THE EFFECT OF CALNEXIN DELETION ON THE EXPRESSION LEVEL OF PDI IN Saccharomyces cerevisiae UNDER HEAT STRESS CONDITIONS

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Abstract: We cultured calnexin-disrupted and wild-type Saccharomyces cerevisiae strains under conditions of heat stress. The growth rate of the calnexin-disrupted yeast was almost the same as that of the wild-type yeast under those conditions. However, the induced mRNA level of the molecular chaperone PDI in the ER was clearly higher in calnexin-disrupted S. cerevisiae relative to the wild type at 37°C, despite being almost the same in the two strains under normal conditions. The western blotting analysis for PDI protein expression in the ER yielded results that show a parallel in their mRNA levels in the two strains. We suggest that PDI may interact with calnexin under heat stress conditions, and that the induction of PDI in the ER can recover part of the function of calnexin in calnexin-disrupted yeast, and result in the same growth rate as in wild-type yeast.

Key words: Calnexin, Molecular chaperone, PDI, Heat stress

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Abbreviations used: ER – endoplasmic reticulum; PAGE – polyacrylamide gel electrophoresis; PDI – protein disulfide isomerase; RT-PCR – reverse transcriptase-polymerase chain reaction; UPR – unfolding protein response
INTRODUCTION

Calnexin is a membrane protein of the endoplasmic reticulum (ER), and it is generally thought to function as a molecular chaperone which associates transiently with numerous newly synthesized glycoproteins during their maturation in the ER [1-3]. In addition to functioning in folding and assembly, calnexin has been proposed to be a component of the ER quality control system that retains misfolded intermediates through their oligosaccharide moieties until these substrates fold properly or until the misfolded proteins are degraded [4-7]. Cne1p, encoded by the CNE1 gene, the homologue of calnexin in the yeast Saccharomyces cerevisiae, is 24% identical and 31% similar at the amino acid level with mammalian calnexin, but it lacks a cytoplasmic tail, and has no calcium-binding capacity [8]. Moreover, S. cerevisiae has no UDP-Glc:glycoprotein glucosyltransferase (GT), which is the key element in the quality control of glycoprotein folding in mammalian cells [9, 10]. Therefore, although the proposal was made in previous papers that calnexin in S. cerevisiae acts in the folding and quality control of glycoproteins, it is unclear whether the model proposed for the quality control of glycoprotein folding in mammalian cells is applicable to S. cerevisiae or not [8]. Interestingly, the disruption of the calnexin gene in S. cerevisiae did not have gross effects on the levels of cell growth, even though the disruption of the gene was lethal for Schizosaccharomyces pombe or mammalian cells [8].

In our previous study, we investigated the level of cell growth of the calnexin-disrupted strain under the internal stress conditions of overexpressing non-glycosylated or glycosylated lysozymes in the ER. There was no evident difference between the growth curve of the calnexin-disrupted strain and that of the wild-type strain [11]. These results indicate that calnexin is not always essential in S. cerevisiae. It also suggests that some other chaperones may act in the function of glycoproteins in S. cerevisiae as backups for calnexin. In this study, in order to obtain a full image of the effect of calnexin deletion on S. cerevisiae under heat stress, we attempt to culture its calnexin-disrupted and wild-type strains under conditions of heat stress.

