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Effects of Calnexin Deletion in *Saccharomyces cerevisiae* on the Secretion of Glycosylated Lysozymes[1]

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Disruption of the calnexin gene in *Saccharomyces cerevisiae* did not lead to gross effects on the levels of cell growth and secretion of wild-type hen egg white lysozymes (HEWL). To investigate the function of calnexin in relation to the secretion of glycoproteins, we expressed both stable and unstable mutant glycosylated lysozymes in calnexin-disrupted *S. cerevisiae*. The secreted amounts of stable mutant glycosylated lysozymes (G49N and S91T/G49N) were almost the same in both wild-type and calnexin-disrupted *S. cerevisiae*. In contrast, the secretion of unstable mutant glycosylated lysozymes (K13D/G49N, C76A/G49N, and D66H/G49N) greatly increased in calnexin-disrupted *S. cerevisiae*, although their secretion was very low in the wild-type strain. This indicates that calnexin may act in the quality control of glycoproteins. We further investigated the expression level of the mRNA of the molecular chaperones BiP and PDI, which play a major role in the protein folding process in the ER, when glycosylated lysozymes were expressed in wild-type and calnexin-disrupted *S. cerevisiae*. The mRNA concentrations of BiP and PDI were evidently increased when the glycosylated lysozymes were expressed in calnexin-disrupted *S. cerevisiae*. This observation indicates that BiP and PDI may be induced by the accumulation of unfolded glycosylated lysozymes due to the deletion of calnexin.

Key words: calnexin, glycosylation of lysozyme, quality control, *Saccharomyces cerevisiae*.

Recent studies have demonstrated that calnexin is a major molecular chaperone associated transiently with numerous newly synthesized glycoproteins during their maturation in the endoplasmic reticulum (ER) (*1–3*). Calnexin was originally found in transient association with assembling of the class I histocompatibility molecules (*4*). Subsequently, calnexin has been found associated with folding and assembly intermediates of a wide array of soluble and membrane proteins (*5, 6*). In addition to folding and assembly, it has been proposed that calnexin is 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 (*7–10*). The isolation and sequencing of cDNA and genomic clones of eukaryotic cells have revealed that the general structural organization of calnexin has been conserved through evolution. The homologue of calnexin (CNE1p) in the yeast *Saccharomyces cerevisiae* has been reported to exhibit similarity to its mammalian counterpart, but it lacks a cytoplasmic tail and has no calcium-binding capacity (*11, 12*). The function of calnexin in *S. cerevisiae* remains to be determined. Interestingly, disruption of the calnexin gene in *S. cerevisiae* did not lead to inviable cells, all cells growing at normal rates, although disruption of the gene in *Schizosaccharomyces pombe* or mammalian cells was lethal (*11*). This makes it possible to directly investigate the function of calnexin in relation to the secretion of glycoproteins using calnexin-disrupted *S. cerevisiae*, while this is impossible for *S. pombe* or mammalian cells.

Hen egg white lysozyme (HEWL) has been well studied with respect to structural and functional properties. Therefore, we have a lot of information on stable and unstable mutant lysozymes. In addition, we have reported that hen egg white lysozyme was glycosylated in a yeast expression system when the *N*-glycosylation signal sequence, Asn-X-Thr/Ser, was introduced by site-directed mutagenesis of lysozyme cDNA (*13*). Thus, in order to elucidate the quality control function of calnexin in relation to the secretion of glycoproteins, we investigated the secretion of both stable and unstable mutant lysozymes having an *N*-glycosylation signal sequence in wild-type and calnexin-disrupted *S. cerevisiae*.

MATERIALS AND METHODS

Materials—Restriction enzyme, T4 DNA ligase, and alkaline phosphatase were purchased from Takara Shuzo (Kyoto). The DNA sequencing kit, DNA blunting kit, PCR *in vitro* mutagenesis kit, competitive DNA construction kit,

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Abbreviations: HEWL, hen egg white lysozyme; ER, endoplasmic reticulum; BiP, binding protein; PDI, protein disulfide isomerase; UPR, unfolding protein response; PAGE, polyacrylamide gel electrophoresis; RT-PCR, reverse transcriptase–polymerase chain reaction.

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competitive RNA transcription kit, and mRNA selective PCR kit were also purchased from Takara Shuzo. Synthetic oligonucleotides were purchased from Kurabo (Osaka). DNA sequencing was carried out using a Thermo Sequenase Core sequencing kit from Amersham Japan. Ethylene glycol chitin for the lysozyme assay was from Sigma. CM-Toyopearl resin was a product of Toso (Tokyo). The Centricon centrifugal concentrator was purchased from Amicon (USA). The RNeasy mini kit was purchased from Qiagen K.K., Tokyo. All other chemicals were of analytical grade for biochemical use.

Bacterial Strains and Plasmids—Escherichia coli TG1 [*K12, Δ(lac-pro), supE. thi, hsd D5/F′ (tra D36, proA⁺B⁺, lacI^q, lacZ ΔM15)*], which was used as the host for propagation of a pUC plasmid (pKK-1 or pUC-118), and M13mp19 were supplied by Amersham Japan. *S. cerevisiae* haploid strain W303-1b (*Mat a ade2-1 can1-100 ura3-1 leu2-3, 112 trp1-1 his3-11, 15*) and W303-1b *Δ(cne1::Leu2)* were provided by Dr. Parlati, McGill University, Canada. *S. cerevisiae* strain AH22 (*MAT a, Leu2, His4, Cir⁺/⁻*) was provided by Dr. I. Kumagai, Tohoku University. Recombinant plasmid pKK-1, which contains a full-length hen egg-white lysozyme cDNA, was also provided by Dr. I. Kumagai, Tohoku University. Plasmid pUC-118 was supplied by Dr. M. Yamada, Department of Biochemistry, Yamaguchi University, Yamaguchi. pYG-100, an *E. coli*–yeast shuttle vector, was provided by Dr. K. Matsubara of Osaka University. Yeast episomal vectors pRS426 and pRS423 were provided by Dr. R. Akada, Department of Applied Chemistry and Chemical Engineering, Yamaguchi University.

