Disulfide-dependent Protein Folding Is Linked to Operation of the Vitamin K Cycle in the Endoplasmic Reticulum

A PROTEIN DISULFIDE ISOMERASE-VKORC1 REDOX ENZYME COMPLEX APPEARS TO BE RESPONSIBLE FOR VITAMIN K$_2$, 2,3-EPOXIDE REDUCTION

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Carboxylation of vitamin K$_1$-dependent proteins is dependent on formation of reduced vitamin K$_1$ (Vit.K$_1$H$_2$) in the endoplasmic reticulum (ER), where it works as an essential cofactor for γ-carboxylation in post-translational γ-carboxylation of vitamin K$_1$-dependent proteins. Vit.K$_1$H$_2$ is produced by the warfarin-sensitive enzyme vitamin K$_1$ 2,3-epoxide reductase (VKOR) of the vitamin K cycle that has been shown to harbor a thioredoxin-like CXXC center involved in reduction of vitamin K$_1$, 2,3-epoxide (Vit.K$_1$O). However, the cellular system providing electrons to the center is unknown. Here data are presented that demonstrate that reduction is linked to dithiol-dependent oxidative folding of proteins in the ER by protein disulfide isomerase (PDI). Oxidative folding of reduced RNase is shown to trigger reduction of Vit.K$_1$O and γ-carboxylation of the synthetic γ-carboxylase peptide substrate FLEEL. In liver microsomes, reduced RNase-triggered γ-carboxylation is inhibited by the PDI inhibitor bacitracin and also by small interfering RNA silencing of PDI in HEK 293 cells. Immunoprecipitation and two-dimensional SDS-PAGE of microsomal membrane proteins demonstrate the existence of a VKOR enzyme complex where PDI and VKORC1 appear to be tightly associated subunits. We propose that the PDI subunit of the complex provides electrons for reduction of the thioredoxin-like CXXC center in VKORC1. We can conclude that the energy required for γ-carboxylation of proteins is provided by dithiol-dependent oxidative protein folding in the ER and thus is linked to de novo protein synthesis.

Vitamin K$_1$-dependent proteins are modified post-translationally to contain γ-carboxyglutamic acid, Ca$^{2+}$ binding amino acid residues (1–3). The modification is carried out by the vitamin K$_1$-dependent γ-carboxylation system located in the endoplasmic reticulum (ER)$^2$ (2, 3). Two essential enzymes of the system are 1) the vitamin K$_1$-dependent γ-carboxylase, a 758-amino-acid-containing integral membrane protein with five transmembrane domains in the ER membrane, which requires reduced vitamin K$_1$ (Vit.K$_1$H$_2$) as cofactor (4) and 2) the warfarin-sensitive enzyme vitamin K$_1$ 2,3-epoxide reductase (VKOR), which produces the cofactor (5). In contrast to γ-carboxylase (4), VKOR has still not been purified to electrophoretic homogeneity. Based on our effort to purify VKOR (5), we arrived at the conclusion that VKOR is a lipid-protein complex in the ER membrane where more than one protein component is needed for expression of warfarin-sensitive vitamin K$_1$, 2,3-epoxide (Vit.K$_1$O) reduction.

A better understanding of VKOR became possible when Rost et al. (6) and Li et al. (7) independently discovered a gene on human chromosome 16 that, when expressed in cell lines, resulted in enhanced warfarin-sensitive VKOR activity. The gene predicts an 18-kDa highly hydrophobic integral protein of the ER membrane with three potential transmembrane domains (8). The gene product was named VKORC1 by Roth et al. (6) to indicate its putative function as a subunit of VKOR.

VKOR has been known for many years to have an active Cys-containing redox center (9), which is essential for reduction of Vit.K$_1$O. The center has also been shown to be the target for the anticoagulant warfarin (10). With bioinformatics analyses of the VKORC1 gene, Goodstadt and Ponting (11) found that VKORC1 harbors a thioredoxin-like CXXC center in the third hydrophobic transmembrane domain of the protein. By mutagenesis of the Cys residues in the center, we (8) and others (12) have provided evidence that this center indeed is the functional redox center in VKOR, and recent data by Rost et al. (12) support the older data that the center is indeed the target for warfarin. On the other hand, the cellular component(s) involved in reduction of the center has never been established with a high degree of certainty (13–15).

Protein disulfide reductase (PDI) in combination with the thioredoxin system has been suggested to donate electrons to VKOR (16). Although thioredoxin was eliminated by Preuschof (15) to be part of the reducing system, we still found the hypothesis attractive that PDI is involved as PDI is a dominant protein in the ER involved in dithiol redox exchange reactions (17). In this report, we present data that strongly suggest that disulfide-

- N-ethylmaleimide; ME, mercaptoethanol; PVDF, polyvinylidene difluoride; RIPA, radioimmune precipitation buffer; IEF, isoelectric focusing; DTT, dithiothreitol; siRNA, small interfering RNA.
dependent folding of reduced RNase by PDI provides electrons for the redox center in VKORC1 for reduction of Vit.K=O by the vitamin K cycle. Furthermore, the experimental results also strongly suggest that PDI and VKORC1 form a tightly associated complex attached to the ER membrane. Based on the acquired data, we propose that post-translational γ-carboxylation of proteins is dependent on disulfide-dependent folding of proteins in the ER as a part of general protein synthesis.

