Kar2 Modification as a Response to Reactive Oxygen Species Generated from Protein Folding Machinery inside the Endoplasmic Reticulum

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Reactive oxygen species are generated as a byproduct of protein folding inside the endoplasmic reticulum. Hydrogen peroxide is a particular form of reactive oxygen that can damage and modify DNA as well as cellular proteins. Previous studies in our lab identified Kar2, a chaperone protein in the family of Heat Shock Protein 70kDa (HSP70), as a target for peroxide modification which also conferred the cell's resistance against oxidative stress. Kar2 contains a conserved cysteine thiol that is susceptible to redox modification by several candidate modifiers including hydrogen peroxide and glutathione, but it still remains elusive which modification of Kar2 provides protection against oxidative stress. In this thesis, I utilized a recently described enzyme-linked immunosorbent assay to detect peroxide and glutathione modifications of Kar2 in vitro. *Results of my experiments show that Kar2 can be modified by glutathione (glutathionylation) through a sulfenic acid intermediate (sulfenylation) that requires the presence of Kar2's conserved cysteine residue. Similar experiments also show that glutaredoxin 6 and 7, thiol oxidoreductases present in the early secretory pathway, have the ability to de-glutathionylate Kar2* in vitro, *and thus may be involved in this signaling pathway. In a related but distinct project, I also show in this thesis that human* CYB5R *genes, sequential homologs of yeast* PGA3 *whose product serves as a potential electron acceptor alternative to oxygen, cannot complement for the loss of* PGA3 *in yeast.*

Reactive oxygen species (ROS) are often depicted as a threat to human health because they are associated with various diseases including cancer, neurodegenerative disease and aging (1-3). ROS can be generated within multiple compartments of the cell, but the endoplasmic reticulum (ER) serves as a special source of ROS in that every disulfide bond broken and formed during protein folding contributes to its generation. This process involves the transfer of electrons from disulfides of substrate proteins to protein disulfide isomerase (PDI), a member of the thioredoxin family that catalyzes the breakage and formation of disulfide bonds. The electrons are then transferred to ER oxidoreductin 1 (Ero1) that catalyzes the oxidation of protein dithiols, and ultimately to molecular oxygen to generate hydrogen peroxide (4). Hydrogen peroxide (H_2O_2) is a ROS that can damage cellular components including DNA and proteins.

Interestingly, generating excessive H_2O_2 in the ER through a constitutively active Ero1 mutant (5) resulted in an unexpected modification of yeast chaperone protein Kar2, a member of the Heat Shock Protein 70kDa family (HSP70). Also called binding immunoglobulin protein (BiP) in humans, Kar2 notably has a single conserved cysteine residue. It has been widely reported that H_2O_2 can oxidize a cysteine thiol group (-SH) into a sulfenic acid (-SOH), and indeed our lab has demonstrated Kar2's peroxide modification *in vivo*. However, sulfenic acid (-SOH) is also very labile, and can undergo further oxidations into sulfinic ($-SO_2H$) and sulfonic acids ($-SO_3H$) in presence of excess H_2O_2. If Kar2 is to be involved in a reversible oxidative stress signaling pathway, such hyper-oxidation can prove detrimental because it can lead to irreversible loss of protein function. Therefore, we sought out for the existence of another mechanism that protects Kar2's cysteine residue. Attachment of glutathione, a tri-peptide reductant present in milli-molar concentrations inside the cell including the ER lumen, to oxidized cysteine residue is a recurring theme in many instances of protein protection against

irreversible oxidation (6). Furthermore, *in vivo* analysis in our lab showed that bulky amino substitutions of Kar2's cysteine residue that did not resemble sulfenic acid but rather glutathione conferred the cells protection against oxidative stress.

In this honors thesis, we sought to test if Kar2 is susceptible to glutathione modification (glutathionylation) in face of H_2O_2 that oxidizes the cysteine thiol into a sulfenic acid (sulfenylation). This hypothesis was tested by utilizing an enzyme-linked immunosorbent assay (ELISA), in which purified proteins were coated onto plastic wells and subsequently treated with different chemicals to induce protein modification. Using ELISA as our *in vitro* system allowed us to eliminate many variables that can be cumbersome to control in a living system, and also to swiftly change modification conditions without having to dialyze the sample after each chemical treatment. Furthermore, the involvement of glutathione in Kar2 modification suggested that glutaredoxins, thiol oxidoreductase proteins that detach glutathione from substrates, may be involved in Kar2's de-modification process. The involvement of glutaredoxins 6 and 7 (Grx6 and Grx7) that are localized to the early secretory pathway (7) was studied by using ELISA and halo assay that detects inhibition of cell growth in presence of chemicals.

In a related second project, we also sought to identify the human homolog of a potential alternate electron acceptor in the ER lumen known as Pga3. If we recollect that each disulfide bond broken and formed contributes to ROS generation, we can imagine how overwhelmingly abundant ROS would be as a result of the protein folding process if oxygen were the only electron acceptor of Ero1. Pga3 is part of the cytochrome b5 reductase family and carries a flavin adenine dinucleotide (FAD) cofactor to function as an electron transporter. Pga3 may act as an alternative electron acceptor of Ero1 to oxygen, but its function is still relatively unknown. Humans have four sequence homologs of Pga3, the cytochrome b5 reductase genes (*CYB5R1-4*),

but it is still unclear from sequence alone which is the functional homolog of *PGA3*. In this thesis, we perform complementation assays to find out which human *CYB5R* gene, if any, can complement for loss of yeast *PGA3*.

