Original Paper

Journal of Biomedical Science

J Biomed Sci 2002;9:223–233

Received: September 5, 2001
Accepted: December 13, 2001

The Effect of C-Terminal Mutations of *HSP60* on Protein Folding

Yi-Chien Fang Mingyuan Cheng

Institute of Genetics, School of Life Sciences, National Yang-Ming University, Taipei, Taiwan, ROC

Key Words

Chaperonin · Hsp60 · Molecular chaperone · Mutation · Protein folding · Rhodanese

Abstract

HSP60 is an essential gene in *Saccharomyces cerevisiae*. The protein forms homotetradecameric double toroid complexes. The flexible C-terminal end of each subunit, which is hydrophobic in nature, protrudes inside the central cavity where protein folding occurs. In order to study the functional role of the C-terminus of Hsp60, we generated and characterized yeast strains expressing mutants of Hsp60 proteins. Most of the yeast strains expressing Hsp60 with C-terminal deletions grew normally, unless the deletion impaired the interaction between neighboring subunits. The cells carrying Hsp60 mutants with an epitope of influenza hemagglutinin (HA) and T7 alone in the C-terminal region grew normally, but the mutant containing both HA and T7 was unable to grow in nonfermentable carbon sources. In vitro biochemical assays were performed using purified Hsp60 proteins. All the mutants examined remained capable of interacting with Hsp10 in a nucleotide-dependent manner. However, binding and/or refolding of denatured rhodanese became defective in most of the *hsp60* mutants. Therefore, the hydrophobic C-terminal tail of Hsp60 plays an important role in the refolding of protein substrates, although it is flexible in structure.

Hsp60 (also known as chaperonin) is a highly conserved protein family existing in eubacteria, eukaryotic mitochondria, chloroplasts and hydrogenosomes [6, 7, 19]. Hsp60s, especially for the bacterial homologue GroEL, have been intensively studied for their function in protein folding. The functional unit of the Hsp60 species is a homotetradecameric complex consisting of two stacked rings with seven subunits each [3, 12, 21, 27]. The denatured polypeptides are known to bind to the central cavity of the complexes via hydrophobic interactions [4]. To refold the bound denatured proteins, multiple rounds of ATP hydrolysis are required [9, 20]. Moreover, Hsp60 has to cooperate with a cochaperonin, Hsp10 (known as GroES in bacteria), to carry out the refolding reaction [19, 31, 38]. The association of GroES with the cis-ring of GroEL is initiated when ATP is bound to GroEL [29]. Subsequently, ATP hydrolysis at the trans-ring induces additional conformational changes in the cis-ring, resulting in a dissociation of GroES from the GroEL complex and releasing the folded protein. More recently, the structures of GroEL, GroEL-GroES-ADP and GroEL-peptide complexes were determined by X-ray crystallography [11, 26, 39], and these structures provide further insight into the folding mechanism. From electron microscopic observation of the crystal structures, the diameter of the central cavity of a GroEL ring is 45 nm and is suitable for a globular protein of 60 kD [3, 12]. Each subunit of GroEL can be divided into apical, intermediate and equatorial domains [3]. The ATP binding site is located in the equatorial

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Mingyuan Cheng, MD, PhD
Institute of Genetics, School of Life Sciences
National Yang-Ming University, 155 Li-nung Street Sec. 2
Shih-Pai, Taipei 112, Taiwan (ROC)
Tel. +886 2 822 8763, Fax +886 2 826 4930, E-Mail mingch@ym.edu.tw

domain, with one site per subunit, and GroES binds to the apical domain of GroEL. Binding of GroES causes a dramatic conformational change in GroEL and deprives the hydrophobic surface of the central cavity for the binding of polypeptides. Consequently, the denatured proteins are sequestered, and spontaneous refolding of the protein takes place in the central cavity [10, 38, 39]. The details of the mechanism of the refolding process have been extensively reviewed [7, 16].

Yeast mitochondrial *HSP60* gene, which is essential for cell growth, encodes a protein of 572 residues with a mitochondrial leader sequence. The amino acid sequence of yeast Hsp60 shares 54% identity with that of GroEL [14, 34]. In particular, the 25 amino acid residues at the C-terminus of yeast Hsp60 consist of predominantly nonpolar residues which are conserved among members of the Hsp60 family, including GroEL [5]. This C-terminal tail protrudes inside the central cavity [2, 3] and is susceptible to proteolytic digestion [29, 30]. Although the crystal structures did not reveal the exact location of the C-terminal region of GroEL [26], these structures did indicate that the C-terminal tail should be located inside the central cavity. The C-terminal region of Hsp60 (circa 20 amino acid residues) is conceivably flexible in structure. Nevertheless, changes in the length or the amino acid composition of the C-terminal tail of Hsp60 might influence the function of Hsp60, since it occupies the central cavity where ATP hydrolysis and protein refolding occurs. Therefore, we generated a series of yeast Hsp60 mutations with C-terminal deletions and insertions. Yeast strains expressing these Hsp60 variants were analyzed genetically and the purified Hsp60 proteins were characterized biochemically. Herein, we report our findings.

Materials and Methods

Yeast Strains

MYD11, a diploid strain of *Saccharomyces cerevisiae,* was derived from NY648 *(a/α, leu2/leu2, ura3–52/ura3–52;* a gift from P. Novick) by inserting a 2.2-kb *LEU2* fragment between two *Eco*RV sites at positions 758 and 1388 of the coding region of the *HSP60* gene on one chromosome.

Plasmid Construction

The plasmid YIpGalHsp60 was constructed by ligating a 5.3-kb *Eco*RI-*Nco*I fragment of pGalHsp60 containing a *GAL1* promoter-*HSP60* coding sequence and part of the *URA3* gene [13] with a 3.7-kb *Eco*RI-*Nco*I fragment of YIp5 containing the remaining part of the *URA3* gene, the replication origin of *Escherichia coli* and a β-lactamase gene. YIpGalHsp60 contains two *Pvu*II sites, one at nucleotide 1674 of the coding region of the *HSP60* gene and the other about 300 bp distal to the coding sequence of the *HSP60* gene. To make further deletion construction more convenient, YIpGalHsp60-1 was constructed by inserting a *Sal*I linker (5′-GGTCGACC-3′) into the *Pvu*II site about 300 bp distal to the coding sequence of the *HSP60* gene of YIpGalHsp60 to destroy the *Pvu*II site.