Growth Media—The *E. coli* cells were grown in LB-broth (1% Bactotryptone, 0.5% yeast extract, 1% NaCl). The medium was supplemented with 60 μg/ml of ampicillin for selection of transformation. For the growth of yeast, YPD (1% yeast extract, 2% bactotryptone, 2% glucose) was routinely used. The *S. cerevisiae* AH22 cells carrying expression vector pRS423/Lz were grown on modified Burkholder minimum medium (BMM) supplemented with 20 μg/ml of histidine. The *S. cerevisiae* W303-1b cells carrying expression vector pRS426/Lz were grown on yeast minimum medium (JMM) supplemented with 40 μg/ml of adenine, 60 μg/ml of leucine, 40 μg/ml of tryptophan, and 20 μg/ml of histidine.

Disruption of the S. cerevisiae AH22 CNE1 Gene—The genomic DNA of *S. cerevisiae* W303-1b disrupted calnexin was extracted and then amplified by PCR with primers from the CNE1 gene. The PCR product was transformed into Leu2⁻ yeast strain AH22. Transformants were selected on BMM minus 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 occurrence of the disruption in parent cells and spores was confirmed by RT-PCR.

PCR Site-Directed Mutagenesis of HEWL cDNA—Site-directed mutagenesis of HEWL cDNA was carried out using a PCR *in vitro* mutagenesis kit (Takara). The five primers mentioned below can be commonly used in a series of different mutagenesis. The primers used for site-directed mutagenesis for constructing various mutants were as follows: 5′-AACACCGATAACAGTACCGAC-3′, 5′-AGCGTGAACGCCGCGAAGAAG-3′, 5′-GCAGCTATGGACCGTCACGGA-3′, 5′-GTGGTGCAACCATGGCAGGAC-3′, and 5′-ATAACAGCGACCGTGAACTGC-3′ for G49N, C76A, K13D, D66H, and S91T, respectively. Single or double mutations were made for the glycosylation of each mutant. After PCR, the products were digested with *Eco*RI/*Hin*dIII. The resulting fragment was purified on a low melting agarose gel and then inserted into pUC118 after digestion with *Eco*RI/*Hin*dIII. The insertion was confirmed by the transformation to *E. coli* TG1. This was selected on a LB agar plate with 60 μg/ml ampicillin. The presence of the mutation was confirmed by the dideoxynucleotide chain termination method using the Thermo Sequenase Core sequencing kit from Amersham Japan.

Construction of S. cerevisiae W303-1b and AH22 Expression Plasmids of the Mutant Hen Lysozymes—The cDNAs of mutant lysozymes were inserted into the *Sal*I site in the pYG100 vector between the GPD promoter and terminator. pYG100 carrying mutant lysozyme genes was treated with *Hin*dIII. Thus, fragments (1.7 kb) comprising from the promoter to terminator region containing the mutant hen lysozyme cDNA were obtained. By using the blunt end approach, these fragments were inserted into the multiclonal site of pRS426, the expression plasmid of yeast *S. cerevisiae* W303-1b, and the multiclonal site of pRS423, the expression plasmid of yeast *S. cerevisiae* AH22.

Expression of the Mutant Hen Lysozymes in S. cerevisiae W303-1b—The expression vector was introduced into *S. cerevisiae* W303-1b according to the lithium acetate procedure (*14*). Ura⁺ transformants were screened by subculturing in yeast minimum medium (JMM) supplemented with 20 μg/ml of histidine, 40 μg/ml of adenine, 60 μg/ml of leucine, and 40 μg/ml of tryptophan at 30°C. The over-expressing subclones with the highest levels of lysozyme activity were screened and propagated from single colonies, and then inoculated into 3 ml of JMM and incubated for 2 days at 30°C with shaking. This preculture was subcultured to 100 ml of the same medium in a flask (500 ml) and incubated for another 2 days at 30°C with shaking, and then 10 ml of the second preculture was transferred to fresh 1 liter of JMM in a 3 liter flask and cultured under the same conditions.

Expression of Mutant Hen Lysozymes in S. cerevisiae AH22—The expression vector was introduced into *S. cerevisiae* AH22 according to the lithium acetate procedure. His⁺ transformants were screened by subculturing in modified Burkholder minimum medium (BMM) supplemented with 60 μg/ml of leucine at 30°C. After cultivation, the same method as described for W303-1b was used for AH22 to express the mutant lysozymes.

Purification of the Mutant Hen Lysozymes—The growth medium of the host cells was centrifuged at 7,000 rpm for 10 min at 4°C. The supernatant was applied to a CM-Toyopearl 650M column (1.5 × 5.0 cm) equilibrated with 50 mM Tris-HCl buffer (pH 7.5), and then the adsorbed proteins were step-eluted using the same buffer containing 0.5 M sodium chloride (*13*). The fraction containing the protein was collected, and then samples which had been desalted and concentrated with Centricon centrifugal concentrators were used in the experiments.

SDS–Polyacrylamide Gel Electrophoresis—SDS–polyacrylamide gel electrophoresis was conducted according to the method of Laemmli (*15*) using a 15% acrylamide separating gel and a 5% stacking gel containing 1% SDS. Sam-

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ples were heated at 100°C for 5 min in Tris-glycine buffer (pH 8.8) containing 1% SDS and 1% 2-β-mercaptoethanol. Electrophoresis was carried out at a constant current of 10 mA for 5 h using an electrophoretic buffer of Tris-glycine containing 0.1% SDS. After electrophoresis, the gel sheets were stained for protein and carbohydrate with a 0.025% Coomassie Brilliant Blue R-250 solution and a 0.5% periodic acid-Fuchsin solution (*16*), respectively.

