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The Yeast *EUG1* Gene Encodes an Endoplasmic Reticulum Protein That Is Functionally Related to Protein Disulfide Isomerase

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The product of the *EUG1* gene of *Saccharomyces cerevisiae* is a soluble endoplasmic reticulum protein with homology to both the mammalian protein disulfide isomerase (PDI) and the yeast PDI homolog encoded by the essential *PDI1* gene. Deletion or overexpression of *EUG1* causes no growth defects under a variety of conditions. *EUG1* mRNA and protein levels are dramatically increased in response to the accumulation of native or unglycosylated proteins in the endoplasmic reticulum. Overexpression of the *EUG1* gene allows yeast cells to grow in the absence of the *PDI1* gene product. Depletion of the *PDI1* protein in *Saccharomyces cerevisiae* causes a soluble vacuolar glycoprotein to accumulate in its endoplasmic reticulum form, and this phenotype is only partially relieved by the overexpression of *EUG1*. Taken together, our results indicate that *PDI1* and *EUG1* encode functionally related proteins that are likely to be involved in interacting with nascent polypeptides in the yeast endoplasmic reticulum.

Protein translocation across membranes requires that proteins assume an unfolded conformation (8, 13). Proteins entering the secretory pathway are translocated across the endoplasmic reticulum (ER) membrane, and these newly synthesized proteins assume their native conformation prior to export from the ER (9, 22). Interactions between the nascent, folding polypeptides and resident ER proteins may aid in newly synthesized proteins achieving a native conformation (23). Proposed interactions include binding to BiP to prevent aggregation and to promote folding and oligomerization (22, 41) and isomerization by protein disulfide isomerase (PDI) to assist in the formation of native disulfide bonds (20, 24).

Many secretory proteins undergo disulfide bond formation in the oxidizing environment of the ER lumen. Several lines of evidence suggest that PDI participates in the folding of proteins containing disulfide bonds. PDI was originally characterized for its ability to catalyze the steps in the in vitro refolding of RNase A (24), bovine pancreatic trypsin inhibitor (10), and collagen (35), all of which require disulfide bond rearrangements. More recently, it was demonstrated that ER microsomes depleted of PDI by a high-pH wash were able to translocate and glycosylate γ-gliadin, yet the formation of disulfide bonds in the translocated γ-gliadin was impaired unless the microsomes were reconstituted with purified PDI (6). In addition, PDI may also be involved in other ER processes including glycosylation (21), prolyl hydroxylation (45), and triglyceride transfer (68), as well as other, non-ER functions (4, 7).

The mechanism by which PDI catalyzes disulfide bond arrangements is not yet understood. Nevertheless, several conserved cysteine residues have been demonstrated to be important for enzyme activity. The sequence Trp-Cys-Gly-His-Cys-Lys (WCGHCK) appears twice in PDI. Alkylation of these cysteine residues leads to inactivation of disulfide isomerase activity, and thus these regions have been defined as the active sites of this enzyme (12). In vitro mutagenesis has shown that the first cysteine residue of each CGHCK repeat is required for in vitro isomerase activity (66). This sequence, with flanking homology, appears in other proteins that may or may not have in vitro disulfide isomerase activity. For example, thioredoxin, a 12-kDa protein that contains the sequence CGPCK, has in vitro protein reductase and disulfide isomerase activity (27, 29), as does the DsbA protein of *Escherichia coli* (1). However, a putative phosphatidylinositol phospholipase C protein that contains two CGHCK sequences does not have in vitro disulfide isomerase activity (11). The sequence also appears in gonadotropic hormones (5), an ER protein of unknown function (39), and a developmentally regulated *Trypanosome* gene product (30). No common activity or function has been found among the proteins that contain the PDI active site sequence, and their ability to functionally substitute for PDI in vivo is unknown.

In *Saccharomyces cerevisiae*, many proteins that traverse the secretory pathway contain disulfide bonds in their final enzyme form. For example, vacuolar carboxypeptidase Y (CPY), a protein that transits the ER and Golgi compartments, contains five disulfide bonds in its native conformation (69). We were interested in examining the role of disulfide bond formation in protein transport and protein sorting in the yeast secretory pathway. An essential gene encoding yeast PDI (*PDI1*) has been previously characterized (19, 25, 38, 54). We report here the cloning of a yeast gene (*EUG1*) that is homologous to *PDI1*. The *EUG1* gene encodes a soluble, resident ER protein (Eug1p) whose synthesis is greatly induced in response to the accumulation of proteins in the ER. Analysis of the roles of PDI and Eug1p in *S. cerevisiae* indicates that these proteins are functionally related.

MATERIALS AND METHODS

Strains, growth media, and materials. Yeast strains SEY2102α (*ura3-52 leu2-3,112 his4 suc2-Δ7*) (18) and CYY7α/a (homozygous for *ura3-52 leu2-3,112 suc2 his4/HIS4 ade6/ADE6*) (70) were used for the characterization of

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EUG1. W303-1Bα (*ade2-1 can1-100 ura3-1 leu2-3,112 trp1-1 his3-11,15*) (37) and YPH274α/a (homozygous for *ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 leu2-Δ1*) (58) were used for characterization of *PDI1* and suppression of Δ*pdi1* by *EUG1* overproduction. Cell density was determined by using a Beckman DU-6 spectrophotometer with samples diluted to read in the 0.1 to 0.5 optical density at 600 nm (OD_{600}) unit range. To determine doubling times, readings were taken at times 2 to 5 h apart, keeping cells in logarithmic growth by diluting into prewarmed medium when the OD_{600} reached 0.3 to 0.9 (approximately 3×10^6 to 9×10^6 cells per ml). Doubling times were calculated by using the following equation:

$$OD_{time2}/OD_{time1} = 2^{[(time\ 2\ -\ time\ 1)/doubling\ time]}$$

Yeast cells were grown in rich or minimal medium with 2% glucose or 2% galactose-1% raffinose with appropriate supplements, prepared as described by Sherman (56). 5-Fluoroorotic acid addition was as described by Sikorski and Boeke (57). Yeast transformations were as described by Ito et al. (31). Sporulation efficiency was calculated by viewing cells in suspension with a light microscope and counting the number of asci per 100 cells after 3 to 5 days on sporulation medium. Sporulation of diploids and dissection of tetrads was as described by Sherman (56) without selection for plasmids when present. The pCT44-containing strain was dissected onto YEPGal; others were dissected onto YEPD.

Plasmids were manipulated according to standard procedures (52). Oligonucleotides for hybridization cloning, polymerase chain reaction (PCR), and site-directed mutagenesis were synthesized by the University of Oregon Biotechnology Lab.

PCR. Oligonucleotides were designed to amplify the *PDI1* gene with 70 bp of upstream sequence and 157 bp of downstream sequence. A *Bam*HI site was added to the upstream end, eliminating a noninitiating ATG, and an *Xba*I site was added to the downstream end. Yeast genomic DNA was amplified by PCR according to the method of Saiki (51).