**EXPERIMENTAL PROCEDURES**

**Construction of an Expression Plasmid with c-myc Epitope-tagged VKORC1**

The oligonucleotides used for rat VKORC1 PCR were as follows. The sense primer was 5′-AAA AAA AAG CTG GCC GCC ACC ATG GCC ACC ACC TGG AGG-3′ (underlined bases were used to generate HindIII site; bases in italics were used to generate a Kozak sequence). The antisense primer was 5′-AAA AAA TCT AGA GGG CTG TTG GAC CTG TTG TTC-3′ (the underlined bases were used to generate a XbaI site; the stop codon was removed by adding the c-myc epitope). The modified cDNA for rat VKORC1 was then cloned into the HindIII and XbaI site of the pBUDCE4.1 vector under the control of a cytomegalovirus promoter. The XbaI site fused the rat c-myc peptide sequence to EQKISKEDIL. The recombinant plasmid with cDNA for rat c-myc tagged-VKORC1 was sequenced on both strands to eliminate any PCR errors.

**Selection of BHK Cell Lines Expressing c-myc-tagged VKORC1**

Plasmid pBUDCE4.1 with c-myc-tagged VKORC1 was used to transfected BHK21 cells, and selection of stable cell lines was carried out as described (18).

**Immunoprecipitation, SDS-PAGE, and Western Blotting**

**Cell Extracts**—Cells were lysed on ice for 30 min in 20 mM MES, 30 mM Tris, 100 mM NaCl, 1% Triton-X 100, 20 mM NEM, pH 7.4, containing 10 μg/ml Sigma protease inhibitor mixture for use with mammalian cell and tissue extracts (protease mixture). After lysis, cell debris was removed by centrifugation at 12,000 × g for 30 min, and the supernatant was collected. The supernatant was preclarified by adding Sepharose-protein G beads for 1 h at 4 °C. The beads were removed by centrifugation, and the supernatant was immunoprecipitated by adding goat anti-c-myc antibodies. For control experiments, preimmune goat IgG was added. The antibody mixtures were allowed to react on a rotating devise overnight at 4 °C. Then, Sepharose-protein G beads were added, and the mixtures were incubated for an additional 2 h at 4 °C. The beads were washed four times in cold lysis buffer without NEM, and immune complexes were released from the beads by boiling them in SDS-PAGE buffer containing 5% mercaptoethanol (ME). Samples were loaded onto 8–16% Criterion SDS-PAGE gels, and electrophoresed proteins were transferred to PVDF membranes for Western blotting. The blots were developed with monoclonal anti-PDI antibodies.

**Rat Liver Microsomes**—Rat liver microsomes were prepared as described by our laboratory (5). For removal of microsomal luminal and peripherally bound membrane proteins, microsomes obtained from 4 g of rat liver were suspended in 8 ml of 100 mM Na2CO3, 1.2 M KCl, 0.025% deoxycholate, pH 11.5, with a Potter Elvehjem glass homogenizer and centrifuged at 100,000 × g for 45 min (19). The pellet was resuspended in 8 ml of 50 mM Tris base with the same homogenizer and centrifuged a second time at 100,000 × g for 45 min. Pellets (extracted microsomes) were stored at −85 °C until used for experiments. Extracted microsomes were resuspended in buffer D (250 mM sodium phosphate, 0.5 mM KCl, 20% glycerol, 0.75% CHAPS, pH 7.85, containing 10 μg/ml protease mixture) using a Dounce homogenizer. For blocking of free thiol groups, 20 mg of NEM was added per ml of buffer D before homogenizing. The protein concentration was 6.8 mg/ml. Prior to immunoprecipitation, the soluble membrane proteins in buffer D were gel-filtrated into RIPA buffer (50 mM Tris, 1% Nonidet-P-40, 0.25% deoxycholate, 150 mM NaCl, 1 mM EDTA, pH 7.4, containing 10 μg/ml protease mixture) by passing it through an affinity column packed with a mixture of Sepharose-protein A and Sepharose-protein G beads equilibrated in RIPA buffer. The various antibodies that were used for immunoprecipitation were added to the precleared samples. The mixtures were allowed to rotate overnight at 4 °C. Depending on the antibody used for immunoprecipitation, Sepharose-protein A or Sepharose-protein G beads, respectively, were added to the samples, and the mixtures allowed to rotate for an additional 1 h at 4 °C for binding of immune complexes to the beads. The beads were washed with RIPA buffer before immune complexes were released from the beads by boiling them in SDS-PAGE buffer containing no reductant. The beads were removed by centrifugation, and the supernatant was prepared for SDS-PAGE either as oxidized samples or as reduced samples prepared by boiling the samples with 5% ME for 2 min.