Determination of ΔG—The Gibbs free energy change (ΔG) was determined from denaturation curves, which were drawn by following the changes in the ellipticity at 222 nm during heating according to the previously described method (*17*). The temperature was controlled during all the measurements by circulating water in the cell holder from a thermostated bath with a heating rate of 1°C/min from 35 to 80°C. Using the data for the thermal denaturation curves, the transition temperature (T_d) and the apparent fraction (f_{app}) of unfolding were represented as a function of temperature to clearly show the denaturation curves. The thermal denaturation of the lysozymes at pH 3.0 was completely reversible, therefore, we can calculate the equilibrium constant between the native and denatured forms by determining the fraction of unfolding, f_d, from the denaturation curves with Eq. 1.

$$K = f_{app}/(1-f_{app}) \quad (1)$$

The unfolding enthalpy change (ΔH) was calculated with the van't Hoff Eq. 2 as a function of temperature near T_d, the transition point of denaturation.

$$\ln K_1/K_2 = -\Delta H^*/R(1/T_1 - 1/T_2) \quad (2)$$

Where ΔH^* is the enthalpy change at T_d, T_1, or T_2 is the temperature near T_d, K_1, or K_2 is the equilibrium constant at T_1 or T_2, respectively, and R is the gas constant. Entropy (ΔS) can be calculated from

$$\Delta G = \Delta H - T\Delta S$$

Because ΔG is 0 at T_d, $\Delta S^* = \Delta H^*/T_d$, where ΔS^* is the entropy change at T_d. The Gibbs energy change (ΔG) of unfolding can be calculated by

$$\Delta G = \Delta H^* - T\Delta S^* + \Delta C_p\,[T - T_d - T \ln (T/T_d)]$$

where ΔC_p is the denaturational increment of the heat capacity and the value for the lysozyme is 1.594 kcal/mol.

Analysis of mRNA by Competitive Reverse Transcriptase–Polymerase Chain Reaction—Analysis of BiP and PDI expression was performed using the competitive reverse transcriptase–polymerase chain reaction (RT-PCR) as described by Siebert and Larrick (*18*). Competitive RT-PCR is the method developed to overcome difficulties, in which the plateau phase of normal RT-PCR indicates almost the same amount of amplified products, regardless of the initial amount of the templates, with sufficient cycles of PCR. A BiP-specific primer pair (5′-GCCCGCTGTAGAAGTAAGTG-3′ and 5′-CTCTTGGTGCTGGTGGAATG-3′), yielding RT-PCR products of 1,099 bp, and a PDI-specific primer pair (5′-ACGTTAAAGCCGCCGAGACT-3′ and 5′-CGTTGGCGTAGGTATCAGCT-3′), yielding RT-PCR products of 1,064 bp, were prepared based on the BiP and PDI cDNA sequences of *S. cerevisiae* (*19, 20*). The internal standard RNA was constructed as follows. 913 bp (for BiP) and 876 bp (for PDI) homologous competitor mRNA fragments (the competitor having the same nucleotide sequence as the target RNA but containing a deletion of about 200 bp), competing for the same set of primers, were obtained as described using a competitive DNA construction kit and a competitive RNA transcription kit (Takara).

Yeast transformants were cultured at 30°C in 50 ml of selective medium (JMM) to the mid-log phase (OD_{600} = 1.5), and then harvested by centrifugation at 1,000 ×*g* for 5 min at 4°C. Total yeast RNA was isolated using RNeasy Mini Kits (Qiagen). 500 ng of total RNA and 2×10^7 copies of competitive mRNA were co-converted into first-strand cDNA by 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 BiP or PDI specific primer pair. The products were then resolved on a 1.5% agarose gel stained with ethidium bromide. Because of the competition, the ratio of the amounts of the two amplified products reflects the ratio of the target mRNA and RNA competitor. The amount of target mRNA is directly proportional to the log (A_t/A_c), where A_t is the amount of amplified product from the target mRNA, and A_c is the amount of amplified product from the competitor RNA. The densities of the target and competitor bands in the gel were determined with a Molecular Imager (Bio-Rad, Tokyo).

RESULTS

Effect of Calnexin Gene Deletion on the Growth of S. cerevisiae W303-1b—The effect of calnexin gene deletion on the growth of *S. cerevisiae* W303-1b was investigated in our previous study (*21*). The growth of the calnexin-deleted strain was almost the same as that of the wild-type strain. We further investigated the growth of calnexin-deleted *S. cerevisiae* W303-1b under the conditions of temperature stress (20°C, 37°C), osmotic stress (0.7 M NaCl, 1 M sorbitol), and over-expressed unstable glycoproteins. As shown in Fig. 1, the growth curve of the calnexin-disrupted strain was almost the same as that of the wild type under any stress condition. A similar phenomenon was observed for another *S. cerevisiae,* AH22 (data not shown). This suggested that disruption of the calnexin gene did not lead to gross effects at the level of growth.

Conformation Stability of Mutant HEWLs Constructed by Genetic Modification—Lysozyme can be glycosylated by the introduction of an Asn-X-Thr/Ser sequence, which is the signal sequence of *N*-glycosylation in eukaryotic cells. We reported that mutant lysozyme G49N, whose glycine at position 49 was substituted with asparagine, was glycosylated in the yeast expression system (*13*). Lysozyme is easily remodeled into stable and unstable forms by site-directed mutagenesis. Mutant lysozymes K13D, whose lysine at position 13 was substituted with aspartic acid, C76A, whose cysteine at position 76 was substituted with alanine (*22, 23*), and D66H, whose aspartate at position 66 was substituted with histidine, were used as unstable forms, while the wild-type and mutant S91T, whose serine at position 91 was substituted with threonine, were used as stable forms (*24*). These stable and unstable mutants were glycosylated by the introduction of *N*-linked glycosylation signal sequences at position 49 (G49N). Thus, mutant lysozymes K13D/G49N, C76A/G49N, and D66H/G49N were used as typical unstable glycosylated proteins, and G49N and S91T/G49N were used as typical stable glycosylated