EXPERIMENTAL PROCEDURES

Plasmid construction – All plasmids used in this thesis are listed in Table 1. The gene sequences for *KAR2*, *GRX6* and *GRX7* were retrieved from the Saccharomyces Genome Database. Primers for PCR were designed based on the gene sequences to amplify the DNA coding for Kar2 amino acids N48-E428, Grx6 amino acids K37-end and Grx7 amino acids V34-end. Primers were used to amplify the desired sequences from genomic DNA prepared from the wild type yeast strain CSY5. Primers were flanked with restriction enzymes used to subclone the PCR fragments into the same restriction sites in the pET28-a vector (Table 1). Final plasmids express proteins with an N-terminal His6 tag. A mutant version of Kar2 (Kar2-C63A) was created by site-directed mutagenesis using the QuikChange protocol.

Protein purification – Plasmids isolated using the standard alkaline lysis miniprep procedure were transformed into BL21 (DE3) competent *E. coli* cells. The cells were cultured overnight in 25mL of Terrific Broth (TB) with 50μg/ml of kanamycin at 37°C, then transferred into 1L of TB with 50μg/ml of kanamycin and grown until the OD_{600} had reached about 2.0. The temperature was brought down to 18°C for an hour, and IPTG was added to a final concentration of 0.5mM to induce protein expression. After IPTG addition, the cells were incubated at 18°C overnight (~ 20 hours). The resulting cell culture was centrifuged at 4000 rpm for 25 minutes,

and resuspended on ice in 5mL lysis buffer (100mM HEPES, 500mM NaCl, 10% glycerol, 10mM imidazole, 0.5mM TCEP, 1 mg/ml lysozyme, 2000 U Benzonase, one tablet of Roche Complete EDTA-free protease inhibitor). The cell suspension was sonicated under a microtip sonicator at maximum the setting in 30 second intervals (rest 2 min) for six times, and centrifuged at 10, 000 x g for 30 minutes at 4°C to remove insoluble material.

The soluble supernatant containing various proteins was put through a 5mL HiTrap Chelating HP column charged with 0.1M $NiSO_4$. The column was then washed first with 5 column volumes of IMAC wash1 buffer (20mM HEPES, 500mM NaCl, 10% glycerol, 10mM imidazole, pH 7.5), then with 5 column volumes of IMAC wash2 buffer (20mM HEPES, 500mM NaCl, 10% glycerol, 25mM imidazole, pH 7.5), and finally eluted with 10~30 column volumes of IMAC elution buffer (20mM HEPES, 500mM NaCl, 10% glycerol, 500mM imidazole, pH 7.5). 2.5mL fractions were collected, and the most concentrated three to six fractions based on SDS-PAGE were combined, and buffers were exchanged using a HiPrep 26/10 desalting column. Kar2 proteins were exchanged into Gel Filtration buffer (20mM HEPES, 300mM NaCl, 10% glycerol, pH7.5), and Grx proteins were exchanged into Grx storage buffer (20mM Tris-HCl pH 8, 50mM NaCl, 10% glycerol). The final protein concentrations were measured using the standard BCA protocol. Proteins were aliquoted into 1mL tubes, flash frozen using liquid nitrogen, and stored at -80°C.

Enzyme-linked immunosorbent assay - 50µl of proteins at 25µg/ml were coated onto Thermo Scientific Nunc MaxiSorp plastic wells in 50mM carbonate/bicarbonate buffer, pH 9.6 (1.59g Na_2CO_3, 2.93g $NaHCO_3$ dissolved in 1L milli-Q water) for 1 hour at room temperature (RT). All subsequent reagents were diluted to appropriate concentrations in phosphate buffered

saline containing 0.05% tween-20 (PBST, 8.00g NaCl, 0.20g KH_2PO_4,1.15g Na_2HPO_4, 20g KCl and 0.5ml Tween-20 dissolved in 1L milli-Q water). All steps were followed by three washes with 200µl PBST for 5 minutes at RT with the samples rocking. Proteins were reduced with 100µl 5mM DTT for 5 minutes at 37°C. After washing, wells were treated with varying concentrations of H_2O_2 or diamide (final 100µl), washed, and then treated with 100µl 1mM GSH. Mouse-derived anti-glutathione antibody purchased from ViroGen was used at 1:2500 dilution for 1 hour at RT (100µl). Goat-derived anti-mouse alkaline phosphatase antibody purchased from Invitrogen™ was used at 1:5000 dilution for 1 hour at RT (100µl). p-Nitrophenyl Phosphate (pNPP) solution was prepared by adding one tablet of Thermo Scientific pNPP into a solution mixture of 4mL water and 1mL 5X diethanolamine substrate buffer (from the Thermo Scientific PNPP substrate kit). Signal was developed in the dark at RT until pale yellow color developed (12~16 hrs using anti-GSH antibody, 15 min using anti-Kar2 antibody). Glutaredoxin proteins were added when necessary.

Halo assay – CSY318 (*GAL2 ura3-52 leu2-3,112 kar2Δ::KanMX* [pCS757]), CSY579 (*GAL2 ura3-52 leu2-3,112 kar2Δ::KanMX grx6Δ::KanMX* [pCS757]) and CSY580 (*GAL2 ura3-52 leu2-3,112 kar2Δ::KanMX grx7Δ::KanMX* [pCS757]) were used, which all contain the *CEN LEU2 KAR2-FLAG* plasmid pCS757. These yeast strains were transformed with pRS316 empty vector or with pRS316-based plasmids encoding for different FLAG-tagged *grx* mutants (pDS21: *grx6-C136A*; pDS22: *grx6-S139A*; pDS26: *grx7-C108A*; pDS27: *grx7-S111A*). Transformed cells were inoculated and grown to saturation in SMM-uracil-leucine liquid medium overnight, and approximately 0.2 OD_{600} equivalent of cells were spread onto SMM-uracil-leucine plates. A paper disc was placed in the center of each plate and soaked with 15µl of 9.8M H_2O_2. Plates

were incubated at 30°C overnight.

