Functional Differences in Yeast Protein Disulfide Isomerases

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Abstract. *PDI1* is the essential gene encoding protein disulfide isomerase in yeast. The *Saccharomyces cerevisiae* genome, however, contains four other nonessential genes with homology to *PDI1*: *MPD1*, *MPD2*, *EUG1*, and *EPS1*. We have investigated the effects of simultaneous deletions of these genes. In several cases, we found that the ability of the *PDI1* homologues to restore viability to a *pdi1*-deleted strain when overexpressed was dependent on the presence of low endogenous levels of one or more of the other homologues. This shows that the homologues are not functionally interchangeable. In fact, *Mpd1p* was the only homologue capable of carrying out all the essential functions of *Pdi1p*. Furthermore, the presence of endogenous homologues with a CXXC motif in the thioredoxin-like domain is required for suppression of a *pdi1* deletion by *EUG1* (which contains two CXXS active site motifs). This underlines the essentiality of protein disulfide isomerase-catalyzed oxidation. Most mutant combinations show defects in carboxypeptidase Y folding as well as in glycan modification. There are, however, no significant effects on ER-associated protein degradation in the various protein disulfide isomerase-deleted strains.

Key words: protein disulfide isomerase • ER • protein folding • carboxypeptidase Y • *Saccharomyces cerevisiae*

Introduction

An important aspect of folding of secretory proteins is the formation of native disulfide bonds, a process catalyzed by protein disulfide isomerase (PDI)[1] (Givol et al., 1995). In the eukaryotic cell, the endoplasmic reticulum is the organelle where folding of secretory proteins occurs, and is the location of PDI as well as other chaperones. PDI-like proteins can be identified by one or more domains with sequence similarity to thioredoxin, a signal sequence, and the presence of an ER retention signal, (K/H)DEL. Yeast PDI (in this study, PDI is used as a more general term for a PDI-like protein, while Pdi1p specifically refers to the protein encoded by the yeast gene *PDI1*), Pdi1p, contains two thioredoxin-like domains with the active site sequence motif CGHC that is found in the majority of eukaryotic PDIs (Edman et al., 1985). The two cysteines are able to cycle between a reduced and an oxidized state. In the latter state, the two cysteines form an intramolecular disulfide bond that in principle enables PDI to convert a pair of sulfhydryl groups in a polypeptide substrate into a disulfide bond. Recently, the yeast protein Ero1p, has been shown to be essential for thiol oxidation in the ER (Frand and Kaiser, 1998; Pollard et al., 1998), and a pathway has been proposed in which oxidizing equivalents flow from Ero1p to Pdi1p and on to the substrate (Frand and Kaiser, 1999). Glutathione, which was previously thought to play the major role in oxidation in the ER, probably acts more as a reductant (Cuozzo and Kaiser, 1999).

In most organisms, a number of genes encoding PDI-like proteins are found. In *Escherichia coli*, at least four proteins with thioredoxin-like domains have convincingly been shown to be involved in various aspects of disulfide bond formation and shuffling in the periplasm (Åslund and Beckwith, 1999). In eukaryotes, the ER fulfills much the same role as the bacterial periplasm. The number of putative PDIs in the mammalian ER is even higher. However, a specific function has been suggested only for a limited number of these proteins (Ferrari and Söling, 1999). One of the PDI-like proteins that has received the most attention is ERp57, which has been shown to be involved in the ER quality control system. It interacts with calnexin/calreticulin in binding glycoprotein folding intermediates. This activity does not seem to depend on its oxidoreductase properties (Oliver et al., 1997). A specific function is also likely for the pancreatic PDI, PDIp, which is the only known PDI-like

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[1]*Abbreviations used in this paper:* 5-FOA, 5-fluoro-orotic acid; AMS, 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid; CPY, carboxypeptidase Y; ONPG, *o*-nitrophenyl-β-D-galactoside; PDI, protein disulfide isomerase.

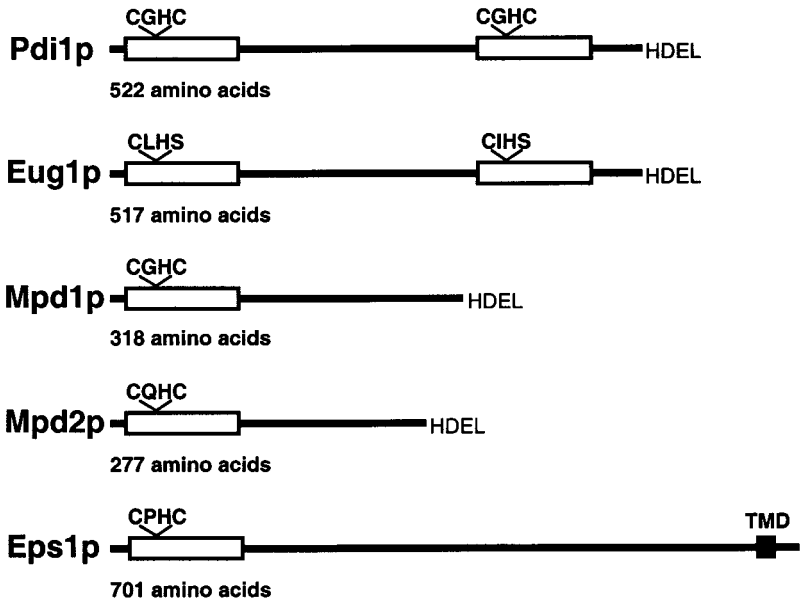

Figure 1. Yeast contains three genes that encode soluble ER proteins with sequence homology to Pdi1p. Open boxes indicate the position and extent of thioredoxin-like domains. Active-site cysteines and the amino acid residues between them are shown. The protein Eug1p has ~40% sequence identity to Pdi1p and contains, like Pdi1p, two regions with sequence homology to thioredoxin. *MPD1* and *MPD2* encode proteins that are smaller than Pdi1p and Eug1p and contain only one thioredoxin-like active site. The protein Eps1p contains one thioredoxin-like region as well as a transmembrane domain (the closed box marked TMD).

protein showing tissue--specific expression (Desilva et al., 1996). The domain organization varies between the different PDIs. For instance, the stress-induced protein ERp72 has three active thioredoxin-like proteins, as compared with the two of Pdi1p (Dorner et al., 1990; Mazzarella et al., 1990). ERp72 and other genes encoding mammalian PDIs are able to suppress the lethal effects of deletion of *PDI1* in yeast (Günther et al., 1993; Laboissière et al., 1995).