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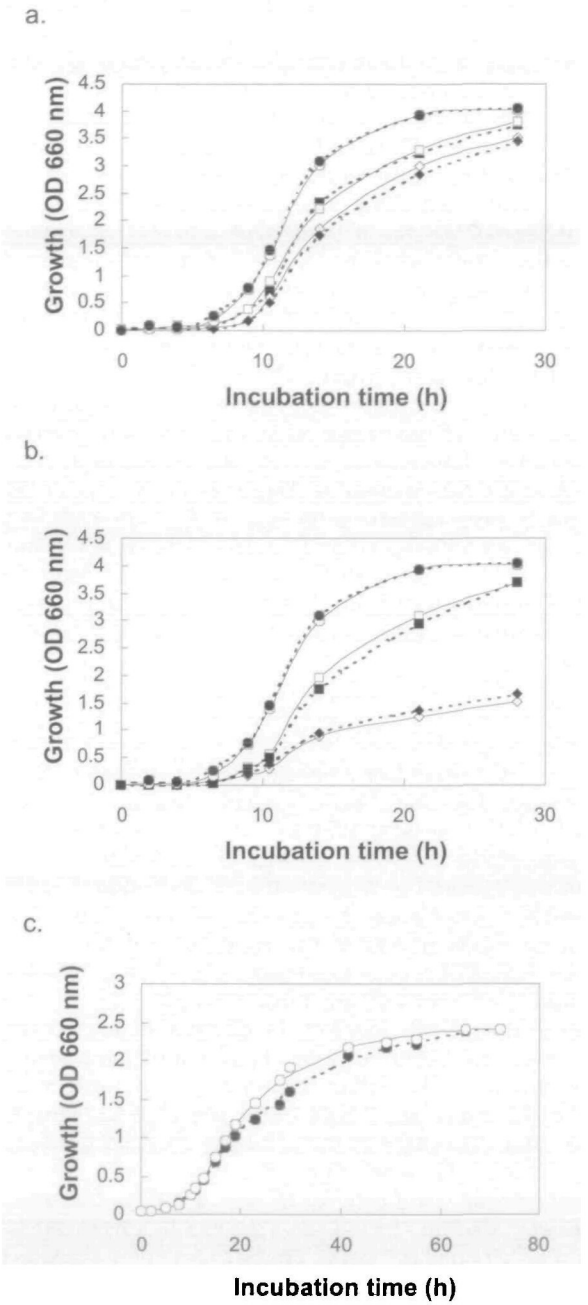

Fig. 1. **Effect of calnexin deletion on the growth of *S. cerevisiae* W303-1b.** a, wild strain W303-1b (open symbols and solid lines) and the calnexin-disrupted strain (solid symbols and dashed lines) were incubated at 30°C in YPD medium for the periods indicated under osmotic stress conditions (1.0 M sorbitol, diamonds; 0.7 M NaCl, squares) and normal conditions (circles); b, wild strain W303-1b (open symbols and solid lines) and the calnexin-disrupted strain (solid symbols and dashed lines) were incubated at 20°C (squares), 30°C (circles), or 37°C (diamonds) in YPD medium for the periods indicated. c, wild strain W303-1b (open symbols and solid lines) and the calnexin-disrupted strain (solid symbols and dashed lines) were incubated when the mutant lysozyme C76A/G49N was expressed. Wild strain W303-1b (open circles) and the calnexin-disrupted strain (solid circles) were incubated at 30°C in yeast minimum medium (JMM) for the periods indicated. The growth curves were drawn by following the turbidity at OD_{660nm}. Values are the means of three measurements.

TABLE I. **Thermodynamic parameters of the wild and mutant glycosylated lysozymes.**

	T_d (°C)	ΔH (kcal·mol^{-1})	$T\Delta S$ (kcal·mol^{-1})	ΔG^a (kcal·mol^{-1})
Wild	68.3	113.4	107.6	5.8
G49N	67.1	112.0	106.4	5.6
S91T/G49N	69.1	114.9	108.4	6.5
K13D/G49N	52.9	90.9	89.4	1.5
C76A/G49N	47.4	82.5	82.5	0
D66H/G49N	46.9	81.7	82.0	–0.3

T_d (transition point of denaturation) was determined from the denaturation curves drawn by following the changes in ellipticity at 222 nm during heating at 40°C to 90°C at pH 3.0. The thermodynamic parameters were determined with the equation under "MATERIALS AND METHODS." [a]Values are extrapolated to 47.4°C.

proteins. The thermodynamic parameters of these glycosylated mutant lysozymes are shown in Table I. The transition points of denaturation (T_d) of glycosylated lysozymes G49N and S91T/G49N are almost the same as that of the wild-type lysozyme, although those of G49N and S91T/G49N are lower and higher by about 1°C, respectively. On the other hand, the transition points of mutants K13D/G49N, C76A/G49N, and D66H/G49N are greatly decreased by more than 15–20°C compared to that of the wild-type lysozyme. The glycosylation of lysozyme at position 49 (G49N) was not affected with regard to conformational stability, as estimated from thermodynamic parameters (Table I). The hydrophobic packing mutant (S91T/G49N) became more stable than the wild type and glycosylated mutant (G49N). On the other hand, the glycosylated mutant lacking a disulfide bridge between cysteines 76 and 94 (C76A/G49N), the glycosylated mutant destabilizing α-helix 5–15 (K13D/G49N), and amyloid-type mutant D66H/G49N were greatly destabilized.

Non-Glycosylated Lysozymes Secreted at Similar Levels in the Wild-Type and Calnexin-Deleted S. cerevisiae W303-1b—The secreted amounts of the wild lysozyme, and mutants K13D and C76A were compared with those of the wild-type and calnexin-deleted *S. cerevisiae* W303-1b. Since most of the lysozymes was secreted into the culture medium, the expressed amounts of the lysozymes can be estimated by measuring the content in the yeast culture medium. Although attempts to detect the lysozymes retained in the cytosol were made, the amounts were too small to detect, suggesting that most of the lysozymes were secreted outside the yeast cells. Figure 2a shows the secreted amounts of the wild-type and typical unstable lysozymes K13D and C76A in the wild-type and calnexin-deleted strains. There was no difference in the secreted amounts of the wild-type lysozyme and mutants K13D and C76A between the wild-type and calnexin-deleted *S. cerevisiae* W303-1b. This suggests that there are no effects of the disruption of calnexin on the secretion of foreign non-glycosylated proteins in this yeast strain.