Cloning the *EUG1* gene. The plasmid containing the *EUG1* gene and surrounding sequences was isolated by screening a yeast genomic library in YCp50 (49) with an oligonucleotide that encoded the amino acids EFYAPWCGHCK (5′-GAA TTC/T TAC/T GCT/C/A CCA/T TGG TGT GGT CAC/T TGT AA-3′) biased for yeast codon preference. Cultures of *E. coli* containing the library plasmids were filtered onto nitrocellulose to give single colonies, plasmids were amplified, and the filters were duplicated by replicon filtering as described by Weis (67) and probed and washed as described by Strauss (61). Plasmids from the positives of a second screen with the same oligonucleotide were restriction digested to characterize the genomic insert and to calculate the insert size. Ten unique plasmids with no overlapping genomic inserts were obtained. These plasmids were analyzed by a Southern blot (52) of restriction digests to determine the smallest possible genomic fragment that hybridized to the probe. Three plasmids had hybridizing genomic fragments, and these fragments were cloned into the Bluescript SK+ vector for sequencing. Only the fragment from pCT1, which contains the open reading frame of *EUG1*, yielded a predicted amino acid sequence with any significant homology to mammalian PDI or the PDI active-site homologs.

DNA sequencing. A 6-kb *Pst*I fragment of pCT1 was cloned into Bluescript SK+ for sequencing. Nucleotide sequence was determined as described by Yamashiro et al. (70). Sequences were analyzed by using the VAX programs of Devereaux et al. (15).

Plasmids and gene deletions. A plasmid containing the 4.5-kb *Sal*I fragment of *YCL313* (54), which contains most of the gene referred to here as *PDI1*, was a gift from B. Scherens. Plasmids Bluescript SK+ and KS+ were from Stratagene (San Diego, Calif.).

Plasmid pCT11, used to make Δ*eug1*::*LEU2* strains, was created by using a *Hin*dIII-*Bam*HI (blunted) fragment of approximately 2 kb containing the *LEU2* gene from pCJR21 to replace from *Hin*dIII to the downstream *Eco*RV of *EUG1* in pCT10, which is the *Pst*I-*Sal*I genomic fragment of pCT1 in SK+. pCT46, a KS+ plasmid used for deletion of the *PDI1* gene, was created by cloning the *HIS3* gene on an *Spe*I to *Sma*I fragment from pDH295 into the *Spe*I to *Stu*I site of *PDI1* in pCT45, which contains a 3.7-kb fragment of *PDI1* from the upstream *Eco*RV to the PCR-introduced *Xba*I in KS+. Genes were disrupted by transformation with pCT11 cut with *Bam*HI and *Xho*I or pCT46 cut with *Eco*RV and *Xba*I.

The *EUG1* product is overexpressed 10- to 20-fold simply by introducing multiple copies of the gene under its own promoter on a high-copy-number 2μm vector. Two different 2μm plasmids were used to overexpress *EUG1* in this way. pCT13 was created by cloning a 4.8-kb *Eco*RI (blunted) to *Sal*I fragment containing the *EUG1* gene into the *Bam*HI (blunted) to *Sal*I sites of pCKR201-1 (which is a *URA3 leu2*-d 2μm plasmid). pCT20 was created by placing the 2.8-kb *Hin*dIII-*Sal*I fragment of *EUG1* into YEp351 (28), a 2μm vector that contains the *LEU2* marker.

A multicopy plasmid with the *EUG1* gene under control of the *GAL1* promoter was constructed by using site-directed mutagenesis as described by Kunkel et al. (36) to eliminate a noninitiating ATG upstream of the *EUG1* gene and put a *Bam*HI site at −36 upstream of the *EUG1* gene. A 3-kb *Bam*HI-*Sna*BI fragment from this clone was ligated into the *Bam*HI to *Ecl*136II site of pDH111 (a gift from C. Boone), which contains the 822-bp fragment of the *GAL1* promoter (32) in the *Eco*RI to *Bam*HI site of pRS316 (58), to create pCT40. pCT44, the multicopy, *LEU2* vector with the *EUG1* gene under galactose-inducible control, was made by cloning the *Hin*dIII to *Pvu*II (blunted) fragment containing the *GAL1* promoter and the *EUG1* gene from pCT40 into the *Hin*dIII-*Sma*I site of YEp351. The *PDI1* gene was placed under *GAL1* promoter control by cloning a *Bam*HI to *Xba*I PCR fragment into pDH111 with the *Sal*I site destroyed. To reduce the possibility of a PCR-introduced mutation in the coding region, a 1.1-kb section of the PCR fragment (from *Hpa*I to *Sal*I) was replaced by the same fragment from the *YCL313* clone. The resulting plasmid is pCT37.

Nucleic acid blots. Southern blots and Northern (RNA) blots were probed with riboprobes made with the Riboprobe kit (Promega) according to the manufacturer's directions. Riboprobe for *EUG1* transcript was made by using plasmid pCT5, which is the *Hin*dIII-*Nru*I fragment of *EUG1* in pSP65, cut with *Hin*dIII. *PDI1* Southern blots were probed with a riboprobe made from pCT35 cut with *Hin*dIII, which hybridizes to the region from *Eco*RI to *Hpa*I upstream of the *PDI1* gene, which is not present in the pCT37 plasmid.

Total yeast RNA for Northern blots was prepared as described by Sprague et al. (59) from untreated cells, cells treated for 2 h with 10 μg of tunicamycin per ml, or *sec18-1* cells at 24 or 30°C. Formaldehyde gels were run and blotted, and nitrocellulose blots were probed as described by Sambrook et al. (52).

Antibodies and immunofluorescence. Antigens for the production and purification of polyclonal antibodies were created by cloning sections of the *EUG1* or *PDI1* open reading

frames into the appropriate pEXP vectors, and antibodies against fusion proteins were made and purified as described by Raymond et al. (46). The *Bgl*II-*Sal*I fragment of *EUG1* was cloned into *Sma*I-*Sal*I of pEXP3 to create pCT14, which produces a fusion protein used for Eug1p antibody production. The plasmid pCT28 contains the *Hpa*I-*Sal*I fragment of *PDI1* from *YCL313* in pEXP2 *Sma*I-*Sal*I and was used to create the yeast PDI antigen. The *Bgl*II (blunted)-*Nru*I fragment of EUG1 cloned into pEXP3 *Sma*I was used to produce a protein that was used for affinity purification of the anti-Eug1p antibodies. pCT29, containing the *Hpa*I to *Bgl*II (blunted) fragment of *PDI1* from *YCL313* in pEXP2 *Sma*I was used to produce a fusion protein for affinity purification of the anti-PDI antibodies. Antibodies against Eug1p were used at a 1:500 dilution for Western immunoblots, and 5 μl was used to immunoprecipitate Eug1 from 0.5 ml of cells at an OD_{600} of 1.0. Antibodies against yeast PDI were used at a 1:1,000 dilution for Western blots, and 2 μl was used for immunoprecipitation. Anti-CPY (331-5/6) and anti-Kar2p sera were used for the analysis of CPY and yeast BiP, respectively.

All immunofluorescence techniques were performed as described by Roberts et al. (47), with anti-Eug1p antibodies affinity purified and absorbed to fixed Δ*eug1* spheroplasts used at dilutions of 1:5 or 1:10. Fixed cells were treated with 1% sodium dodecyl sulfate (SDS) for 1 min, and anti-Eug1p samples went through one round of amplification to enhance the Eug1p signal.

Western blotting and immunoprecipitation. Protein gel electrophoresis and Western blotting were performed as described by Kane et al. (34), except that Western blot lysates were made by boiling in 2× Laemmli sample buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 μg (each) of leupeptin and pepstatin per ml. Immunoprecipitations, electrophoresis of radiolabeled protein samples, and fluorography were as described by Yamashiro et al. (70), with the following exceptions: SD-met medium was not buffered to pH 5.0, and spheroplasts were lysed by heating at 100°C for 5 min in 100 μl of 1% SDS and then brought up to 1 ml with 1.1× immunobuffer without SDS before preadsorption with IgGSorb. The ER block in *sec18-1* cells was induced by incubation for 10 min at 30°C before labeling. All protein gels were 10% polyacrylamide.

