharmacogenetic drug response.

The VKORC1 gene encodes a 18 kDa protein of the endoplasmic reticulum (ER) with extreme hydrophobic properties (7). Homology searches with VKORC1 detected a paralogous protein with 50% identity, assigned as VKORC1-like1 (VKORC1L1). The function of VKORC1L1, especially its involvement in the vitamin K cycle, is still unknown. Except for this paralogous gene, VKORC1 does not share significant sequence homology with any other known human gene or protein domain (7). However, orthologous genes have been identified in all phyla including archaebacteria (17) raising the question of the physiological role other than vitamin K reduction of these homologues. A thioredoxin-like CXXC motif is common to all VKORC1-homologuous genes, which together with two other cysteine pairs and a serine/threonine residue

is believed to be part of the active centre (17, 18). Topology prediction algorithms and the recently determined three-dimensional structure of a bacterial homologue of VKORC1 predict four transmembrane helices and a single luminal loop for the human enzyme (18) which could be confirmed by Schulman et al. (19).

Early functional studies of the vitamin K cycle have suggested a membrane-bound multi-enzyme complex to perform the reaction steps of complete vitamin K epoxide reduction (20, 21) but the components of this postulated complex could never be purified (22). Among other proteins, calumenin has been proposed to be a member of this complex (23). On the other hand, recombinant VKORC1 was found capable of catalysing both steps of vitamin K epoxide reduction to vitamin KH_2 and to be sensitive to warfarin *in vitro* (24). More recently, it was shown that reduction of vitamin K is linked to dithiol-dependent oxidative folding of proteins in the ER by protein disulfide isomerase (PDI). By immunoprecipitation and two-dimensional (2D)-SDS-PAGE of microsomal membrane proteins PDI and VKORC1 were demonstrated to form a tightly associated complex. It was proposed that PDI provides electrons for the reduction of the thioredoxin like CXXC center in VKORC1. Thus, the energy required for γ-carboxylation of vitamin K-dependent proteins was postulated to be provided by dithiol dependent oxidative protein folding in the ER and linked to *de novo* protein synthesis (25). This model is supported by the three-dimensional (3D)-structure of a bacterial VKOR (18). Schulman et al. showed recently, that VKORC1 interacts strongly with TMC, an ER membrane-anchored Trx-like protein and weaker with TMX4 and PDIA16, the smallest Trx-like protein of the ER. They could also prove, that human VKORC1 uses the same electron transfer pathway as its bacterial homolog (19).

The 3D-model also provided a basis for understanding the mechanism of some of the warfarin-resistance mutations found in patients and rodents (18). However, using an *in vitro* enzyme assay to recombinantly express wild-type and mutant VKORC1, the warfarin resistance observed *in vivo* could not be recapitulated for all mutations investigated (9, 26). Furthermore, the warfarin concentrations required for inhibition of the wild-type enzyme were three orders of magnitude higher than *in vivo*. It was speculated that this *in vitro* system may be lacking as yet unknown interaction partners of VKORC1.

We, therefore, applied the split-ubiquitin yeast two-hybrid system to identify potential interaction partners of VKORC1.

Materials and methods

Yeast two-hybrid analysis

The screening of possible interaction partners for VKORC1 was carried out by using the split-ubiquitin system of Dualsystems Biotech AG (Zürich, Switzerland) (27, 28).

The bait construct was made by subcloning full-length human VKORC1 cDNA (NM_024006) into the vector pBT-STE (Dualsystems Biotech AG) and transformed into the yeast strain NMY32 (MATa his3Δ200 trp1–901 leu2–3,112 (lexAop)8–ADE2 LYS2::(lexAop)4–HIS3 URA3::(lexAop)8–lacZ GAL4) using standard procedures. The bait construct was co-transformed together with a human adult liver NubG-X cDNA library (Clontech, Mountain View, CA, USA) into NMY32 cells grown on a selective medium.

Ninety positive clones yielded a HIS+/ADE+/lacZ+ phenotype and were identified by sequencing on an ABI 3130XL automatic sequencer (Applied Biosystems Inc, Foster City CA, USA) and homology searches using basic local alignment search tool (BLAST).

Like any genetic selection system, the yeast two- hybrid analysis produces a certain number of false positive clones. These clones result in a HIS+/ADE+/lacZ+ phenotype independent of a true interaction between the bait and a prey. In order to identify and eliminate false positive clones a few isolated prey plasmids were retransformed with the original bait plasmid (VKORC1). Only preys that yielded a HIS+/ADE+/lacZ+ phenotype when co-expressed with our bait but not with the control baits were considered as true positives and are analysed further.

Generation of plasmids

For expression in eukaryotic cell lines, polymerase chain reaction (PCR)-amplified human full-length cDNAs of VKORC1 (NM_024006.4), VKORC1L1 (NM_173517), EBP (NM_006579.2) and SERP1 (NM_014445.3) were cloned into the *HindIII* and *EcoRI* sites whereas PDIA6 (NM_005742.2) was cloned into the *HindIII* and *NotI* sites of the pcDNA3.1Myc/His-B vector (Invitrogen, Karlsruhe, Germany) which allows expression of Myc/his-tagged proteins. VKORC1 was also cloned into the *HindIII* and *EcoRI* site of pcDNA3.1 (Invitrogen, Karlsruhe, Germany). The EcoRI site fused the Flag-epitope (DYKDDDDK) in frame to the N-terminus of VKORC1, the sequence of the Flag tag was provided by the forward cloning primer (primer sequences on request). All plasmids with c-Myc tag and Flag tag were sequenced on both strands to eliminate clones with PCR errors.