Complementation assay – cDNAs encoding for the human *CYB5R* genes from the Mammalian Genome Collection were purchased from Thermo Scientific. PCR primers were designed to amplify the DNA encoding for the entire open reading frame (ORF) or a portion of the ORF (*CYB5R1*, *CYB5R2*, *CYB5R3*, *CYB5R4*, truncated *CYB5R4* encoding for amino acid residues G164–end, and truncated *CYB5R4* encoding for amino acid residues K260-end). These genes and yeast *PGA3* gene (amplified by PCR from our lab plasmid pHS17) were cloned into a yeast expression vector that contained both the *GPD(TDH3)*-promoter and CYC1 terminator. This expression vector was generated by ligating the insert from pPM28 (P_{TDH3}-*kar2ss-roGFP2-HDEL (eroGFP)*) into the pRS315 vector backbone using SacII and HindIII restriction enzymes (Table 1). Resulting plasmids (pDS48-52, 56, 57) contained the *PGA3* or *CYB5R* genes in place of the *eroGFP* ORF. Plasmids were transformed into CSY421 (*GAL2 ura3Δ0 leu2Δ0 his3Δ1 pga3Δ::KanMX* [*URA3 CEN PGA3*]) and CSY542 (*GAL2 ura3-52 leu2-3,112 pga3-L77V M262K*) which contains a temperature sensitive *pga3* gene. Transformed CSY421 strains were inoculated into SMM-leucine media. incubated at 30°C overnight, and frogged onto SMM plates containing 5-FOA to select against cells that have kept the original *URA3 PGA3* plasmid. Plates were incubated at 30°C overnight. Transformed CSY542 strains were inoculated in SMM-leucine media, incubated at 24°C overnight, and frogged onto SMM-leucine plates and incubated at 24°C, 30°C and 37°C overnight.

Table 1. Plasmids used in this thesis

Plasmid Name	Plasmid Backbone	Product	Markers	Restriction Enzyme used Cloning
pET28-a	pET28-a	Empty	*kanR*	-
pJO1	pET28-a	Kar2-(N48-E428)	*kanR*	-
-	pET28-a	Kar2-(N48-E428) –C63A	*kanR*	-
pDS1	pET28-a	Grx6-(K37-N231)	*kanR*	NheI / XhoI
pDS3	pET28-a	Grx7-(V34-A203)	*kanR*	NdeI / XhoI
pRS316	pRS316	Empty	*URA3 CEN ampR*	-
pDS21	pRS316	Grx6-C136A-FLAG	*URA3 CEN ampR*	-
pDS22	pRS316	Grx6–S139A-FLAG	*URA3 CEN ampR*	-
pDS26	pRS316	Grx7–C108A-FLAG	*URA3 CEN ampR*	-
pDS27	pRS316	Grx7–S111A-FLAG	*URA3 CEN ampR*	-
pPM28	pRS316	Kar2ss-eroGFP2-HDEL (eroGFP)	*URA3 CEN ampR*	-
pRS315	pRS315	Empty	*LEU2 CEN ampR*	-
pDS47	pRS315	Kar2ss-eroGFP2-HDEL (eroGFP)	*LEU2 CEN ampR*	SacII / HindIII
pDS48	pDS47	CYB5R1	*LEU2 CEN ampR*	NheI / XbaI
pDS49	pDS47	CYB5R2	*LEU2 CEN ampR*	NheI / XbaI
pDS50	pDS47	CYB5R3	*LEU2 CEN ampR*	NheI / XbaI
pDS51	pDS47	CYB5R4	*LEU2 CEN ampR*	NheI / XbaI
pDS52	pDS47	Pga3	*LEU2 CEN ampR*	NheI / XbaI
pDS56	pDS47	CYB5R4-(G164-end)	*LEU2 CEN ampR*	NheI / XbaI
pDS57	pDS47	CYB5R4-(K260-end)	*LEU2 CEN ampR*	NheI / XbaI

RESULTS

Kar2 can be glutathionylated through a sulfenic acid intermediate – Previously, it has been shown that actin undergoes S-glutathionylation, a process by which glutathione becomes attached to a protein's free thiol group (8,9). There are two proposed mechanisms for this reaction, one involving the interaction between reduced glutathione (GSH) and protein sulfenic acid (P-SOH), and another involving a direct disulfide exchange between oxidized glutathione (GSSG) and protein free thiol (10). Under physiological condition that favors an abundance of GSH over GSSG in the cytoplasm, it has been proposed that the former pathway with a protein

sulfenic acid intermediate better reflects the actual molecular mechanism (8). Similarly, there is an over-abundance of GSH over GSSG in the endoplasmic reticulum where Kar2 resides (11), which suggests that Kar2 may also be glutathionylated via the pathway that involves sulfenic acid (Figure 1A).