In *Saccharomyces cerevisiae*, the complete genome sequence shows that the number of putative PDIs is most likely limited to five, of which the *PDI1* gene is the only essential gene (Farquhar et al., 1991). Overexpression of any of the nonessential homologues *EUG1*, *MPD1*, *MPD2*, or *EPS1*, however, has been found to suppress the lethality caused by deletion of *PDI1* (Tachibana and Stevens, 1992; Tachikawa et al., 1995, 1997; Wang and Chang, 1999). Eug1p shows an overall sequence identity of ~40% to Pdi1p and contains two thioredoxin-like domains (Fig. 1). Unlike all known PDIs, the active sites of Eug1p have a CXXS motif (Tachibana and Stevens, 1992). Thus, the active sites of Eug1p are not able to form intramolecular disulfide bonds. Therefore, Eug1p alone is unable to carry oxidizing equivalents. *MPD1* and *MPD2* encode proteins containing only a single thioredoxin-like domain (Fig. 1). Apart from the thioredoxin domain, they share no further sequence homology with any protein of known function. Overexpression of *EUG1*, *MPD1*, or *MPD2* does not fully complement a *pdi1* deletion since such strains show a reduced folding rate of procarboxypeptidase Y (proCPY) (Tachibana and Stevens, 1992; Tachikawa et al., 1995, 1997).

Recently, a novel PDI-like protein named Eps1p was characterized. It contains a single thioredoxin-like domain and is localized to the ER membrane (Fig. 1). It has been proposed to be involved in ER quality control, as deletion of the gene suppresses a dominant-negative *pma1* mutant. *PMA1* encodes a plasma membrane proton ATPase, which is essential for growth. The mutant in question prevents wild-type Pma1p from being transported to the plasma membrane. In a strain deleted for *eps1*, both mutant and wild-type Pma1p are transported to the plasma membrane, thereby allowing growth (Wang and Chang, 1999).

In previous genetic analyses of *pdi1* complementation, be it heterologous or homologous, the potential contributions from the Pdi1p homologues have been disregarded. With the present work, we wish to determine the role of the homologues in thiol oxidation and, in particular, to determine how important these enzymes are when PDI activity is compromised. To do this, we have constructed yeast strains simultaneously deleted for two or more of these genes. The success of this approach is illustrated by the observation that a mutant form of Pdi1p, $Pdi1p_{CGHS\text{-}CGHS}$, (this nomenclature is adopted for describing the sequence of the two active sites of the protein; the sequence of the more NH_2-terminal active site is written first), which is defective in oxidation, is dependent on an endogenous homologue for its ability to complement the *pdi1* deletion. We have also revealed differences in the functionality between the PDI-like proteins, as *MPD1* is the only *PDI1* homologue that, when overexpressed, is able to suppress a strain deleted for all genes of the *PDI1* family.

Materials and Methods

Media and Materials

Yeast cells were grown in standard YPD and SC media (Sherman, 1991). *E. coli* was grown in LB medium supplemented with the appropriate antibiotic (Sambrook, 1989). Restriction enzymes, T4 DNA polymerase, T4 polynucleotide kinase, and T4 DNA ligase were from Promega. Oligonucleotides were synthesized by DNA Technology (Aarhus). Sequencing was performed using a *Taq* Dye DeoxyTM terminator cycle sequencing kit on an API 373A DNA Sequencer, both from Applied Biosystems. 1,4-Dithiothreitol, 5-fluoro-orotic acid (5-FOA), and *o*-nitrophenyl-β-D-galactoside (ONPG) were from Merck. *N*-acetyl-DL-phenylalanine β-naphtyl ester and calcofluor white were from Sigma-Aldrich. 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (AMS) was from Molecular Probes. [^{35}S]Methionine was from DuPont.

Strains and Plasmids

All plasmids used in this study are listed in Table I. The yeast strains used in this study are listed in Table II.

Table I. Plasmids Used

Name	Gene*	Marker	Source
Plasmids used for complementation‡			
pCT38	PDI1	URA3	This study
pCT58	$EUG1_{CLHC\text{-}CIHC}$	TRP1	Holst et al., 1997
pBH1464	PDI1	TRP1	Holst et al., 1997
pBH1711	$PDI1_{CRRC\text{-}SGHS}$	TRP1	Holst et al., 1997
pBH1800	MPD1	TRP1	This study
pBH1806	MPD2	TRP1	This study
pBH1857	PDI1	TRP1	Holst et al., 1997
pBH1865	EUG1	TRP1	This study
pBH1966	$PDI1_{CGHS\text{-}CGHS}$	TRP1	Holst et al., 1997
	Used for		
Plasmids used for deletion of genes			
pCT19	Deletion of *EUG1*		This study
pPN100	Deletion of *EPS1*		This study
pPN330	Deletion of *MPD1*		This study
pFA6-kanMX3	Deletion of *MPD2*		Wach et al., 1994
	Promoter		
Plasmids containing promoter-*lacZ* fusions			
pPN379	*MPD1* promoter		This study
pPN382	*MPD2* promoter		This study
pPN410	*PDI1* promoter		This study
pPN575	*EUG1* promoter		This study

*In all cases, the gene is placed under control of the *PDI1* promoter.
‡All self-replicating plasmids listed are centromere based and are therefore present at a low copy number.

Table II. Yeast Strains

Name	Chromosomal deletions	Plasmid	Source
M 4129	—	—	Tachibana and Stevens, 1992
M 4426	*eug1*	—	This study
M 4427	*mpd1*	—	This study
M 4419	*mpd2*	—	This study
M 4360	*eps1*	—	This study
M 4174	*pdi1*	pCT38	Tachibana and Stevens, 1992
M 4741	*ero1-1 pdi1*	pCT38	This study
M 4172	*pdi1 eug1*	pCT38	This study
M 4496	*pdi1 mpd1*	pCT38	This study
M 4418	*pdi1 mpd2*	pCT38	This study
M 4837	*mpd1 mpd2 eug1 eps1*	—	This study
M 4497	*pdi1 eug1 mpd1*	pCT38	This study
M 4417	*pdi1 eug1 mpd2*	pCT38	This study
M 4508	*pdi1 mpd1 mpd2*	pCT38	This study
M 4478	*pdi1 eug1 mpd1 mpd2*	pCT38	This study
M 4477	*pdi1 eug1 mpd1 mpd2 eps1*	pCT38	This study

All strains are isogenic with W303 MATα *ade2-1 can1-100 ura3-1 leu2-3,112 trp1-1 his3-11,15* (Kurjan, 1985).

Deletion of the *PDI1* gene was described in Tachibana and Stevens (1992). The *EUG1* gene was deleted with the plasmid pCT19, using the "pop-in, pop-out" method (Rothstein, 1991). A 4.8-kb EcoRI-SalI fragment of *EUG1* was inserted into the *URA3*-based plasmid pRS306 (Sikorski and Hieter, 1989). The HindIII-NruI fragment was subsequently deleted, resulting in plasmid pCT19, which was digested with SnaBI, introduced into yeast, and integrated into the chromosome. Integrants were spread on plates containing 5-FOA, which selects cells that have lost the *URA3* gene. The desired pop-out results in a deletion of the HindIII-NruI region of the *EUG1* locus. To confirm the correct pop-in, pop-out, PCR was performed using the primers oCT3 (5′ CCA ACA GTA AAG CAC TGT CC 3′) and oCT5 (5′ CCC CTG AAT CTA CTT CGG CG 3′). Correct deletion of the *EUG1* gene resulted in a PCR product of ~330 bp.