Co-immunoprecipitation experiments (IPs)

HEK293 cells were transiently transfected with the indicated constructs using FuGeneHD™ (Roche, Basel, Switzerland). For double transfections, the transfection protocol was optimised to provide equal expressions of the proteins.

Cells were lysed as described by Wajih et al. (25). After lysis, cell debris was removed by centrifugation at 12,000 g for 30 minutes (min), and the supernatant was collected. The supernatant was precleared by absorption to Sepharose CL-6B beads (Sigma-Aldrich, Steinheim, Germany) by overhead rotation for 1 hour (h) at 4°C. The beads were removed by centrifugation, and the supernatant was incubated either with Anti-C-Myc Agarose Affinity Gel (Sigma-Aldrich) or with Flag M2 Agarose Affinity Gel (Sigma-Al-

drich) and rotated overhead for 2 h at 4 °C. The beads were washed eight times in ice cold phosphate-buffered saline (PBS).

For gel analysis, 4 x NuPAGE LDS Sample buffer and 10 x NuPAGE Reducing Agent (Invitrogen) was added to the beads and IP eluates were heated for 10 min at 70°C. IP eluates were subjected to 4–12% gradient SDS-PAGE (Invitrogen) and analysed by Western blotting. The blots were developed with rabbit polyclonal Anti-C-Myc antibody (Sigma-Aldrich) or mouse monoclonal Anti-Flag M2 antibody (Sigma-Aldrich). Anti-mouse and anti-rabbit secondary antibodies conjugated to horseradish peroxidase were purchased from Dianova (Hamburg, Germany). Detection was accomplished with ECL Plus Western Blotting Detection Reagents from GE healthcare (Freiburg, Germany).

Co-localisation experiments

HeLa cells were co-transfected with Flag-tagged VKORC1 and one of the Myc-tagged interaction proteins, grown on coverslips and after 36 h fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS). Then, cells were washed with PBS followed by permeabilisation in 0.1% Triton X-100 in PBS for 10 min and incubation in 5% bovine serum albumin (BSA) in PBS (blocking solution) for 20 min. The cells were incubated for 1 h at room temperature with rabbit polyclonal anti-c-Myc or mouse monoclonal anti-Flag M2 primary antibodies (Sigma-Aldrich). After three washing steps with PBS and 10 min incubation with blocking solution, cells were incubated with anti-mouse and anti-rabbit secondary antibodies (Dianova) which were conjugated with horseradish peroxidase and fluorescence labelled with Alexa Fluor® 488 carboxylic acid, succinimidyl ester or Alexa Fluor® 594 carboxylic acid, succinimidyl ester (Invitrogen) for 1 h at room temperature. After several washing steps and 5 min incubation with Hoechst 33342 nucleic acid stain (Invitrogen), coverslips were mounted onto glass slides in 10% (w/v) Moviol 4–88 (Carl Roth, Karlsruhe, Germany) in PBS. Imaging was performed using a Leica 6000 DMI microscope.

Results

Putative VKORC1 interaction partners

In this study, we used the split- ubiquitin yeast two-hybrid system (28) to identify potential interaction partners of VKORC1. Extensive sequence analyses of VKORC1 failed to reveal significant homologies to protein domains with a known structure and function. Therefore, the full-length human VKORC1 cDNA was chosen to screen a human adult liver cDNA library.

We found 191 clones with strong β-galactosidase activity as indicated by consistent blue colour intensities (data not shown). We characterised all clones by sequencing and BLAST homology searches. After elimination of multiple-hit clones, we finally identified 90 unique candidates as interactors of VKORC1 (see ▶Table 1). Interestingly, calumenin which was previously supposed to interact with VKOR (23) was not identified by this approach, neither other suggested candidates such as γ-glutamyl-carboxylase (29).

Next, we excluded all proteins known to be spurious false positive interactors in yeast-two hybrid assays (K. Rutschmann, Dualsystems Biotech, see ▶Table 1).

We ordered the remaining candidates by the number of positive hits (the three candidates where among the first four hits) and focused on proteins known or suspected to be localised in the ER membrane, which could be involved in redox processes or are known to deal with vitamin K and oxidative stress, respectively. Three candidate proteins, VKORC1 itself with the possibility to form homomeric complexes, the emopamil binding protein (EBP), a high affinity receptor of phenylalkylamine Ca^{2+} antagonists (30, 31) and the stress-associated endoplasmic reticulum protein 1 (SERP1) (32), showed β-galactosidase activity when co-expressed with the bait but not with the control baits and thus were considered to be bait-dependent positive interactors (▶Fig. 1). PDIA6, was the only member of the PDI family which had been suggested as potential redox partner of VKORC1 (20) which was positive in our screening. However, it was not confirmed in the bait dependency test (data not shown).