Newly synthesized secretory and transmembrane proteins traverse the ER, where they fold into correct tertiary and quaternary structures. The productive folding process of these proteins is assisted by molecular chaperones and folding enzymes localized in the ER [12, 13]. When unfolded proteins are accumulated in the ER under a variety of stress conditions (ER stress), synthesis of these chaperones and enzymes is known to be induced at the level of transcription [14, 15]. This means that eukaryotic cells possess an intracellular signaling pathway from the ER to the nucleus, called the unfolded protein response (UPR) pathway [10]. Protein disulfide isomerase (PDI) is one of the main molecular chaperones in the ER, and it plays a major role in the protein-folding process. It has been reported that PDI also plays an important role in glycoprotein folding in mammalian cells [16]. In our previous study, it appeared that the deletion of
calnexin resulted in the induction of the unfolded protein response (UPR), and
increased the levels of other ER molecular chaperones (PDI) to fold the unfolded
glycosylated lysozymes (internal stress) [11].
ERp57 is a member of the PDI oxidoreductase family. It is a PDI isoform that
functions naturally in association with the lectins calnexin and calreticulin [17].
There is a known interaction between ERp57 and calnexin [18]. Unlike the
mammalian calnexin gene, the \textit{S. cerevisiae} \textit{CNE1} gene is not essential for cell
viability, although it has an effect on the retention of mutant proteins in the ER
[19], and there are no other chaperones such as the mammalian calreticulin which
may act instead of calnexin. In a previous study, Parlati \textit{et al.} found that \textit{Cne1p}
has similar functions to mammalian calnexin [8], but whether the PDI interacts
with \textit{Cne1p in vivo} was still not clarified. A recent study identified that Mpd1p,
one of 4 homologues of Pdi1p in yeast, can interact with \textit{Cne1p in vitro} [26].
Interestingly, a study recently done on \textit{Caenorhabditis elegans} yielded the
suggestion that the increment of PDI family members in the absence of calnexin
can be an alternative compensatory way for the chaperone machinery \textit{in vivo}
[27].
In this study, we attempt to investigate the induced mRNA level of PDI in
calnexin-disrupted yeast under external heat stress conditions. This may offer
some insights as to why calnexin deletion does not lead to gross effects on the
levels of cell growth under stress conditions. Moreover, the protein expression
level of PDI in the ER will be further investigated using western blot analysis.
This paper also compares the concentration of molecular chaperones (PDI) in the
ER when the wild-type and calnexin-disrupted \textit{S. cerevisiae} strains are cultured
under normal and heat stress conditions. These observations may shed light on
the role of calnexin in the ER of \textit{S. cerevisiae}.

\textbf{MATERIALS AND METHODS}

\textbf{Materials}
The DNA sequencing kit, competitive DNA construction kit, competitive RNA
transcription kit, and mRNA selective PCR kit were purchased from Takara
Shuzo Co., Ltd. (Japan). Synthetic oligonucleotides were purchased from
Kurabo Co., Ltd. (Japan). The RNeasy mini kit was purchased from Qiagen
K.K., Japan. The DNA sequencing was carried out using a Thermo Sequenase
Core sequencing kit from Amersham (Japan). The peroxidase-conjugated rabbit
anti-rat IgG was purchased from Sigma and the peroxidase-conjugated goat anti-
rabbit IgG was purchased from Biosource International (USA). All the other
chemicals were of analytical grade for biochemical use.

\textit{S. cerevisiae} strains and growth media
The \textit{S. cerevisiae} haploid strains W303-1b (\textit{Mat a ade2-1 can1-100 ura3-1 leu2-3,
112 trp1-1 his3-11, 15}) and W303-1b \textit{\Delta (cne1::leu2)} were provided by
Dr. Parlati, McGill University, Canada. The \textit{S. cerevisiae} strain AH22 (\textit{MAT a,
Leu2, His4, Cir^-}) was provided by Dr. I. Kumagai, Tohoku University. For the
growth of the *S. cerevisiae* W303-1b strain, YPD (1% yeast extract, 2% peptone, 2% glucose) was routinely used.

**Disruption of the *S. cerevisiae* AH22 CNE1 gene**

The genomic DNA of *S. cerevisiae* W303-1b with disrupted calnexin was extracted and then amplified by PCR with primers from the CNE1 gene. The PCR product was transformed into the Leu² yeast strain AH22. Transformants were selected on BMM (Burkholder minimum medium) minus the leucine plates. Disruption of the CNE1 gene was confirmed by PCR with genomic DNA and RT-PCR with total mRNA using the same primers. For further genetic analysis, diploids were sporulated and tetrad dissection was performed by standard procedures, and the presence of the disruption in parent cells and spores was confirmed by RT-PCR.

**Analysis of mRNA by competitive reverse transcriptase-polymerase chain reaction**

The analysis of PDI expression was performed using the competitive reverse transcriptase-polymerase chain reaction (RT-PCR) as described by Siebert and Larrick [20]. Competitive RT-PCR is a method developed to overcome difficulties with basic RT-PCR. In it, the plateau phase of normal RT-PCR indicates almost the same amount of amplified products with sufficient cycles of PCR, regardless of the initial amount of the templates. A PDI-specific primer pair (5'-ACGTTAAAGCCGCGAGACT-3' and 5'-CGTTGCGTAGGTATCAGCT-3'), yielding RT-PCR products of 1064 bp, were prepared based on the PDI cDNA sequences of *S. cerevisiae* [21, 22]. The internal standard RNA was constructed as follows. An 876-bp (for PDI) homologous competitor mRNA fragment was obtained as described using a competitive DNA construction kit and a competitive RNA transcription kit (Takara, Japan). The homologous competitor mRNA fragment is a competitor that has the same nucleotide sequences as the target RNA, but contains a deletion of about 200 bp. Therefore, both compete for the same set of primers.