Stable Glycosylated Lysozymes Secreted at Similar Levels in the Wild-Type and Calnexin-Deleted S. cerevisiae W303-1b—The secretion of the stable glycosylated wild-type lysozyme and mutant S91T was attempted in the wild-type strain and calnexin-disrupted strain W303-1b. As shown in Fig. 2b, the secreted amounts of the mutant G49N and S91T/G49N lysozymes were nearly the same for the wild-type and calnexin-deleted *S. cerevisiae* W303-1b. This was

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also confirmed by the SDS-PAGE patterns of the glycosylated mutant lysozymes, as shown in Fig. 3. We have reported that the large molecular sized *N*-glycosylated lysozyme with a polymannose chain is predominantly expressed in the yeast carrying the expression plasmid inserted into the mutant lysozyme (G49N) cDNA (*13*). After the initial core glycosylation in the ER, the hyperglycosylation of the mutant lysozyme occurs in the Golgi apparatus in *S. cerevisiae* due to elongation of the branched outer chains made up of more than 300 mannose residues (*13*). As shown in Fig. 3, all glycosylated mutant lysozymes were secreted in polymannosylated forms of large molecular size. Thus, it was confirmed that the secretion of stable glycosylated lysozymes was similar in the wild-type and calnexin-deleted strains.

Unstable Glycosylated Lysozymes Are Secreted in a Calnexin-Deleted Strain Much More Than in the Wild-Type Strain W303-1b—The secretion of unstable glycosylated mutants C76A/G49N, K13D/G49N, and D66H/G49N was very low in wild-type *S. cerevisiae* W303-1b, while it was greatly increased in a calnexin-deleted strain (Fig. 2c). It seems likely that unstable glycosylated lysozymes are retained by calnexin in the ER because of the poor folding

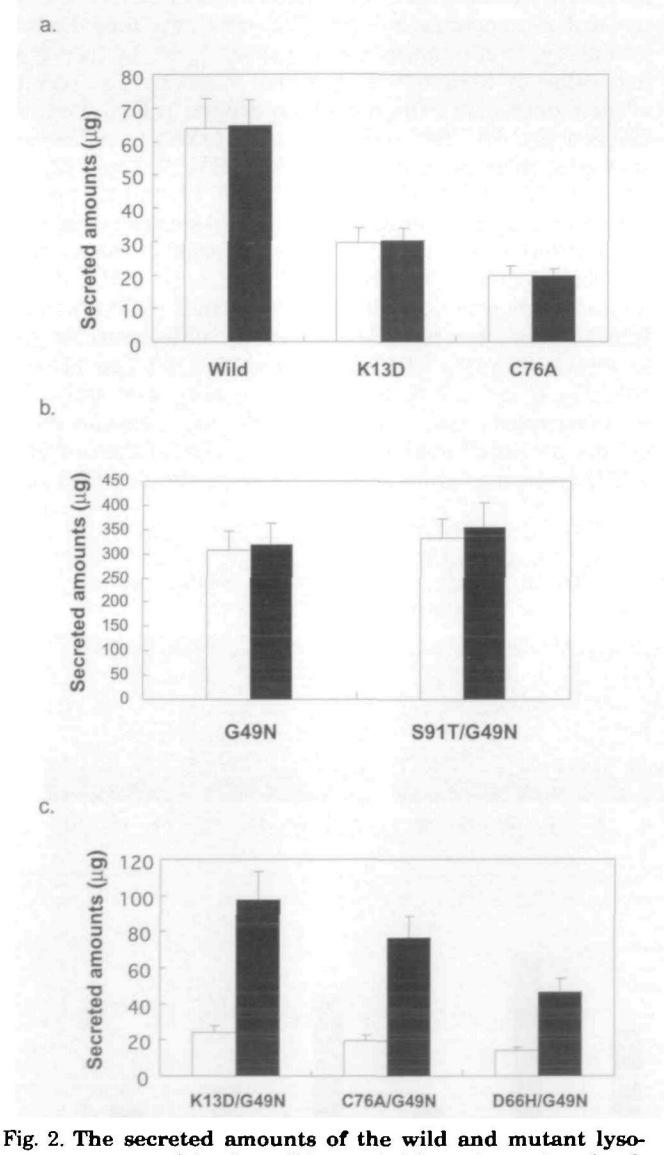

Fig. 2. **The secreted amounts of the wild and mutant lysozymes expressed in the wild-type (white columns) and calnexin-disrupted (black columns) *S. cerevisiae* W303-1b.** a, non-glycosylated lysozymes (wild-type, K13D and C76A); b, stable mutant glycosylated lysozymes (G49N and S91T/G49N); c, unstable mutant glycosylated lysozymes (K13D/G49N, C76A/G49N, and D66H/G49N). The growth medium supernatants were adsorbed on a CM-Toyopearl 650M column equilibrated with 50 mM Tris-HCl buffer (pH 7.5) and then eluted with the same buffer containing 0.5 M NaCl. The vertical bars indicate the standard deviation (n = 3).