In order to test if sulfenylation of Kar2's thiol primes Kar2 for glutathionylation, we performed an ELISA by sequentially treating Kar2 with H_2O_2 of varying concentrations and 1mM GSH. Sequential treatment with H_2O_2 and GSH allowed us to circumvent the formation of GSSG through the interaction of the two molecules. In this assay, mouse derived anti-GSH antibody, followed by goat derived anti-mouse antibody conjugated with alkaline phosphatase were used to detect the presence of glutathione on samples. The strength of the Kar2 glutathionylation signal was detected by measuring the absorbance of light at 405nm that developed through the reaction of alkaline phosphatase with p-Nitrophenyl Phosphate. The result of the assay showed that the extent to which Kar2 was glutathionylated was positively correlated with H_2O_2 concentration up to 5mM, after which the glutathionylation signal actually decreased (Figure 1B). The decrease in Kar2 glutathionylation signal at high peroxide concentrations was suggestive of irreversible over-oxidation of the protein, in line with our model that ROS overabundance results in protein hyper-oxidation, which can lead to loss of protein activity. The slight difference between no GSH and GSH without H_2O_2 could possibly be attributed either to unspecific binding of GSH to plastic wells, or to the miniscule presence of peroxide in the buffer used. This result suggests that Kar2 can be glutathionylated via a sulfenic acid intermediate.

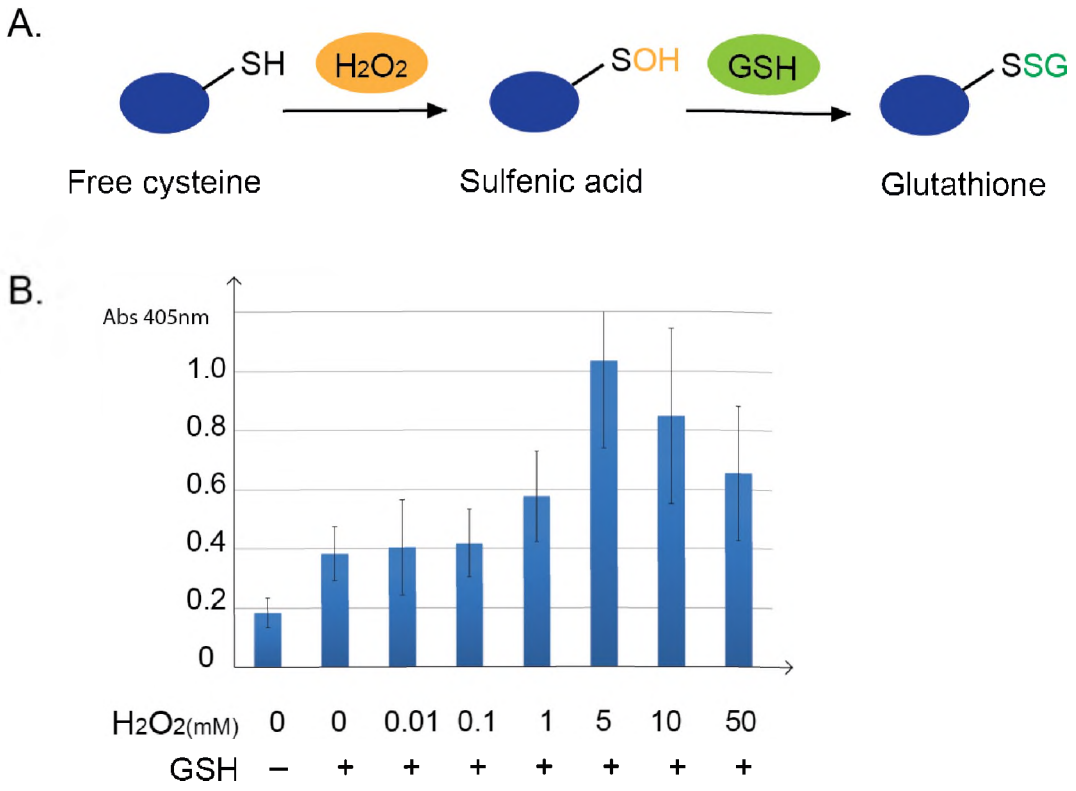

Figure 1. Kar2 glutathionylation involves a sulfenic acid intermediate. (A) Schematic diagram showing Kar2 modification. Kar2 is thought to be modified at its cysteine residue by the sequential formation of sulfenic acid and glutathionylated protein. **(B)** ELISA showing glutathionylation of purified Kar2 protein. Coated Kar2 protein was sequentially treated with varying concentrations of H_2O_2 for 10 minutes, then with 1mM GSH for 15 minutes at room temperature. Glutathionylation signal was detected by using mouse derived anti-GSH antibody and subsequently goat derived anti-mouse antibody conjugated to alkaline phosphatase.

Kar2 glutathionylation is dependent on the protein's conserved cysteine residue – Kar2 has only one cysteine residue, but interestingly it is at the same time highly conserved among species (Figure 2A). The fact that this cysteine is highly conserved suggests that an important regulatory function can be attributed to this residue. One possible role of this residue may be to act as a switch sensor for oxidative stress by getting reversibly oxidized and glutathionylated, because glutathionylation requires the presence of a free thiol.

To test that Kar2 glutathionylation is absolutely dependent on its cysteine residue, we performed an ELISA using a purified a Kar2 cysteine-to-alanine (Kar2-C63A) mutant. When we treated wild type Kar2 and its C63A mutant with 5mM H_2O_2 and 1mM GSH, only the wild type but not the cysteine mutant exhibited glutathionylation (Figure 2B). When treated with a strong reducing agent dithiothreitol (DTT), all signals were decreased suggesting that these signals were indeed from disulfide bonds and not from unspecific attachments. This result was not an artifact of unequal protein coating, because the wild type and mutant Kar2 showed equal anti-Kar2 antibody signals, as compared to the negative control using bovine serum albumin that does not contain Kar2-specific antigen motif (Figure 2C). These results verify that Kar2 glutathionylation is absolutely dependent on its conserved cysteine residue.