Yeast transformants were cultured at 30°C in 50 ml of selective medium JMM (yeast minimum medium) to the mid-log phase (OD₆₆₀ = 1.5), and then harvested by centrifugation at 1,000 g for 5 min at 4°C. Total yeast RNA was isolated using RNasy Mini Kits (Qiagen K.K., Japan). 500 ng of total RNA and 2 × 10⁷ copies of competitive mRNA were co-converted into first-strand cDNA using the antisense specific primers. Subsequently, equal portions of cDNA (a mixture of the target and a competitor) were co-amplified by PCR with the PDI-specific primer pair. The products were then resolved on a 1.5% agarose gel stained with ethidium bromide. Due to the competition, the ratio of the amounts of the two amplified products reflects the ratio between the target mRNA and RNA competitor. The amount of target mRNA is directly proportional to the log (Aᵣ/Aᶜ), where Aᵣ is the amount of amplified product from the target mRNA, and Aᶜ is the amount of amplified product from the competitor RNA. The densities
of target and competitor bands in the gel after RT-PCR were analyzed using a Molecular Imager (Bio-Rad, Japan). The entire gel was digitized and the relevant band scanned. The lane scanning width was 4 mm (80-90% of the total lane width). This scanning width was adjusted slightly in some lanes to compensate for artifacts. The DNA levels corresponded to density absorbance units (OD × mm).

Analysis of the induction of molecular chaperones by western blot
Cell lysates were prepared from a total of 2.0 A_{660} units of exponentially growing cells that were pelleted and washed once with 10 mM NaN_{3} before the cell pellet was frozen at -20ºC and thawed on ice. A 20-μl aliquot of sample buffer (80 mM Tris, pH 6.8, 2% SDS, 0.1% bromphenol blue, 100 mM dithiothreitol, and 10% glycerol) was then added, and the samples were incubated at 95ºC for 2 min. After a total of 0.12 g of glass beads (Sigma G-9268) was added, the cells were disrupted by agitation on a vortex mixer for 1 min. 80 μl of sample buffer was added before the aliquots were reheated and applied to SDS-polyacrylamide gels. After the transfer of the electrophoresed proteins to nitrocellulose membranes, antibodies against yeast PDI (1:1,000, Stressgen Biotechnologies Corp. SPA-890) were used to probe the cell lysates and HRP-conjugated goat anti-rabbit IgG (1:10,000, for PDI) to detect the primary antibodies. The loading and transfer control (load) on the PVDF membrane was stained by Amido Black. The resulting blots were washed, and then exposed and processed by ECL analysis. The densities of the PDI and loading bands in the gel were analyzed using a Molecular Imager (Dolphin gel analysis, USA). The entire gel was digitized and the relevant band scanned. The protein levels correspond to the integration optical density.

RESULTS AND DISCUSSION
Calnexin gene deletion has no effect on the generation time of S. cerevisiae under heat stress conditions
The effect of calnexin gene deletion on the growth of S. cerevisiae W303-1b was investigated in our previous study. The growth of the calnexin-deleted strain was almost the same as that of the wild-type strain [23]. Here, we further investigated the generation time of calnexin-deleted S. cerevisiae W303-1b under the conditions of temperature stress (20ºC, 37ºC). As shown in Fig. 1 and Tab. 1, we observed that the calnexin-deleted and wild-type strains grew less well under the stress conditions than under normal conditions. Interestingly, there is no evident difference between the colonies of calnexin-deleted and wild-type strains even under heat stress conditions. A similar manner of growth was obtained for another strain, S. cerevisiae AH22 (data not shown). We again noted that the calnexin-deleted and wild-type strains have similar generation times under any conditions.
Fig. 1. The effect of calnexin deletion on the generation time of *S. cerevisiae* W303-1B under heat stress conditions. A wild strain W303-1b (black column) and a calnexin-disrupted strain (white column) were incubated at 20°C, 30°C and 37°C in YPD medium for the period indicated. The generation time was calculated by following the turbidity at OD660 nm. The vertical bars indicate the standard deviations (n = 3).