Northern blots and immunoprecipitations were quantitated by using an AMBIS Radioanalytic Imaging System. Quantitation of the immunoprecipitations in Fig. 5A was corrected for inequalities in loading by standardizing to CPY, run on another part of the gel. Endoglycosidase H (endo H) (Boehringer Mannheim) treatment was as described by Orlean et al. (42). Tunicamycin treatment was as described by Rose et al. (48).

Nucleotide sequence accession number. The *EUG1* nucleotide sequence has been assigned accession number M84796.

RESULTS

Cloning the *EUG1* gene. Approximately 15,000 bacterial colonies containing plasmids with yeast genomic inserts of 10 to 20 kb were screened for hybridization to an oligonucleotide encoding the PDI active-site sequence. Plasmids were isolated from colonies that passed a second hybridization screen, and their genomic inserts were analyzed with restriction enzymes and for their ability to hybridize to the probe. Hybridizing genomic fragments were sequenced, and the translated amino acid sequences were examined for homology to rat PDI (17), thioredoxin (29), Erp72 (40), and PI-PLC (3). The genomic fragment from one of the plasmids, pCT1, had an open reading frame that showed amino acid homology to the PDI active-site sequence. A restriction map of the section of the pCT1 genomic insert containing the gene that we refer to as *EUG1*, for ER protein that is unnecessary for growth under standard laboratory conditions, is shown in Fig. 1.

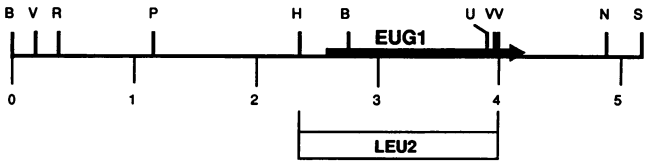

FIG. 1. Restriction map of the genomic fragment containing *EUG1*. A plasmid with an 8-kb genomic insert was cloned from a library of *S. cerevisiae* DNA in YCp50 (49). Five kilobases of the insert containing the *EUG1* open reading frame are shown here. The arrow indicates the direction and extent of the *EUG1* open reading frame, and the region deleted by the Δ*eug1*::*LEU2* construct is shown below. Distances in kilobases are indicated below. Restriction sites: B, *Bgl*II; H, *Hin*dIII; N, *Sna*BI; P, *Pst*I; R, *Eco*RI; S, *Sal*I; U, *Nru*I; V, *Eco*RV.

Figure 2 contains the complete nucleotide and predicted amino acid sequence of the *EUG1* gene. The open reading frame predicts a protein of 517 amino acids (Eug1p) that has 21.3% amino acid identity to rat PDI. The percent identity of Eug1p to other PDI-like proteins such as *E. coli* thioredoxin (29), rodent PI-PLC (3), and Erp72 (39) is lower. Homology to mammalian PDI is concentrated primarily around the two regions corresponding to the PDI active-site sequence (WCGHCK), which are underlined in Fig. 2 and 3. However, unlike PDI and the other PDI homologs, Eug1p has only one cysteine per site instead of two (the sequences at the two sites are WCLHSQ and WCIHSK, respectively). There are five sites for N-linked glycosylation, four Asn-X-Thr and one Asn-X-Ser. The amino acid sequences of Eug1p and yeast PDI (19, 25, 38, 54) are 43% identical, with the introduction of five gaps in each sequence, with no gaps longer than seven amino acids (Fig. 3).

EUG1 encodes an ER protein. Features of the translated amino acid sequence suggest that Eug1p is translocated into the ER. The 29 amino acids at the N terminus are sufficiently hydrophobic to function as an ER signal sequence (65). Affinity-purified polyclonal antiserum against the *EUG1* gene product immunoprecipitated proteins of 65 and 67 kDa that were more abundant when the *EUG1* gene was present in multiple copies and appeared as a single protein of 55 kDa when treated with the deglycosylating enzyme Endo-H (Fig. 4A). The size of deglycosylated Eug1 protein is consistent with the size of the *EUG1* open reading frame, and the shift of 10 to 12 kDa upon deglycosylation suggests that Eug1p is modified by the addition of four to five N-linked oligosaccharides. Furthermore, the presence of glycosyl groups indicated that Eug1p was indeed translocated into the ER.

The predicted C terminus of Eug1p contains the yeast ER retention sequence His-Asp-Glu-Leu (HDEL), suggesting that this protein resides in the lumen of the ER (44). The anti-Eug1p antibodies were used to determine the steady-state intracellular location of Eug1 protein by indirect immunofluorescence. As shown in Fig. 4B, Eug1p was localized to the ER as defined by its perinuclear and subplasma membrane immunofluorescence pattern, which was indistinguishable from the immunolocalization pattern of yeast BiP (48),