VKORC1 co-precipitates with itself and its paralog VKORC1L1

Because VKORC1 as a bait had identified itself as a prey in the split-ubiquitin-system we wanted to confirm the interaction by co-immunoprecipitation and co-localisation. Because of the high homology between VKORC1 and its paralog VKORC1L1, we were also interested whether VKORC1 may also interact with VKORC1L1. To study both homo- and heterocomplex formation of VKORC1 with itself or VKORC1L1, we performed co-immunoprecipitation studies in HEK 293 cells co-transfected with VKORC1 together with VKORC1 or VKORC1L1 constructs carrying different epitope tags (e.g. VKORC1-Flag together with VKORC1-Myc). The tagged proteins were still active in the VKOR enzyme assay of Rost et al. (7) (data not shown).

After cell lysis, lysates were immunoprecipitated with anti-Flag-tagged agarose. The immunoprecipitates were subjected to SDS-PAGE and Western blotting and analysed with anti-Myc IgG. These experiments showed that VKORC1 co-precipitates with itself (▶Fig. 2, lane 2) and its paralog VKORC1L1 (▶Fig. 2, lane 3).

In parallel assays, PDIA6 could not be co-immunoprecipitated together with VKORC1 (▶Fig. 2, lane 6).

As negative controls, Western blots of cells transfected with Myc-VKORC1, Myc-VKORC1L1 or Myc-PDIA6 co-expressed with an empty Flag-vector and precipitated by anti-Flag could not be stained by anti-Myc (▶Fig. 2, lanes 4, 5 and 7). Similarly, Flag-VKORC1 co-expressed with an empty Myc-vector could not be detected by anti-Myc after immunoprecipitation using anti-Flag (▶Fig. 2, lane 1), showing the specificity of the antibodies used for immunoprecipitation.

Table 1: VKORC1 split-ubiquitin hits. The table is listed in order of the number of hits. Bold hits were analysed further. The hits which are detected in many different screens are in italic (K. Rutschmann, Dualsystem Biotech).

Number of hits	Definition	Accession
19	Homo sapiens CD63 molecule (CD63)	NM_001040034.1
17	Homo sapiens emopamil binding protein (sterol isomerase) (EBP)	NM_006579.1
16	Homo sapiens stress-associated endoplasmic reticulum protein 1 (SERP1)	NM_014445.3
8	Homo sapiens vitamin K epoxide reductase complex, subunit 1 (VKORC1), transcript variant 1	NM_024006.4
6	Homo sapiens proteolipid protein 2 (colonic epithelium-enriched) (PLP2)	NM_002668.1
6	Homo sapiens VAMP (vesicle-associated membrane protein)-associated protein B and C (VAPB)	NM_004738.3
5	Homo sapiens transferrin (TF)	NM_001063.2
4	Homo sapiens mitochondrion, complete genome	NC_001807.4
4	Homo sapiens protein disulfide isomerase family A, member 6 (PDIA6)	NM_005742.2
4	Homo sapiens serum amyloid A1 (SAA1), transcript variant 2	NM_199161.2
3	Homo sapiens haptoglobin (HP)	NM_005143.2
3	Homo sapiens signal transducer and activator of transcription 6, interleukin-4 induced (STAT6)	NM_003153.3
3	Homo sapiens transmembrane protein 134 (TMEM134), transcript variant 1	NM_025124.2
2	Homo sapiens serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1 (SERPINA1), transcript variant 1	NM_000295.3
2	Homo sapiens surfeit 4 (SURF4)	NM_033161.2
2	Homo sapiens homer homolog 2 (Drosophila) (HOMER2), transcript variant 1	NM_004839.2
2	Homo sapiens asialoglycoprotein receptor 1 (ASGR1)	NM_001671.2
2	Homo sapiens chromosome 14 open reading frame 147 (C14orf147)	NM_138288.3
2	Homo sapiens fibrinogen-like 1 (FGL1),mRNA	NM_004467.3
2	Homo sapiens hepcidin antimicrobial peptide (HAMP)	NM_021175.2
2	Homo sapiens haptoglobin-related protein (HPR)	NM_020995.3
2	Homo sapiens asialoglycoprotein receptor 2 (ASGR2), transcript variant H2'	NM_080912.2
2	Homo sapiens ADP-ribosylation-like factor 6 interacting protein 5 (ARL6IP5)	NM_006407.3
2	Homo sapiens receptor accessory protein 5 (REEP5)	NM_005669.4
2	Homo sapiens calponin 3, acidic (CNN3)	NM_001839.2
2	Homo sapiens required for meiotic nuclear division 5 homolog A (S.cerevisiae) (RMND5A).	NM_022780.2
2	Homo sapiens dolichyl-phosphate mannosyltransferase polypeptide 2, regulatory subunit (DPM2)	NM_003863.2
2	Homo sapiens peptidylprolyl isomerase B (cyclophilin B) (PPIB)	NM_000942.4
2	Homo sapiens B-cell receptor-associated protein 31 (BCAP31)	NM_005745.6
1	Homo sapiens protein C receptor, endothelial (EPCR) (PROCR)	NM_006404.3
1	Homo sapiens gap junction protein, beta 1, 32kDa (GJB1), transcript variant	NM_001097642.1
1	Homo sapiens ATP synthase, H+ transporting, mitochondrial F0 complex, subunit C3	NM_001689.4
1	Homo sapiens ATPase, H+ transporting, lysosomal accessory protein 2 (ATP6AP2)	NM_005765.2
1	Homo sapiens spermidine/spermine N1-acetyltransferase 1 (SAT1)	NM_002970.1
1	Homo sapiens complement component 1, s subcomponent (C1S), transcript variant 2.	NM_201442.1
1	Homo sapiens CD9 molecule (CD9)	NM_001769.2
1	Homo sapiens apolipoprotein C-IV (APOC4)	NM_001646.1
1	Homo sapiens Yip1 interacting factor homolog A (S. cerevisiae) (YIF1A)	NM_020470.1
1	Homo sapiens transmembrane protein 176A (TMEM176A)	NM_018487.2
1	Homo sapiens ferritin, light polypeptide (FTL)	NM_000146.3
1	Homo sapiens apolipoprotein A-I (APOA1)	NM_000039.1
1	Homo sapiens fission 1 (mitochondrial outer membrane) homolog (S. cerevisiae)	NM_016068.2
1	Homo sapiens transmembrane protein 45B (TMEM45B)	NM_138788.3