Tab. 1. The effect of calnexin deletion on *S. cerevisiae* under different conditions.

| Strains          | Conditions       | Relative induced mRNA levels of PDI | Generation time [h] b |
|------------------|------------------|-------------------------------------|-----------------------|
| W303-1b wild-type| Normal (control) | 1.00                                | 1.61                  |
| W303-1b wild-type| 37°C             | 2.29                                | 4.32                  |
| W303-1b wild-type| LzWT (stable)    | 1.09 a                              | 1.72                  |
| W303-1b wild-type| LzG49N (stable)  | 1.54 a                              | ND                    |
| W303-1b wild-type| LzK13D (unstable)| 3.08 a                              | ND                    |
| W303-1b wild-type| LzK13D/G49N (unstable, glycoprotein) | 2.31 a | 1.78 |
| W303-1b Δ CNE    | Normal           | 1.00                                | 1.65                  |
| W303-1b Δ CNE    | 37°C             | 3.28                                | 4.22                  |
| W303-1b Δ CNE    | LzWT (stable)    | 1.07 a                              | 1.71                  |
| W303-1b Δ CNE    | LzG49N (stable)  | 2.33 a                              | ND                    |
| W303-1b Δ CNE    | LzK13D (unstable)| 3.00 a                              | ND                    |
| W303-1b Δ CNE    | LzK13D/G49N (unstable, glycoprotein) | 3.59 a | 1.75 |

The values are the means of three measurements. a Revised data from our previous study [11]. b Growth rates were measured in liquid YPD at 30°C except for those for the heat stress conditions (37°C). ND, not determined; Lz, lysozyme; wt: wild type; K13D, G49N, K13D/G49N: point mutations on lysozyme.

**The induced mRNA levels of the molecular chaperones (PDI) increased in calnexin-disrupted *S. cerevisiae* under heat stress conditions**

Protein disulfide isomerase (Pdi1p) constitutes a family of structurally related enzymes that catalyze disulfide bond formation, reduction, or the isomerization of newly synthesized proteins in the lumen of the endoplasmic reticulum (ER). They also act as chaperones, and are a part of a quality-control system for the correct folding of the proteins in the same subcellular compartment [24].
Therefore, it seems likely that the deletion of calnexin induces the unfolded protein response (UPR) [25], and increases the level of PDI to fold the unfolded glycoprotein. To investigate whether the molecular chaperones in the ER are induced as a result of calnexin disruption, we measured the mRNA concentration of molecular chaperones (PDI) in wild-type and calnexin-disrupted *S. cerevisiae* W303-1b. The levels of the induction of PDI are shown in Fig. 2. The yeast cells showed a similar amount of PDI (Fig. 2A, lanes 1 and 2) in the wild-type and calnexin-disrupted strains under normal conditions, from which we speculate that the calnexin deletion does not induce PDI in *S. cerevisiae* under normal conditions. By contrast, significant differences in the inductions of PDI (Fig. 2A, lanes 3 and 4) were observed when the wild-type and calnexin-disrupted *S. cerevisiae* were cultured under heat stress conditions. Interestingly, both of the PDI chaperones were induced in higher concentrations in the calnexin-disrupted strain than in the wild strain. The quantitative analysis of the RT-PCR patterns was further measured with a densitometer. The graphs in Fig. 2B show the ratios of the density of the target band (above) to that of the competitor band (below) in calnexin-disrupted (black column) and wild-type *S. cerevisiae* (white column).

Fig. 2. Quantitative analysis of the PDI mRNA level when the wild-type and calnexin-disrupted *S. cerevisiae* strains were grown under heat stress conditions. The electrophoresis of RT-PCR products separated in 1.5% agarose gel was stained with ethidium bromide (A). The 1064-bp products (above) from the yeast RNA and the 876-bp products (below) from the PDI competitor RNA are indicated. In order to quantitatively analyze the mRNA levels, the concentrations of the RT-PCR patterns were measured with a densitometer (B). The graph shows the ratios of the density of the target band (above) to that of the competitor band (below) in calnexin-disrupted (white column) and wild-type *S. cerevisiae* (black column). D_t, the density of the band from the target mRNA; D_c, the density of the band from the competitor RNA. The vertical bars indicate the standard deviations (n = 3).