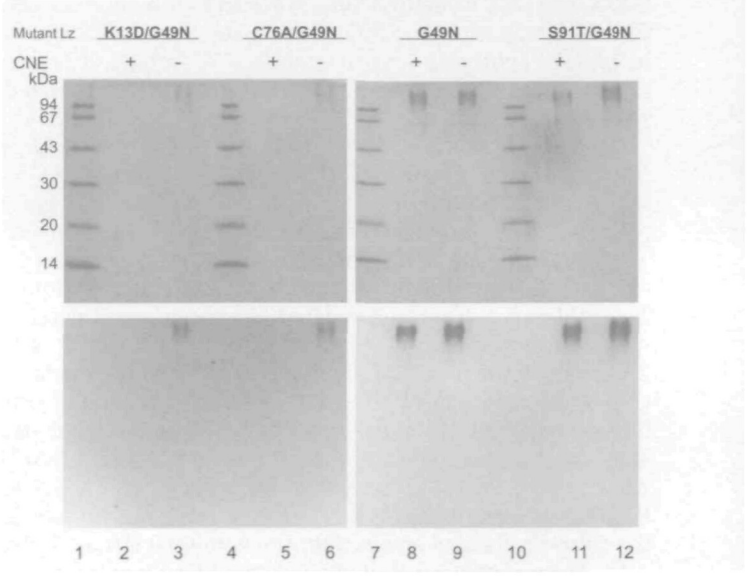

Fig. 3. **SDS-PAGE patterns of glycosylated mutant lysozymes secreted by wild-type (CNE$^+$) and calnexin-disrupted (CNE$^-$) *S. cerevisiae*.** The peak materials obtained on CM-Toyopearl column chromatography were subjected to SDS-PAGE. The eluates of unstable mutants (K13D/G49N and C76A/G49N) were concentrated five times and those of stable mutants (G49N and S91T/G49N) were not concentrated before electrophoresis. The gel sheets were stained for proteins and carbohydrates with Coomassie Brilliant (upper panels) and periodic acid–Fuchsin (lower panels). Lanes 1, 4, 7, and 10, molecular markers (94 kDa, phosphorylase *b*; 67 kDa, bovine serum albumin; 43 kDa, ovalbumin; 30 kDa, carbonic anhydrase; 20 kDa, trypsin inhibitor; 14 kDa, lysozyme).

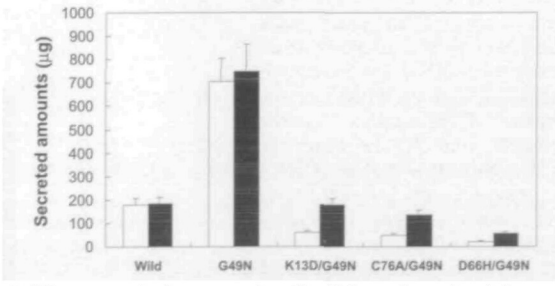

Fig. 4. **The secreted amounts of wild and mutant lysozymes expressed in wild-type (white columns) and calnexin-disrupted (black columns) *S. cerevisiae* AH22.** Non-glycosylated lysozymes (wild-type), a stable mutant glycosylated lysozyme (G49N), and unstable mutant glycosylated lysozymes (K13D/G49N, C76A/G49N, and D66H/G49N) secreted in the growth medium supernatants were adsorbed on a CM-Toyopearl 650M column equilibrated with 50 mM Tris-HCl buffer (pH 7.5) and then eluted with the same buffer containing 0.5 M NaCl. The vertical bars indicate the standard deviation (n = 3).

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capacity and are subsequently degraded by the quality control system. On the other hand, stable glycosylated lysozymes are not degraded due to their stable conformation, as shown in Fig. 2b. As shown in Fig. 3, the SDS-PAGE patterns of C76A/G49N and K13D/G49N also showed that the secretion of the glycosylated lysozyme was very low in the wild-type strain, while that of both mutant lysozymes showed the secretion of a considerable amount of glycosylated lysozyme in the calnexin-deleted strain. The secreted glycosylated proteins were shown to all be the same polymannosyl forms in C76A/G49N, K13D/G49N, and S91T/G49N secreted in both strains with and without calnexin. This also suggests that there are no significant differences in protein synthesis and modification of foreign unstable glycoproteins between the wild-type and calnexin-deleted yeasts.

Comparison of the Secretion of Stable and Unstable Glycosylated Lysozymes in a Different Yeast Strain, S. cerevisiae AH22—The interesting observation made for *S. cerevisiae* W303-1b was further made in another yeast strain. The calnexin gene of *S. cerevisiae* AH-22 was disrupted to evaluate the secretion of stable and unstable glycoproteins. The wild and mutant glycosylated lysozymes were secreted in the wild-type and calnexin-disrupted *S. cerevisiae* AH-22. As shown in Fig. 4, a similar observation was made to that for *S. cerevisiae* W303-1b. The wild lysozyme and stable glycosylated lysozyme (G49N) were secreted at the same level in the wild-type and calnexin-deleted strain AH-22, while the unstable glycosylated lysozymes (C76A/G49N, K13D/G49N, and D66H/G49N) were secreted much more in the calnexin-deleted strain than the wild-type strain. Thus, it has been confirmed that the deletion of calnexin in yeast *S. cerevisiae* resulted in enhancement of the secreted amounts of unstable glycoproteins.

BiP and PDI Are Induced at High Concentrations When the Glycosylated Lysozymes Are Secreted by Calnexin-Disrupted S. cerevisiae W303-1b—Binding protein (BiP) and protein disulfide isomerase (PDI) play a major role in the protein folding process in the ER. It has been reported that BiP and PDI also play a role in glycoprotein folding in mammalian cells (*25*). It seems likely that the deletion of calnexin results in induction of the unfolded protein response (UPR) (*26*), and increases in the levels of BiP and PDI to fold the unfolded protein. This possibility was investigated as follows. To investigate whether the molecular chaperones located in the ER are induced as a result of calnexin-disruption or not, the mRNA concentrations of both molecular chaperones BiP and PDI were determined when various types of lysozymes were expressed in the wild-type and calnexin-disrupted *S. cerevisiae* W303-1b. The results of the induction of PDI and BiP are shown in Figs. 5 and 6, respectively. The host cells without lysozyme expression showed similar concentrations of PDI (Fig. 5, lanes 12, 13, 14, and 15) and BiP (Fig. 6, lanes 12, 13, 14, and 15) for the wild and calnexin-disrupted types. This suggests that calnexin deletion does not affect the expression of the molecular chaperones in the ER in *S. cerevisiae* W303-1b. In addition, when non-glycosylated lysozymes (wild-type and K13D) were secreted, there were no differences in the expression levels of PDI (Fig. 5, lanes 7, 8, 10, and 11) and BiP (Fig. 6, lanes 7, 8, 10, and 11) between wild and calnexin-disrupted host cells, although the concentrations greatly increased in response to expression of the unstable K13D lysozyme, which may result from the unfolded pro-