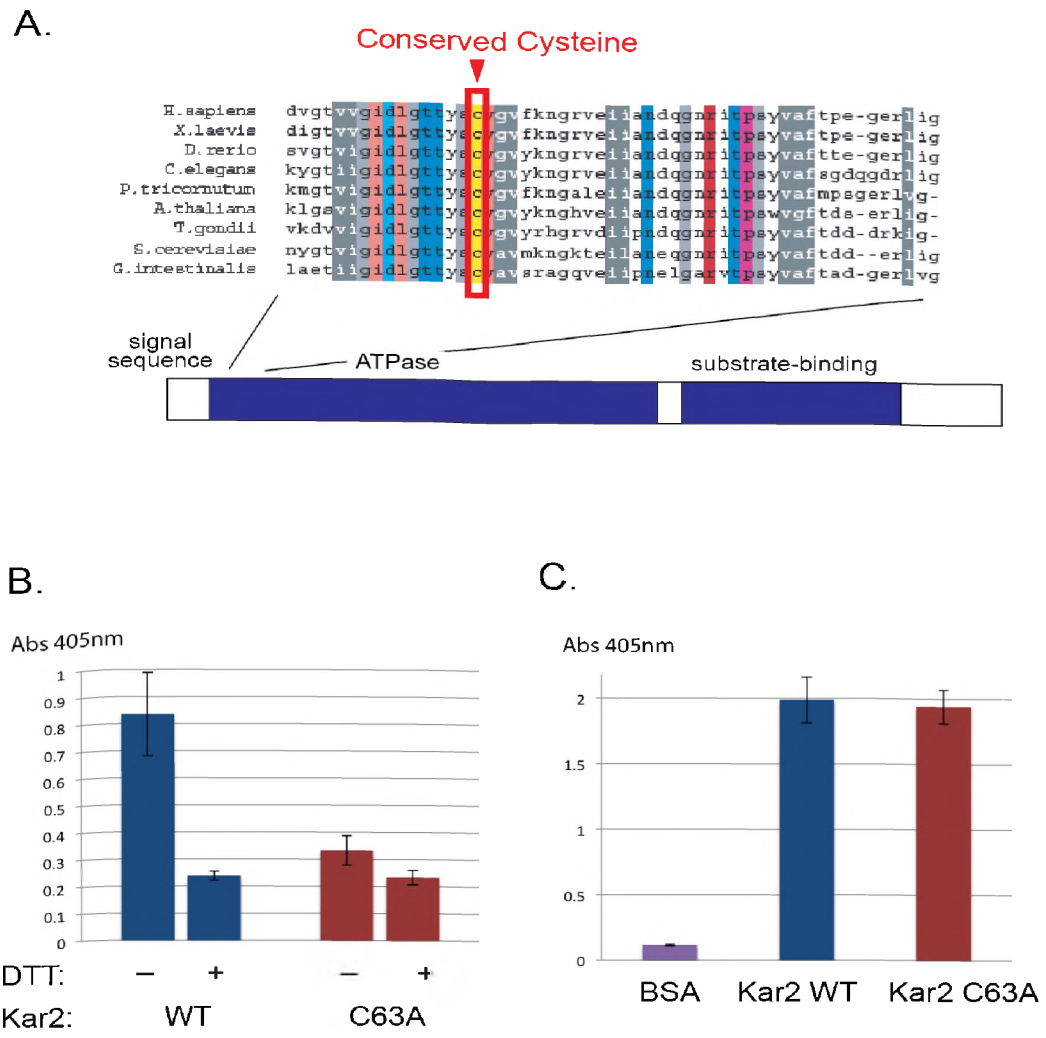

Figure 2. Kar2 modification is dependent on its conserved cysteine residue. (A) Diagram showing the conservation of a cysteine residue in the ATPase domain of Kar2. **(B)** ELISA showing glutathionylation of wild type versus C63A mutant versions of Kar2. Proteins were glutathionylated by treating with 5mM H_2O_2 and 1mM GSH for 15 minutes at room temperature. DTT was used at 5mM for 5 minutes to reduce all disulfide bonds. **(C)** ELISA showing equal coating of Kar2 wild type and C63A mutant. BSA and Kar2 proteins at 25µg/ml were coated, and then detected using anti-Kar2 antibody used at 1:1000 dilution.

Kar2 glutathionylation can be reversed by the actions of Grx6 and 7 in vitro – *In vivo* analysis in our lab showed that mutation of the conserved cysteine residue into bulky amino acids that may mimic a glutathionylated cysteine disrupts Kar2's normal ATPase activity (unpublished data). In order for Kar2 to be recycled after the termination of oxidative stress and to resume its normal chaperone function, it is crucial that the cysteine residue be de-modified. Glutaredoxins are thiol oxidoreductases that reduce disulfide bonds by using GSH, and it was recently discovered that two members of this family, Grx6 and Grx7, reside in the early secretory pathway (7). Interestingly, *GRX6* and *GRX7* deleted yeast strains show increased sensitivity towards H_2O_2 and diamide (12), implying the genes' involvement in oxidative stress.

Therefore, we tested whether purified Grx6 and Grx7 proteins can de-glutathionylate Kar2 by using the ELISA. Kar2 was first glutathionylated by adding 1mM diamide and 1mM GSH and incubating for 20 minutes at room temperature. Treating glutathionylated Kar2 with DTT, 1μM Grx6 and 1μM Grx7 proteins for 5 minutes at 37°C visibly reduced the glutathionylation signal (Figure 3). Although no GSH was added with Grx proteins to regenerate their free thiols after one enzymatic activity, enough proteins were added to de-glutathionylate Kar2. Interestingly, Grx7 exhibited a higher enzymatic activity than Grx6, in accordance with our previous result (data not shown) and with previous findings from the literature (7). The result of this experiment demonstrates that both Grx6 and 7 have the ability to de-glutathionylate Kar2 *in vitro*.