**Two-dimensional SDS-PAGE**

Extracted microsomes were dissolved in the standard SDS-PAGE buffer containing 2% SDS and 5% ME and boiled for 2 min. Solubilized proteins were precipitated with cold acetone and washed with trichloroacetic acid and ether/ethanol as described previously (5). The final dried protein precipitate was dissolved in the 8M urea containing isoelectric focusing (IEF) sample buffer 1 obtained from Bio-Rad. Adding a small amount of 0.1 M NaOH to the sample buffer to bring the pH to 8 resulted in improved solubilization of the hydrophobic ER membrane proteins. This pH adjustment did not have any noticeable effect on IEF and significantly increased the amount and intensity of proteins spots seen in the gels after silver or Coomassie Blue staining. Prior to IEF, insoluble material was removed by centrifugation, and protein samples were absorbed onto 11-cm agarose/plastic strips with immobilized amphyolite gradients from pH 3 to 10 (Bio-Rad). IEF of the proteins was carried out on a Bio-Rad PROTEAN II IEF apparatus according to the supplier's instructions and separated in the second dimension on 8–16% SDS Criterion gels (Bio-Rad). Transfer of the two-dimensional SDS-PAGE separated proteins to PVDF membranes for Western blotting was carried out at reduced intensity by our laboratory (8). Following immunostaining of the two-dimensional blots, the membranes were washed briefly in distilled water and
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stained for 2 min in 50% ethanol, 10% acetic acid containing 0.2% Coomassie Brilliant blue. When the stained membranes were dried, the proteins appeared as blue spots against a white background. It was found that this staining procedure could only be carried out if the PVDF membrane had been blocked with fat-free milk powder. Proteins separated in SDS-PAGE gels identical to those used for two-dimensional Western blotting were silver-stained (Dodeca silver stain, Bio-Rad), and gel spots containing proteins of interest identified on the two-dimensional Western blots were cut from the gel for protein identification by MS/MS.

siRNA Silencing of PDI

An siRNA SMART pool against human PDI containing 50 nmol of a mixture of four oligonucleotides with potential for PDI mRNA destruction by RISC complexes was designed by and purchased from Dharmacon RNA Technologies, Lafayette, CO. The nucleotide sequences of the four siRNA oligomers were not released by the company for this kit. Transfection of HEK 293 cells with 100 and 150 nM PDI siRNA SMART pool was carried out with Lipofectamine (Invitrogen) according to the protocol provided by the company. As recommended by Dharmacon, siRNA against human glyceraldehyde-3-phosphate dehydrogenase was used as a positive control, and a negative control consisted of a mixture of four scrambled siRNAs. Following transfection, cells were grown positive control, and a negative control consisted of a mixture of four scrambled siRNAs. Following transfection, cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum for 48 h. The attached cells were then scraped from the plates, pelleted, and washed in phosphate-buffered saline and prepared for VKOR assay in buffer D as described (20). Portions of the various buffer D cell extracts with the same protein content were prepared for SDS-PAGE and Western blotting with PDI antibodies. Stripping of the PVDF membrane and reprobing with an antibody against the peptide epitope KAARARNEDYRALC were custom made by Alpha Diagnostic, San Antonio, TX and affinity-purified by our laboratory as described (8). Affinity-purified rabbit peptide antibodies made against the peptide epitope YALHVKAAARRDREDYRALC from the human VKORC1 sequence were a gift from Dr. Allan Rettie, Department of Medicinal Chemistry, University of Washington, Seattle, WA, and c-myc antibodies and anti-human glyceraldehyde-3-phosphate dehydrogenase antibodies were from Abcam Inc., Cambridge, MA. Sepharose-protein A, Sepharose-protein G, and monoclonal anti-a-Actin antibodies were from Sigma. Bovine pancreas RNase A was from Worthington. Recombinant human PDI was from Affinity Bioreagents, Golden, CO. All other chemicals used were of highest quality. Mass spectrometry was carried out by the Proteomics LC-MS/MS Core Facility at Virginia Polytechnic Institute and State University, Blacksburg, VA. Digitized images of immunoreactivity bands on Fuji medical x-ray film SuperRX (Fisher) and stained protein bands in SDS-gels were analyzed with Kodak one-dimensional software (Eastman Kodak Co.) to determine the integrated areas representing the protein bands. Human liver microsomes were from CellzDirect, Pittsboro, NC.

Trapping and Concentration of the Putative PDI-VKORC1 Enzyme Complex by Immunoabsorption with Immunomagnetic Beads

In this experiment, we brought extracted microsomes into solution with buffer B (25 mM phosphate, 25 mM KCl, 20% glycerol, 0.75% CHAPS, pH 7.85, containing 10 µg/ml Sigma protease inhibitor mixture), which we (5) previously have reported partially solubilizes the VKOR enzyme complex in an active form. Insoluble proteins were removed by filtering the Dounce tease inhibitor mixture), which we (5) previously have reported

Enzyme Assays

Warfarin-sensitive VKOR activity was measured as described (22). VKOR activity was triggered either with 5 mM DTT or with 0.32 mM reduced RNase. γ-Carboxylase activity was assayed as described (19) as 14CO2 incorporation into the synthetic peptide FLEEL (FLEEL γ-carboxylation). The reaction was triggered either by adding the chemically reduced vitamin K1H2 (100 µg/ml) to the assay mixture or by adding 0.5 mM reduced RNase plus 40 µM Vit.K>O for VKOR production of reduced Vit.K1H2. Reduced RNase was prepared according to a method originally described by Rupp et al. (23). One hundred mg of RNase was dissolved in 500 µl of 0.2 M Tris acetate, 6 M guanidinium hydrochloride, pH 8.0. Seventy-two mg of DTT was added, and the solution was left at 37 °C for 1 h. Then, the pH was adjusted to 4.0 with acetic acid and DTT, and Tris and guanidinium hydrochloride was removed by gel filtration on a column of Sephadex G-25 equilibrated in 0.1 M acetic acid. Prior to chromatography, the column resin and the 0.1 M acetic acid were saturated with N2. The void volume fraction containing reduced RNase was distributed into sealed 0.75-ml serum flasks filled with N2 that were stored at 4 °C until use.