To generate the plasmid pET-HSP10, the coding region of the *Hsp10* gene was first amplified by polymerase chain reaction (PCR) using yeast DNA as a template and the two primers hsp10-5′ (5′-GGATCCATATGTCCACCCTTTGAAGTCTGC) and hsp10-3′ (5′-ACACATAGT GTCCCTAAAATTCC). The PCR products were purified and ligated with the vector pGEM5zf(+) (Promega, Madison, Wisc., USA), previously linearized with *Eco*RV, resulting in plasmid pGEM-Hsp10. The plasmid was sequenced, and the insert was then excised by *Nde*I and cloned into the vector pET23a (Novagen, Madison, Wisc., USA), previously treated with *Nde*I to obtain pET-HSP10.

In vitro Mutagenesis

The plasmid YIpGalHsp60-1 was linearized with *Pvu*II treatment (the *Pvu* II site is located at nucleotide 1674 of the coding region) and then digested by Bal-31 exonuclease. At different time points, aliquots were withdrawn and the reaction was stopped by adding 20 m*M* EGTA to the mixtures. Both ends were rendered blunt by treatment with the Klenow fragment of DNA polymerase. Subsequently, an *Xba*I linker (5′-CTAGTCTAGACTAG-3′; NEB, Beverly, Mass., USA) containing stop codons in all three forward reading frames was inserted at the deletion site, resulting in the Hsp60 C-terminal deletion mutants del14, del19, del25, del32 and del36. Six additional Hsp60 C-terminal deletion mutations, del26, del27, del28, del29, del30 and L1, as well as four Hsp60 C-terminal insertion derivatives, CHA, C25HA, CT7 and DT7, were generated by oligonucleotide-directed mutagenesis using the U-template method to enrich mutants [28]. All the deletions and extensions were confirmed by DNA sequencing. Primers used for mutagenesis are listed in table 1.

Construction of Yeast Strains Harboring Hsp60 with C-Terminal Deletion and Insertion Mutations

The deletion plasmids derived from YIpGalHsp60-1 were linearized at the *URA3* coding region by *Nco*I digestion. They were transformed into MYD11 to generate strains carrying the C-terminal deletion in *HSP60*p. To construct yeast strains with chromosomal *hsp60* insertional mutations driven by the endogenous promoter, the insertion plasmids (CHA, C25HA, CT7 and DT7) derived from YIpGalHsp60-1 were digested by *Eco*RI and *Eco*RV to obtain the 6.8-kb *Eco*RI-*Eco*RV fragment. Both ends of this fragment were blunted by treatment with the Klenow fragment of DNA polymerase and then self-ligated. The resulting plasmids were linearized by *Xba*I at the *HSP60* coding region and transformed into a diploid strain MYD10 *(a/α, leu2/leu2, ura3–52/ura3–52).* The transformants were selected by growth on medium lacking uracil. Subsequently, tetrad dissection was performed to isolate haploid cells expressing only Hsp60p with a C-terminal deletion or insertion.

Preparation of Cell Lysates and Mitochondria

Yeast mitochondria were isolated by differential centrifugation as previously described [1]. Yeast cell lysates were prepared by the methods of Cheng et al. [13]. Briefly, purified yeast mitochondria were resuspended in a minimal volume of buffer A (20 m*M* Tris, pH 7.4, 0.5% Triton X-100, 1 m*M* phenylmethylsulfonyl fluoride), incubated at 4 °C for 20 min and then centrifuged at 10,000 *g* for 20 min

Table 1. Nucleotide sequences of the primers used for site-directed mutagenesis

Mutant	Nucleotide
del26	5′-CCGAAGTTGCTATT**TAG**TTGATGCCCCA-3′
del27	5′-CTACCGAAGTTGCT**TAA**TTGTTGATGCCCC-3′
del28	5′-CTACTACCGAAGTT**TGA**TTGTTGATGCCCC-3′
del29	5′-CTATTAGCTACTACCGAA**TGA**TTGTTGATGCCCC-3′
del30	5′-TATTAGCTACTACC**TAA**GTTGCTATTGTTG-3′
L1	5′-TATTAGCTACTACCGATGCCCCAGAACCAC-3′
CHA	5′-ATGCCAGGTATGATGTACCCATACGACGTCCCATACTACGCT**TAA**CGACCGCCTTAATTC-3′
C25HA	5′-CCGAAGTTGCTATTGTTTACCCATACGACGTCCCAGACTACGCTGATGCCCCAGAACCAC-3′
CT7	5′-ATGCCAGGTATGATGGCTAGCATGACTGGTGGACAGCAAATGGGTCGC**TAA**CGACCGCCTTAATTC-3′

Stop codons are shown in bold and extra sequences are underlined.

at 4°C. The supernatant was collected as mitochondrial lysates for the purification of Hsp60.

Purification of Yeast Hsp60

To isolate Hsp60 from yeast mitochondria, a 10–30% linear sucrose density gradient in SW41 centrifuge tubes containing buffer B (100 m*M* KCl, 5 m*M* $MgCl_2$, 20 m*M* Tris, pH 7.4) was first obtained using a gradient master (Biocomp, New Brunswick, Canada) with a design program. Mitochondrial lysates were loaded onto the top of the tubes and were centrifuged at 30,000 rpm for 18 h. Fractions were then collected with an automatic liquid collector (Gilson, Middleton, Wisc., USA). Aliquots of the fractions were analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). The gels were either stained by Coomassie brilliant blue for visualization or subjected to immunoblotting analysis using anti-Hsp60 antibody. Fractions containing predominantly Hsp60 complex were collected.