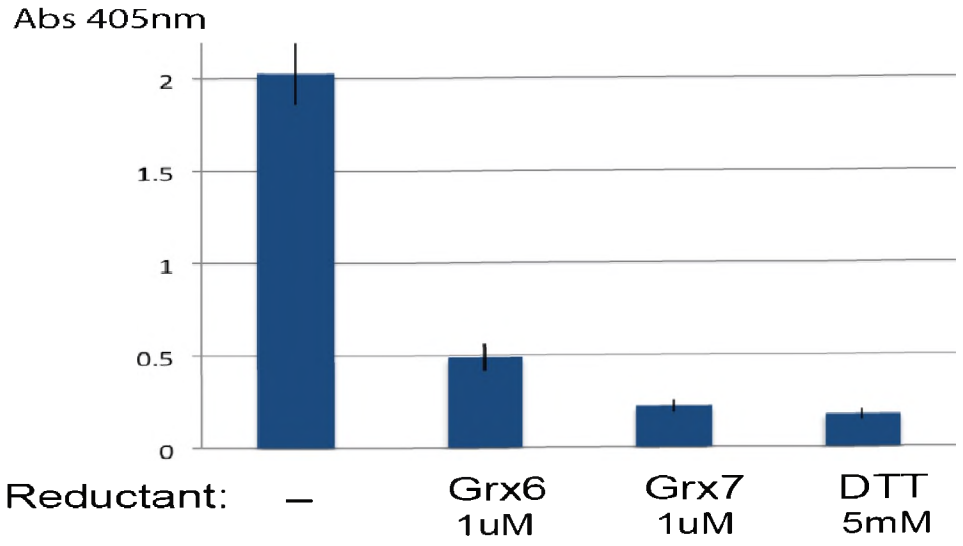

Figure 3. Grx6 and Grx7 can de-glutathionylate Kar2. ELISA showing de-glutathionylation of Kar2 by the activities of Grx6 and Grx7. Kar2 was fully glutathionylated by treating 1mM diamide and 1mM GSH for 20 minutes at room temperature. Subsequently, 1μM Grx6, 1μM Grx7 or 5mM DTT was added for 5 minutes at 37 °C.

GRX6 deletion strain's sensitivity towards oxidative stress is associated with Grx6 catalytic activity – By using ELISA, we were able to show that Grx6 and Grx7 have the ability to detach glutathione from Kar2 *in vitro*. This suggested that the glutaredoxin proteins may be involved in oxidative stress regulation. In fact, previous studies have shown that deleting *GRX6* and *GRX7* genes in yeast strains can induce increased sensitivity to different oxidants including H_2O_2 and diamide (12). However, the studies did not proceed to verify that the *GRX* deletion strains' sensitivity towards oxidants was associated with Grx catalytic activity. In order to test that this sensitivity towards oxidative stress is associated with Grx catalytic activity, we introduced mutations in the catalytic Cys-X-X-Ser motifs (where X = any amino acid) of *GRX6* and *GRX7* genes contained on pRS316-derived plasmids (pDS21:Grx6 C136A; pDS22: Grx6 S139A; pDS26: Grx7 C108A; pDS27: Grx7 S111A).

By inoculating cells on plates with paper discs loaded with 15µl of 9.8M H_2O_2, we could observe different sizes in the zone of growth inhibition called halos. The assay allowed us to verify that the *grx6Δ* strain was more sensitive to H_2O_2 than the wild type, and that introducing *GRX6* back into the strain mitigated this sensitivity (Figure 4). Among the *grx6Δ* strains transformed with different *grx6* mutants, the catalytic cysteine mutant showed increased sensitivity to H_2O_2, suggesting that this sensitivity was associated with protein catalytic activity. *GRX7* deletion strain, however, did not show much difference regardless of whether wild type or mutant *GRX7* genes were introduced back into the strain. This was an unexpected result that should be addressed through further studies. In summary, the halo assay provides evidence that at least grx6Δ strain's sensitivity towards oxidative stress is related to Grx6 catalytic activity.