Table 1 continued

Number of hits	Definition	Accession
1	Homo sapiens apolipoprotein A-II (APOA2)	NM_001643.1
1	Homo sapiens hydroxysteroid (17-beta) dehydrogenase 7 (HSD17B7)	NM_016371.2
1	Homo sapiens interferon induced transmembrane protein 3 (1–8U) (IFITM3)	NM_021034.2
1	Homo sapiens chromosome 19 genomic contig, alternate assembly (based on HuRef SCAF_110327	NM_001838476.1
1	Homo sapiens histone deacetylase 6 (HDAC6)	NM_006044.2
1	Homo sapiens alpha-2-HS-glycoprotein (AHSG)	NM_001622.2
1	Homo sapiens asialoglycoprotein receptor 2 (ASGR2), transcript variant 2	NM_080913.2
1	Homo sapiens transmembrane BAX inhibitor motif containing 4 (TMBIM4)	NM_016056.2
1	Homo sapiens jagunal homolog 1 (Drosophila) (JAGN1)	NM_032492.2
1	Homo sapiens pyrophosphatase (inorganic) 2 (PPA2), nuclear gene encoding mitochondrial protein, transcript variant 2.	NM_006903.4
1	Homo sapiens chromosome 20 open reading frame 30 (C20orf30), transcript variant 4	NM_001009925.1
1	Homo sapiens protein-L-isoaspartate (D-aspartate) O-methyltransferase (PCMT1)	NM_005389.1
1	Homo sapiens phosphatidylinositol glycan anchor biosynthesis, class H (PIGH)	NM_004569.3
1	Homo sapiens chromosome 12 genomic contig, alternate assembly (based on HuRef SCAF_1103	NW_001838059.1
1	Homo sapiens sterol-C4-methyl oxidase-like (SC4MOL), transcript variant 1	NM_006745.3
1	Homo sapiens chromosome 17 open reading frame 61 (C17orf61)	NM_152766.2
1	Homo sapiens chromosome 10 open reading frame 56 (C10orf56)	NM_153367.2
1	Homo sapiens chromosome 6 open reading frame 35 (C6orf35)	NM_018452.3
1	Homo sapiens chromosome 6 open reading frame 129 (C6orf129)	NM_138493.2
1	Homo sapiens methyltransferase like 7B (METTL7B)	NM_152637.1
1	Homo sapiens small nucleolar RNA, H/ACA box 12 (SNORA12) on chromosome 10	NR_002954.1
1	Homo sapiens enoyl Coenzyme A hydratase domain containing 1 (ECHDC1), transcript variant 1	NM_001002030.1
1	Homo sapiens translocation associated membrane protein 1 (TRAM1)	NM_014294.4
1	Homo sapiens selenoprotein X, 1 (SEPX1)	NM_016332.2
1	Homo sapiens three prime repair exonuclease 1 (TREX1)	NM_033629.2
1	Homo sapiens amyloid beta (A4) precursor-like protein 2 (APLP2)	NM_001642.1
1	Homo sapiens apolipoprotein C-I (APOC1)	NM_001645.3
1	Homo sapiens metallothionein 1X (MT1X)	NM_005952.2
1	PREDICTED: Homo sapiens similar to ZMYM6 protein (LOC100130633)	XM_001725169.1
1	Homo sapiens solute carrier family 31 (copper transporters), member 2 (SLC31A2)	NM_001860.2
1	Homo sapiens chromosome 20 open reading frame 30 (C20orf30), transcript variant 4, mRN	NM_001009925.1
1	Homo sapiens coxsackie virus and adenovirus receptor (CXADR)	NM_001338.3
1	Homo sapiens solute carrier family 15, member 3 (SLC15A3)	NM_016582.1
1	Homo sapiens fibrinogen gamma chain (FGG), transcript variant gamma-A	NM_000509.4
1	Homo sapiens potassium channel, subfamily K, member 17 (KCNK17)	NM_031460.3
1	Homo sapiens asialoglycoprotein receptor 1 (ASGR1)	NM_001671.2
1	Homo sapiens apolipoprotein A-II (APOA2)	NM_001643.1
1	Homo sapiens transmembrane protein 120A (TMEM120A)	NM_031925.1
1	Homo sapiens apolipoprotein C-III (APOC3)	NM_000040.1
1	Homo sapiens solute carrier family 25 (mitochondrial carrier; citrate transporter), member 1 (SLC25A1), nuclea	NM_005984.1
1	Homo sapiens CD81 molecule (CD81)	NM_004356.3
1	Homo sapiens coiled-coil domain containing 12 (CCDC12)	NM_144716.2
1	Homo sapiens fibrinogen beta chain (FGB)	NM_005141.2
1	Homo sapiens claudin 10 (CLDN10), transcript variant 2	NM_006984.3
1	Homo sapiens protocadherin 1 (PCDH1)	NM_032420.2
1	Homo sapiens catechol-O-methyltransferase (COMT), transcript variant MB-COMT	NM_000754.2
1	Homo sapiens family with sequence similarity 82, member B (FAM82B)	NM_016033.2