To isolate Hsp60 from heat-treated cell lysates, yeast cell lysates in buffer A were incubated at 60°C for 30 min and were then centrifuged in an SS34 rotor at 10,000 rpm for 15 min. The supernatant was collected and applied to a diethylaminoethyl (DEAE)-cellulose column equilibrated with buffer C (10 m*M* Tris, 50 m*M* NaCl, pH 7.4). Hsp60 was then eluted with a 150–600 m*M* linear NaCl gradient. Fractions were collected and analyzed by SDS gel electrophoresis. Those fractions containing Hsp60 were pooled, and concentrated by Centricon-10 (Millipore, Bedford, Mass., USA). The concentrated Hsp60 samples were further purified with 10–30% linear sucrose density gradient centrifugation as described in the previous paragraph.

Purification of Recombinant Hsp10

The plasmid pET-HSP10 was transformed into *E. coli* strain BL21(DE3)(pLysS), and the expression of Hsp10 was induced by the addition of isopropyl β-*D*-thiogalactopyranoside. Thereafter, bacteria were lysed in buffer A and centrifuged at 10,000 *g* for 15 min at 4°C. The proteins in the supernatant were precipitated by ammonium sulfate. The precipitants appearing between 30 and 70% saturation were collected, dissolved in a small amount of buffer D (50 m*M* NaCl, 10 m*M* Tris, pH 8.0) and applied to a Sephadex G-75 column (Amersham Pharmacia, Uppsala, Sweden) equilibrated with the same buffer. Fractions containing Hsp10 were then applied to a DEAE-cellulose column equilibrated with buffer D. Since Hsp10 is highly basic, it will not bind to the resin under the experimental conditions. The flow-through fraction containing Hsp10 was then applied to a CM-52 column equilibrated with buffer D. The flow-through fraction containing Hsp10 was collected and concentrated by Centricon-10 (Millipore). Subsequently, using a Sephacryl S-300 column (Amersham Pharmacia) equilibrated with buffer D, the heptameric Hsp10 complexes were further purified by fractionating the material flow through the DEAE-cellulose resin.

Coimmunoprecipitation of Hsp60 and Hsp10

Purified Hsp60 (7.5 pmol) was mixed with Hsp10 (15 pmol) in 600 µl of buffer B with 0.1% Triton X-100 and 5 m*M* of the specified nucleotides. They were incubated on ice for 30 min. Then, anti-Hsp10 antiserum was added to the mixtures and the incubation was continued at 4°C for 2 h with constant rocking. At the end of the incubation, protein A-Sepharose was added to the mixtures. The proteins bound to the resins were collected by centrifugation and were analyzed by Western blot analysis.

Rhodanese Binding Assay

Rhodanese was labeled with tritium by reductive methylation using [^{3}H]-$NaBH_4$ (Amersham Pharmacia; specific activity 11.6 Ci/mmol). In most cases, [^{3}H]-rhodanese was denatured in 6 *M* guanidine chloride to a final concentration of 10 µ*M* (circa 10^5 cpm). For del14, 20 µ*M* [^{3}H]-rhodanese was used for the binding assay. Then, purified Hsp60 (40 pmol) was mixed with the denatured [^{3}H]-rhodanese in 100 µl of buffer E (30 m*M* Tris, pH 7.5, 100 m*M* KCl, 5 m*M* $MgCl_2$) and incubated at 4°C for 15 min. At the end of the incubation, the mixtures were subjected to sucrose gradient centrifugation and were fractionated. The fractions were analyzed by SDS-PAGE and liquid scintillation counting to determine the distribution of proteins and the [^{3}H] radioactivity.

Rhodanese Refolding Assay

Rhodanese was denatured in 6 *M* guanidine chloride to a final concentration of 50 µ*M*. Two hundred picomoles of purified Hsp60 was mixed with 50 pmol of denatured rhodanese in buffer E (50 m*M* KCl, 10 m*M* Tris, pH 7.4) with 1 m*M* DTT and incubated at 4°C for

15 min. Two hundred picomoles of Hsp10 was then added to the mixtures and incubated at 30°C for 15 min. Subsequently, ATP was added to a final concentration of 2 m*M* in a total volume of 50 μl. The refolding of rhodanese was allowed to continued for an additional 15 min. At the end of the incubation, the reaction was stopped by adding trichloroacetic acid to a final concentration of 10%, and the rhodanese activity was measured following methods described previously [24].

Other Methods

Tetrad dissection and analysis of mutants have been described previously [17]. Briefly, the diploid transformants, in an MYD11 background, were sporulated, and tetrads were dissected onto galactose-containing plates. Viable spores were tested for their leucine and uracil requirements for growth. Those mutants were further confirmed by immunoblotting. Rabbit anti-Hsp60 and anti-Hsp10 antibodies were prepared by methods described previously [18]. The amount of protein was determined using a BioRad protein assay kit (BioRad, Hercules, Calif., USA).

Results

The Effect of Hsp60 C-Terminal Mutations on Cell Growth

Since the C-terminus of yeast Hsp60 is hydrophobic and protrudes inside the central cavity [3, 4, 29], the length or the content of the C-terminal region conceivably may affect the function of Hsp60. Therefore, we generated and characterized yeast strains bearing *hsp60* mutants with C-terminal deletions or insertions. The deletion mutants are summarized in figure 1A. In yeast cells, the expression of the mutated Hsp60 proteins was under the control of *GAL1* promoter. As shown in figure 1B, in diploid cells, the mutated Hsp60 proteins were expressed after galactose induction. The amounts of the mutant proteins were about 2–3 times greater than that of the endogenous Hsp60.