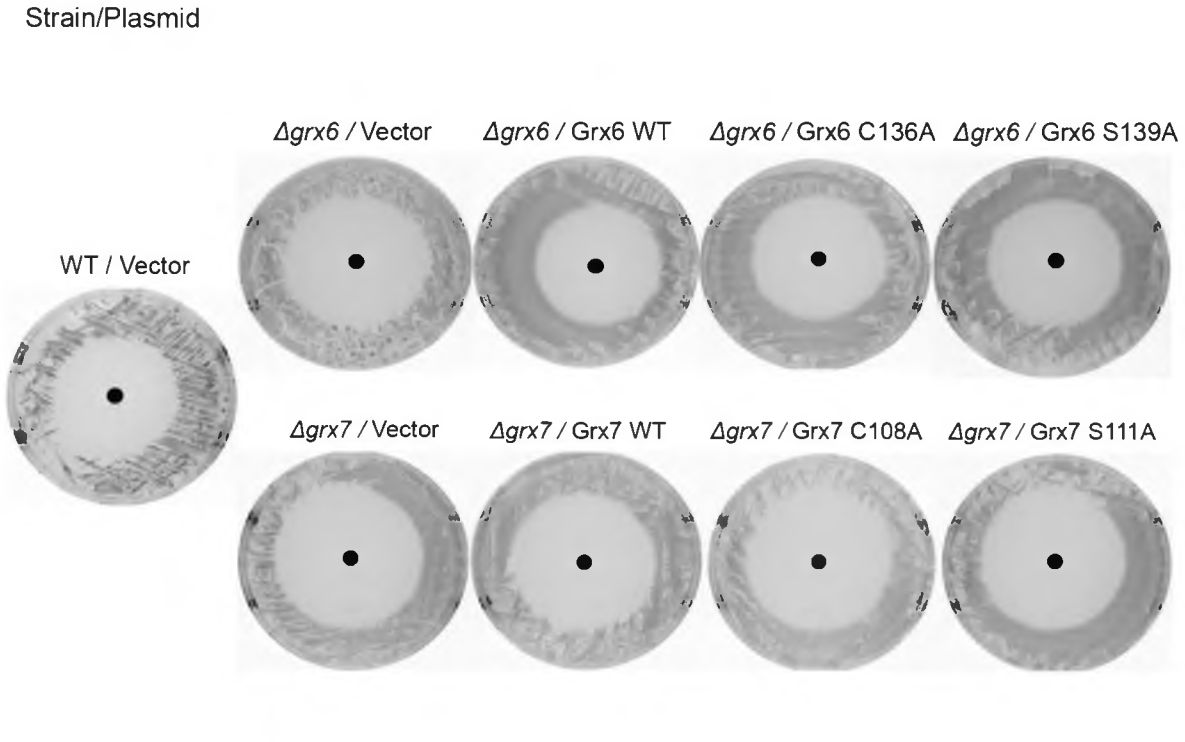

Figure 4. *GRX6* deletion strain is sensitive to H_2O_2 in a manner dependent on Grx6 catalytic activity. Wild type yeast strain was compared to GRX6 deletion strain transformed with wild type *GRX6* and *grx6* catalytic site mutants including C136A and S139A, and to *GRX7* deletion strain transformed with wild type *GRX7* and *grx7* catalytic site mutants including C108A and S111A. 15µl of 9.8M H_2O_2 was added to the filter discs in the middle, and the plates were incubated at 30°C overnight. Sizes of the zones of growth inhibition were observed.

***No single human* CYB5R *gene efficiently complements yeast* PGA3** – Due to the incredible amount of electron flow during disulfide formation and reduction inside the endoplasmic reticulum, other sources of electron acceptor likely exist beside oxygen to accept electrons from Ero1 to. Pga3 is a member of the cytochrome b5 reductase family, and is a potential electron transporter with a FAD cofactor. Furthermore, a large scale genetic study showed that Pga3 has a similar drug sensitivity profile to Ero1 (13). Therefore, Pga3 is among the potential electron acceptors of Ero1, and our lab is working to explore the relationship between the two proteins. Interestingly, there are four homologs of *PGA3* in humans, *CYB5R1-4*. Numerous studies suggest that *CYB5R* genes are associated with oxidative stress (14-16), but it is unclear which gene can actually complement Pga3 function, which is essential in yeast.

To test which of the human *CYB5R* genes can complement loss of *PGA3*, we cloned *CYB5R* genes and yeast *PGA3* into yeast expression vectors, then transformed them into two different yeast strains. One of the strains was *pga3Δ* transformed with a *PGA3* plasmid with a *URA3* marker, and another had temperature sensitive *pga3*, whose gene product showed a drastic drop in activity at higher temperatures. Truncated pieces of *CYB5R4* were separately cloned, because this gene is larger than other members, and encodes for the oxidoreductase domain in its C-terminal region. We used SMM + 5-FOA plates to actively select for cells that have lost the original *PGA3* copy and kept only the newly transformed *CYB5R1-4* or *PGA3* plasmids. The result from this complementation assay showed that none of the human *CYB5R* genes but only the yeast *PGA3* could sustain cell survival (Figure 5A). Similarly, complementation assay using temperature sensitive pga3 showed that none of the human *CYB5R* genes but only the yeast *PGA3* could sustain cell survival at non-permissive temperatures of 30°C and 37°C (Figure 5B). These results show that none of the *CYB5R* genes can complement loss of *PGA3* by itself.

