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) (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 proteins can be found in 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
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 procaryoxypeptidase 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, Pdi1PCGHS-CGHS, (this nomenclature is adopted for describing the sequence of the two active sites of the protein; the sequence of the more NH2-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 PDI 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 Tag 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 4-aminomethyl-2,2'-disulfonic acid (AMS) was from Sigma-Aldrich. 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid (AMS) was from Molecular Probes. [35S]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.
Deletion of the PDI1 gene was described in Tachibana and Stevens (1992). The EUG1 gene was deleted with the plasmid pCT9, using the "pop-in, pop-out" method (Rothstein, 1991). A 4.6 kb EcoRI-Sall fragment of EUG1 was inserted into the URA3-based plasmid pRS306 (Sikorski and Hieter, 1989). The HindIII-NruI fragment was subsequently 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 plasmid. This resulted in a fragment containing the URA3 region. To confirm the correct pop-out, pop-out, PCR was performed using the primers oCT3 (5' CCA ACA GTA AAG CAC CGC TG 3') and oCT5 (5' CCC CGT AAT CTA CTT CGG CG 3'). Correct deletion of the EUG1 gene resulted in a PCR product of ~330 bp. The plasmid pPN146 is derived from pFA6-anMX3 (Wach et al., 1994) by exchanging the kan gene with the URA3 gene. Two DNA fragments (the HincII-HindII and the EcoRV-PvuII fragment from pBH137) with sequence identity to the MDP1 gene were inserted into pPN146 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 MDP1 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 MDP1 ORF. Cells that had lost URA3 were selected by plating the integrants on medium containing 5-FOA. To confirm the correct deletion of MDP1, PCR was performed using oPN13 (5' CCT TGC GAG GCG TTT AAA 3') and oBH64 (5' CCT GAG TAC ACT TCA TCG GC 3'). The desired deletion gave a PCR product of ~700 bp.

The vector pFA6-anMX3 (Wach et al., 1994) was used as template in a PCR reaction using the primers oLA4 (5' CAC ACG GAG TCT AGT GCA AGT ACG TCG GCA AAG TAA AAC ACA AAG GAG ATG GGA TCC CGG GGA TTA TAT ACG CCG G 3') and oLA5 (5' CGT CTG TAG TCG GTA TCT GAA TAA AAG ACG CAG AGC TTA TGT CTA CTT ATC TCA TCG GAC AGC 3'). This resulted in a fragment containing the krt gene flanked by two direct repeats and two MDP2 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 TGG GGG GAA 3') and oLA8 (5' CCC ATA TAA ATC AGC ATC C 3'). The desired deletion of the MDP2 gene gave a PCR product of ~1,480 bp. The mating type of the MDP2 deleted strain was changed from a to a by crossing to an isogenic strain. This strain was subsequently crossed with a Spd1p Leu2p strain (Spd1p Leu2p [PDI1] strain [Spd1p Leu2p [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 ATCC CTCC AGG 3') and oBH3 (5' TCC CGG GGA TTT TTA TGG TAG GCG TGC 3'), and the PCR product was cloned into the EcoRV site of pBCSK+ (Stratagene).

The Sall-XhoI and EcoRV-EcoRV fragments of a plasmid containing the EPS1 ORF were inserted into the Sall and SmaI sites, respectively, of Ydp1-L (Berben et al., 1991). The resulting plasmid, pPN100, was digested with Sphi and Sall, 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 oPN060 (5' AGC GGA AAT AGG TCG GAA 3') and oPN001 (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, pBH1446, 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. 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. MDP2 was amplified using the oligonucleotides oBH59 (5' CGC GGA TCC GCG GCA GAG GTG CTA GTG TCG 3') and oBH60 (5' TCC CGG GGA TTT TTA TGG TAG GCG TGC 3'). The use of oBH59 and oBH60 also introduced a BamHI site upstream of the ORF and a SacII site downstream of MDP1, respectively. 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.
The plasmid pPN379 contains 1,500 base pairs of the MPD1 promoter fused to lacZ. A PCR product, made using the primers oPN25 (5'-ACG CGG GGA TCC ATG GTA TTA TAT GAG CGG G-3') and oPN49 (5'-ACG CGC GGA TCC AGT CAT CCT CTG ATT AAC TTT CC-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 EU11I promoter, was made by cloning a PCR product from the primers oPN48 (5'-ACG CGG GGA TCC AGT CAT CCT CTG ATT AAC TTT CC-3') and oPN49 (5'-ACG CGG 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 spo11 (43237) were made prc1 (by integration of the wild-type allele of prc1 using the plasmid pPRC). 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-naphthyl ester overlay assay (Jones, 1977). Strains were made spo11Δ, his3Δ to 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. Hilt and D. Wolf (Institut für Biochemie, Universität Stuttgart, Stuttgart, Germany).