Because *HSP60* is an essential gene in yeast, complete or partial loss of function of Hsp60 may bring about abnormal growth. To determine whether yeast cells bearing solely truncated Hsp60p are viable, tetrads of diploid transformants were dissected and analyzed. The results of the growth phenotype of these haploid yeast cells are tabulated in figure 1A. Evidently, haploid cells with Hsp60 deletion up to 25 amino acids from the C-terminus grew normally at both 25 and 37°C. Cells containing Hsp60 without the last 26 amino acids exhibited normal growth at 25°C but showed slow growth at 37°C. Cells were no longer viable if more than 27 residues were deleted. Moreover, the mutant strain L1 in the del14 background with additional deletion from amino acids 27 to 32 from the C-terminus also failed to survive (fig. 1A). A similar observation was reported for bacteria expressing the C-terminal truncated GroEL. Therefore, deletion of the C-terminal tails of GroEL also resulted in a growth defect [8, 33].

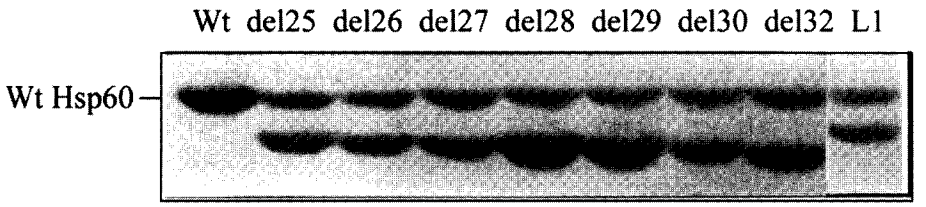
The next question was whether the presence of the *hsp60* mutants influences the normal function of wild-type (Wt) *HSP60*. The diploid transformants carrying both wild type *HSP60* and the mutants were grown at 25 and 37°C on media containing either glucose or galactose. On glucose-containing medium, yeast strains behaved like the Wt at both temperatures, since the mutant proteins were not expressed (data not shown). However, on galactose-containing medium, the colonies of the diploid strains Wt/del27, Wt/del28, Wt/del29, Wt/del30 and Wt/del32 were smaller than those of the Wt strain (fig. 1C). Among the diploid mutants, Wt/del30 exhibited the most severe phenotype. Nevertheless, Wt/del36 showed normal growth at both 25 and 37°C. Suffice it to say, small colonies were observed at both temperatures on diploid cells containing Wt protein and mutants with C-terminal deletions including the amino acids 27–32 from the C-terminus, except that Wt/L1 grew normally at 25°C, but exhibited small colonies on galactose-containing medium at 37°C.

Fig. 1. Characterization of the C-terminal deletion mutants of *HSP60*. **A** The deletion mutants and growth of haploid cells. The C-terminal deletion mutants of *HSP60* were produced as described in Materials and Methods. Wt Hsp60 contains 572 amino acids; only the C-terminal region of the proteins is shown. The numerical indexes of the C-terminal truncated mutants del14, del19, del25, del26, del27, del28, del29, del30, del32 and del36 represent the numbers of residues which were deleted from the C-terminus of Hsp60. Mutant L1 with a del14 background contains an additional internal deletion from amino acids 543 to 547. In a few cases, addition of a few residues to the C-terminus resulted from the cloning process, and they are underlined. Growth of haploid cells expressing only C-terminal truncated Hsp60 proteins is also given. ++ = Normal growth; + = slow growth; – = no growth. **B** Expression of the C-terminal deleted HSP60 proteins in yeast cells. Diploid yeast cells (in an MYD11 background) harboring plasmids containing *HSP60* and *HSP60* with C-terminal truncations were grown in galactose medium, and the cell lysates were analyzed by SDS-PAGE and immunoblotted with rabbit anti-Hsp60 antiserum. The strains examined were Wt/Wt, Wt/del25, Wt/del26, Wt/del27, Wt/del28, Wt/del29, Wt/del30, Wt/del32 and Wt/L1. **C** Growth of diploid strains expressing both Wt Hsp60 and C-terminal deleted Hsp60 proteins. The diploid transformants were streaked onto galactose-containing plates for 3 days at 25 or 37°C. The plates were then photographed, and the growth of C-terminal deletion mutants on galactose-containing plates is shown here.

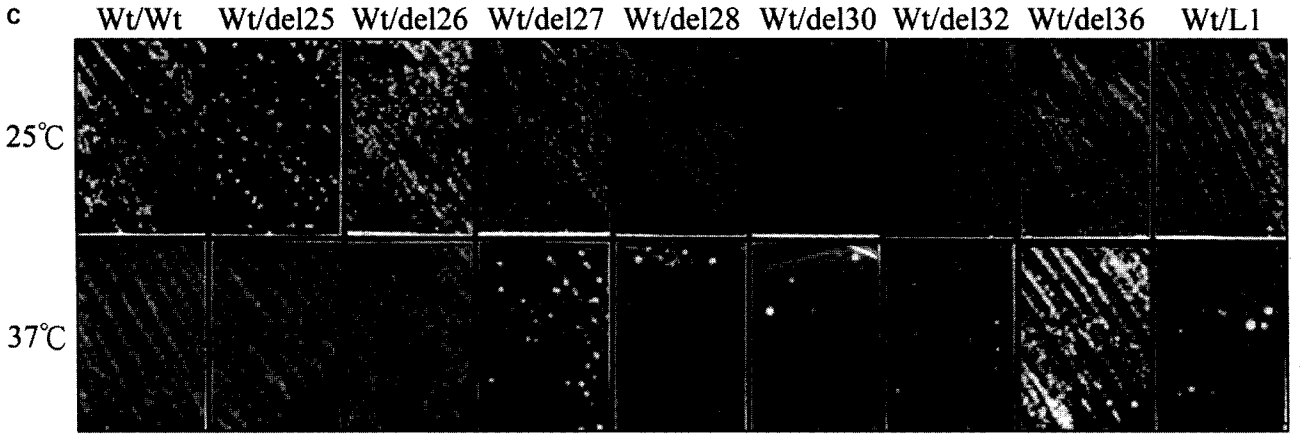
A

		Haploid growth 25 ℃	37℃
Wt	VASLLATTEVAIVDAPEPPAAAGAGGMPGGMPGMPGMM	++	++
del14	VASLLATTEVAIVDAPEPPAA—SLD	++	++
del19	VASLLATTEVAIVDAP—SLD	++	++
del25	VASLLATTEVA—LY	++	++
del26	VASLLATTEVAI—	++	+
del27	VASLLATTEVA—	-	
del28	VASLLATTEV—	-	
del29	VASLLATTE—	-	
del30	VASLLATT—	-	
del32	VASLLA—	-	
del36	VA—	-	
L1	VASLLATT—DAPEPPAA—SLD	-	