The PDI protein expression parallels its mRNA level in both wild-type and calnexin-disrupted *S. cerevisiae*

To identify the effect of calnexin deletion on PDI expression at the translational level, we performed SDS-PAGE and western blot analysis to detect PDI proteins in the cell lysates. These were prepared from a total of 2.0 A_{660} units of exponentially growing cells. The cell pellets were disrupted with glass beads by agitation on a vortex mixer, and used for SDS-PAGE. After transferring the
electrophoresed proteins to nitrocellulose membranes, antibodies against yeast PDI were used to probe the cell lysates, and HRP-conjugated goat anti-rabbit IgG (for PDI) were used to detect the primary antibodies. The resulting blots were washed, and then exposed and processed by ECL analysis (Fig. 3). The yeast cells showed a similar protein concentration of PDI (Fig. 3, lanes 1 and 2) in the wild-type and calnexin-disrupted strains under normal conditions, while the protein levels of PDI in the ER were evidently increased in the calnexin-disrupted \textit{S. cerevisiae} under the heat stress conditions (Fig. 3, lane 4). Therefore, the data from the western blotting analysis for proteins showed similar results to those for mRNAs. This indicates that PDI protein expression parallels their mRNA level in both strains.

Fig. 3. Western blot analysis of the PDI expression level when the wild-type and calnexin-disrupted \textit{S. cerevisiae} strains were grown under heat stress conditions. A – Whole cell lysates of wild-type (CNE⁺) and calnexin-disrupted (CNE⁻) yeasts under the conditions indicated at the top were subjected to Western blotting analysis probing for PDI as indicated. The bottom section shows portions of membranes stained by Amido Black as loading and transfer controls (input). B – The concentrations of the bands were measured with a densitometer. The graph shows the ratios of the density of the PDI band (above) to that of the load band (below) in calnexin-disrupted (white column) and wild-type \textit{S. cerevisiae} (black column). The vertical bars indicate the standard deviations (n = 3).

These results indicate that the high level induction of the mRNA of the molecular chaperones (PDI) occurs only in the calnexin-disrupted strain under the heat stress conditions. The significant increases in the induction of the mRNA in the calnexin-disrupted yeast suggest calnexin is involved in the folding of glycoproteins and that the increased PDI seems likely to fold unfolded glycoproteins instead of calnexin in calnexin-deleted yeast under the heat stress conditions. In other words, it seems likely that when the \textit{S. cerevisiae} was cultured under heat stress, the deletion of calnexin brought about the inhibition of unfolded glycoproteins undergoing the degradation pathway. Thus, the accumulation of unfolded glycoproteins enhanced the expression level of ER PDI via the unfolded protein response (UPR) pathway in order to help the folding of unfolded glycoproteins.
In our previous study, we obtained evidence of quality control of calnexin by comparing the secreted amounts of the wild-type and various mutant glycosylated lysozymes in both wild-type and calnexin-disrupted yeast strains. The calnexin-disrupted *S. cerevisiae* exhibited a great increase in the secretion of unstable glycosylated lysozyme mutants, despite the slight secretion by the wild type strain. In addition, we also found that high-level induction of PDI only occurs for those glycosylated proteins over-expressed in the calnexin-disrupted strain [11]. Combined with the results that we obtained in this study, it seems that both the external and internal ER stress (overexpressing glycoproteins in the ER) could lead to an increase in the induced level of ER molecular chaperones (PDI) in calnexin-disrupted *S. cerevisiae* (Tab. 1). The significant increases in the expression of PDI in the calnexin-disrupted yeast suggest that Cne1p is involved in the folding of glycoproteins associated with PDI, and can increase the efficiency of PDI, and that PDI seems likely to fold unfolded glycoproteins instead of Cne1p in the calnexin-deleted yeast. In other words, it seems likely that the deletion of calnexin protects unfolded glycoproteins from the degradation pathway, thus the accumulation of unfolded glycoproteins enhances the expression levels of the ER molecular chaperones (PDI) via the UPR pathway to facilitate the folding of unfolded glycoproteins.

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