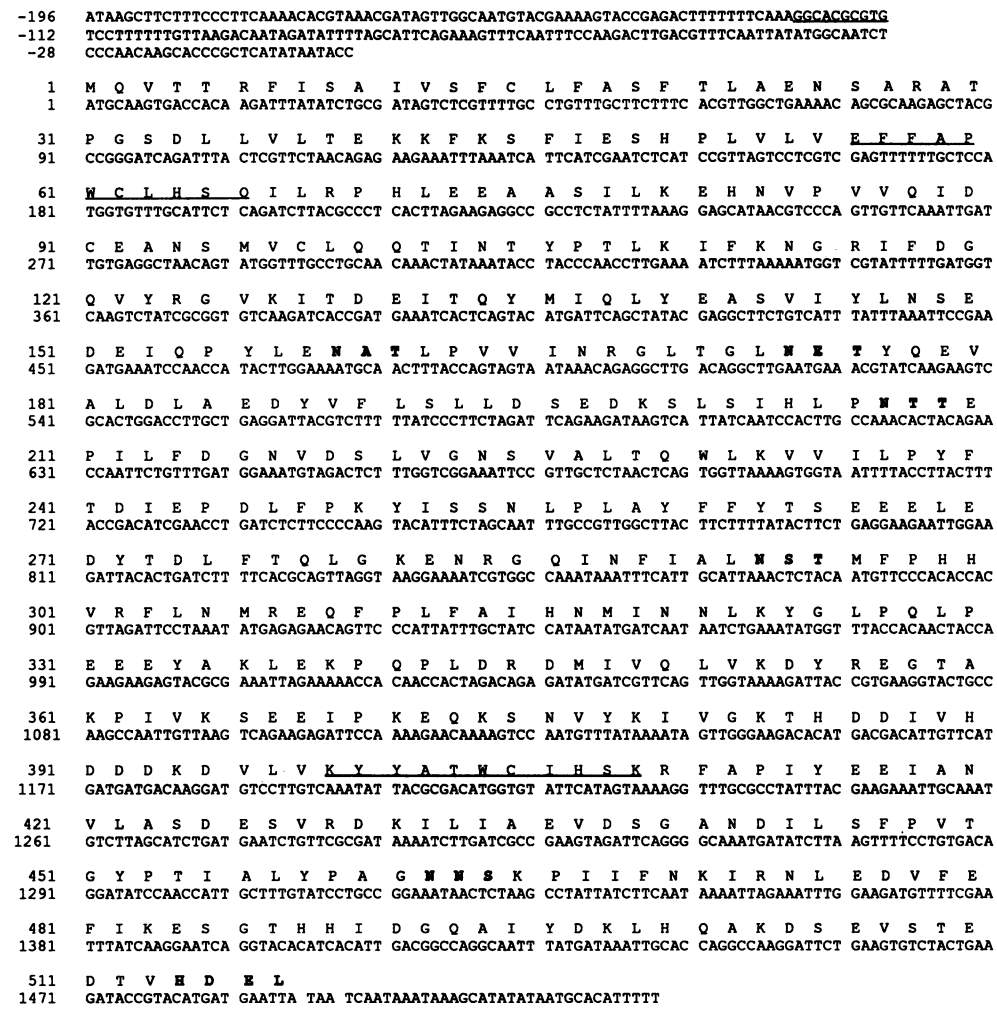

FIG. 2. Nucleotide and corresponding amino acid sequence of *EUG1*. Nucleotide number 1 is the first nucleotide of the predicted initiation codon, and amino acid number 1 is the initiating methionine. The unfolded protein-response element described by Mori et al. (40), from nucleotides −113 to −122, is underlined. The PDI active-site homologies are underlined, and the HDEL ER retention signal and the sites for N-linked glycosylation are shown in bold type.

and the *SEC62* gene product (14), both known residents of the ER.

To test further whether Eug1p is retained in the secretory pathway, we investigated the fate of newly synthesized Eug1p. Pulse-chase immunoprecipitations indicated that Eug1p was not secreted by wild-type cells (63). Consistent with the retention of Eug1p in the ER, we found that Eug1p was secreted from *erd2* mutant cells (63), which are defective in the retention of HDEL-containing proteins (55). Interestingly, the secreted Eug1p received modifications that resulted in an 8- to 9-kDa increase in apparent molecular mass (relative to the ER form); however, only 6 kDa of this was Endo-H sensitive (63). The nature of the Endo-H resistant modification to secreted Eug1p is unknown but could correspond to O-linked mannose addition (26).

Absence or overproduction of Eug1p does not affect growth. The *EUG1* gene was cloned into multicopy, yeast 2μm-based plasmids. Cells carrying these plasmids overproduced Eug1p 10-fold or more relative to that of wild-type levels, as estimated by Western blot (62). A null allele of *EUG1*, constructed as shown in Fig. 1, was used to make chromosomal deletions of *EUG1* in both haploid and diploid cells, which were confirmed by Southern blot (62). Haploid Δ*eug1* strains, or strains overproducing Eug1p via the multicopy plasmid, grew in liquid culture at growth rates identical to that of the isogenic wild-type strain (63). Heterozygous Δ*eug1*/*EUG1* diploids and homozygous Δ*eug1*/Δ*eug1* diploids showed sporulation efficiencies (26 and 28%, respectively) comparable to that of the wild type (26%), and tetrads from the heterozygous Δ*eug1*/*EUG1* diploid showed spore viability that was indistinguishable from that of an isogenic wild-type diploid. No unilateral or bilateral mating defect was seen when Δ*eug1* strains were tested in qualitative mating assays (62).

Haploid Δ*eug1* and Eug1p overproducing strains showed no apparent growth rate difference when compared with isogenic wild-type strains at temperatures ranging from 18 to 40°C. They showed no increased sensitivity or resistance to the presence of up to 100 mM $CaCl_2$ (53), 10 mM EGTA, tunicamycin, or anaerobic conditions. Furthermore, the absence or overproduction of Eug1p did not affect the rate at which CPY moved through the secretory pathway (63). Although deletion of the *EUG1* gene caused a slight defect in the sorting of the soluble vacuolar proteins (CPY) and

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Eug1   1 MQVTTRFISAIVSFC..LFASFTLAENSARATPGSDLLVLTEKKFKSFIE 48
         |        | |    | ||   |  | |   |    |    |   |
yPDI   1 MKFSA...GAVLSWSSLLLASSVFAQQEAVAPEDSAVVKLATDSFNEYIQ 47

Eug1  49 SHPLVLVEFFAPWCLHSQILRPHLEEAASILKEHNVPVVQIDCEANSMVC 98
         || ||| ||||||| |      |    ||  | | |    ||||  |   |
yPDI  48 SHDLVLAEFFAPWCGHCKNMAPEYVKAAETLVEKNITLAQIDCTENQDLC 97

Eug1  99 LQQTINTYPTLKIFKNGRIFDGQVYRGVKITDEITQYMIQLYEASVIYLN 148
            |   | ||||||         | |       | | ||     |
yPDI  98 MEHNIPGFPSLKIFKNSDVNNSIDYEGPRTAEAIVQFMIKQSQPAVAVV. 146

Eug1 149 SEDEIQPYLENATL..PVVINRGL..TGLNETYQEVALDLAEDYVFLSLL 194
               || | |   ||     |       | |    |      || | |
yPDI 147 ..ADLPAYLANETFVTPVIVQSGKIDADFNATFYSMANKHFNDYDFVSAE 194

Eug1 195 DSEDK.SLSIHLPNT.TEPILFDGNVDSLVGNSVALTQWLKVVILPYFTD 242
            |   ||| ||    ||    |          |    || |  ||||
yPDI 195 NADDDFKLSIYLPSAMDEPVVYNGKKADIADADV.FEKWLQVEALPYFGE 243

Eug1 243 IEPDLFPKYISSNLPLAYFFYTSEEELEDYTDLFTQLGKENRGQINFIAL 292
         |    |  |  | ||| | ||  ||||| |  ||| | | |||  ||
yPDI 244 IDGSVFAQYVESGLPLGYLFYNDEEELEEYKPLFTELAKKNRGLMNFVSI 293

Eug1 293 NSTMFPHHVRFLNMREQFPLFAIHNMINNLKYGLPQLPEEEYAKLEKPQP 342
            |  |   ||| |||||||||  |   ||||||| ||    |
yPDI 294 DARKFGRHAGNLNMKEQFPLFAIHDMTEDLKYGLPQLSEEAFDELSDKIV 343

Eug1 343 LDRDMIVQLVKDYREGTAKPIVKSEEIPKEQKSNVYKIVGKTHDDIVHDD 392
         |    |  ||||   | | ||||| ||   | | |   ||| || || |
yPDI 344 LESKAIESLVKDFLKGDASPIVKSQEIFENQDSSVFQLVGKNHDEIVNDP 393

Eug1 393 DKDVLVKYYATWCIHSKRFAPIYEEIANVLASDESVRDKILIAEVDSGAN 442
          ||||| ||| || | || || | | |   |   |     |||  |   |
yPDI 394 KKDVLVLYYAPWCGHCKRLAPTYQELADTYANATS...DVLIAKLDHTEN 440

Eug1 443 DILSFPVTGYPTIALYPAGNNSKPIIFNKIRNLEDVFEFIKESGTHHIDG 492
         |       ||||| ||| |  |        | |   | |||| |    ||
yPDI 441 DVRGVVIEGYPTIVLYPGGKKSESVVYQGSRSLDSLFDFIKENGHFDVDG 490

Eug1 493 QAIYDKLHQAKDSEVST.......EDTVHDEL* 518
          | |        |          ||  ||||
yPDI 491 KALYEEAQEKAAEEADADAELADEEDAIHDEL* 523
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FIG. 3. Homology between the translated amino acid sequences of *EUG1* and *PDI1*. The sequence analysis programs of Devereaux et al. (15), used to align and compare the amino acid sequences of Eug1p and yeast PDI (yPDI), calculated 42.9% amino acid identity between the sequences. Gaps introduced by the program are indicated by dotted lines, the initiating methionines are designated amino acid 1, vertical lines indicate identical amino acids, and asterisks indicate stop codons. The PDI active-site sequence homologies are underlined.