B



C



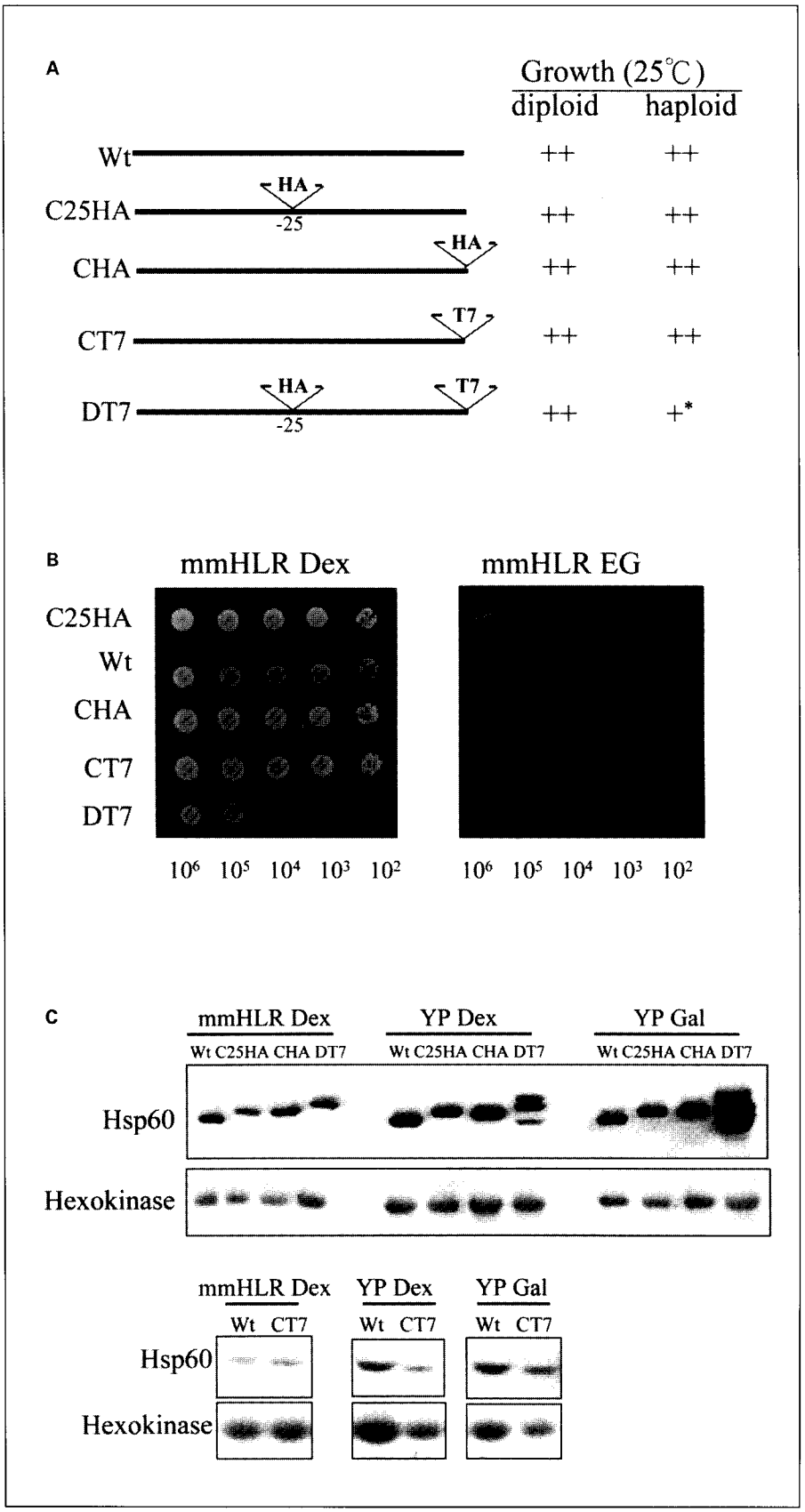


Fig. 2. The effect of Hsp60 C-terminal insertion on the growth of yeast cells. **A** The location of the insertion and the effect on cell growth. The schematic drawings illustrate the position of the insertion of epitopes at the C-terminus of Hsp60 proteins. The amino acid sequences for the HA and T7 tags are YPYDVPDYA and MASMTGGQQMG, respectively. ++ = Normal growth; + = slow growth. * No growth on YPEG medium. **B** Growth of yeast strains expressing the C-terminal insertion mutants of Hsp60. The diploid transformants were sporulated. Tetrads were dissected and viable spores were tested for their nutrient requirements. Yeast cells were subjected to tenfold serial dilution and then spotted onto minimal plates containing histidine, leucine and arginine (mmHLR) and various carbon sources [2% glucose (Dex) or 2% ethanol and 3% glycerol (EG)]. The cells were allowed to grow for 3 days at 30 °C, and were then photographed. The growth of C-terminal insertion mutants on media containing glucose or ethanol and glycerol is shown here. **C** Expression of Hsp60 mutant proteins with C-terminal extension in yeast cells. Haploid yeast cells expressing Wt Hsp60 protein or C-terminal extension derivatives were grown on minimal plates containing histidine, leucine and arginine (mmHLR) and glucose (Dex), rich medium (YP) containing glucose or rich medium containing galactose (Gal). The cell lysates were prepared and displayed by SDS-PAGE. The expression of Hsp60 proteins was detected by immunoblotting with anti-Hsp60 antiserum. Besides the mature form, there are other forms of DT7 proteins.