For Western blots, the cells (~4 OD600 units 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 (10 mM MOPS, pH 7.0, 2% SDS) containing 200 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. CYP was subsequently immunoprecipitated as described by Horazdovsky and Emr (1993). Horseradish peroxidase blots were developed using ECL+ substrate (Amer sham 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 0.25 mM DTT. The plates were subsequently incubated in a CO2 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 an OD600 of ~0.1 in a total volume of 25 ml in 100-ml flasks. The culture was grown at 30°C until an OD600 of 0.3. An appropriate amount of culture was harvested by centrifugation and the pellet was resuspended in 450 μl buffer Z (Guarente, 1983) containing 0.1% 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 Na2CO3. The activity is calculated as described by Guarente (1983). Each result is the mean ± SEM of the data resulting from two independent transfectants 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 multicyclop suppressors of a pdil 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 pdil 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 Δpdi1 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 mixed.
Table III. Rescue of PDI Deficiencies by Overexpression of PDI Homologues

| Introduced gene | ∆pdi1 | ∆pdi1/∆eug1 | ∆pdi1/∆mpd1 | ∆pdi1/∆mpd2 | ∆pdi1/∆eug1 | ∆pdi1/∆mpd1 | ∆pdi1/∆mpd2 | ∆pdi1/∆eug1 | ∆pdi1/∆mpd1 | ∆pdi1/∆mpd2 | ∆pdi1/∆eug1 | ∆pdi1/∆mpd1 | ∆pdi1/∆mpd2 |
|-----------------|-------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|
| PDI1            | ++    | ++          | ++          | ++          | ++          | ++          | ++          | ++          | ++          | ++          | ++          | ++          | ++          | ++          |
| EUG1            | +     | +           | +           | +           | +           | +           | +           | +           | +           | +           | +           | +           | +           | +           |
| MPD1            | +     | +           | +           | +           | ++          | ++          | ++          | ++          | ++          | ++          | ++          | ++          | ++          | ++          |
| MPD2            | +     | +           | -           | +           | +           | +           | +           | +           | +           | +           | +           | +           | +           | +           |
| PDI1_GCBS-GCBS  | +     | ++          | ++          | ++          | ++          | ++          | ++          | ++          | ++          | ++          | ++          | ++          | ++          | ++          |
| EUG1_CLHC-CBHC  | +     | ++          | +           | +           | ++          | ++          | ++          | ++          | ++          | ++          | ++          | ++          | ++          | ++          |
| PDI1_CLHC-CBHC  | +     | +           | +           | +           | +           | +           | +           | +           | +           | +           | +           | +           | +           | +           |

++ , 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-CBHC, rescues all strains, while the gene encoding a Eug1p-like form of PDI, PDI1_CQHC-CQHA, 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 ∆pdi1 strain complemented by MPD1 under control of the PDI1 promoter (∆pdi1 [MPD1]) showed a small reduction in growth rate compared with the wild-type strain, while the ∆pdi1 [MPD2] and ∆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 ∆pdi1 strain (Holst et al., 1997). We found that overexpression of the corresponding gene EUG1_CLHC-CBHC 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                          |
and incubated at 30°C in a CO2 atmosphere. The plating plates were freshly made 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 CO2 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 PDIICRRC-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 PDIICRRC-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 (Gettign 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 in strains depleted for PDIs. Cells were labeled with [35S]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 homologue 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 ∆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 sulphydryl 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 3. DTT sensitivity of PDI-deficient yeast strains overexpressing PDI homologues. Cultures were diluted and applied to SC + 5xLeu + 5xAde 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 CO2 atmosphere.](image-url)
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 Δpdi1 [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 Δpdi1 [EUG1], the Δpdi1 [MPD2], and the Δpdi1 Δeug1 Δmpd1 Δmpd2 Δeps1 [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 Δpdi1 [EUG1], the Δpdi1 [MPD2], and the Δpdi1 Δeug1 Δmpd1 Δmpd2 Δeps1 [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 Δmpd1 Δmpd2 Δeug1 Δeps1 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 Δpdi1 [PDI1SGHS-CGHC] strain used by Gillece et al. (1999). Consistent with their find-
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 (Frands 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-

**Figure 5.** Western blot showing the steady state pool of CPY. (A) A wild-type and a Δpdi1 [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 the steady state pool of CPY.
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 pdl1-deleted strain. Also, a pdl1-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 (Grausoph, 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 COHC. While changing this sequence to CGHC did increase the growth rate of Δpdl1 [MPD2CGHC], the mutation did not enable the mutant to complement a Δpdl1 Δeug1 Δmpd1 Δmpd2 Δeps1 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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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 Δprc1 and contain the prc1-1 allele on the plasmid pHIT341.
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