Materials

Anti-PDI mouse monoclonal antibodies, catalog number SPA-891, lot number B503461 were from Stressgen Bioreagents, Victoria, British Colombia, Canada. Rabbit anti-rat VKORC1 antibodies against the peptide epitope KAARARNEDYRALC were custom made by Alpha Diagnostic, San Antonio, TX and affinity-purified by our laboratory as described (8). Affinity-purified rabbit peptide antibodies made against the peptide epitope YALHVKAARRDREDYRALC from the human VKORC1 sequence were a kind gift from Dr. Allan Rettie, Department of Medicinal Chemistry, University of Washington, Seattle, WA, and c-myc antibodies and anti-human glyceraldehyde-3-phosphate dehydrogenase antibodies were from Abcam Inc., Cambridge, MA. Sepharose-protein A, Sepharose-protein G, and monoclonal anti-a-Actin antibodies were from Sigma. Bovine pancreas RNase A was from Worthington. Recombinant human PDI was from Affinity Bioreagents, Golden, CO. All other chemicals used were of highest quality. Mass spectrometry was carried out by the Proteomics LC-MS/MS Core Facility at Virginia Polytechnic Institute and State University, Blacksburg, VA. Digitized images of immunoreactive bands on Fuji medical x-ray film SuperRX (Fisher) and stained protein bands in SDS-gels were analyzed with Kodak one-dimensional software (Eastman Kodak Co.) to determine the integrated areas representing the protein bands. Human liver microsomes were from CellzDirect, Pittsboro, NC.

RESULTS

Reduced RNase Triggers Warfarin-sensitive Vitamin K Epoxide Reduction by the Vitamin K Cycle

As shown in Fig. 1, we found that when reduced RNase (black bar) was added to our test system prepared from extracted micro-
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FIGURE 1. \(\gamma\)-Carboxylase activity triggered by reduced RNase. Rat liver microsomes extracted from luminal and peripherally bound membrane proteins (see "Experimental Procedures") were tested for \(\gamma\)-carboxylation when the reaction was triggered by 0.5 mM reduced RNase in the presence of 40 \(\mu\)M Vit.K\(>\)O (black bar) or chemically reduced vitamin K\(_1\)H\(2\) (open bar). No DTT was present in the assays. Microsomal protein concentration in each assay mixture was 6.8 mg/ml. Activities measured in control incubations without reduced RNase (300 cpm/mg) and DTT (245 cpm/mg) were subtracted from the activities shown as bar graphs. Each bar represents the average activity of three parallel incubations. Standard deviation is shown on each bar.

FIGURE 2. Bacitracin inhibition of reduced RNase triggered VKOR activity. A shows reduced RNase (0.32 mM) triggered VKOR activity in the presence of various concentrations of bacitracin. VKOR activities are presented as the percentage of controls (100%), which contained no PDI inhibitor. B shows that a high concentration of bacitracin (5.5 mM) had no effect on DTT-triggered VKOR activity. Each bar is the average activity of three parallel incubations, and standard deviations are indicated as error bars. Microsomal protein concentrations in the assays shown in A and B were 3.4 and 6.8 mg/ml, respectively.

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Previously, we have shown that the pH 11, deoxycholate, Tris base extraction procedure used in this work to extract microsomes completely removed the ER chaperone calumenin (20), and Rustaeus et al. (28) have reported that the method strips the membrane for proteins not integral to the membrane. As PDI is a dominant ER protein in the lumen of the ER, these observations made us determine how much PDI was left in the

somal membranes, oxidative folding of RNase could “drive” Vit.K\(>\)O reduction by the vitamin K cycle to produce reduced Vit.K\(_1\)H\(_2\) cofactor for \(\gamma\)-carboxylase. The activity triggered with reduced RNase was close to this activity triggered with chemically reduced vitamin K\(_1\)H\(_2\) (open bar). This finding indicated efficient reduction of the redox center in VKORC1 related to RNase dithiol oxidation and refolding. The reduced RNase and the Vit.K\(_1\)H\(_2\) triggered Vit.K\(>\)O reductions were inhibited 90% by 25 \(\mu\)M warfarin (not shown).

Our experiments with Vit.K\(>\)O added to the reduced RNase-containing \(\gamma\)-carboxylase test system was more specifically focused on the vitamin K cycle than similar experiments reported by Soute et al. (16), who used reduced RNase and vitamin K\(_1\) quinone in a test system prepared from crude microsomes. Vitamin K\(_1\) quinone-reducing enzymes have been shown to be present in the ER and cytosol (2), whereas Vit.K\(>\)O reduction appears to be specific for VKORC1 (2, 4).

A commonly used inhibitor with high specificity for PDI is bacitracin (24–27). To acquire data that would indicate that PDI was involved in reduced RNase-triggered Vit.K\(>\)O reduction, we added bacitracin to the test samples. As shown in Fig. 2A, there was a dose-dependent inhibition of VKOR activity by bacitracin when the activity was triggered by reduced RNase. On the other hand and importantly, bacitracin had no effect on VKOR activity when Vit.K\(>\)O reduction was triggered with DTT (Fig. 2B). This important control experiment showed that bacitracin did not affect any of the components of the vitamin K-dependent \(\gamma\)-carboxylation system (2). This finding prompted the ideas that either PDI by itself was the reductase of the VKORC1 redox center or PDI worked as an essential enzyme in a larger redox system present in our \textit{in vitro} \(\gamma\)-carboxylation test system needed for VKORC1 reduction. Thioredoxin has been proposed to be an intermediate redox partner between PDI and VKORC1 (16), but Preusch (15) showed that immunodepletion of thioredoxin from a microsomal \textit{in vitro} system did not affect VKOR activity. We were unable to detect thioredoxin in our extracted microsomal \(\gamma\)-carboxylation system, which supports the data by Preusch (15).