We next investigated the effect of Hsp60 mutants with C-terminal extensions on cell growth. Using site-directed mutagenesis, four epitope-tagged *HSP60* genes (C25HA, CHA, CT7 and DT7) with C-terminal extensions were generated. C25HA contains a hemagglutinin (HA) epitope inserted in front of the 25th amino acid from the C-terminus. CHA contains an HA epitope at the C-terminal end. CT7 contains a T7 epitope at the C-terminal end. DT7 contains two epitope tags, one HA tag in front of the C-terminal 25th amino acid and one T7 tag at the C-terminal end (fig. 2A). The viability of the cells bearing solely epitope-tagged proteins was examined by tetrad analysis of diploid transformants. The results demonstrated that yeast strains containing only C25HA, CHA or CT7 behaved like the Wt yeast. On the other hand, those containing DT7 grew slower at both 25 and 37°C in glucose-containing medium, and they failed to grow in medium containing nonfermentable carbon sources (ethanol and glycerol) at both temperatures (fig. 2B).

Immunoblotting analysis was performed to determine whether the growth defect in DT7 cells might be due to a reduction in the level of DT7 protein. For comparison, we also examined the expression of Hsp60 and other insertion mutants under different growth conditions. As shown in figure 2C, except for a significantly higher amount of DT7 in medium containing galactose, the amounts of CHA, C25HA, CT7 and DT7 were similar to that of Wt Hsp60 in all the media examined. In any case, the slow growth phenotype of yeast harboring the DT7 mutation is not due to a low level of protein expression. It is likely that the insertion of tags into Hsp60 brings about certain functional defects.

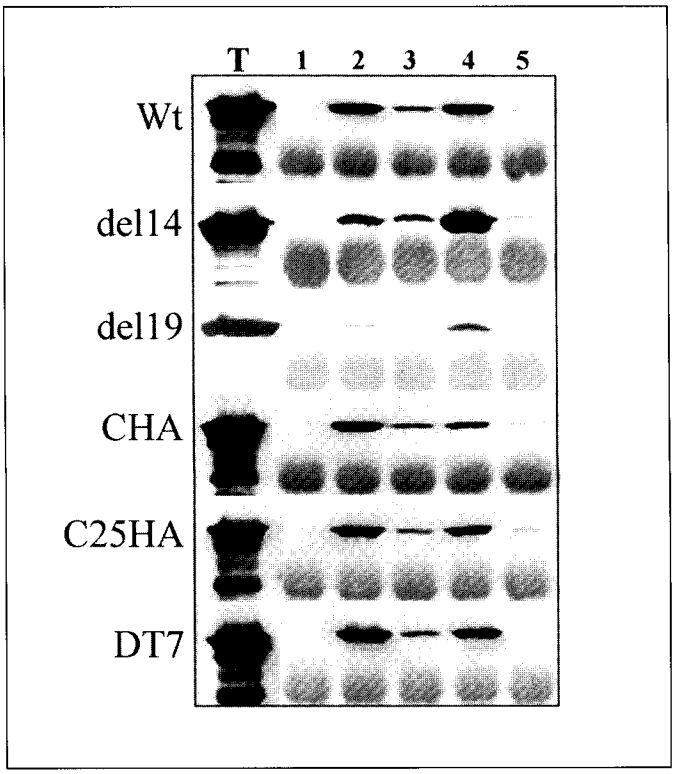


Fig. 3. The effect of Hsp60 mutations on complexing with Hsp10. Purified Hsp60 and Hsp10 were incubated with various nucleotides. Then, the complexes formed by Hsp60 and Hsp10 were examined by coimmunoprecipitation of Hsp60 with anti-Hsp10 antiserum as described in Materials and Methods. Hsp60 was visualized by immunoblotting using anti-Hsp60 antibodies. Lane T: total amount of Hsp60 added; lane 1: reaction mixtures without the addition of nucleotides; lane 2: with 5 m*M* ATP; lane 3: with 5 m*M* ADP; lane 4: with 5 m*M* ATP-γS; lane 5: with 5 m*M* AMP-PNP.

The Effect of Mutations on the Hsp60/Hsp10 Complexes

Association of Hsp60 with its cochaperonin Hsp10 is nucleotide dependent [23, 29]. Since the nucleotide-binding site of the Hsp60 complex is topologically near the proposed C-terminal end of the polypeptides, deletion or insertion of the C-terminus, therefore, might affect nucleotide binding and association with Hsp10. To investigate if this is the case, purified Hsp60 species, including the mutants, were incubated with Hsp10 with or without the nucleotides. Subsequently, the complex was immunoprecipitated with anti-Hsp10 antibodies. Under the experimental conditions, virtually all the Hsp10 in the reaction mixtures was precipitated (data not shown). Clearly, in the absence of nucleotides, Hsp60 and its mutants did not appear to associate with Hsp10 (lane 1, fig. 3). Moreover, AMP-PNP would not support the formation of Hsp60/Hsp10 complexes (lane 5, fig. 3). In the presence of ATP, ADP or ATP-γS, Hsp60 as well as its mutants appeared to form complexes with Hsp10 (lanes 2–4, fig. 3), although the amount of Hsp60 proteins associated with Hsp10 was dependent on the nucleotide species added. Compared with ATP-bound Hsp60s, ADP-bound Hsp60 proteins were less effective in forming complexes with Hsp10. Therefore, the Hsp60 C-terminal mutants that we examined behaved more or less like the Wt protein and remained capable of forming complexes with Hsp10.