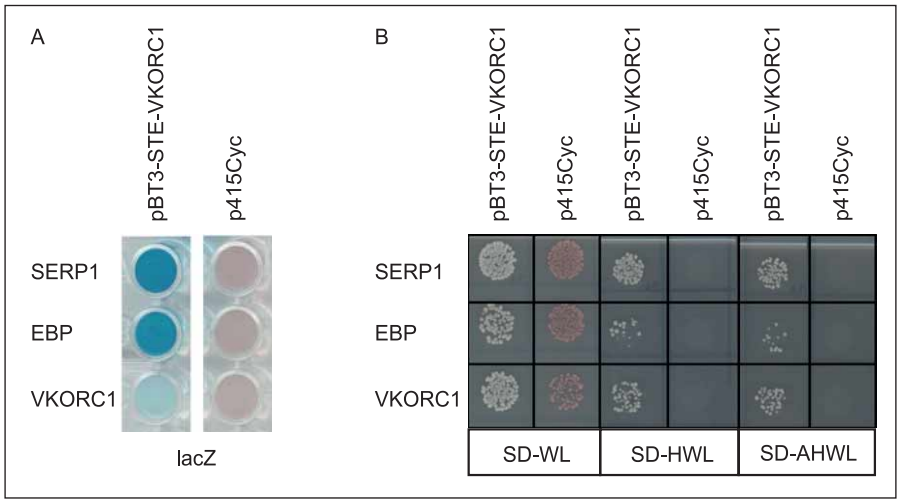

Figure 1: Bait-dependency test. Plasmids expressing putative interactors identified in the screening were retransformed into reporter strains along with plasmids expressing either the bait of interest or an unrelated bait (p415Cyc = neg. control). A) HTX β-galactosidase (lacZ) assay results of the mated yeast colonies and the p415Cyc control. B) Cells were spotted on media to select for transformation (SD-WL) and reporter gene activation (SD-HWL and SD-AHWL).

As positive controls, HEK 293 cells were transfected with either Myc-tagged or Flag-tagged proteins alone, immunoprecipitated and blotted with the same antibodies (anti-Myc or anti-Flag, respectively, data not shown).

Thus, co-immunoprecipitation experiments revealed the existence of complexes between the two VKOR paralogs and VKORC1 itself.

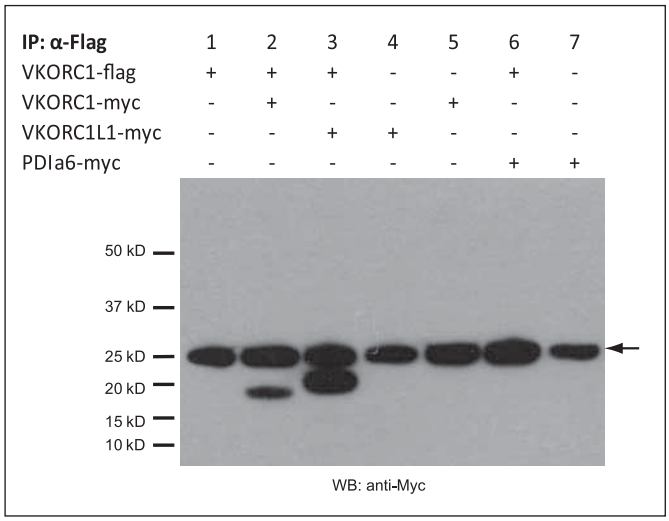

Figure 2: VKORC1 forms complexes with VKORC1 and VKORC1L1. Co-immunoprecipitation studies (IP) using HEK 293 cells transiently co-expressing Flag-VKORC1 and Myc-VKORC1, Myc-VKORC1L1 or Myc-PDIa6, respectively, as indicated above for each lane. IPs were performed with anti-Flag M2 agarose affinity gel (Sigma) and analysed by Western blot (WB) with anti-Myc antibody. All lanes were loaded with immunoprecipitates derived from the same amount of cell lysates (10-cm dish). VKORC1 (with tag) migrates at ~20.5 kDa and VKORC1L1 (with tag) migrates at ~22 kDa. Co-transfections of the individual Myc-tagged plasmids with empty Flag-vector and also the Flag-tagged VKORC1 co-expressed with empty Myc-vector were used as negative controls (lanes 1, 4, 5 and 7). Flag-tagged VKORC1 specifically interacted with Myc-VKORC1 (lane 2) and with its paralog VKORC1L1 (lane 3) while Myc-PDIa6 could not be detected in the assay (lane 6). IgG light chain bands at 25kDa (arrow) served as loading controls.