The Amount of PDI Present in Extracted Microsomes

Previously, we have shown that the pH 11, deoxycholate, Tris base extraction procedure used in this work to extract microsomes completely removed the ER chaperone calumenin (20), and Rustaeus et al. (28) have reported that the method strips the membrane for proteins not integral to the membrane. As PDI is a dominant ER protein in the lumen of the ER, these observations made us determine how much PDI was left in the
extracted membranes. To determine the percentage of distribution of PDI in the extract and the remaining extracted membranes used in our experiments, aliquots of the extract and SDS-PAGE buffer solubilized extracted membranes were subjected to SDS-PAGE and Western blotted with PDI antibodies. Images of the Coomassie Blue protein bands present in the two aliquots are shown in Fig. 3. Consistent with previously published data (20), we calculated that 75% of total microsomal proteins were removed by the extraction procedure and 25% remained in the extracted membranes. The large amount of proteins removed by extraction is also obvious from the protein-stained lane (Extract) in Fig. 3.

Quantitative estimation by densitometry of PDI in the aliquots subjected to SDS-PAGE and Western blotting showed that 68% of the microsomal PDI was removed by extraction and 32% remained in the extracted membranes. These results convincingly demonstrate that a smaller portion of the microsomal PDI pool is tightly associated with the ER membrane and were present in our ΔH9253-carboxylation test system, the components of which are completely embedded in the membrane (2).

Additional Experiments Supporting a Role for PDI in Reduction of Vit.K>0 by VKORC1

Silencing of PDI in HEK 293 Cells with siRNA Reduces VKOR Activity—Silencing of an mRNA gene product by complementary siRNA oligomers has become an important technology to determine whether the translated protein product is functional in the cellular system under study (29). Since the bacitracin inhibitor data pointed toward an involvement of PDI in cellular VKORC1 function, we also determined the effect of silencing PDI in cultured cells that express the vitamin K-dependent ΔH9253-carboxylation system. For these experiments, we selected HEK 293 cells that have been used extensively to study the ΔH9253-carboxylation system. Fig. 4A shows that HEK 293 cells treated with a 100 and 150 nM siRNA SMART pool against human PDI reduced VKOR activity to 38% (117 pmol/mg) and 25% (98 pmol/mg), respectively, when compared with control cells 100% (390 pmol/mg). Fig. 4B shows a Western blot of PDI in the control cells (Cont.) and the cells treated with 100 and 150
Extracted rat liver microsomal membranes were solubilized in buffer B and incubated with antibodies (Abs.) to PDI and VKORC1, respectively. Control incubations to VKORC1 antibody-containing samples contained rabbit IgG. Control incubations to PDI antibody-containing samples contained mouse IgG. The immune complexes were absorbed onto immunomagnetic beads containing immobilized protein A or protein G (see “Experimental Procedures”), and the washed particle suspensions in buffer D were tested for VKOR activity. The bar graph shows specific VKOR activity measured in the various samples. Standard deviation of three parallel incubations is indicated by error bars.

nm of the siRNA SMART pool. The concentrations of PDI in the various samples were determined by immunofluorescent band intensity integration (see “Experimental Procedures”). The integration estimated the concentration of PDI to be reduced 20 and 32% in the 100- and 150-nmol siRNA-treated cells, respectively (Fig. 4C). Western blots of α-actin among the proteins in each lane showed equal protein loading (Fig. 4D). Cells treated with the positive siRNA GADPH and the negative scrambled siRNA controls worked as predicted by Dharmacon. These control experiments provided evidence for the specificity of the PDI siRNA SMART pool supplied by the company. When estimated by dot-blot analysis, the concentration of VKORC1 was found also to be unchanged in each sample loaded into each lane on the gel shown in Fig. 4B. These results provided evidence for a quantitative relationship between available PDI in the HEK 293 cells and an active VKOR enzyme.

VKORC1 and PDI Antibodies Recognize and Bind the Active VKOR Enzyme Complex—Trapping and concentration of the VKOR enzyme complex was carried out with VKORC1 and PDI antibodies followed by capturing the complexes with magnetic beads linked to protein A and protein G, respectively, as described in detail under “Experimental Procedures.” Fig. 5 shows VKOR activities in suspensions of captured beads from buffer B solubilized-extracted membranes incubated with VKORC1 and PDI antibodies, respectively. Suspensions of beads from buffer B-extracted microsomal membranes incubated with rabbit IgG and mouse IgG served as controls. When compared with the controls, significant enrichment of the active VKOR enzyme was found in the suspensions containing VKORC1 and PDI antibodies. These results suggested that PDI and VKORC1 were physically associated in the buffer B extract and potentially represented our previously hypothesized VKOR lipid-protein enzyme complex.