Binding and Refolding of Denatured Rhodanese by the Hsp60 Species

It has been well documented that the central cavity of Hsp60 binds denatured proteins and provides a seclusive

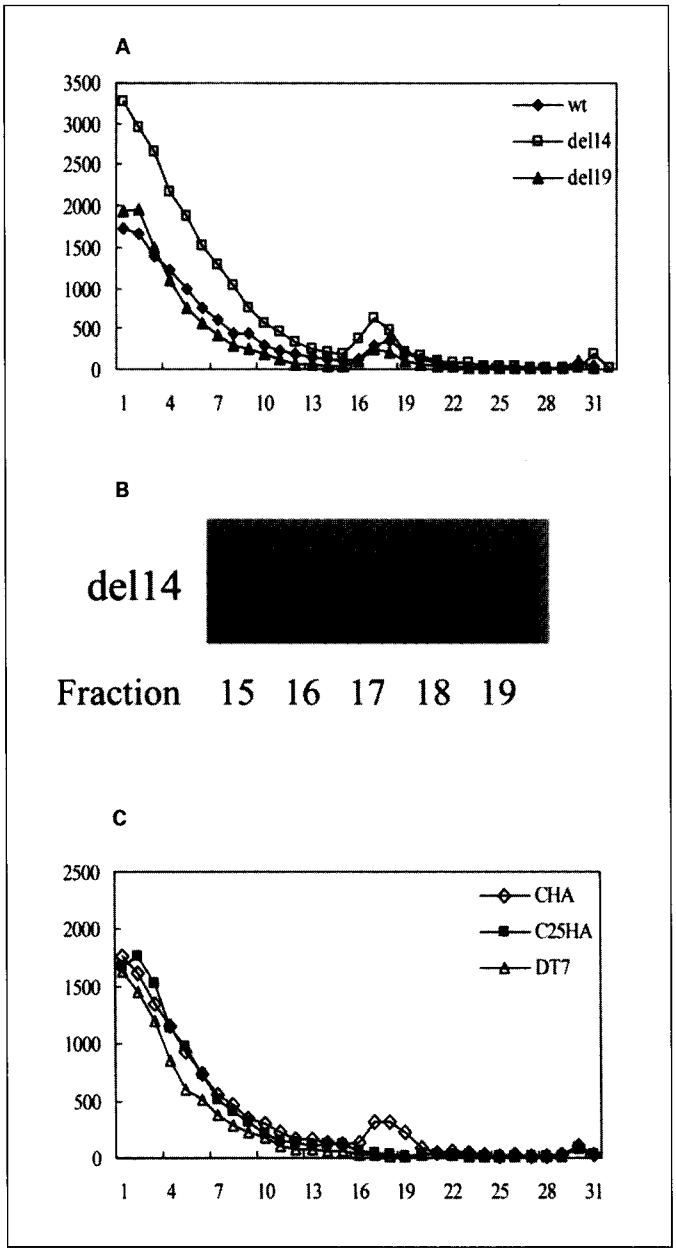


Fig. 4. Binding of denatured bovine rhodanese by Hsp60 species. [^{3}H]-rhodanese was denatured in 6 *M* guanidine chloride and was diluted into solution with purified Hsp60. The mixtures were subjected to 10–30% linear sucrose density gradient centrifugation. Samples were then fractionated and analyzed. **A**, **C** The results of liquid scintillation counting for several Hsp60 mutants with C-terminal deletions or insertions. **B** The results of immunoblotting using anti-Hsp60 antibodies on fractions 15–19 of Hsp60 from del14 mutants.

place for the refolding reaction [7, 39]. Changes in the central cavity due to mutations at the C-terminal end of Hsp60 may therefore affect its substrate binding and refolding activities. Since rhodanese has been used as an in vitro model system in studying the chaperonin-mediated folding reaction [24, 36, 37], it was chosen here to examine the substrate binding and refolding activities of the mutants of Hsp60. We first investigated if denatured rhodanese binds to the mutants. Therefore, [^{3}H]-labeled rhodanese was treated with 6 *M* guanidine hydrochloride and diluted into renaturation buffer containing Hsp60 proteins. The mixtures were subjected to sucrose density gradient centrifugation. Fractions were then collected to determine the distribution of rhodanese and Hsp60. Without Hsp60, [^{3}H]-rhodanese appeared at the top of the sucrose gradient (data not shown). In the presence of Wt Hsp60, a portion of rhodanese was detected in fractions 16–19 (fig. 4A). Similar results were observed for mixtures containing rhodanese and del14 or del19 (fig. 4A). The distribution of Hsp60 proteins was determined by immunoblotting analysis. Using del14 as an example, Hsp60 protein species were found predominantly in fractions 16–19 (fig. 4B). The distribution of Wt and del19 proteins were similar, if not identical, to that of del14 (data not shown). Cosedimentation of denatured rhodanese and Hsp60 proteins in fractions 16–19 indicates that denatured rhodanese indeed bound to Hsp60 proteins. For C-terminal extension mutants, cosedimentation of rhodanese and Hsp60 proteins only occurred when denatured rhodanese was incubated with CHA (fig. 4C). For mixtures containing rhodanese and DT7 or C25HA, rhodanese predominantly appeared at the top of the sucrose gradient, but the Hsp60 proteins were found in fractions 16–19. Under the experimental conditions, DT7 and C25HA were evidently not capable of forming complexes with denatured rhodanese.

The last question considered in this study was whether or not those mutants with binding activities are capable of refolding rhodanese. A ratio of chaperonin to substrate of 4:1 was used to examine the Hsp10-dependent refolding activity for the mutants. The results shown in figure 5 demonstrate that, at this ratio, 80% of the rhodanese activity can be restored. Under these experimental conditions, the del14 mutant remained capable of refolding denatured rhodanese, although the recovery of rhodanese activity was reduced to 50% (fig. 5). As expected, C25HA and DT7, which were incapable of binding denatured rhodanese, did not show any refolding capability. However, del19 and CHA, which were capable of binding denatured rhodanese, also failed to restore any significant level of

rhodanese activity (fig. 5). Therefore, binding of denatured substrates (i.e. rhodanese) by Hsp60 mutant proteins would not automatically lead to renaturation thereof.