VKORC1 co-precipitates with EBP and SERP1

We further studied by co-immunoprecipitation the candidates EBP and SERP1 which had been identified by the split-ubiquitin-system as potential interaction partners of VKORC1. HEK 293 cells were transiently transfected with Flag-VKORC1 and co-expressed either with Myc-EBP or Myc-SERP1, and anti-Flag co-immunoprecipitation was performed (▶Fig. 3A). The interaction of VKORC1 with itself and VKORC1L1 was used as positive control (▶Fig. 3A, lanes 1 and 3), and Myc-VKORC1, Myc-VKORC1L1, Myc-EBP or Myc-SERP1 co-expressed with an empty Flag-vector served as negative controls (▶Fig. 3A, lanes 2, 4, 5 and 6). Western blot analyses showed that Flag-tagged VKORC1 co-precipitates with Myc-EBP and Myc-SERP1 (▶Fig. 3A, lanes 7 and 8). SERP1 migrates at ~9.7 kDa (with tag) and EPB migrates at 28,65 kDa, close to the IgG light chain band at 25 kDa which served as loading control (arrow).

Because of the poor resolution of the EBP and the IgG light chain bands and because an alternative Flag antibody raised in a different species had shown cross-reactivity with the antibody used for antigen detection, independent confirmation of the interaction was provided by co-immunoprecipitation using anti-Myc agarose (▶Fig. 3B). Therefore, we co-expressed Flag-VKORC1 with Myc-VKORC1 as positive controls (▶Fig. 3B, lane 1) and Flag-VKORC1 with either Myc-PDIa6, Myc-EBP or Myc-SERP1 (▶Fig. 3B, lanes 3, 5, 7) as well as all of the Myc-tagged proteins with an empty Flag-vector (▶Fig. 3B, lanes 2, 4, 6, 8) and Flag-VKORC1 with an empty Myc-vector (▶Fig. 3B, lane 9). Co-immunoprecipitation was performed using anti-Myc agarose. The N-tagged Flag-VKORC1 migrates at ~19.3 kDa well separated from the IgG heavy chain bands at 50 kDa (▶Fig. 3B).

Co-localisation of the putative complexes

To study whether human VKORC1L1 is also expressed in the ER membrane like VKORC1, we co-expressed the same constructs that were used for interaction screening in HeLa cells. Primary antibodies against the epitope tags and fluorochrome- labelled secondary antibodies were used to visualise the proteins. The green immunofluorescence of VKORC1L1 decorated the mesh-like structures of the ER in the cytoplasm and perfectly co-localised with the red-labelled VKORC1 (▶Fig. 4) (7). Thus, immunofluorescence analyses demonstrated co-localisation of Myc-VKORC1L1 and Flag-VKORC1 (▶Fig. 4) in the ER.

We further showed the co-localisation of VKORC1 with EBP or SERP1, which were already known to be localised in the ER (33, 34).

Discussion

Yeast-two-hybrid (Y2H) systems are widely used to identify protein-protein interactions *in vivo*. In classical Y2H systems, it is essential that both hybrid proteins are imported into the cellular nucleus (35). Thus, membrane proteins like VKORC1 are not suited for classical Y2H. The DUALmembrane system which is based on the split-ubiquitin-system is able to search for interaction partners especially of membrane proteins (28). Using this approach to identify potential interaction partners of VKORC1, we detected 90 singular clones by screening of a human adult liver cDNA library.

We further analysed four proteins, which were interesting because of their biochemical function, hit multiple times and confirmed when re-assaying with the bait-dependency test.

VKORC1 found itself in the DUALmembrane screening eight times. The interaction was verified by co-immunoprecipitation experiments. Because VKORC1L1 shares high homology with VKORC1, we assumed that VKORC1L1 may also bind to VKORC1. Indeed we could show a specific interaction of the two paralogous proteins in our co-immunoprecipitation analyses. The co-immunoprecipitation experiments, however, do not allow analysing the stoichiometry of the homomeric or heteromeric complexes. Further studies, e.g. analysis by mass spectroscopy, are required for a final clarification of the complex composition.

PDIA6 was the only potential thioredoxin-like redox protein identified in the DUALmembran screening, and therefore one of the most promising candidates since PDI had been proposed to supply the reduction equivalents for VKORC1 (25). However, we could not confirm the interaction of VKORC1 and PDIA6 either in the bait dependency test or by immunoprecipitation. Schuman et al. recently reported that VKORC1 strongly interacts with TMX, an ER membrane-anchored Trx-like protein and weaker with TMX4 and ERp18, also called PDIA16. These authors also investigated PDIA6 as a potential partner of VKORC1 by immunoprecipitation studies using either a Cys58Ala or Cys193Ala mutated PDIA6 and mutated or wild-type VKOR but they could not detect any interaction either (19).