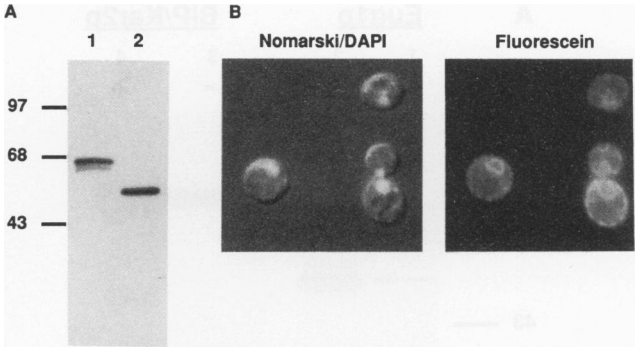

FIG. 4. Eug1p is an ER glycoprotein. (A) Eug1p was immunoprecipitated from SEY2102α overproducing Eug1p from the pCT13 multicopy plasmid labeled for 30 min and chased for 45 min. Half of the sample received no further treatment (lane 1), and the other half was deglycosylated with Endo-H (lane 2). Eug1p immunoprecipitated from nonoverproducing strains comigrates with the bands shown here. Migration of protein standards is shown at left. (B) Immunofluorescence of Eug1p. The strain used in panel A was fixed and stained with anti-Eug1p antibodies. Nomarski/DAPI, cells visualized simultaneously by Nomarski optics, which shows whole cells, and fluorescence conditions which show nuclei stained with DAPI as a bright spot within the cell; Fluorescein, the same field of cells stained to show Eug1p. Wild-type cells containing one chromosomal copy of *EUG1* gave the same pattern of localization.

proteinase A, *eug1* is not a member of the *vps* or *pep* mutant complementation groups (50, 63). Only 8% of the newly synthesized CPY was secreted from a Δ*eug1* strain (64), and this low level of CPY mislocalization did not appear to be the result of cell lysis (63).

Eug1p and *EUG1* mRNA levels increase after tunicamycin treatment. BiP, GRP94, and other ER proteins, including the PDI active-site homolog Erp72, are induced under conditions that cause proteins to be delayed or obstructed in transport from the ER, such as treatment of cells with tunicamycin, an antibiotic that inhibits N-linked glycosylation (16, 41, 48). To test for similar regulation of *EUG1*, the protein was immunoprecipitated from cultures treated for 2 h with 10 μg of tunicamycin per ml and from untreated cultures. The *KAR2* gene product (yeast BiP) and the vacuolar protein CPY were immunoprecipitated from parallel cultures as positive and negative controls, respectively, for induction after tunicamycin treatment. To obtain uniformly treated, deglycosylated samples, all immunoprecipitations were treated with the deglycosylating enzyme endo-H after immunoprecipitation. As seen in Fig. 5A, tunicamycin treatment caused increased production of the Eug1 protein (10-fold over untreated cultures), similar to its effect on BiP (13-fold over untreated cultures). The level of CPY was unaffected (63).

The upstream region of the *EUG1* gene from −113 to −122 bp contains the consensus element responsible for the increased message levels of several genes, including mammalian and yeast BiP, that are induced in response to unfolded proteins in the ER (Fig. 2) (36). Northern blot analysis of the mRNA levels of *EUG1* confirmed that, consistent with the presence of the regulatory element, *EUG1* transcript level increased after tunicamycin treatment, comparable to a parallel analysis of *KAR2* mRNA (Fig. 5B, lanes 1 and 2) (41, 48). As expected, no *EUG1* transcript is seen in a Δ*eug1* strain (Fig. 5B, lanes 5 and 6).

At the nonpermissive temperature, *sec18* conditional mutants are blocked in protein transport from the ER to the Golgi, causing the accumulation of proteins in the ER (33). Both *KAR2* and *EUG1* exhibited elevated levels of transcript in the *sec18* strain at the permissive temperature, and the already induced levels increased further upon shift to the nonpermissive temperature (Fig. 5B, lanes 3 and 4).

The protein and mRNA levels indicate that the expression of *EUG1* is induced by the same conditions that cause induction of the protein-binding enzyme BiP. This common regulation of expression suggests that Eug1p may also interact with nascent and/or unfolded polypeptides in the ER lumen.

Construction of Δ*pdi1* strains. The homology between Eug1p and yeast PDI, and the induction of *EUG1* in response to the accumulation of proteins in the ER, suggested that Eug1p and PDI may overlap in function or activity or may both be involved in the folding of proteins in the ER. Such an overlap in function could be revealed by genetic interactions between *EUG1* and *PDI1*. To test for such interactions it was necessary to obtain the *PDI1* gene and construct a chromosomal deletion. A plasmid containing the *HIS3* replacement of the *PDI1* gene (Δ*pdi1*) was constructed. To control the level of PDI in yeast cells, the *PDI1* gene was placed under the control of the *GAL1* promoter in a *URA3*-CEN plasmid (pCT37). Genes controlled by the

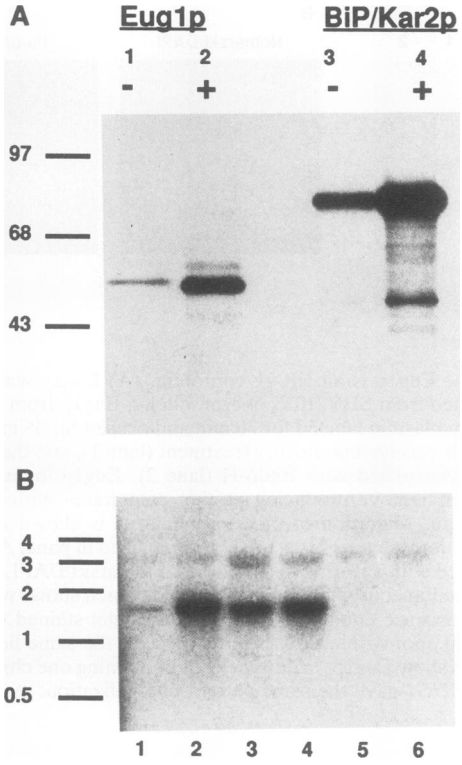

FIG. 5. Expression of *EUG1* increases after tunicamycin treatment. (A) Immunoprecipitations. SEY2102α cells were labeled for 30 min and immunoprecipitated with anti-Eug1p or anti-Kar2p antibodies. +, samples were treated with 10 μg of tunicamycin per ml for 2 h prior to, and during, labeling. All samples were treated with Endo-H after immunoprecipitation. Lanes 1 and 2, Eug1p; lanes 3 and 4, yeast BiP. Protein size standards are indicated on the left. (B) Northern blot of *EUG1* mRNA. Samples treated with tunicamycin received 10 μg of tunicamycin per ml for 2 h prior to RNA extraction. Sizes in kilobases as measured by denatured, radioactive 1-kb ladder are indicated at left. The band at 3 kb is probably nonspecific hybridization to abundant rRNA. Lanes 1 and 2, SEY2102α without and with tunicamycin; lanes 3 and 4, *sec18-1* cells at 24 and 37°C, respectively; lanes 5 and 6, SEY2102α *Δeug1*::*LEU2*, without and with tunicamycin.