The plasmid pPN196 is derived from pFA6-kanMX3 (Wach et al., 1994) by exchanging the *kan*^{r} gene with the *URA3* gene. Two DNA fragments (the HincII-HincII and the Eco47III-PvuII fragment from pBH1800) with sequence identity to the *MPD1* gene were inserted into pPN196 on each side of the *URA3* gene, resulting in the plasmid pPN330. The fragment containing the *URA3* gene flanked by two direct repeats and the regions homologous to the *MPD1* gene was excised from plasmid pPN330 with SspI, introduced into yeast, and integrated into the chromosome. This leads to deletion of base pairs 101–387 of the *MPD1* ORF. Cells that had lost *URA3* were selected by plating the integrants on medium containing 5-FOA. To confirm the correct deletion of *MPD1*, PCR was performed using oPN13 (5′ CCT TGC GAG GCG TAT AAA 3′) and oBH64 (5′ CCT GAG TAC ACT TCA TTG GC 3′). The desired deletion gave a PCR product of ~700 bp.

The vector pFA6-kanMX3 (Wach et al., 1994) was used as template in a PCR reaction using the primers oLA4 (5′ CAC AGC GAG TCT AGT GCA AGT ACG TCG GCA AAG TAA AAC ACA AAG GAG ATG GGA TCC CCG GGT TAA TTA AGG CGC 3′) and oLA5 (5′ CGT CTG TAG TCG GTA TTC GTA AAG TAA AAG ACA GAG CGA AGC TTA TGT TCA CTG ATA TCA TCG ATG AAT TCG AGC 3′). This resulted in a fragment containing the *kan*^{r} gene flanked by two direct repeats and two *MPD2* fragments represented by the oligonucleotides. The fragment was integrated into the chromosome and correct substitution was confirmed by PCR using the primers oLA1 (5′ GGT CAA CGG GAA TGA GGG GAA 3′) and oLA8 (5′ CCC ATA TAA ATC AGC ATC C 3′). The desired deletion of the *MPD2* gene gave a PCR product of ~1,480 bp. The mating type of the *MPD2* deleted strain was changed from α to **a** by crossing to an isogenic strain. This strain was subsequently crossed with a Δ*pdi1* Δ*eug1* [PDI1] strain (Δ*pdi1* Δ*eug1* [PDI1] denotes a yeast strain deleted for *PDI1* and *EUG1* and complemented by *PDI1* on a plasmid).

The *EPS1* gene was amplified by PCR using the primers oBH61 (5′ CGC GGA TCC GCG CAA GAA ATT CTA TCC AGG 3′) and oBH62 (5′ TCC CCG CGG GGA TTT TTA TGG TAG GCG TGC 3′), and the PCR product was cloned into the EcoRV site of pBCSK+ (Stratagene). The SalI-XhoI and EcoRV-EcoRV fragments of a plasmid containing the *EPS1* ORF were inserted into the SalI and SmaI sites, respectively, of YDp-L (Berben et al., 1991). The resulting plasmid, pPN100, was digested with SphI and SalI, and the fragment containing the *LEU2* gene flanked by regions homologous to *EPS1* was integrated into the chromosome leading to deletion of base pairs 499–978 of the ORF of *EPS1*. To confirm correct integration, PCR was performed using the primers oPN006 (5′ AGC GGA AAT AGG TGC GAA 3′) and oPN012 (5′ AAC AGT ACC ACC GAA GTC 3′). The desired deletion of *EPS1* resulted in a PCR product of ~800 bp.

Of the plasmids listed in Table I, pBH1464, pBH1711, pBH1857, pBH1966, and pCT58 have been described elsewhere (Holst et al., 1997). pBH1865 was constructed by subcloning a BamHI-NaeI fragment from pCT40 (Tachibana and Stevens, 1992) into the corresponding sites of pBH1692 (Holst et al., 1997). pCT38 is derived from pRS316 and contains a 2.6-kb KpnI-XbaI fragment extending from 879-bp upstream of the *PDI1* gene to 160-bp downstream of *PDI1*.

MPD1 and *MPD2* used to construct the plasmids for complementation were amplified by PCR from yeast chromosomal DNA. *MPD1* was amplified using the oligonucleotides oBH57 (5′ CGC GGA TCC GCG TCC ACT TAA CAC AAT TAG G 3′) and oBH58 (5′ TCC CCG CGG GGA CTT ATT CTT ATG CCC C 3′) as primers, which introduced a BamHI site upstream of the ORF and a SacII site downstream of *MPD1*, respectively. After digestion with BamHI and SacII, the PCR-amplified fragment was inserted between the corresponding sites of pBH1692, resulting in plasmid pBH1800. The sequence of the cloned ORF was found to be identical to the published sequence, except for a silent C to T mutation at nucleotide position +168 relative to the ATG codon. *MPD2* was amplified using the oligonucleotides oBH59 (5′ CGC GGA TCC GCG GCG AGT CTA GTG CAA GTA CG 3′) and oBH60 (5′ TCC CCG CGG GGA CTT ATA TTG CGG CTA ACG 3′). The use of oBH59 and oBH60 also introduced a BamHI site upstream and a SacII site downstream, respectively, of the ORF. After digestion with BamHI and SacII, the PCR-amplified fragment was inserted between the corresponding sites of pBH1692, giving pBH1806. The sequence of the cloned ORF was found to be identical to the published sequence.

The genes encoding the Pdi1p homologues were introduced via a plasmid shuffle procedure (Sikorski and Boeke, 1991). The *TRP1*-containing plasmids were introduced into the desired strains carrying *PDI1* on a *URA3*-based plasmid. Clones lacking the plasmid carrying *PDI1* were selected on medium containing 5-FOA.

pPN379 contains 1,500 base pairs of the *MPD1* promoter fused to *lacZ*. A PCR product, made using the primers oPN25 (5′ ACG CGC GGA TCC ACA GTC TTA GGG AAG TAA CC 3′) and oPN26 (5′ ACG ACG GGA TCC ATT ATA TGT CAA ATT TTG TCT CTC C 3′), was digested with BamHI and inserted into the corresponding site of pFN8 (Nagawa and Fink, 1985). The plasmid pPN382 contains 1,500 base pairs of the *MPD2* promoter fused to *lacZ*. A PCR product, made using the primers oPN27 (5′ ACG CGC GGA TCC GAA GCC TGA GTT CAT GCC CG 3′) and oPN28 (5′ ACG ACG GGA TCC ATC TCC TTT GTG TTT TAC 3′), was digested with BamHI and cloned into the corresponding site of pFN8. The primers oVW201 (5′ CGT CAG CTG AGG ATC CGG TAC CAC CCA GG 3′) and oVW202 (5′ TAG CTG ACT GGG ATC CTT CAT AAC GGG 3′) were used to make plasmid pPN410, which contains 880 base pairs of the *PDI1* promoter fused to the *lacZ* gene. The plasmid pPN575, containing 268 base pairs of the *EUG1* promoter, was made by cloning a PCR product from the primers oPN48 (5′ ACG CGC GGA TCC AGT CAT CCT CTG ATT AAC TTT CC 3′) and oPN49 (5′ ACG ACG GGA TCC ATG GTA TTA TAT GAG CGG G 3′). The BamHI-digested PCR product was cloned into BamHI-digested pFN8.

