Interactions among Yeast Protein-Disulfide Isomerase Proteins and Endoplasmic Reticulum Chaperone Proteins Influence Their Activities*

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We previously reported that the reductive activities of yeast protein-disulfide isomerase (PDI) family proteins did not completely explain their contribution to the viability of Saccharomyces cerevisiae (Kimura, T., Hosoda, Y., Kitamura, Y., Nakamura, H., Horibe, T., and Kikuchi, M. (2004) Biochem. Biophys. Res. Commun. 320, 359–365). In this study, we examined oxidative refolding activities and found that Mpd1p, Mpd2, and Epg1p exhibit activities of 13.8, 16.0, and 2.16%, respectively, compared with Pdi1p and that activity for Eps1p is undetectable. In analyses of interactions between yeast PDI proteins and endoplasmic reticulum molecular chaperones, we found that Mpd1p alone does not have chaperone activity but that it interacts with and inhibits the chaperone activity of Cne1p, a homologue of mammalian calnexin, and that Cne1p increases the reductive activity of Mpd1p. These results suggest that the interface between Mpd1p and Cne1p is near the peptide-binding site of Cne1p. In addition, Eps1p interacts with Pdi1p, Epg1p, Mpd1p, and Kar2p with dissociation constants (KD) in the range of 10⁻⁷ to 10⁻⁹ M. Interestingly, co-chaperone activities were completely suppressed in Eps1p-Pdi1p and Eps1p-Mpd1p complexes, although only Eps1p and Pdi1p have chaperone activity. The in vivo consequences of these results are discussed.

An important event in the folding of secretory proteins is the formation of disulfide bonds. Protein-disulfide isomerase (PDI), which is a resident of the endoplasmic reticulum (ER), catalyzes the formation, reduction, and isomerization of disulfide bonds (1). PDI contains four thioredoxin-like domains (a, b, b', and a') and an ER retention signal (KDEL) at its carboxyl terminus (1, 2). The a and a' domains contain the thioredoxin-like motif CGHC (2). PDI is active in the ER, which contains a high concentration of both reduced and oxidized glutathione (GSH and GSSG) (3). Recently, Ero1p and flavin adenine dinucleotide complex was shown to reoxidize the reduced state of the active sites of PDI (4). Moreover, PDI has been reported to have chaperone and anti-chaperone activities (5) and may be involved in a quality control system that targets misfolded proteins for degradation (6). PDI is also known to facilitate the secretion of human lysozyme from Saccharomyces cerevisiae cells, a reflection of its chaperone activity (7). Recently, many homologues similar in structure to PDI have been identified. These PDI homologues have two or more CXCC motifs (where X is a variable amino acid), each of which is predicted to function as a site for the formation, reduction, or isomerization of a disulfide bond.

S. cerevisiae PDI (Pdi1p), encoded by the PDI1 gene, is essential for growth (8). The critical function of Pdi1p may be the isomerization of non-native disulfide bonds (8), and its chaperone activity is thought to depend on this isomerase activity (9). Moreover, S. cerevisiae contains four nonessential genes with homology to PDI: MPD1 (10), MPD2 (11), EUG1 (12), and EPS1 (13). Genetic analyses of strains lacking PDI genes indicated that Mpd1p is the only homologue that can fully compensate for the absence of Pdi1p (14). Furthermore, mutant Eup1p, which contains two mutated CXCC active site motifs, is functionally equivalent to Pdi1p (15).

Recently, the interactions of ERp57 with calnexin (CNX) and calreticulin (16, 17), of PDI with peptidyl-prolyl cis-trans isomerase (18) and ERp57 (19) and of JPD1 (a domain-containing protein-disulfide isomerase-like protein) with BiP (immunoglobulin heavy chain-binding protein) (20) have been reported in mammalian cells. However, interactions among yPDI proteins and between yPDI proteins and ER chaperone proteins have not yet been described. Previously, we found that Mpd1p interacts with Mpd2p but that the two proteins do not influence the reductive or chaperone activities of each other (21).

Here, we report interactions involving yPDI proteins and ER chaperone proteins. Among yPDI proteins, only Mpd1p interacts with Cne1p. The reductive activity of Mpd1p is increased in the presence of Cne1p, and the chaperone activity of Cne1p is quenched by interaction with Mpd1p. Mpd1p may function in yeast as a counterpart to mammalian ERp57 because Cne1p corresponds to mammalian CNX. Moreover, in this study we examined Eps1p and Kar2p (22), because Eps1p corresponds to mammalian TMX, a transmembrane thioredoxin-related PDI protein (23), and Kar2p corresponds to mammalian BiP, respectively. Eps1 interactions with Pdi1p, Eup1p, Mpd1p, and Kar2p have different effects on their activities.