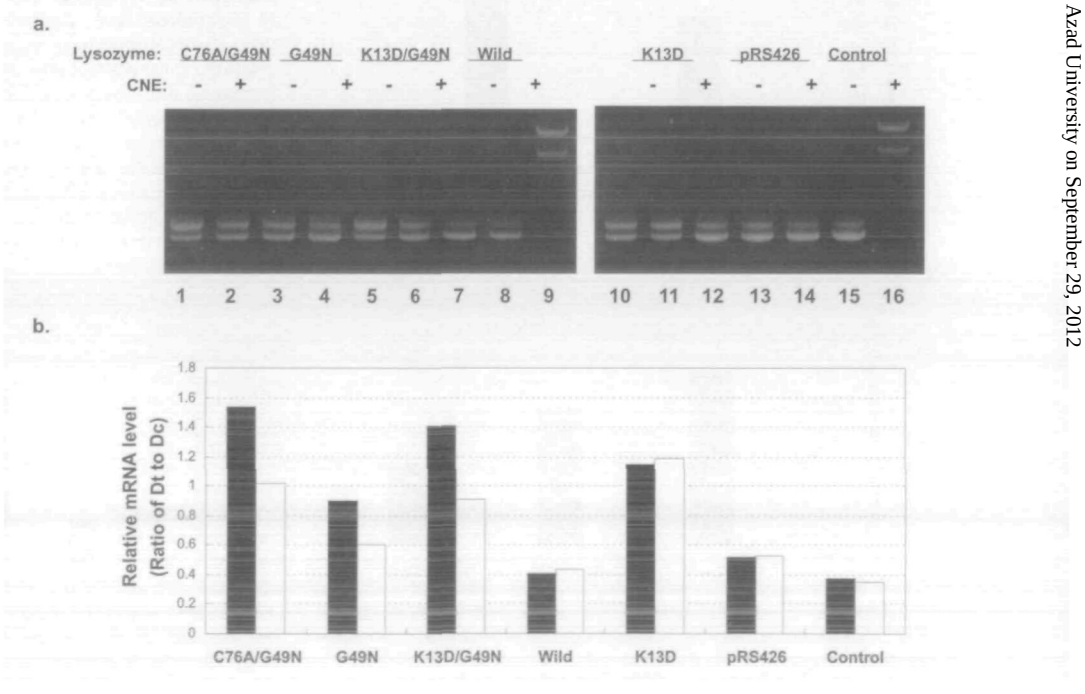

Fig. 5. **Quantitative analysis of the PDI mRNA level when the mutant lysozymes were secreted by wild-type and calnexin-disrupted *S. cerevisiae* W303-1b.** a, electrophoresis of RT-PCR products separated in a 1.5% agarose gel stained with ethidium bromide. The RT-PCR products from a mixture containing 500 ng of yeast total RNA plus 2×10^7 molecules of PDI competitor RNA after amplification with 27 cycles were used. The 1,064 bp products (upper) from yeast RNA and the 876 bp products (below) from PDI competitor RNA are indicated. Lanes 1, 3, 5, and 10, when mutant lysozymes C76A/G49N, G49N, K13D/G49N, and K13D were secreted by calnexin-deleted (CNE^-) strains, respectively; lanes 2, 4, 6, and 11, when mutant lysozymes C76A/G49N, G49N, K13D/G49N, and K13D were secreted by wild (CNE^+) strains, respectively; lanes 7 and 8, when the wild-type lysozyme was secreted by the calnexin-deleted (CNE^-) and wild (CNE^+) strains, respectively; lanes 12 and 13, when plasmid pRS426 was transformed in the calnexin-deleted (CNE^-) and wild (CNE^+) strains, respectively; lanes 14 and 15, when the host cells without the secretion of lysozyme were cultivated, *i.e.* calnexin-deleted (CNE^-) and wild (CNE^+) strains, respectively; lanes 9 and 16, marker DNA. b, in order to quantitatively determine the mRNA levels, the intensities of the RT-PCR patterns were measured with a densitometer. The graph shows the ratios of the density of the target band (upper) to that of the competitor band (lower) for calnexin-disrupted *S. cerevisiae* (black columns) and wild-type *S. cerevisiae* (white columns). D_t, density of the band from target mRNA; D_c, density of the band from competitor RNA.

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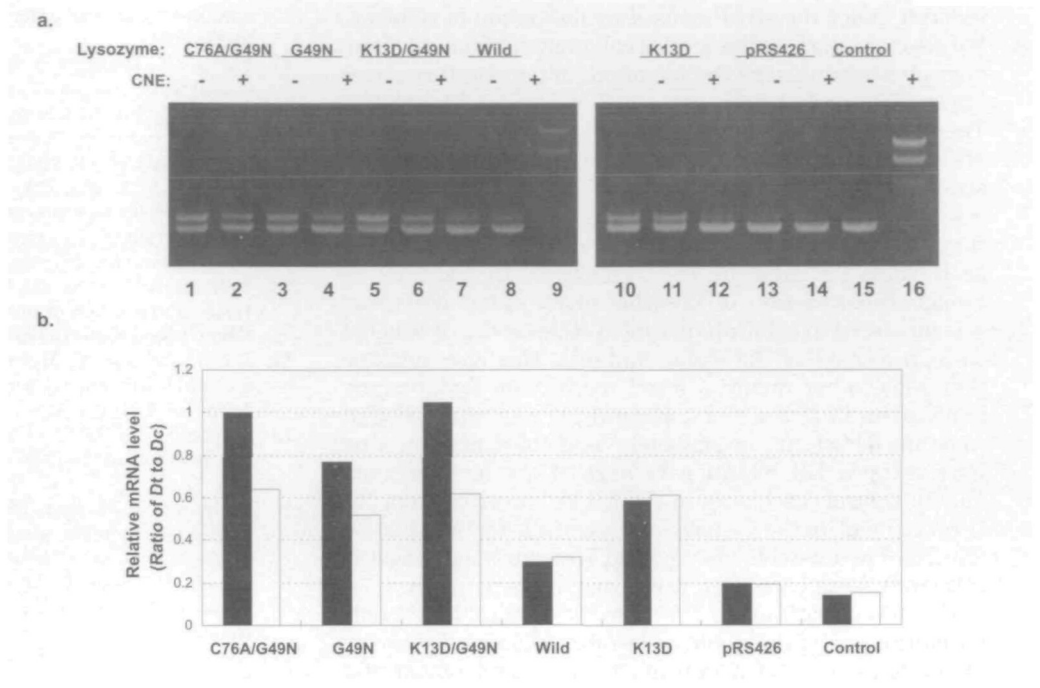