Pulse Labeling and Immunoprecipitation

The pulse-chase and immunoprecipitation experiments were carried out essentially as described by Horazdovsky and Emr (1993). Yeast strains that were Δ*prc1* were made *PRC1* by integration of the wild-type allele of *PRC1* using the plasmid pIPRC. This plasmid is an EcoRI shrink of pTSY3 (Stevens et al., 1986). Digestion with SacI facilitates integration into the chromosome. The transformed yeast strains were tested for their PRC1$^+$ phenotype by testing for CPY activity using an *N*-acetyl-DL-phenylalanine β-naphtyl ester overlay assay (Jones, 1977). Strains were made Δ*prc1*::*HIS3* using the plasmid described in Blachly-Dyson and Stevens (1987). The *prc1-1* allele encoding CPY* was introduced on the CEN-*URA3* plasmid pHIT341, a gift from R. Hitt and D. Wolf (Institut für Biochemie, Universität Stuttgart, Stuttgart, Germany).

For Western blots, the cells (~4 OD_{600} of cells per sample) were collected by centrifugation and resuspended in 5% (wt/vol) TCA. After centrifugation, the pellet was washed twice in 100% acetone and subsequently resuspended in 100 μl of lysis buffer (100 mM MOPS, pH 7.0, 2% SDS) containing 20 mM AMS to modify free thiols. Also, a protease inhibitor cocktail (Boehringer) was included, according to the supplier's instructions. Approximately 50 μl of acid-washed glass beads (212–300 μm; Sigma-Aldrich) were added to each sample. The samples were then vortexed for 30 min in an Eppendorf Mixer 5432 at room temperature, and incubated for a further 30 min at room temperature to complete the sulfhydryl modification. CPY was subsequently immunoprecipitated as described by Horazdovsky and Emr (1993). Horseradish peroxidase blots were developed using ECL+ substrate (Amersham Pharmacia Biotech), and scanned using Storm system image analysis (Molecular Dynamics).

Growth Phenotypes

Cultures grown overnight in SC + 5×Leu + 5×Ade were diluted to a concentration of ~5,000 cells/ml (5×Leu or 5×Ade denotes five times of the normal SC concentration of the compound). 20 μl were applied on SC + 5×Leu + 5×Ade plates. The plates were incubated for 2 d at 30°C before being photographed. The ability of yeast strains to grow in the presence of DTT was determined by spotting cells in the same way onto freshly prepared SC + 5×Leu + 5×Ade plates buffered to pH 5 and containing 0–2.5 mM DTT. The plates were subsequently incubated in a CO_2 atmosphere to minimize air oxidation of DTT. After 2 d at 30°C, the plates were photographed.

Measurement of β-Galactosidase Activity

β-galactosidase assays were performed using a protocol modified from Guarente (1983). For preparation of the cell suspension, an overnight culture was diluted to a OD_{600} of ~0.1 in a total volume of 25 ml in 100-ml flasks. The culture was grown at 30°C until an OD_{600} ~ 0.3. An appropriate amount of culture was harvested by centrifugation and the pellet was resuspended in 450 μl buffer Z (Guarente, 1983) containing 0.01% Triton X-100. The solution was transferred to a 2-ml Eppendorf tube and the old tube was washed with another 450 μl buffer Z + Triton X-100. The cell suspension was vortexed for 10 s and immediately frozen in dry ice/ethanol before storage overnight at −80°C. For the assay, the cell suspension was thawed, centrifuged at 10,000 *g* for 10 min to collect the cells. For each assay, 950 μl buffer Z and 200 μl ONPG (from 4 mg/ml stock) were mixed. 575 ml of buffer Z/ONPG mixture was added to the cells and vortexed for 5 s. Another 575 ml of buffer Z/ONPG mixture was added and vortexed. After incubation at 30°C, the assay was stopped by addition of 500 μl 1 M Na_2CO_3. The activity is calculated as described by Guarente (1983). Each result is the mean ± SEM of the data resulting from two independent transformants each measured five times.

Results

MPD1 Is the Only PDI1 Homologue that, on Its Own, Is Able to Ensure Cell Viability

From combined in vivo and in silico analysis, five genes can be identified as being members of or obvious candidates for the PDI family: *PDI1*, *EUG1*, *MPD1*, *MPD2*, and *EPS1* (Tachibana and Stevens, 1992; Tachikawa et al., 1995, 1997; Wang and Chang, 1999). Of these genes, *PDI1* stands out as being the only essential gene. All nonessential *PDI1* homologues have been shown to be multicopy suppressors of a *pdi1* deletion. Thus, these proteins, in principle, have the ability to carry out the minimum Pdi1p function necessary for cell survival, but they are either present in too low amounts and/or do not have the necessary catalytic potency when produced at normal levels.

We were interested in studying why yeast and other eukaryotes are equipped with such an array of PDI-like proteins. More specifically, we wished to uncover putative synthetic phenotypes by systematic disruption of several *PDI1* homologues in the same cell, and to investigate each homologue's effectiveness in suppression of a *pdi1* deletion when expressed at roughly the same levels as Pdi1p. To do so, we constructed null mutations of *PDI1* *EUG1*, *MPD1*, *MPD2,* and *EPS1*, alone and in combinations. The first important result of this experiment is that simultaneous deletion of all four Pdi1p homologues causes no obvious growth defect on synthetic complete medium under normal laboratory conditions. Thus, no synthetic phenotypes are displayed as long as *PDI1* is present. We then placed the *MPD1*, *MPD2*, *EUG1*, and *EPS1* open reading frames under the control of the *PDI1* promoter on a low-copy *TRP1*-based plasmid. These plasmids were introduced into a Δ*pdi1* strain carrying *PDI1* on a *URA3*-based plasmid. Growth on 5-FOA, which selects for loss of the *PDI1*-containing *URA3*-based plasmid, was used as a measure of the ability of a gene to suppress the *PDI1* deletion. The result is that Eug1p, Mpd1p, and Mpd2p are all able to replace Pdi1p when produced

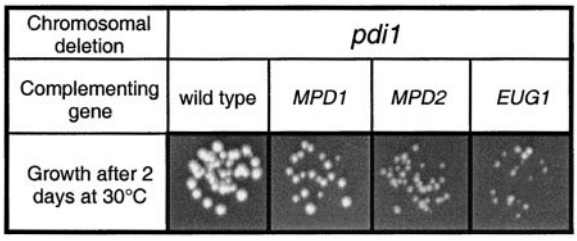

Figure 2. Growth of PDI-deficient yeast strains overexpressing *PDI1* homologues. Cell cultures were diluted, applied to SC + 5×Leu + 5×Ade plates and incubated at 30°C for 2 d. The wild-type growth rate (defined as the reciprocal of the doubling time) was ~0.7 h^{-1} in SC medium, while the most extreme reduction in growth rate was seen for a Δ*pdi1* [EUG1] strain (~0.3 h^{-1}; data not shown).