EXPERIMENTAL PROCEDURES

Strains and Plasmids—Escherichia coli AD494 (DE3) (Δara*, leu7967, ΔlacX74, ΔphoA, Pru1h, phoR, amalF3, F[lac+, lacF]), pro,
Expression and Purification of His-tagged Human and Yeast PDI Proteins—E. coli AD494(DE3) was transformed with derivatives of pET15b that express yPDI proteins and Cne1p. Pdi1p, Mpd1p, and Eps1p to immobilized yPDI proteins was measured in a surface plasmon resonance assay (Biacore 3000, Biacore Inc., Uenchatel, Switzerland). Pdi1p, Eug1p, Mpd1p, Mpd2p, and Eps1p were covalently coupled to the matrix of a CM5 sensor chip via amine groups according to the manufacturer’s instructions. Cne1p, Kar2p, and PDI proteins were delivered to the protein-coated flow cell to observe binding. In the reverse experiment, Cne1p, Kar2p, and PDI proteins were used as analytes. The data were analyzed with Biacore evaluation software (version 3.1).
Cne1p, with a $K_D$ value of $2.27 \times 10^{-7}$ M (Table I). Mpd1p has no chaperone activity (see Fig. 5), and Mpd2p has 68% of the chaperone activity of Pdi1p (21). Mpd1p interacts with Mpd2p but does not affect its chaperone activity, and Mpd2p does not affect the reductive activity of Mpd1p (data not shown). Therefore, the interaction of Mpd1p with Cne1p will be described below. As shown in Table I, Kar2p interacted with Pdi1p and Eps1p, and the $K_D$ values of these interactions were $5.83 \times 10^{-6}$ and $1.03 \times 10^{-6}$ M, respectively. Eps1p was found to bind Pdi1p, Eug1p, Mpd1p, Kar2p, and Cne1p, with $K_D$ values of $7.33 \times 10^{-6}, 6.06 \times 10^{-6}, 3.88 \times 10^{-6}, 1.03 \times 10^{-6}$, and $3.88 \times 10^{-6}$ M, respectively (Table I). The Eps1p-Cne1p interaction was not examined further because of its low affinity. These data suggest that the role of Eps1p, which interacts with the four yeast PDI-like proteins, is significant in yeast. In contrast, Pdi1p interacted with all yPDI proteins, but none of these interactions had an effect on the activities of Pdi1p and Pdi1p-binding proteins (data not shown).

**Effect of the Interaction of Mpd1p and Cne1p on Their Reductive and Chaperone Activities**—As described above, Mpd1p, which has a weak reductive activity but no chaperone activity (21), specifically interacts with Cne1p. To understand the roles of Mpd1p and Cne1p, we measured their co-reductase and co-chaperone activities. Co-oxidative refolding activity was not determined because Cne1p was unstable in the refolding buffer for an undetermined reason. Although Cne1p alone lacked reductive activity, the addition of two molar equivalents of Cne1p to a reaction mixture containing Mpd1p appeared to increase the reductive activity of Mpd1p (Fig. 4). In contrast, Mpd1p, which has no chaperone activity, completely inhibited the chaperone activity of a molar equivalent of Cne1p (Fig. 5). This inhibition could be overcome by increasing the Cne1p concentration, with 98% Cne1p chaperone activity observed at a Cne1p to Mpd1p ratio of 7:1. These results suggest that the binding interface between Mpd1p and Cne1p is near the peptide-binding site of Cne1p and that the chaperone function requires the peptide-binding site.