A Putative Protein Complex Formed between PDI and VKORC1

The in vivo electron donor for Vit.K>Ο reduction by the vitamin K cycle has never been identified. Based on our finding of an apparent close cooperation between PDI and VKORC1 for expression of VKOR activity and the knowledge that PDI is a subunit of enzyme complexes (30) and tightly binds hydrophobic protein sequences (26, 31), we hypothesized the existence of a PDI-VKORC1 enzyme complex and designed experiments aimed at identifying the complex. Our first set of experiments focused on identifying proteins in the extracted membranes that were recognized by the VKORC1 and PDI.
antibodies when the proteins were separated by two-dimensional SDS-PAGE. Successful separation of significant amounts of the integral membrane proteins solubilized in the IEF sample buffer was only obtained when the pH was raised to 8. Adding thiourea to the sample without raising pH did not improve solubilization. Fig. 6B shows Coomassie Blue-stained proteins on the PVDF membrane obtained after protein transfer from an 8–16% SDS-PAGE Criterion gradient gel and Western blotting of these proteines with PDI antibodies (Fig. 6A) and VKORC1 antibodies (Fig. 6C). Fig. 6E, lane 3, shows the same membrane proteins used for IEF (Fig. 6B) when separated by one-dimensional SDS-PAGE. Fig. 6A shows that the PDI monoclonal antibodies identified four protein spots with molecular masses equal to and higher than 55 kDa. The protein shown in Fig. 6, A and B, named PDI(a), was excised from an identical silver-stained gel and determined to be PDI by MS/MS. Fig. 6C shows that the VKORC1 antibodies recognized four proteins with molecular masses of about 28 kDa, which previously has been determined by our laboratory for rat liver VKORC1 after one-dimensional SDS-PAGE and Western blotting (18). The protein named VKORC1(b) and the more acidic forms VKORC1(a) and VKORC1(b)? were excised from a silver-stained gel and subjected to MS/MS. We were unsuccessful in obtaining MS/MS spectra of this highly hydrophobic VKORC1 protein (8). The reason for the more acidic migration of some of the 28-kDa proteins reacting with the VKORC1 antibodies (VKORC1(b)?) is unknown but could be the result of modifications of VKORC1. VKORC1 has a consensus sequence for glycosylation (11). However, the highest molecular mass protein (Fig. 6, a+b) (molecular mass about 80 kDa) was recognized by both antibodies (Fig. 6, A and C) and identified as the same protein (a+b) shown on the stained blot in Fig. 6B. Identification of specific protein bands on the stained blot (Fig. 6B) reacting with antibodies is 100% accurate because the film obtained after exposure of the ECL blot has an image that is identical to the protein-stained blot.

To obtain more confidence in the data obtained from the two-dimensional SDS-PAGE experiments suggesting the existence of a PDI-VKORC1 complex, we carried out immunoprecipitation experiments with the two antibodies to determine whether they both would immunoprecipitate the complex. PDI is organized into five domains (a, b, b’, a’, and c) where the a and a’ domains contain thioredoxin homologous CGHC redox centers that are involved in the protein folding and refolding activities of PDI as an isomerase and oxido/reductase (32, 33). The b’ domain is the dominant protein binding domain (26). If PDI was a hydrophobically bound subunit of the putative PDI-VKORC1 protein-lipid complex, we hypothesized that the thiol redox exchange reaction between the two proteins would be carried out by the Cys residues present in the redox centers in the two proteins (34). To prevent rapid disruption of the expected S–S linked intermediate (35), we blocked free thiols with NEM. Blocking of the C-terminal Cys residue in the CXXC center has been shown to be important for preservation of such reduction/oxidation intermediates (26). Fig. 6F, lanes 4 and 5, show Western blots of immunoprecipitates obtained with the rat VKORC1 antibodies and developed with the PDI antibodies as the first antibody. The individual samples loaded in lanes 4...
and 5 were not reduced and reduced with ME, respectively, prior to SDS-PAGE. Both lanes demonstrated an immunoreactive protein band with an 80-kDa molecular mass identical to the molecular mass of the a+b protein seen in Fig. 6B. Surprisingly, this putative PDI-VKORC1 complex did not dissociate when reduced with ME prior to SDS-PAGE (lane 5). This finding could indicate a stable association between the two proteins that could not be broken in the reducing SDS-PAGE buffer. Stable associated protein fragments resistant to dissociation in reducing SDS-PAGE buffer have been described and studied at the amino acid sequence level (36). Fig. 8, lane Rat Micro., shows two forms of VKORC1 with different molecular masses, whereas lane Human Micro. shows that the rat has the form with the higher molecular mass and is recognized by the human peptide antibodies. The rat antibodies did not recognize the human protein. The putative 80-kDa PDI-VKORC1 protein complex was apparent as a faint immunoreactive band in both preparations. Based on densitometry, we estimated that 7% of total VKORC1 was tied up as a complex in the rat membrane, whereas 2% was tied up in the human ER membrane.

DISCUSSION

The present study strongly suggests that PDI, a thioredoxin-like oxidoreductase and chaperone present at high concentration in the ER, associates with VKORC1 discovered by Rost et al. (6) and Li et al. (7) to form a warfarin-sensitive VKOR enzyme complex capable of reducing Vit.K>O to Vit.KH2 (2). Our data provide a new working hypothesis for operation of the vitamin K cycle. We (8) and Rost et al. (12) have shown by mutagenesis that the Cys residues present in the CXXC center in VKORC1 are needed for Vit.K>O reduction by the vitamin K cycle, and Rost et al. (12) have also shown that VKORC1 appears to harbor a Vit.K>O binding site in a location distant from the CXXC center (12). Both findings are consistent with earlier kinetic data aimed at answering these questions (9,10). The finding that reduced RNase can drive Vit.K>O reduction and support γ-carboxylation presents evidence that dithiol-dependent protein folding in the ER, as part of de novo protein synthesis, is an electron-providing system for production of reduced vitamin KH2, the essential cofactor needed for γ-carboxylation of the members of the vitamin K-dependent protein family.