Table III. Rescue of PDI Deficiencies by Overexpression of PDI Homologues

	Yeast strain								
Introduced gene*	Δ*pdi1*	Δ*pdi1* Δ*eug1*	Δ*pdi1* Δ*mpd1*	Δ*pdi1* Δ*mpd2*	Δ*pdi1* Δ*eug1* Δ*mpd1*	Δ*pdi1* Δ*eug1* Δ*mpd2*	Δ*pdi1* Δ*mpd1* Δ*mpd2*	Δ*pdi1* Δ*eug1* Δ*mpd1* Δ*mpd2*	Δ*pdi1* Δ*eug1* Δ*mpd1* Δ*mpd2* Δ*eps1*
PDI1	++	++	++	++	++	++	++	++	++
EUG1	+	+	−	−	−	−	−	−	−
MPD1	+	+	+	+	+	+	+	+	+
MPD2	+	+	−	+	−	+	−	−	−
$PDI1_{CRRC-SGHS}$	++	++	++	++	++	++	++	++	++
$EUG1_{CLHC-CIHC}$	++	++	++	++	++	++	++	++	++
$PDI1_{CGHS-CGHS}$	+	+	+	−	+	−	−	−	−

++, viable strain with near wild-type growth. +, viable strain with a reduced growth rate compared with a wild-type strain. −, viable strain could not be obtained. Overexpression of *PDI1* and *MPD1* rescues all strains, while overexpression of *MPD2* only rescues strains containing *MPD1*. Furthermore, rescue by overexpression of *EUG1* requires the presence of both *MPD1* and *MPD2*. The gene encoding a PDI-like mutant form of Eug1p, $EUG1_{CLHC-CIHC}$, rescues all strains, while the gene encoding a Eug1p-like form of PDI, $PDI1_{CGHS-CGHS}$, is not able to rescue when *MPD2* is deleted.
*The gene was under control of the *PDI1* promoter and was introduced on a low-copy number plasmid.

to roughly the same level. However, their effectiveness at doing so varies greatly. A Δ*pdi1* strain complemented by *MPD1* under control of the *PDI1* promoter (Δ*pdi1* [MPD1]) showed a small reduction in growth rate compared with the wild type, while the Δ*pdi1* [MPD2] and Δ*pdi1* [EUG1] strains showed larger reductions in growth rates (Fig. 2). We next wanted to see whether the ability of these plasmids to complement the *pdi1* deletion was dependent on chromosomal copies of the other *PDI1* homologues, or whether synthetic effects could be revealed. This was accomplished by shuffling the various plasmids containing the homologues into strains containing a *pdi1* deletion in combination with deletions of the homologues. The pattern of viability in this array of strains did reveal functional differences between the *MPD1*, *MPD2*, and *EUG1* gene products (Table III). Deletion of *EUG1* affected neither viability nor growth rate in any of the yeast strains investigated. Overexpression of *EUG1*, on the other hand, was able to complement the *pdi1* deletion only if *MPD1* and *MPD2* were both present. Differences in the functionality of Mpd1p and Mpd2p were seen, as overexpression of *MPD2* was no longer able to rescue a *pdi1* deletion if *MPD1* was also deleted, while overexpression of *MPD1* rescued all strains. Not even expression of *MPD2* from a high-copy plasmid rescued a *pdi1* deletion in the absence of *MPD1*. The difference could, in principle, reflect very low steady state levels of Mpd2p compared with Mpd1p, caused by differences in protein stability. To test this, we constructed plasmids containing *MPD1* and *MPD2*, in which the sequence encoding a COOH-terminal *myc*-tag followed by the ER retention signal HDEL were added. Both constructs were found to phenocopy the wild-type genes in their ability to complement a *pdi1*-deleted strain. A Western blot of extracts from these strains probed with *myc*-antibodies shows that Mpd2p-*myc* is present at roughly the same level as Mpd1p-*myc* when both tagged genes are expressed under the control of the *PDI1*-promoter (data not shown). This indicates that in a strain completely depleted of other Pdi1p-like proteins, Mpd1p is the only homologue able to provide all Pdi1p functions required for survival.

EPS1 was not able to rescue a *pdi1*-deleted strain when expressed under control of the *PDI1*-promoter. It has previously been shown, however, that it can complement, when expressed from a 2 μm plasmid (Wang and Chang, 1999). Since Eps1p may have a specialized role in ER quality control, we did not pursue the investigation of this protein.

The importance of the oxidizing CXXC motif was shown with specific *EUG1* and *PDI1* mutants. Mutation of the active sites of Eug1p to CXXC converts Eug1p to a more Pdi1p-like enzyme as judged by its improved ability to rescue a Δ*pdi1* strain (Holst et al., 1997). We found that overexpression of the corresponding gene $EUG1_{CLHC-CIHC}$ was able to rescue the simultaneous deletion of *PDI1*, *MPD1*, *MPD2*, *EUG1*, and *EPS1* (Table III). Conversely, mutation of the active sites of Pdi1p to the more Eug1p-like sequence CXXS restricted the ability of the gene to rescue a *pdi1* deletion to situations where *MPD2* was present (Table III). This might reflect a higher oxidative capacity of Mpd2p than Mpd1p.

The protein Ero1p is required for ER oxidation of protein thiols (Frand and Kaiser, 1998; Pollard et al., 1998). It has been shown that when a mutant form of Mpd2p containing a CQHC-to-CQHA active site mutation is overexpressed, mixed disulfides with Ero1p can be trapped (Frand and Kaiser, 1999). We wanted to test whether Ero1p is involved in delivering oxidizing equivalents not only to Pdi1p and Mpd2p, but also to Mpd1. To address this question, we crossed a *pdi1*-deleted strain with the temperature-sensitive *ero1-1* mutant. If redox equivalents passed only through Pdi1p, while a second pathway was used when one of the homologues was overexpressed, no synthetic phenotype would be observed. However, in the double mutant, none of the homologues was able to rescue the *pdi1* deletion when expressed under control of the *PDI1* promoter. The simplest explanation for this result is that Ero1p is also able to transfer oxidizing equivalents to the homologues.

Table IV. Expression Levels

Promoter	β-Galactosidase activity
	Miller units
MPD2	0.0466 ± 0.0038
MPD1	0.972 ± 0.076
EUG1	1.173 ± 0.056
PDI1	26.28 ± 2.34

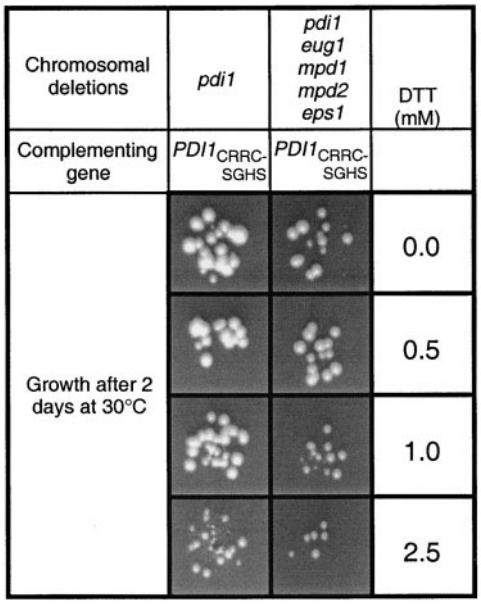

Figure 3. DTT sensitivity of PDI-deficient yeast strains overexpressing *PDI1* homologues. Cultures were diluted and applied to SC + 5×Leu + 5×Ade plates buffered to pH 5 and supplemented with different concentrations of DTT. To prevent oxidation of DTT by molecular oxygen, the plates were freshly made and incubated at 30°C in a CO_2 atmosphere.