Fig. 6. **Quantitative analysis of BiP mRNA levels when the mutant lysozymes were secreted by wild-type and calnexin-disrupted *S. cerevisiae* W303-1b.** a, electrophoresis of RT-PCR products separated in a 1.5% agarose gel stained with ethidium bromide. The RT-PCR products from a mixture containing 500 ng of yeast total RNA plus 2×10^7 molecules of BiP competitor RNA after amplification with 27 cycles were used. The 1,099 bp products (upper) from yeast RNA and the 913 bp products (below) from BiP competitor RNA are indicated. Lanes 1, 3, 5, and 10, when mutant lysozymes C76A/G49N, G49N, K13D/G49N, and K13D were secreted by calnexin-deleted (CNE^-) strains, respectively; lanes 2, 4, 6, and 11, when mutant lysozymes C76A/G49N, G49N, K13D/G49N, and K13D were secreted by wild (CNE^+) strains, respectively; lanes 7 and 8, when the wild-type lysozyme was secreted by the calnexin-deleted (CNE^-) and wild (CNE^+) strains, respectively; lanes 12 and 13, when plasmid pRS426 was transformed in the calnexin-deleted (CNE^-) and wild (CNE^+) strains, respectively; lanes 14 and 15, when the host cells without the secretion of lysozyme were cultivated, *i.e.* calnexin-deleted (CNE^-) and wild (CNE^+) strains, respectively; lanes 9 and 16, marker DNA. b, in order to quantitatively determine the mRNA levels, the intensities of the RT-PCR patterns were measured with a densitometer. The graph shows the ratios of the density of the target band (upper) to that of the competitor band (lower) for calnexin-disrupted *S. cerevisiae* (black columns) and wild-type *S. cerevisiae* (white columns). D_t, density of the band from target mRNA; D_c, density of the band from competitor RNA.

tein response (UPR). This result suggests that calnexin has no relation to the expression of non-glycosylated proteins. In contrast to non-glycosylated lysozymes, significant differences in the concentrations of PDI (Fig. 5, lanes 1, 2, 3, 4, 5, and 6) and BiP (Fig. 6, lanes 1, 2, 3, 4, 5, and 6) were observed when the glycosylated lysozymes were expressed by the wild-type and calnexin-disrupted *S. cerevisiae* W303-1b. Both chaperones BiP and PDI were induced at higher concentrations in the calnexin-disrupted strain than in the wild strain. A similar result was obtained for the wild and calnexin-disrupted *S. cerevisiae* AH-22 (data not shown). This indicates that high level induction of molecular chaperones BiP and PDI only occurs for the glycosylated proteins over-expressed in the calnexin-disrupted strain. The significant increases (1.5–2.0 times) in the expression of the mRNA in the calnexin-deleted yeast suggest that calnexin is involved in the folding of glycoproteins, and that BiP and PDI seem likely to fold unfolded glycoproteins instead of calnexin 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 ER molecular chaperones BiP and PDI *via* the UPR pathway to facilitate the folding of unfolded glycoproteins.

DISCUSSION

CNE1p, the homologue of calnexin in *S. cerevisiae*, is about 23% identical to that in mammalian cells, lacks a cytoplasmic tail, and has no calcium-binding capacity (*11*). Moreover, there is no UDP-Glc:glycoprotein glucosyltransferase (GT), which is the key element in the quality control of glycoprotein folding in mammalian cells, in *S. cerevisiae* (*12*). Therefore, although calnexin in *S. cerevisiae* was proposed to act in the folding and quality control of glycoproteins in previous papers, it is unclear whether the model proposed for the quality control of glycoprotein folding in mammalian cells is applicable to *S. cerevisiae* or not (*11*). The experimental methods used for mammalian cells are not applicable to *S. cerevisiae*, so far there have been no papers on the quality control of calnexin in *S. cerevisiae*. In this 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 slight secretion by the wild strain. We reported in a previous paper (*21*) that the disruption of calnexin is effective in increasing the secretion of unstable glycosylated lysozyme H5/G49N, in which a hydrophobic pentapeptide (Phe-Phe-Val-Ala-Pro) was genetically inserted into the C-terminus and the signal sequence of *N*-linked glycosylation at position 49 was introduced into the lysozyme. This result suggests that calnexin disruption is effective for avoiding quality control of the unstable glycoprotein in *S. cerevisiae*, thereby increasing the secreted amounts of unstable glycoproteins. Thus, calnexin seems to act in the quality control for glycoproteins folding in *S. cerevisiae*, although the mechanism is not always the same as that in mammalian cells.

Moreover, the calnexin-disrupted *S. cerevisiae* was viable and grew at a normal rate. The molecular chaperones located in the ER, such as BiP and PDI, are not always

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induced under the stress caused by disruption of calnexin. We also investigated the level of cell growth of the calnexin-disrupted strain under the conditions of temperature stress (20°C, 37°C) and osmotic stress (0.7 M NaCl, 1 M sorbitol). There was no evident difference between the growth curve of the calnexin-disrupted strain and that of the wild-type strain. These results indicate that calnexin is not always essential in *S. cerevisiae*. It also suggests that some other chaperones may act in the quality control for glycoproteins, as backups for calnexin, in *S. cerevisiae*. In addition, although the secretion of unstable glycosylated lysozymes was increased in calnexin-disrupted *S. cerevisiae*, it was not as high as that of the stable mutants. This also suggests that some other quality control mechanism for glycoproteins exists in *S. cerevisiae*. Recently, it was reported that some misfolded and incompletely assembled proteins that have escaped ER retention because of the less stringent quality control machinery in the ER are diverted from the Golgi complex to the vacuole in *S. cerevisiae* for degradation (*27, 28*). The unstable glycosylated lysozymes examined in this study seem to be degraded through this pathway.

The observation made here may be applicable not only for elucidation of the quality control mechanism for unstable proteins in the ER but also for the development of a high secretion system for unstable mutant glycoproteins in yeast.

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