Several experiments were carried out to test the hypothesis that PDI plays an essential role in providing the electrons for the

FIGURE 9. Dithiol-dependent oxidative protein folding in the ER is linked to post-translational γ-carboxylation of proteins, an hypothetical model. The thioredoxin-related redox center CGHC (S–S) in PDI is reduced (SH–SH) when PDI catalyzes dithiol-dependent oxidative folding of newly synthesized proteins in the ER. Reoxidation of the redox centers by endoplasmic reticulum oxidase1 (ERO1) is a major pathway for the continuous operation of PDI in oxidative folding. Our data suggest that some of the PDI proteins in the ER form stable complexes with the VKORC1 integral membrane protein. We propose that the PDI-VKORC1 protein complex is responsible for warfarin-sensitive reduction of Vit.K>O by the vitamin K cycle to form reduced Vit.KH2, the essential cofactor for γ-carboxylation of vitamin K-dependent proteins.
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CXXC center in VKORC1. Initially, we expected PDI to form a VKORC1-S–S-PDI intermediate redox complex that, when stabilized by NEM labeling of the C-terminal Cys residue in the center, would dissociate upon reduction with ME prior to SDS-PAGE (34). On the contrary, we found the complex to be stable in SDS-PAGE both with and without disulfide reduction by ME. Resistance of protein complexes associated by strong hydrophobic interactions to be separated in SDS-PAGE under oxidizing and reducing conditions is known (36).

By immunoprecipitation with anti-PDI and VKORC1 antibodies, we found a protein with molecular mass of 80 kDa that was recognized by both antibodies. We also identified this complex as a c-myc-tagged VKORC1-PDI complex from cells transfected with a c-myc-VKORC1 construct. In two-dimensional SDS-PAGE gels of proteins from extracted microsomal membranes treated with NEM and reduced with DTT prior to electrophoresis, we also found the 80-kDa protein identified by both antibodies. MS/MS of in-gel PDI and VKORC1 easily identified PDI, but we were unable to obtain reliable MS/MS spectra of the hydrophobic VKORC1 protein. Indeed, MS/MS spectra of both PDI and VKORC1 to be found in the 80-kDa (a+b) spot shown in Fig. 6B would have provided undisputable evidence for the existence of the complex. However, we know that the VKORC1 affinity-purified antibodies used in these studies show high specificity on Western blots of rat liver microsomes, which adds support to the hypothesis of the existence of a PDI-VKORC1 complex. The strongest evidence for PDI involvement in triggering VKOR activity is the siRNA PDI silencing data, which coincided with a significant drop in VKOR activity when the activity was triggered by reduced RNase. Bacterial inhibition of reduced RNase-triggered but not DTT-triggered VKOR activity strengthened this evidence.

PDI is organized into five domains (a, b, b', a', and c) where the a and a' domains are homologous to thioredoxin and contain CGHC redox centers that mediate some of the activities of PDI including isomerase and oxidative/reductase (17). The U-shaped b-b' domain acts as a hydrophobic binding site for PDI substrates (33) and is likely to be the interaction site with the hydrophobic VKORC1 protein. It is well established that binding of PDI to other proteins does not necessarily involve its thiol redox centers (38), which then could be involved in redox exchange with the VKORC1 subunit of a PDI-VKORC1 enzyme complexes in rat and human livers.

PDI is known to be a subunit of NAD(P)H oxidase in vascular smooth muscle cells (37), prolyl hydroxylase (38), and triglyceride transferase (39). Based on this knowledge and our findings that indicate that a complex between VKORC1 and PDI is formed in the ER, we propose that part of the PDI protein pool in the ER becomes strongly linked to part of the VKORC1 pool and that the complex is the active warfarin-sensitive Vit.K–O reducing enzyme complex of the vitamin K cycle. Based on Western blotting of the proteins in extracted microsomal membranes, we estimated that <7% of the 28-kDa VKORC1 pool in rat liver microsomes and <2% in human microsomes are parts of the PDI-VKORC1 complex in rat and human livers. Based on our finding of a putative protein complex formed between VKORC1 and PDI, we propose that operation of the vitamin K cycle and post-translational γ-carboxylation of proteins is linked to protein synthesis and dithiol-dependent oxidative folding of proteins in the ER. As shown in the schematic in Fig. 9, we propose that new proteins become oxidatively folded by PDI, which subsequently acquires reduced CGHC centers. A known pathway for PDI thiol reoxidation in the ER is oxidation by the ER oxidase ERO1, which ultimately leads to formation of H₂O₂ (34, 40). Our data support the notion that part of the reduced PDI protein pool becomes associated with part of the VKORC1 pool in the ER membrane. We hypothesize that the reduced CGHC center in PDI provides electrons for the CXXC center in VKORC1 for Vit.K–O reduction. On the other hand, our data provide conclusive evidence that abnormal concentrations of PDI in the ER can affect synthesis of functional vitamin K-dependent proteins.