The PDI1 Homologues Are Expressed at a Much Lower Level than PDI1

Since the *PDI1* homologues are able to complement the *pdi1* deletion only when expressed under control of the *PDI1* promoter, their normal levels are likely to be substantially lower than that of the *PDI1* gene. To estimate expression levels, we measured the β-galactosidase activity from reporter constructs in which the promoter regions of all the genes in question were fused to *lacZ*. In an otherwise wild-type yeast, the β-galactosidase activity obtained from the *MPD1* and *EUG1* reporters were 4–5% of the activity from the *PDI1-lacZ* reporter, while that of the *MPD2-lacZ* reporter was only 0.2% (Table IV). This indicates that the homologous proteins are present at a much lower concentration than Pdi1p.

The question was posed as to whether the inability of *MPD1* to complement a *pdi1* deletion is due only to the normally low abundance of the gene product or whether it also is a consequence of reduced catalytic potency compared with Pdi1p. To test this, we placed *PDI1* under control of the weak *MPD1* promoter. Interestingly, such a plasmid was able to rescue a strain simultaneously deleted for *pdi1*, *eug1*, *mpd1*, *mpd2*, and *eps1* (data not shown). This shows that it is not only the lower expression levels of the *PDI1* homologues that make them incapable of fulfilling PDI function.

The Effects of Mutations in PDI1 Are Enhanced in Strains Lacking Pdi1p Homologues

DTT sensitivity is closely linked to the effectiveness of the oxidizing apparatus of the ER (Jämsä et al., 1994; Frand and Kaiser, 1998). DTT penetrates the ER and interferes with the formation of disulfide bonds in nascent proteins (Braakman et al., 1991). Mutants of Pdi1p mutated at the two central residues of the active site CGHC sequence furthermore show increased DTT sensitivity (Holst et al., 1997). We were interested in determining to what extent the oxidizing apparatus of the various deletion mutants was affected. A wild-type strain is able to grow in the presence of 5 mM DTT under otherwise normal conditions. To amplify possible differences in DTT sensitivity with respect to growth, we used the mutant $PDI1_{CRRC\text{-}SGHS}$, which had previously been shown to cause increased sensitivity towards DTT (Holst et al., 1997). We found that deletion of all genes encoding the PDI homologues increased the DTT sensitivity of the strain rescued by the mutant gene $PDI1_{CRRC\text{-}SGHS}$, most clearly seen by the difference in growth at 0.5 and 1.0 mM DTT (Fig. 3).

The rate of intracellular folding of proCPY can be monitored, as only folded proteins are allowed to exit the ER (Gething et al., 1986). Since proCPY is converted to forms of different molecular mass as it traverses the different compartments of the secretory pathway, the rate of ER exit can be determined (Stevens et al., 1982). Upon translocation into the ER, the protein receives four core *N*-glycosyl residues, resulting in the 67-kD ER form of proCPY called p1. The carbohydrate structure is further modified in the Golgi, giving the 69-kD p2 form. Finally, when proCPY reaches the vacuole, the propeptide is cleaved off, resulting in the 63-kD mature, active form of CPY (Stevens et al., 1982).

We followed the maturation of proCPY to CPY in strains depleted for PDIs. Cells were labeled with [^{35}S]methionine for 15 min and chased for 0, 15, and 60 min. After immunoprecipitation with CPY antibody, the samples were subjected to SDS-PAGE and analyzed using a PhosphorImager. As seen in Fig. 4 A, the rate of proCPY folding was strongly dependent on the gene that was used to rescue the *pdi1* deletion. The half-time of proCPY maturation in the *PDI1*-complemented strain is 5–10 min, whereas the half time increased to ~30 min in strains rescued by overexpression of *MPD1*. When the *pdi1* deletion is rescued by overexpression of *EUG1*, proCPY maturation is almost arrested, accompanied by accumulation of the p1 ER form of proCPY. This effect is even more pronounced in the strain rescued by overexpression of *MPD2*. Deletion of the genes encoding the various homologues had no, or very little, effect on the rate of proCPY maturation as long as the strains were complemented by *PDI1* (Fig. 4 B), indicating that the Pdi1p homologues do not normally make a significant contribution to proCPY folding. However, the homologues do recognize proCPY as a substrate in the absence of Pdi1p, because maturation of proCPY takes place, although at a reduced rate.

As Eug1p is not on its own able to carry out oxidation reactions, oxidation should be less efficient in a Δ*pdi1* strain complemented by *EUG1*. We tested this assumption by looking at the redox state of proCPY under steady state. Extracts were made in the presence or absence of the thiol modifying agent AMS. AMS modifies free sulfhydryl groups only, and the modification causes a mobility shift on SDS-PAGE due to the bulkiness of the AMS group. The cell extracts were separated on an SDS-PAGE, blotted onto a nitrocellulose membrane, and probed with CPY antibodies. As seen in Fig. 5 A, proCPY in a wild-type strain is not modified by AMS, while treatment of the