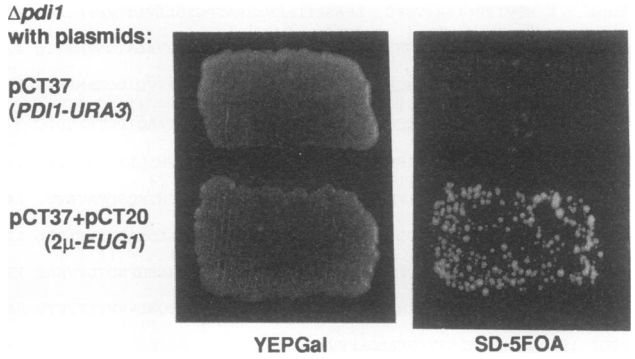

FIG. 6. Overproduction of Eug1p allows growth of a *Δpdi1* strain under conditions that require loss of the *PDI1* complementing plasmid. Patches of haploid *Δpdi1* strains rescued by the *PDI1* gene on a *URA3*-marked plasmid were replica plated onto YEPGal or SD containing 5-FOA and grown for 3 to 10 days at 30°C. pCT37 is the *URA3-PDI1* plasmid; pCT20 is the multicopy *LEU2-EUG1* plasmid. YEPGal is rich galactose-containing medium; SD-5FOA is minimal glucose medium containing 5-FOA, which selects against cells carrying the *URA3* plasmid.

GAL1 promoter are highly expressed in cells grown on galactose but are repressed more than 1,000-fold in the presence of glucose. A *Δpdi1*::*HIS3* construct was used to delete the *PDI1* gene in haploid and diploid strains. Haploid cells were cotransformed with both this deletion construct and pCT37 and grown on galactose-containing media while selecting for His^+ Ura^+ prototrophs. The genotypes of the resulting yeast strains were confirmed by Southern blot (62). The haploid strain carrying a chromosomal deletion of *PDI1*, in which PDI expression is controlled by the *GAL1* promoter, is referred to as *Δpdi1*/pCT37 and was dependent on galactose for growth.

To monitor levels of yeast PDI protein, antibodies specific for yeast PDI were generated. Polyclonal antibodies were obtained and affinity purified against *E. coli* fusion proteins containing segments of yeast PDI. The sizes and glycosyl modifications of the protein recognized by the antibodies corresponded with the predicted and published sizes of yeast PDI (19, 25, 38, 54, 63).

Transferring the *Δpdi1*/pCT37 strain from galactose medium to glucose medium caused the cells to slow in growth commensurate with depletion of PDI. In minimal galactose medium, the *Δpdi1*/pCT37 cells grew with a doubling time of 2.2 h and had approximately 10-fold the PDI found in wild-type yeast cells, as assayed by Western blot (62), because of high expression from the *GAL1* promoter. After 4 h in glucose, PDI protein levels decreased to wild-type levels. After 20 h in glucose, *Δpdi1*/pCT37 cells had approximately 5 to 10% of wild-type levels of PDI, and the doubling time had slowed to 9 h, demonstrating that growth of the *Δpdi1*/pCT37 strain was dependent on the *GAL1* promoter-controlled *PDI1* gene on the pCT37 plasmid.

Overproduction of Eug1p allows growth in the absence of PDI. Multicopy, extragenic suppression of essential genes is not uncommon in *S. cerevisiae*, and genes that interact in this way are often involved in common processes in the cell (2). To test for genetic interaction between *EUG1* and *PDI1*, multicopy *EUG1* plasmids were transformed into strains deleted for the *PDI1* gene to test for suppression of the lethal phenotype. Suppression of the phenotypes of PDI loss by overproduction of Eug1p was tested in two experiments. In the first, *Δpdi1*/pCT37 cells were grown under conditions that overproduced Eug1p while also being subjected to conditions that forced the loss of pCT37, the complementing *PDI1* plasmid. The second approach tested the ability of multicopy *EUG1* plasmids to rescue haploid spores that had inherited the *Δpdi1* allele.

The *Δpdi1*/pCT37 strain, in which the chromosomal *PDI1* deletion is complemented by the *PDI1* gene on a *URA3* plasmid, was transformed with a yeast multicopy *LEU2* plasmid with *EUG1* under control of its own promoter (pCT20) or under control of the *GAL1* promoter (pCT44). The presence of *EUG1* multicopy plasmids resulted in approximately 10- to 20-fold overproduction of Eug1p. Transformants were cultured and plated onto medium containing 5-fluoroorotic acid (5-FOA), which strongly selects for *ura3* mutant cells (57), thus forcing the *Δpdi1*/pCT37 cells to lose the *PDI1-URA3* plasmid in order to grow. Under these conditions, only the strains that were overproducing Eug1p formed colonies. Isogenic strains without the *EUG1* plasmid, or carrying only the parent *LEU2* plasmid, were unable to grow on medium containing 5-FOA (Fig. 6) (62).

TABLE 1. Tetrad analysis of Δ*pdi1*/*PDI1* diploids and diploids with multicopy *EUG1* plasmids

Diploid strain and plasmid	No. of tetrads analyzed	No. with following viable:nonviable segregation:				No. of viable spores	
		4:0	3:1	2:2	1:3	His^+/Leu^+	His^+/Leu^-
Δ*pdi1*/*PDI1*	15	0	0	14	1	0	0
Δ*pdi1*/*PDI1* + YEP351[a]	12	0	0	12	0	0	0
Δ*pdi1*/*PDI1* + pCT20[b]	41	3	6	29	3	12	0
Δ*pdi1*/*PDI1* + pCT44[c]	47	7	9	24	7	31[d]	0

[a] YEp351 is a multicopy *LEU2* plasmid.
[b] pCT20 is YEp351 carrying the *EUG1* gene controlled by its own promoter.
[c] pCT44 is YEp351 carrying the *EUG1* gene under *GAL1* promoter control.
[d] Three of the 3:1 tetrads had 2 His^+ and one His^- spore and five of the 2:2 tetrads had one His^+ and one His^- spore.

The Δ*pdi1*/pCT37 strain transformed with pCT44, in which *EUG1* expression is dependent on galactose, was able to form colonies on galactose 5-FOA but not on glucose 5-FOA, demonstrating that growth without the *PDI1*-containing plasmid required expression from the *EUG1* gene (63). Colonies picked from the 5-FOA plates were His^+ Leu^+ Ura^-, indicating the presence of the Δ*pdi1*::*HIS3* allele, the Eug1p overproducing plasmid, and loss of the *URA3*-containing *PDI1* plasmid.

The second approach to test for extragenic suppression involved transforming the heterozygous Δ*pdi1*::*HIS3*/*PDI1* diploid with the *LEU2* multicopy *EUG1* plasmid pCT20 or with the parent YEp351 plasmid as a control. These strains were sporulated and the tetrads were analyzed for spore viability, segregation of the Δ*pdi1*::*HIS3* allele (His^+), segregation of the *EUG1* plasmid (Leu^+), and mating type of the spores. As shown in Table 1, the Δ*pdi1*/*PDI1* diploid with or without the YEp351 control plasmid gave tetrads with 2:2 viable:nonviable spores. No viable His^+ spores were obtained from either dissection, confirming previous reports that *PDI1* is essential for cell growth (19, 25, 38, 54).