**Effects of Interactions of Eps1p with Pdi1p, Eug1p, and Mpd1p on Reductive and Chaperone Activities**—Eps1p recognizes Pdi1p, Eug1p, and Mpd1p (Table I), and we investigated the effect of these interactions on their co-activities. The co-reductive activity of Eps1p-Pdi1p was equal to the sum of their individual activities (data not shown), but the co-reductive activity of Eps1p-Mpd1p decreased relative to the sum of their individual activities (Fig. 6), although the system was not still saturated with Mpd1p alone (21). In contrast, the co-reductive activity of Eps1p-Eug1p was somewhat increased, although Eug1p alone had no reductive activity. In contrast, the co-

![Fig. 4. The co-reductase activity of Mpd1p and Cne1p. The activity of 0.10 µM Mpd1p was normalized to 100%. Each value shown is the mean of data from two independent experiments.](http://www.jbc.org/)

![Fig. 5. The co-chaperone activities of Mpd1p and Cne1p. The activity of 0.23 µM Cne1p was normalized to 100%. Each value shown is the mean of data from two independent experiments.](http://www.jbc.org/)

![Fig. 6. The co-reductase activities of Eps1p-Eug1p and Eps1p-Mpd1p. The activity of 0.10 µM Eps1p was normalized to 100%. Each value shown is the mean of data from two independent experiments.](http://www.jbc.org/)
chaperone activities of Eps1p-Pdi1p and Eps1p-Mpd1p were completely suppressed (Fig. 7), although Pdi1p and Eps1p individually have chaperone activity. These results suggest that Pdi1p and Mpd1p bind to the peptide-binding site of Eps1p and that Mpd1p binds to the peptide-binding site of Pdi1p. The co-reductive and co-chaperone activities of the Eps1p-Eug1p complex were greater than the activities of either protein alone (Figs. 5 and 6), suggesting that Eug1p enhances the reductive and chaperone activities of Eps1p. Thus, we propose that Eps1p functionally interacts with Pdi1p, Mpd1p, and Eug1p in vivo.

**DISCUSSION**

Our previous study indicated that the reductive activity of Pdi1p is remarkably strong and that other PDI proteins in yeast apparently have no significant activity (21). In this study, we found that Pdi1p, Mpd1p, Mpd2p, and Eug1p, but not Eps1p, have oxidative refolding activity (Fig. 3). Although overexpression of Mpd1p rescues the growth of cells lacking PDI, overexpression of Mpd2p does not (14). However, Mpd1p cannot compensate for the absence of Mpd2p, and 33% of the cells lacking Pdi1p are rescued when Eug1p is overexpressed (14). These results suggest that oxidative refolding activities are important for viability and that Mpd1p and Mpd2p act as oxidases in vivo. With respect to the functions of Mpd1p and Mpd2p, we hypothesize that Pdi1p controls proteins essential for growth and that the functions of Mpd1p and Mpd2p overlap with Pdi1p. Eug1p substitutes for Pdi1p because it alone rescues viability when overexpressed.

Eps1p has strong chaperone activity, extremely weak reductive activity, and no oxidative activity and like Pdi1p it interacts with many ER proteins including Kar2p (yeast BiP). Pdi1p and Eps1p interact with Kar2p result in a decrease in co-chaperone activities but had no effect on co-reductive activities (data not shown). Although Pdi1p interacted with many proteins examined in this study, it exhibited co-chaperone activity only with Kar2p (data not shown). Mammalian PDI and BiP were reported to assist protein folding in a concerted manner (27, 28), suggesting that Pdi1p may interact with Kar2p. Several PDI proteins, such as JPDI (19) and ERp44 (29), apparently lack reduct activity. These observations collectively suggest that Eps1p may function as a molecular chaperone rather than as a reduct enzyme in vivo. Nevertheless, we cannot completely rule out the possibility that Eps1p acts as a reduct enzyme, because its reductive activity increased in the presence of Eug1p. We found that Eps1p affects the reductive and chaperone activities of Eps1p-binding proteins, that Mpd1p specifically interacts with Cne1p, and that the interaction between Mpd1 and Cne1p affects the activities of these two proteins. Cne1 corresponds to mammalian CNX, and ERp57 binds to the P domain (30), which is not a peptide-binding domain in CNX and calreticulin. The ERp57-CNX complex is involved in folding nascent glycoproteins (31, 32), suggesting that in *S. cerevisiae* Mpd1p and Cne1p play roles in glycoprotein folding. These observations indicate that yPDI proteins and ER chaperones may form complexes and influence each other’s activity in vivo.

Recently, PDI and ERp57 were identified in nucleoli isolated from cultured human cells (33), and Pdi1p and Eug1p were found in *S. cerevisiae* mitochondria (34). Therefore, determination of the intracellular locations of yPDI proteins such as Mpd1p and Mpd2p may provide insights into their roles in *S. cerevisiae*.

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