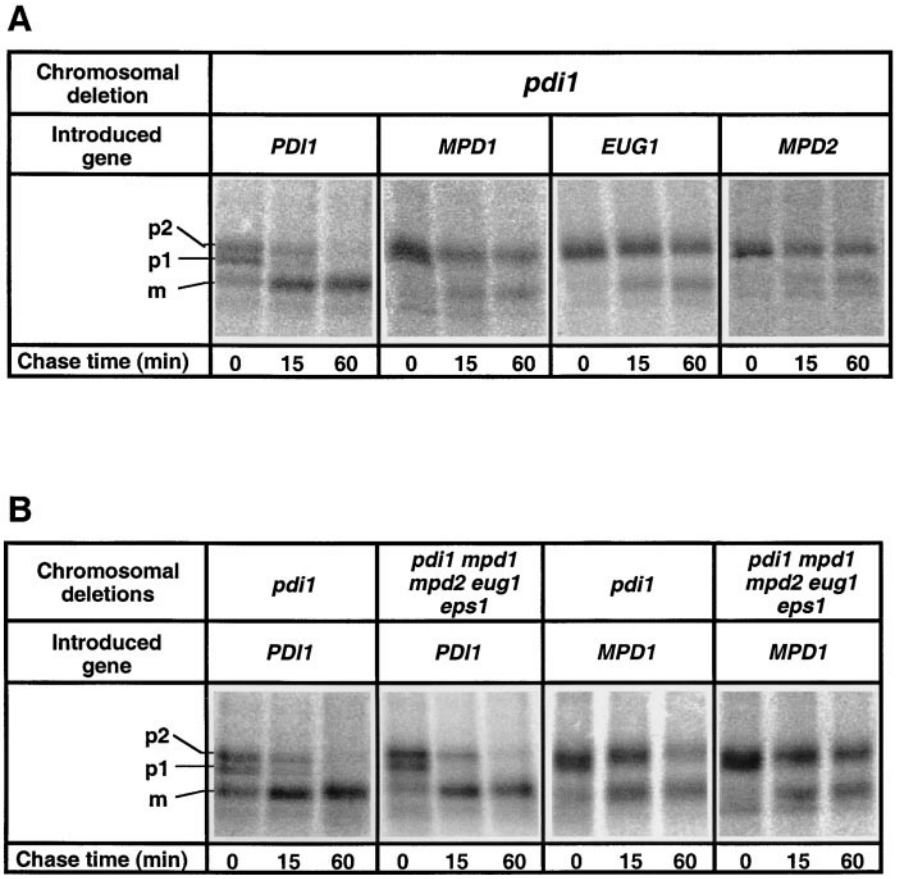

Figure 4. Intracellular folding and maturation of CPY followed by immunoprecipitation from pulse-labeled cells separated on SDS-PAGE. Cells were labeled for 15 min and chased for 0, 15, and 60 min. (A) A Δ*pdi1* strain rescued by the indicated genes. (B) Comparison of a Δ*pdi1* and a Δ*pdi1* Δ*mpd1* Δ*mpd2* Δ*eug1* Δ*eps1* strain containing plasmids with *PDI1* or *MPD1*.

cells with DTT before the AMS modification causes a large mobility shift on the gel. This corresponds to modification of all eleven cysteines in the fully reduced proCPY. proCPY in the Δ*pdi1* [EUG1] strain is partially AMS modifiable, suggesting that a significant portion of the proCPY is not completely oxidized at steady state. This indicates that oxidation is in fact compromised in the *EUG1* complemented strain.

Pdi1p Depletion Affects Protein Glycosylation

In the Δ*pdi1* [EUG1], the Δ*pdi1* [MPD2], and the Δ*pdi1* Δ*eug1* Δ*mpd1* Δ*mpd2* Δ*eps1* [MPD1] strains, the p1 and the p2 forms of proCPY are not fully separated (Fig. 4, A and B). The p1 form in these strains appears to migrate slower than wild type. Also, the mature form of CPY shows a faster mobility than in the wild-type strain. That these differences in mobility originate from glycosylation defects is confirmed by their disappearance upon treatment of the samples with endoglycosidase H (data not shown). Since the p1 form migrates slower (most clearly seen in Fig. 5 B), this defect must originate in the ER.

The compound calcofluor white interferes with cell wall assembly, and hypersensitivity towards this compound has therefore been used to indicate defects in cell-wall biogenesis. Several mutants showing hypersensitivity towards calcofluor white are affected in enzymes involved in the glycosylation apparatus (Ram et al., 1994; Lussier et al., 1997). Consistent with the observed glycosylation defects was the finding that the Δ*pdi1* [EUG1], the Δ*pdi1* [MPD2], and the Δ*pdi1* Δ*eug1* Δ*mpd1* Δ*mpd2* Δ*eps1* [MPD1] strains all were extremely sensitive towards calcofluor white (data not shown).

Pdi1p Homologues Play No Significant Role in ER-associated Degradation

PDI-like proteins have been implicated in quality control and ER-associated degradation of misfolded ER proteins (Oliver et al., 1997; Gillece et al., 1999; Wang and Chang, 1999). In yeast, Pdi1p has been shown to play a role in degradation via the ubiquitin-proteasome pathway (ERAD) of two model substrates: an unglycosylated mutant form of prepro-α-factor and CPY with a single amino acid mutation, CPY* (Gillece et al., 1999). The degradation of the former appeared to be independent of disulfide chemistry, but rather involved a putative peptide-binding domain of Pdi1p. CPY* degradation on the other hand appeared to be dependent on Pdi1p activity, but not the binding domain. As it was suggested that other additional components might be involved, we wished to investigate whether any of the Pdi1p homologues played a role. We found that the homologues were not important for efficient degradation of CPY*, as demonstrated by the fact that the degradation rate was not reduced in a Δ*mpd1* Δ*mpd2* Δ*eug1* Δ*eps1* strain (Fig. 6 A). Surprisingly, however, substitution of *PDI1* with *MPD1, MPD2,* or *EUG1* also had no significant effect on CPY* degradation (Fig. 6 B). To rule out any possible inconsistencies as compared with the experimental procedures used by Gillece et al. (1999), we tested the CPY* degradation in a Δ*pdi1* [$PDI1_{SGHS\text{-}CGHC}$] strain used by Gillece et al. (1999). Consistent with their find-

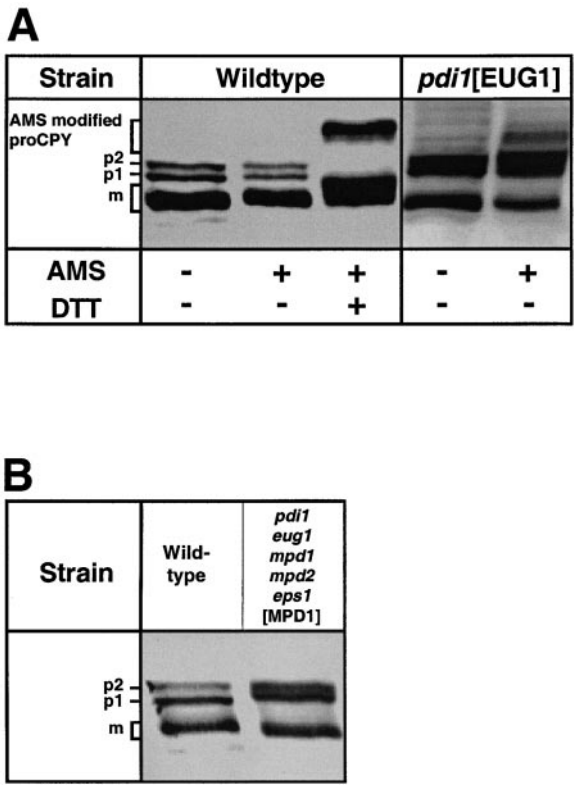

Figure 5. Western blot showing the steady state pool of CPY. (A) A wild-type and a Δ*pdi1* [EUG1] strain. Cells were lysed in the presence or absence of 20 mM AMS. In the third lane, the wild type was treated with 40 mM DTT before lysis and AMS modification. (B) Western blot showing glycosylation defects of CPY in a strain with Mpd1p as only PDI source. A wild-type and a Δ*pdi1* Δ*eug1* Δ*mpd1* Δ*mpd2* Δ*eps1* [MPD1] strain show differences in the mobility of the various forms of CPY.

ings, the rate of CPY* degradation in this strain was significantly reduced (data not shown). Based on these observations, we must conclude that the homologues can substitute for any putative role played by Pdi1p in the degradation of this substrate.

Discussion

When cysteine residues enter the lumen of the ER as part of a translocated polypeptide chain, they encounter a change in the redox environment that ultimately promotes formation of intrachain and/or interchain disulfide bonds. In the generally accepted view, PDI is involved in two reactions during the oxidative folding of a protein: (a) the introduction of disulfide bonds in a polypeptide substrate by transfer of a disulfide bond between the two PDI active site cysteines (i.e., in the oxidized state) to the substrate, and (b) rearrangement of the incorrect disulfide bonds that most likely form during complex folding reactions.