Dissection of asci resulting from sporulation of the diploid carrying the multicopy *EUG1* plasmid yielded nine tetrads that showed more than two viable spores. In these tetrads, two of the spores grew quickly, while the remaining one or two, which were visible without magnification only after 3 to 4 days, gave rise to slowly growing but viable His^+/Leu^+ colonies. Marker analysis showed that all slowly growing colonies were His^+ and all His^+ colonies were also Leu^+. Nonviable (presumably Δ*pdi1* without the *EUG1* plasmid) spores arrested as microcolonies of 2 to 15 cells.

The four spores from a 4:0 tetrad from the Δ*pdi1*/*PDI1*/pCT20 dissection were analyzed by Western blot for steady-state levels of PDI and Eug1p. The results, shown in Fig. 7, show the complete absence of PDI in the His^+ spores. These spores had highly induced levels of Eug1p, in fact, even higher levels than the His^- spores that were also carrying the multicopy *EUG1* plasmid, suggesting that overproduction of Eug1p was responsible for the growth of the Δ*pdi1* spores and that very high levels were required to rescue the lethality due to the *PDI1* deletion.

The Δ*pdi1*/*PDI1* diploid was also transformed with pCT44, a multicopy *LEU2* plasmid containing the *EUG1* gene under control of the *GAL1* promoter. This strain was sporulated as described above, but tetrads were dissected onto galactose-containing medium to induce *EUG1* overexpression. As

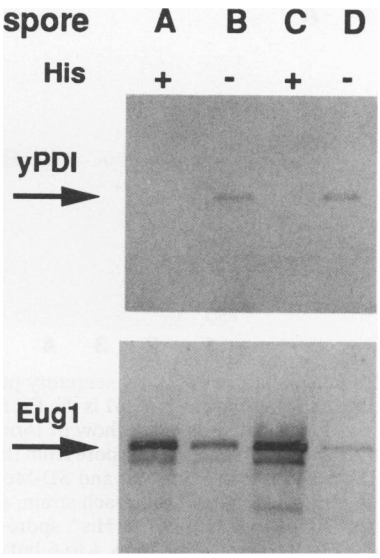

FIG. 7. PDI and Eug1 protein levels in viable Δ*pdi1* spores. Lysates were made from all four viable spores (A to D) of a tetrad that was 2:2 His^+:His^- and 4:0 Leu^+ from a diploid Δ*pdi1*::*HIS3*/*PDI1* carrying the multicopy *EUG1-LEU2* plasmid (Table 1). Equal amounts of lysate from each spore grown in SD-Leu were loaded onto an SDS-polyacrylamide gel to produce duplicate Western blots, one of which was probed with antibodies against yeast PDI, the other with antibodies against Eug1p. PDI levels in His^- spores (B and D) are comparable to amounts seen in lysates from other haploid wild-type cells, while Eug1 levels are approximately 10- to 20-fold higher in the His^- spores and greater than 20-fold higher in His^+ (Δ*pdi1*) spores.

described above, dissection produced spores with variable growth rates, with the slowly growing spores giving rise to His^+ colonies. As shown in Table 1, all His^+ spores were also Leu^+. Spores that were His^+ showed no PDI protein by Western blot and were dependent on galactose for growth. Culturing in glucose medium caused the cells to slow in growth and stop doubling after 20 to 25 h.

These data show that cells capable of growth in the absence of PDI have an absolute dependence on high levels of *EUG1* expression for viability. The tetrad analysis also provided haploid strains from spores that inherited the Δ-pdi1::*HIS3* allele, that had never been exposed to the presence of the *PDI1* gene on a plasmid, and that were completely lacking in PDI but rescued from lethality by the multicopy *EUG1* plasmid. These strains, called Δ*pdi1*/pCT20 and Δ*pdi1*/pCT44, were used in the analyses below, which examined the fate of a secretory pathway protein in the absence of PDI, and when the Δ*pdi1* strain was rescued from lethality by overproduction of Eug1p.

The pCT44 plasmid, which overexpressed *EUG1* from the *GAL1* promoter, allowed the optimal growth of Δ*pdi1* strains. In YEPGal rich medium, the Δ*pdi1*/pCT44 strain had a doubling time of 3.8 h, while a congenic $PDI1^+$/pCT44 strain had a doubling time of 3 h. The similar growth rates indicate that cells that lack PDI can grow almost as well as wild-type cells if Eug1p is present at very high levels.

Absence of PDI causes CPY to accumulate in an early secretory pathway precursor form. Since many different functions have been proposed for mammalian PDI, the intracellular effects of yeast PDI depletion were examined by using CPY, a vacuolar protein whose carbohydrate modifications and proteolytic processing provide a good marker for

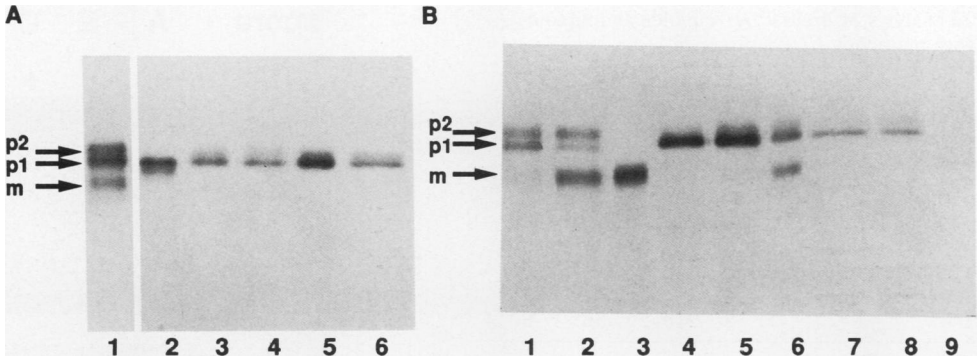

FIG. 8. CPY accumulates as an early secretory pathway precursor in the absence of PDI. (A) CPY was immunoprecipitated from cultures labeled for 10 min and chased for 5 min. p1 is the ER form of proCPY, p2 is the Golgi form, and m is mature, vacuolar CPY. Lane 1, W303-1Bα (exposed twice as long as other lanes to show p1 form more clearly); lane 2, *sec18-1* at 30°C; lane 3, Δ*pdi1*/pCT37 grown in glucose-containing medium for 18 h; lane 4, a Δ*pdi1*/pCT20 spore from the tetrad described in Table 1 and Fig. 7; lanes 5 and 6, a Δ*pdi1*/pCT44 spore from a tetrad described in Table 1 grown in SGal-Met and SD-Met, respectively, for 18 h. (B) CPY was immunoprecipitated from cultures labeled for 10 min and chased for 0, 5, or 60 min. For each strain, samples for increasing chase times are loaded from left to right. Lanes 1 to 3, W303-1Bα wild type; lanes 4 to 6, Δ*pdi1*/pCT44, a His^+ spore lacking PDI from the tetrad characterized in Table 1, grown in SGal to induce Eug1p synthesis; lanes 7 to 9, same as for lanes 4 to 6 but grown in SD for 18 h to deplete Eug1p.

translocation into the ER, N-linked glycosylation, and passage through the secretory pathway. The core glycosylated, ER form of proCPY can be distinguished from Golgi-modified proCPY and mature, vacuolar CPY by gel electrophoresis (60). CPY has five disulfide bonds and one free cysteinyl that are essential for activity and stability (69).