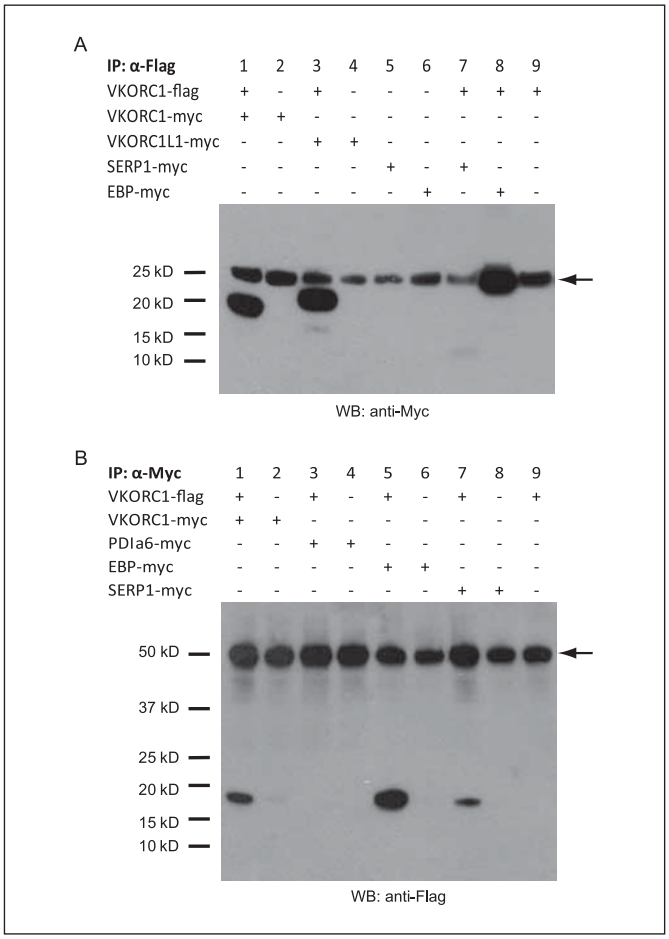

Figure 3: Co-immunoprecipitation studies of VKORC1 with putative interaction partners. Co-immunoprecipitation studies (IP) using HEK 293 cells transiently co-expressing Flag-VKORC1 and Myc-VKORC1, Myc-VKORC1L1, Myc-SERP1, Myc-EBP or Myc-PDIa6, respectively, as indicated above for each lane. A) IPs were performed with Anti-Flag M2 Agarose Affinity Gel (Sigma) and analysed by Western blot with anti-Myc antibody. All lanes were loaded with immunoprecipitates derived from the same amount of cell lysates (10-cm dish). VKORC1 and VKORC1L1 were used as positive controls (lanes 1 and 3). Co-transfections of the individual Myc-tagged plasmids with empty Flag-vector and also the Flag-tagged VKORC11 co-expressed with empty Myc-vector were used as negative controls (lanes 2, 4, 5, 6 and 9). Flag-tagged VKORC1 specifically interacted with Myc-SERP1 which migrates at 9.7 kDa (with tag) (lane 7) and with Myc-EBP which migrates at 28.65 kDa (lane 8). The Myc-EBP band is overlapping with the IgG light chain bands at 25 kDa which are also indicated as loading controls (arrow). B) IPs were performed with Anti-c-Myc Agarose Affinity Gel (Sigma) and analysed by Western blot with anti-Flag antibody, detection was performed by ECL. All lanes were loaded with immunoprecipitates derived from the same amount of cell lysates (10-cm dish). VKORC1 with the Flag-tag migrates at ~19.3 kDa. Co-transfections of the individual Myc tagged plasmids with empty Flag-vector and also the Flag-tagged VKORC1 co-expressed with empty Myc-vector were used as negative controls (lanes 2, 4, 6, 8 and 9). Flag-tagged VKORC1 specifically interacted with Myc-VKORC1, Myc-EBP and Myc-SERP1 (lanes 1, 5 and 7) but not with Myc-PDIa6 (lane 3). IgG heavy chain bands at 50 kDa (arrow) served as loading controls.

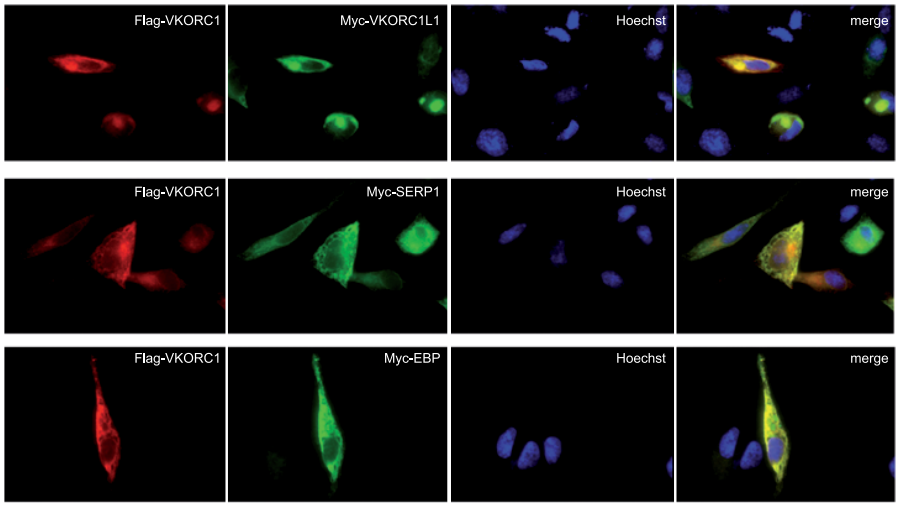

Figure 4: Co-immunofluorescence of VKORC1 with VKORC1L1, SERP1 and EBP. VKORC1 co-localises with VKORC1L1, SERP1 and EBP in HeLa cells. Cells overexpressing Flag-VKORC1 together with Myc-VKORC1L1, Myc-SERP1 or Myc-EBP, respectively, were co-stained with anti-mouse Alexa488 (Flag-VKORC1, red) and anti-rabbit 488 (Myc-tag, green). The DNA was stained with Hoechst (blue). The merged images indicate a co-localisation of the co-expressed proteins.