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REFERENCES

1. Furie, B., and Furie, B. C. (1988) Cell 53, 505–518
2. Wallin, R., and Hutson, S. M. (2004) Trends Mol. Med. 10, 299–302
3. Furie, B., and Furie, B. C. (1992) N. Engl. J. Med. 326, 800–806
4. Stafford, D. W. (2005) J. Thromb. Hemostasis 3, 1873–1878
5. Wallin, R., Hutson, S. M., Cain, D., Sweatt, A., and Sane, D. C. (2001) FASEB J. 15, 2542–2544
6. Rost, S., Fregin, A., Ivasekvičius, V., Conzelmann, E., Hortnagel, K., Pelz, H. J., Lappegard, K., Seifried, E., Scharrer, I., Tuddenham, E. G., Muller, C. R., Strom, T. M., and Oldenburg, J. (2004) Nature 427, 537–541
7. Li, T., Chang, C.-Y., Jin, D.-Y., Lin, P.-J., Khvorova, A., and Stafford, D. (2004) Nature 427, 541–544
8. Wajih, N., Sane, D. C., Hutson, S. M., and Wallin, R. J. (2005) J. Biol. Chem. 280, 10540–10547
9. Lee, J. I., and Fasco, M. J. (1984) Biochemistry 23, 2246–2252
10. Fasco, M. J., Principe, L. M., Walsh, W. A., and Friedman, P. A. (1983) Biochemistry 22, 5655–5660
11. Goodstadt, L., and Ponting, C. P. (2004) Trends Biochem. Sci. 29, 289–292
12. Rost, S., Fregin, A., Hunerberg, M., Bevans, C. G., Muller, C. R., and Oldenburg, J. (2005) J. Thromb. Hemostasis 94, 780–786
13. Thijsen, H. H., Janssen, Y. P., and Vervoort, L. T. (1994) Biochem. J. 297, 277–280
14. Silverman, R. B., and Nandi, I. N. (1988) Biochem. Biophys. Res. Commun. 155, 1248–1254
15. Preusch, P. C. (1992) FEBS Lett. 305, 257–259
16. Souté, B. A., Groenen-van Dooren, M. M., Holmgren, A., Lundstrom, L., and Vermeer, C. (1992) Biochem. J. 281, 255–259
17. Wilkinson, B., and Gilbert, H. F. (2004) Biochim. Biophys. Acta. 1699, 35–44
18. Wajih, N., Hutson, S. M., Owen, J., and Wallin, R. (2005) J. Biol. Chem., 280, 3160–31607
19. Wallin, R., Sane, D. C., and Hutson, S. M. (2002) Thromb. Res. 108, 221–226
20. Wajih, N., Sane, D. C., Hutson, S. M., and Wallin, R. (2004) J. Biol. Chem. 279, 25276–25283
21. Wajih, N., Borras, T., Xue, W., Hutson, S. M., and Wallin, R. (2004) J. Biol. Chem. 279, 43052–44306
22. Wallin, R., and Martin, L. F. (1985) J. Clin. Investig. 76, 1879–1884
23. Rupp, K., Birnbach, U., Lundstrom, J., Nguyen, Van, P. N., and Soling, H.-D. (1994) J. Biol. Chem. 269, 2501–2507
24. Mandel, R., Ryser, Hugues, J.-P., Ghani, F., Wu, M., and Peak, D. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 4112–4116
25. Orlandi, P. A. (1997) J. Biol. Chem. 272, 4591–4599
26. Tu, B. P., Ho-Schleyer, S. C., Travers, K. J., and Weissman, J. S. (2000)
27. Ahamed, J., Versteeg, H. H., Kerver, M., Chen, V. M., Muller, B. M., Hogg, P. J., and Ruf, W. (2006) Proc. Natl. Acad. Sci. U. S. A. 103, 13932–13937
28. Rustaeus, P., Stillemark, K., Lindberg, Gordon, D., and Olofsson, S. O. (1988) J. Biol. Chem. 273, 5196–5203
29. Schlee, M., Hornung, V., and Hartman, G. (2006) Mol. Ther. 14, 463–470
30. Ellgaard, L., and Ruddock, L. W. (2005) EMBO Rep. 6, 28–32
31. Koivunen, P., Salo, K. E. H., Myllyharju, J., and Ruddock, L. W. (2005) J. Biol. Chem. 280, 5227–5235
32. Papp, E., Nardai, G., Mandl, J., Banhegyi, Y., and Cermely, P. (2005) Biochim. Biophys. Res. Commun. 338, 938–945
33. Tian, G., Xiang, S., Noiva, R., Lennarz, W. J., and Schindelin, H. (2006) Cell 124, 61–73
34. Dias-Gunasekara, S., Gubbens, J., Van Lith, M., Dunne, C., Williams, J. A. G., Kataky, R., Scoones, D., Lapthorn, A., Bulleid, N., and Benham, A. M. (2005) J. Biol. Chem. 280, 33066–33075
35. Benham, A. M., Cabibbo, A., Fassio, A., Bulleid, N., Sitia, R., and Braakman, I. (2000) EMBO J. 19, 4493–4502
36. Asundi, V. K., and Carey, D. J. (1995) J. Biol. Chem. 270, 26404–26410
37. Janiszewski, M., Lopes, L. R., Carmo, A. O., Perro, M. A., Bradnes, R. P., Santos, C. X. C., and Laurindo, F. R. M. (2005) J. Biol. Chem. 280, 40813–40819
38. Pihlajaniemi, T., Helaakoski, T., Tasanen, R., Myllyla, M. L., Huhtala, J., and Kivirikko, K. (1987) EMBO J. 6, 643–649
39. Wetterau, J. R., Coms, K. A., Spinner, S. N., and Joiner, B. J. (1990) J. Biol. Chem. 265, 9800–9807
40. Gross, E., Sevier, C. S., Heldman, N., Vitu, E., Bentzur, M., Kaiser, C. A., Thrope, F., and Fass, D. (2006) Proc. Natl. Acad. Sci. U. S. A. 103, 299–304