As shown in Fig. 8A, after a 10-min pulse and 5-min chase, the ER, Golgi, and vacuolar CPY were present in wild-type cells. Only the ER form was present in *sec18* mutant cells shifted to 30°C, since the transport of proteins from the ER to the Golgi complex is blocked in these mutants (Fig. 8A, lanes 1 and 2). The PDI-depleted cells accumulated a form of CPY with a mobility that was the same as, or slightly slower than, that of ER-accumulated CPY (Fig. 8A, compare lanes 2 and 3). The same ER form of CPY accumulated in Δ*pdi1* cells that were lacking PDI but rescued from lethality by overproduction of Eug1p (lanes 4 to 6). These data suggested that depletion of PDI caused CPY to become slow in movement from the ER, consistent with the proposed role of PDI in protein folding. Furthermore, Eug1p overproduction allowed growth in the absence of PDI but did not suppress the phenotype of accumulation of an early secretory pathway form of CPY.

To test this hypothesis further, CPY was examined after longer chase times from a wild-type strain and from a Δ*pdi1* strain that contained the plasmid pCT44, the multicopy plasmid with *EUG1* under *GAL1* promoter control, under conditions that caused either overproduction or repression of Eug1p synthesis. As quantitated from the immunoprecipitations shown in Fig. 8B, wild-type cells contained all three forms of CPY after a 10-min pulse label, with 28% of total CPY in the mature vacuolar form. This increased to 61% after 5 min of chase, with complete vacuolar delivery of CPY after an hour of chase (Fig. 8B, lanes 1 to 3). The Δ*pdi1*/pCT44 strain grown in galactose to induce overexpression of Eug1 contained less than 1% mature CPY after 5 min of chase and only 40% in the vacuolar form after 1 h of chase (Fig. 8B, lanes 4 to 6). The half-time for vacuolar delivery of CPY was 7.5 min for wild type and at least 75 min for Δ*pdi1*/pCT44. The growth rate of the Δ*pdi1* strain (4.8-h doubling time) under these conditions was less than twofold slower than that for wild-type cells (2.6-h doubling time), yet the transit of CPY through the secretory pathway was at least 10 times slower. This result demonstrated that Eug1p overproduction allowed growth of Δ*pdi1* strains, without restoring the rate at which CPY was transported through the early secretory pathway. In cells that lacked PDI and were not overproducing Eug1p (e.g., yeast strain Δ*pdi1*/pCT44 grown on glucose), CPY accumulated in its ER form and disappeared altogether, possibly because of degradation, after 1 h of chase.

DISCUSSION

A new gene of *S. cerevisiae*, called *EUG1* for ER protein that is unnecessary for growth under standard laboratory conditions, has been cloned and characterized. The *EUG1* DNA sequence indicates that Eug1p has several features in common with yeast PDI, the product of the *PDI1* gene (19, 25, 38, 54). Eug1p has a hydrophobic N-terminal signal sequence, contains the yeast ER retention signal (HDEL) at the C terminus, and exhibits 43% overall amino acid identity to yeast PDI. Eug1p also contains two PDI-like active-site sequences (WCGHCK); however, in Eug1p each site contains one cysteine and one serine (WCLHSQ and WCIHSK), instead of the two cysteines found in PDI and other proteins with homology to the active site region of PDI.

Eug1p was found to be a 65- to 67-kDa glycoprotein localized to the ER lumen, a compartment known to contain enzymes such as BiP that interact with nascent polypeptides (48). Subcellular fractionation indicated that yeast PDI is an ER protein (25), and we found that yeast PDI received Asn-linked oligosaccharides and was retained within the yeast secretory pathway (63). However, in *erd2* mutants, which are defective for retention of HDEL-containing proteins in the ER (55), yeast PDI was secreted (63). Thus, both yeast Eug1p and PDI reside in the ER lumen, where they would encounter newly translocated nascent polypeptides.

The upstream region of the genes for the ER proteins GRP94 and BiP (GRP78) contains an unfolded protein response element that is responsible for induction of these genes in response to the presence of unfolded proteins in the ER (40). This regulation is consistent with the proposed function of these proteins in binding to misfolded secretory

pathway proteins and nascent polypeptides in the ER (43). The unfolded protein response element is found upstream of the *EUG1* gene, and *EUG1* message and protein levels increase in response to the accumulation of proteins in the ER, suggesting a role in the same process. Although PDI is also proposed to interact with newly translocated proteins that are folding in the ER, the yeast *PDI1* gene does not have the unfolded protein response element. Quantitative immunoprecipitation experiments indicate that yeast PDI is at least 10-fold more abundant than Eug1p (63), suggesting the possibility that PDI may be expressed at sufficiently high levels in yeast cells to obviate the need for its induction in response to unfolded proteins in the ER.

Deletion or overproduction of *EUG1* in an otherwise wild-type yeast strain caused no measurable growth defects. In contrast, yeast PDI has been shown to be essential for growth (19, 25, 38, 54). Cells lacking PDI are viable if *EUG1* is present on a multicopy plasmid. That is, in the absence of any PDI, Eug1p performs an essential function in *S. cerevisiae*. Suppression of the lethal phenotype of *PDI1* gene deletion required significant overproduction of Eug1p, but this could merely reflect the fact that PDI is a much more abundant protein than Eug1p (63). The possibility that Eug1p possesses a PDI activity in vitro has not been tested. Nevertheless, if the in vitro isomerase activity of yeast PDI (19) reflects its essential in vivo function, then Eug1p, in spite of its single-cysteine active sites, probably has disulfide isomerase activity as well. Interestingly, several studies have suggested that the first cysteine of the CGHCK sequence, which is present in both active-site homologies of Eug1p, is the residue that is most reactive at physiological pH and possibly the most essential for in vitro isomerase activity (27, 66).

Several studies have found that proteins fold to their native conformation before they exit the ER (23). The rate at which vacuolar CPY moved through the yeast secretory pathway was unaffected by reduced levels of Eug1p. In contrast, PDI depletion caused yeast cells to accumulate the ER form of CPY (25) (Fig. 8), indicating that transport of this soluble vacuolar glycoprotein was slowed in these cells. The accumulation of the ER form of CPY when PDI is depleted is consistent with the model that PDI catalyzes folding in the ER by acting as a PDI. However, since normal CPY transport is not restored to Δ*pdi1* cells when lethality is suppressed by overproduction of Eug1p, it is possible that the essential activity of PDI may be something other than disulfide isomerization. Alternatively, it is possible that Eug1p possesses only a weak disulfide isomerase activity relative to that of PDI and thus cannot fully compensate for its loss.

It is also possible that Eug1p overproduction may suppress lethality because of a loss of PDI via a bypass mechanism, in which Eug1p does not perform the same function as PDI but permits the cells to grow without the essential enzymatic activity of PDI. For example, Eug1p, which is regulated like the ER binding protein BiP, may bind to nascent unfolded proteins in the ER lumen. Overproduction could result in an increase in the capacity of the ER for unfolded proteins that result from PDI loss and may sufficiently prevent protein aggregation to allow growth in the absence of PDI. This model implies that, in spite of their amino acid homology, Eug1p and PDI may not catalyze the same reaction in vivo.

Given the regulation of the *EUG1* gene in response to the accumulation of proteins in the ER, Eug1p may play a role in the folding of nascent proteins in the lumen of the ER. Further studies on the in vivo function of Eug1p, as well as a thorough phenotypic analysis of yeast strains carrying temperature-sensitive alleles of *pdi1*, are needed to distinguish among the possible functional roles for both Eug1p and PDI in yeast secretion.

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