SERP1 was found 16 times in the library screening, while most of the other proteins were found once or twice. SERP1, which is identical to ribosome-associated membrane protein 4 (RAMP4), is a protein of the ER translocation site that assists the membrane insertion of proteins and is involved in the ER quality control system and in stress response (32, 34). According to Yamaguchi et al., overexpression of SERP1 in HEK 293 cells suppressed the aggregation and/or degradation of newly synthesised integral membrane proteins which are observed after oxidative stress of the ER (32). One of the reasons for oxidative stress to the endoplasmic-reticulum is the generation of reactive oxygen species (ROS) during oxidation of PDI by the flavoprotein Ero1. This oxidation happens through disulphide exchange that results in the formation of reduced Ero1 plus ROS (36). Ero1 can be oxidised in the presence of flavin adenine dinucleotide (FAD) and oxygen (37). Glutathione is believed to protect the cell from the damage caused by ROS (38). A number of studies have described antioxidant properties of vitamin K quinone (39–42). Since VKORC1 is able to generate the reduced vitamin K-hydroquinone, it may also be acting as an antioxidant (24). The recently published interaction between PDIA16 and/or TMX and VKORC1 (19) adds further support to a potential role of VKOR proteins in prevention of oxidative stress. Our data suggest SERP1 as another component of the complex protection mechanisms against oxidative damage in the ER.

Further, we have identified an interaction between VKORC1 and EBP. EBP was found 17 times in the split-ubiquitin assay. EBP serves as a binding protein for emopamil ((S)-Emopamil ((2S)-2-isopropyl-5-(methylphenylethylamino)-2-phenylvaleronitrile hydrochloride), a compound of the phenylalkylamine group of Ca^{2+} antagonists (30, 31). EBP is a high-affinity acceptor for several anti-ischaemic drugs. It also acts as a 3β-hydroxy-steroid-Δ8–Δ7 sterol isomerase (30, 33). Mutations in EBP cause an X-linked dominant form of chondrodysplasia punctata (CDPX2), also known as Conradi-Hünermann-Happle syndrome, a rare type of genodermatosis with heterogeneous clinical phenotypes (43). Chondrodysplasia punctata is characterised by the association of usually bilateral and symmetrical Blaschko-linear cutaneous lesions, ocular involvement, morphological and characteristic punctuate epiphyseal calcifications leading to skeletal abnormalities (44). Interestingly, warfarin embryopathy – resulting from warfarin overdosage in the mother – has been considered a phenocopy of chondrodysplasia punctata (10, 45). Furthermore, children suffering from VKCFD2 – a complete functional loss of VKORC1 activity – sometimes show developmental skeletal abnormalities such as nasal hypoplasia, distal digital hypoplasia, epiphyseal stippling and mild conductive hearing loss, symptoms resembling those of warfarin embryopathy (46). The link between vitamin K metabolism and calcium homeostasis may be the vitamin K-dependent proteins osteocalcin and matrix Gla protein which are involved in bone mineralisation (47–49).

Like the vitamin K-dependent coagulation factors, osteocalcin and matrix Gla protein require γ-carboxylation to gain their full activity in calcium binding. High levels of undercarboxylated osteocalcin have a negative effect on bone health (50). Developmental skeletal defects such as those seen in CDPX2, warfarin embryopathy and VKCDF2 are associated with inappropriate calcium deposition and abnormal calcification. A physical interaction between EBP and VKORC1 – as suggested by our results – could explain why mutations in either gene may lead to reduced VKOR activity, similar to warfarin overdosage, which in turn could have a negative effect on Ca^{2+} metabolism. This would explain why mutations in EBP cause a phenotype resembling warfarin embryopathy. The detailed mechanism of interaction of VKORC1 and EBP could provide a new approach to understanding the basis of warfarin embryopathy, VKCFD2 and the different subtypes of chondrodysplasia punctata as well as the relation between these phenotypes. However, because of their similarity it cannot be excluded that some of all of these interaction occur with the VKORC1L1 protein which shows 50% homology and a similar structure with VKORC1.

In conclusion we have identified four proteins that potentially interact with VKORC1: VKORC1 itself may form a homodimer, VKORC1L1 that may function with VKORC1 in a heterodimer, and EBP, a high-affinity receptor of phenylalkylamine Ca^{2+} antag-

What is known about this topic?

- VKORC1 is a component of the vitamin K cycle. VKOR regenerates vitamin KH_2 and is the target of warfarin.
- VKOR has been suggested to be a multi-enzyme-complex. However, components other than VKORC1 have not yet been identified.

What does this paper add?

- In this study we screened a liver cDNA library for interaction partners of VKORC1 and identified three proteins.
- Especially the interaction with emopamil-binding protein (EBP) is highly interesting as it provides a new understanding of warfarin embryopathy, VKCFD2 and chondrodysplasia punctata and the correlation between these phenotypes.

onists that – when mutated – causes chondroplasia punctata, a phenotype resembling warfarin embryopathy. SERP1 may point to a role of the vitamin K pathway in the regulation of antioxidant stress. Further studies are needed to clarify the role of these proteins in the vitamin K pathway.

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