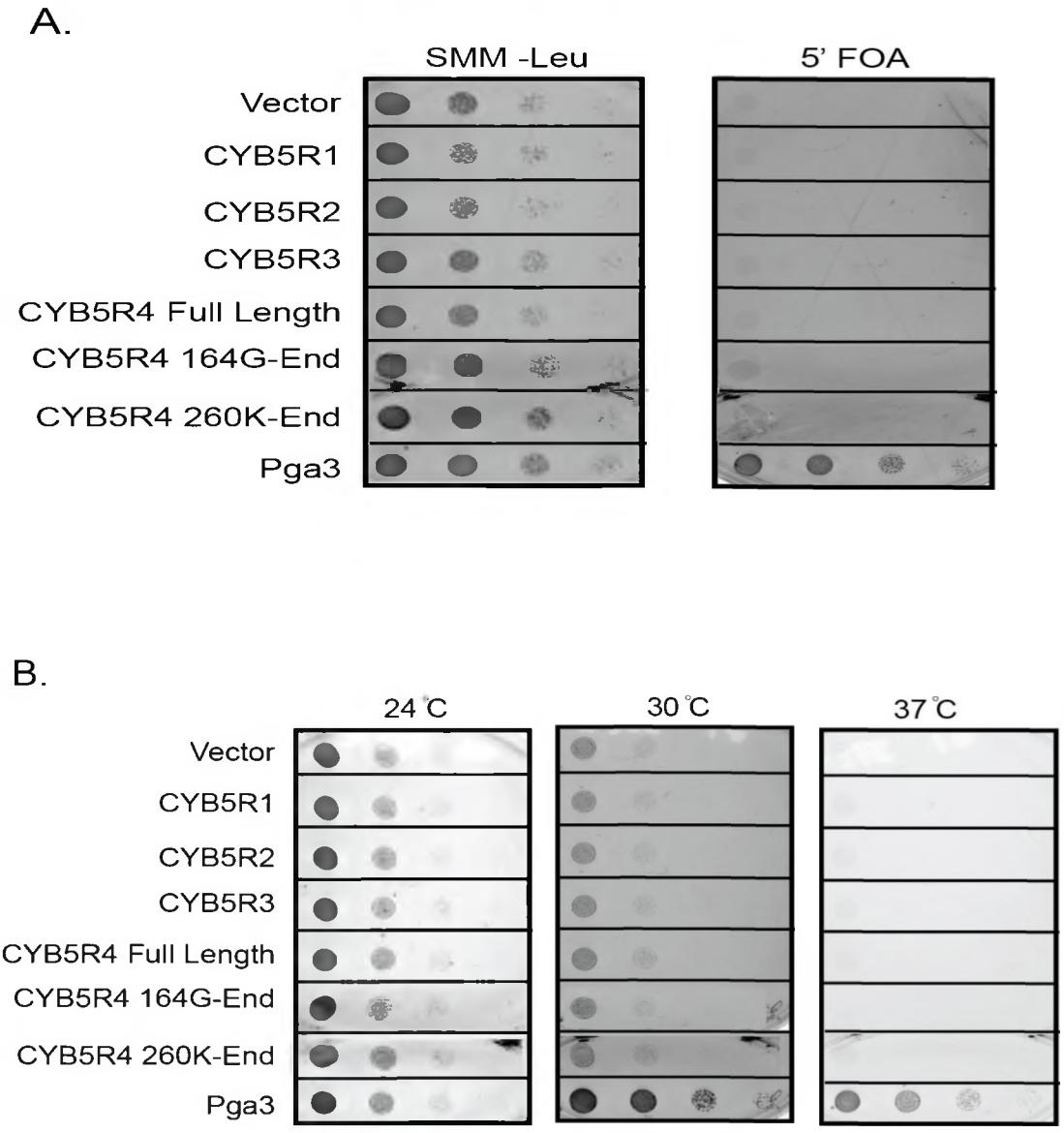

Figure 5. No human *CYB5R* gene can complement yeast *PGA3* on its own. **(A)** *PGA3* delete yeast strain transformed with a *PGA3* plasmid (*URA3* marker) was transformed with different human *CYB5R* genes and with yeast *PGA3*. After selecting for cells that have lost the original *PGA3* plasmid by plating on 5-FOA plates, growth was observed. **(B)** *pga3* temperature sensitive strain was transformed with different human *CYB5R* genes and with yeast *PGA3*. Growth was observed at a permissive (24°C) and at non-permissive temperatures (30°C and 37°C).

DISCUSSION

Protein folding is a crucial process in normal cell function but it can also generate reactive oxygen species as a byproduct and can present a threat to the stability of intracellular contents including DNA and proteins. Specifically, a flow of electrons is initiated in the ER when folding proteins pass electrons from disulfide bonds onto PDI, Ero1, and finally to oxygen. Therefore, understanding how the cell regulates this flow of electrons becomes essential in our study of oxidative stress regulation.

In this honors thesis, we show that hydrogen peroxide generated in the ER can oxidize the protein Kar2, a chaperone protein of the HSP70 family. By utilizing an ELISA, we showed that Kar2's conserved cysteine residue can be sulfenylated, and then subsequently glutathionylated. In support of this result, treatment with sodium arsenite and 5,5-Dimethylcyclohexane-1,3-dione that specifically reduce or trap sulfenic acid and prevent glutathionylation indeed seemed to reduce glutathionylation signal when added in addition to H_2O_2 (data not shown). Importantly, glutathionylation may protect Kar2 from being over-oxidized, as high peroxide concentrations led to a decrease in glutathionylation, probably due to over-oxidation of the protein that results in irreversible protein modification (Figure 6). How glutathionylation of Kar2 actually confers cells protection against oxidative stress remains a question to be addressed in further studies.

Using the ELISA, we also showed that Kar2 modification could be reversed by the action of Grx6 and Grx7 proteins that are in the oxidoreductase family and reside in the early secretory pathway. However, our data could only confirm the possibility of modified Kar2 interacting with Grx6 and Grx7, as the ELISA is an *in vitro* assay. Further *in vivo* analysis would be required to show that these proteins actually interact inside the cell. In line with the idea that

Grx6 and Grx7 may be involved in oxidative stress response, a *GRX6* deletion strain exhibited increased sensitivity towards oxidative stress in a halo assay in a manner associated with protein catalytic activity. The fact that the *GRX6* deletion strain had a more distinctive phenotype in the face of oxidative stress than the *GRX7* deletion strain may be related to the fact that Grx6 may have a bigger role than Grx7 in the cell due to its higher abundance, even though Grx7 shows higher enzymatic activity *in vitro (12)*.

Pga3 is a member of the cytochrome b5 reductase family, and a potential candidate as an alternate electron acceptor to oxygen. There are four sequence homologs of Pga3 in humans, and we tried to find if any could complement yeast *PGA3* function. The results from our complementation assay suggested that none of the four human *CYB5R* genes could complement for loss of *PGA3*. This may be due to the presence of four, instead of one genes in humans that perform the activities of yeast *PGA3*. During the course of evolution, the four CYB5R proteins could have assumed different functions of Pga3 so that no single protein can any longer complement Pga3. This hypothesis could be addressed by studying the complementation of *PGA3* by a combination of human *CYB5R* genes. Alternatively, it could be the case that the human *CYB5R* genes were not properly expressed in yeasts due to differences specific to the species. This hypothesis could be tested first by checking for gene expression, then seeking other ways to better express the human genes in yeast if they are indeed not ideally expressed.

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