The discovery of Ero1p as important for oxidative folding suggests a pathway for the flow of oxidizing equivalents from yet unknown donors to Ero1p, to the CXXC-motif of Pdi1p and further to the polypeptide substrate (Frand and Kaiser, 1999). The present work has demonstrated that the cell has an absolute requirement for at least one CXXC-containing PDI. While otherwise wild-type cells are able to grow if the CGHC-sites of Pdi1p are exchanged with CGHS, this is not the case if the gene encoding the auxiliary CXXC-containing PDI *MPD2* is deleted. Similarly, *EUG1* rescues a *pdi1*-deleted strain only if *MPD1* and *MPD2* both are present, while an Eug1p mutant form containing CXXC active sites can circumvent the requirement for other CXXC-containing Pdi1p homologues. This also strengthens the notion that the context of this motif might be of lesser importance (Chivers et al., 1996). It is remarkable that yeast has evolved a Pdi1p homologue, Eug1p, with only one cysteine residue in each active site, rendering it incapable of forming the internal disulfide bond that is required for oxidation, but suited for shuffling reactions. The finding that the steady state proCPY pool in a Δ*pdi1* [EUG1] strain is partially reduced (Fig. 5 A) clearly demonstrates the decreased oxidative capacity of this strain. Frand and Kaiser (1999) propose that oxidizing equivalents may flow via Pdi1p or Mpd2p to protein substrates, as shown by cross-linking experiments using CXXS/A mutant forms. As a Δ*pdi1 ero1-1* strain is not rescued by any of the homologues, they must also be dependent on the Ero1p pathway. A weak allele of *ERO1* would not affect the ability of the homologues to complement had they used a different pathway. This is consistent with the model that Ero1p not only transfers oxidizing equivalents to Pdi1p and Mpd2p but also to Mpd1p.

As *pdi1* deletion is lethal, the question arises as to what essential cellular process or processes are compromised by Pdi1p depletion, or, in this case, substitution of Pdi1p with one of the homologues. We find that mature CPY in some of our strains migrates differently than CPY in a wild-type strain, indicating a changed outer chain glycosylation (Figs. 4 and 5 B). Defects in the ER folding apparatus could give rise to diminished steady state levels of the enzymes involved in glycosylation. Furthermore, these cells show hypersensitivity to calcofluor white, which is probably due to cell wall defects. Consistent with this, the morphology of the cells is changed as a consequence of Pdi1p depletion. It is most prominent in the Δ*pdi1* [EUG1] strain, where odd-shaped cells often are encountered.

There are several indications from both yeast and higher eukaryotes that PDI may be involved in quality control and degradation of misfolded secretory proteins. However, we find no effect of deleting the homologues in the presence of a wild-type copy of *PDI1*. Likewise, there is no reduction in the rate of CPY* degradation on substitution of Pdi1p with any of the homologues Mpd1p, Mpd2p, or Eug1p (Fig. 6). It therefore appears that neither Pdi1p nor the homologues play a unique, irreplaceable role in degradation of CPY*. This is surprising in view of the previous observation that substitution of Pdi1p with a $Pdi1p_{SGHS\text{-}CGHC}$ mutant form results in significant stabilization of CPY* (Gillece et al., 1999). At the present time, we cannot rationalize these observations. However, it is possible that a different degradation pathway is invoked under conditions of severe PDI depletion.

Mpd1p ranks as the most Pdi1p-like of the homologues, as it can act as the sole PDI in a Δ*pdi1* Δ*eug1* Δ*mpd1* Δ*mpd2* Δ*eps1* strain and, under these conditions, support an almost normal growth. However, the observation that expression of *PDI1* from the weak *MPD1* promoter is sufficient for survival, in conjunction with the multiple defects seen in the *pdi1*-deleted strains rescued by overex-

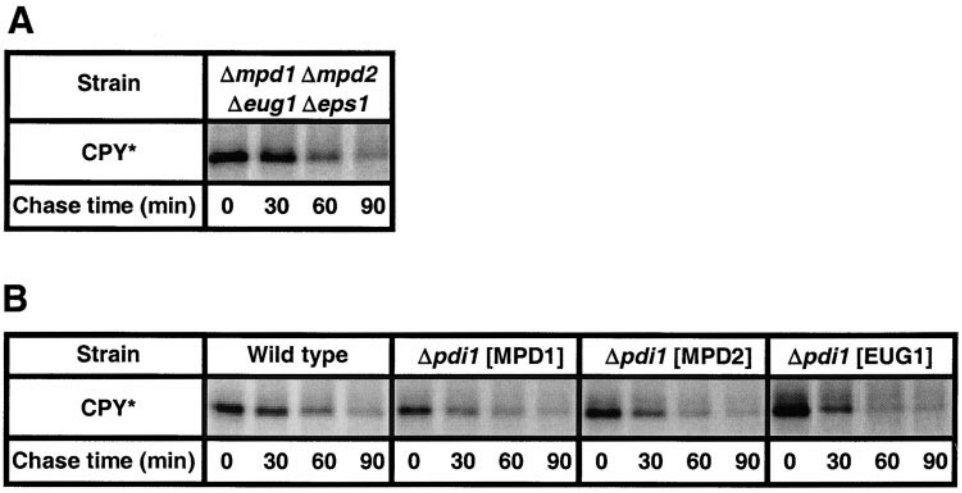

Figure 6. Degradation of CPY*. Cells were labeled for 10 min and chased for 0, 30, 60, and 90 min. The results were consistent in two to four independent experiments. All strains are Δ*prc1* and contain the *prc1-1* allele on the plasmid pHIT341.

pressed homologues, demonstrates the superior catalytic potency of Pdi1p. In the absence of Pdi1p, both Mpd1p and/or Mpd2p appear to be important contributors to protein oxidation in the ER. However, *MPD1* and *MPD2* do not seem equally well suited to carry out protein oxidation, since *MPD2* requires *MPD1* to be present to restore viability of a *pdi1*-deleted strain. Also, a *pdi1*-deleted strain rescued by *MPD2* is severely affected in proCPY folding. The redox potential of the PDI-like protein from *E. coli*, DsbA, is strongly dependent on the nature of the two amino acid residues between the active site cysteines (Grauschopf et al., 1995). The active site of Mpd1p contains the same sequence as Pdi1p, CGHC, while the active site of Mpd2p contains the sequence motif CQHC. While changing this sequence to CGHC did increase the growth rate of Δ*pdi1* [$MPD2_{CGHC}$], the mutation did not enable the mutant to complement a Δ*pdi1* Δ*eug1* Δ*mpd1* Δ*mpd2* Δ*eps1* strain (data not shown).

In this paper, we have addressed the functionality of the Pdi1p homologues using an approach that focuses on the ability of the homologues to replace Pdi1p in various strain backgrounds. We have seen that there are clear differences in the ability of the Pdi1p homologues to ensure growth when Pdi1p function is compromised, suggesting different in vivo responsibilities. Some of the genes are targets of the unfolded protein response (Mori et al., 1998). Differences in the in vivo function might therefore include responding to different stress conditions. Currently, we are investigating the regulation of the genes encoding the homologues.

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