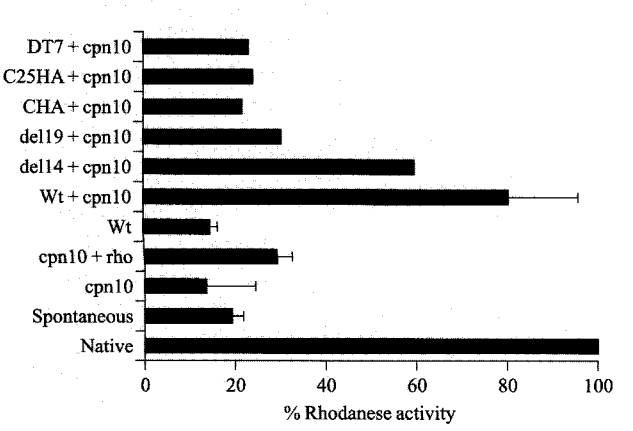


Fig. 5. The effect of Hsp60 mutation on the refolding of rhodanese. The refolding of denatured rhodanese with the chaperonin systems was described in Materials and Methods. The activity of native rhodanese represents 100%. The arrow bars represent the standard deviation of the measurements. cpn = Chaperonin; rho = rhodanese.

Discussion

When comparing Hsp60 protein sequences in different organisms, one finds that the length of the structurally flexible C-terminus protruding into the central cavity ranges from 19 to 29 amino acids [5]. Herein, we demonstrated that deletion of 25 amino acids from the C-terminus of mitochondrial Hsp60 did not affect the growth of yeast cells (fig. 1A). However, further deletion from the C-terminus (mutants del27–del36) rendered the haploid yeast cells unable to grow. In diploid yeast cells, coexpression of these deleted Hsp60s (del27–del36) with the Wt Hsp60 exhibited a dominant negative growth effect (fig. 1C). This region of yeast Hsp60 (amino acids 540–546) corresponds to amino acids 516–522 of GroEL [34]. In a GroEL subunit, these residues are known to interact with one of their neighbors at the equatorial domain [3], and bacteria expressing GroEL C-terminal truncation up to amino acid 521 also show growth defects [8, 32, 33]. Therefore, it is likely that the amino acids 540–546 in yeast Hsp60 may also be involved in subunit interaction. Consequently, deleting these amino acids should cause a severe defect in the assembly of Hsp60 complexes. The involvement of residues 540–546 in Hsp60 assembly is in agreement with the observation that truncated Hsp60 isolated from del27 even at a permissive temperature of 25 °C contained a much higher level of disassembled proteins then Hsp60 isolated from Wt cells (data not shown). Therefore, one possible explanation of the dominant negative effect (fig. 1C) is that the chaperonins in these cells were destabilized due to the coassembly of the mutated and Wt Hsp60s into tetradecameric complexes, although this scenario remains to be proven. In any event, it appears that deletion of the flexible C-terminal tail of Hsp60 would not have a significant effect on the growth of yeast cells as long as it does not affect the stability of Hsp60 complexes.

We also investigated whether adding extra amino acids at the C-terminal region of Hsp60 might affect the function thereof, since previous studies showed that both the size and the environment of the central cavity of Hsp60 determine the repertoire of the protein substrates [5, 15, 25]. Among the mutants examined, only DT7, containing an extra 20 amino acids due to insertion of both a T7 epitope and an HA epitope, showed an abnormal growth phenotype. Yeast cells expressing DT7 exhibited a respiratory-deficient phenotype (fig. 2). Therefore, increasing the size of the C-terminal tail could make Hsp60 lose some of its function, although the tail is flexible in its structure. Moreover, we carried out in vitro studies to examine whether the DT7 mutant remained capable of associating with Hsp10 and refolding rhodanese. As shown in figure 3, the capability of the DT7 mutant to bind Hsp10 is similar, if not identical, to that of Wt Hsp60. However, the mutant failed to associate with denatured rhodanese (fig. 4) and was not capable of refolding rhodanese (fig. 5). In this case, the defect in growth caused by the DT7 mutation is correlated with inability to perform protein folding.

Surprisingly, yeast strains carrying several *hsp60* mutations (e.g. del14, del19, CHA, C25HA and CT7) showed a normal growth phenotype (fig. 1, 2), yet the chaperonins isolated from these mutants had impaired activities with respect to rhodanese binding and/or refolding (fig. 4, 5). However, there should be no doubt that rhodanese is a proper model system for assaying these activities, since it has the correct structural motifs as a substrate for chaperonin [22, 25]. Nevertheless, it is clear that Hsp60 must have a number of other substrates. Conceivably, a marginal Hsp60 system would be sufficient to produce enough of the proteins important for yeast growth. The defect therefore was not evident by examining the growth

phenotype. A similar situation has been reported previously. For instance, although the E214D mutation in Ste14p retained only about 10% of the methyltransferase activity, the mutant appeared normal with respect to mating efficiency [35]. Therefore, biochemical assays could be more stringent.

The results of the in vitro assays in the present study provide some important insights into the mechanism of protein folding by the chaperonin system. Clearly, the C25HA mutant could not bind denatured rhodanese, while CHA remained capable of associating with denatured rhodanese. The effect of insertion of an HA tag on rhodanese binding was position dependent. Moreover, in CHA, C25HA and DT7, addition of a hydrophilic HA tag in the flexible C-terminal region of Hsp60 compromised its rhodanese refolding activity. If so, the hydrophobicity of the central cavity of Hsp60 might be essential for rhodanese refolding, although we cannot rule out the possibility that extra amino acids occupy more space in the cavity, since yeast carrying the CT7 mutant behaves like the Wt. Unfortunately, the contribution of the T7 tag alone could not be assessed biochemically, since the purified CT7 protein was unstable. However, it is not likely that the severe defect of the DT7 mutant is due to insertion of the T7 tag alone. In addition, the Hsp60 protein species from the del14 mutant also retained the ability to bind denatured rhodanese, but it had a reduced refolding activity (fig. 4, 5). Indeed, the amino acids which were deleted were predominantly hydrophobic (fig. 1A). Taken together, these results suggest that the hydrophobicity of the central cavity of chaperonin is essential for refolding of rhodanase. Thus, the flexible C-terminal tail of Hsp60 may have an important role in the folding of denatured proteins.

Acknowledgment

This work was supported by the National Science Council, ROC (NSC 85-2311-B-010-002-B13).

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