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Direct evidence for the intracellular localization of Hsp104 in *Saccharomyces cerevisiae* by immunoelectron microscopy

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Abstract To reveal the intracellular localization of Hsp104 in the yeast *Saccharomyces cerevisiae* before and after heat-shock, we performed immunoelectron microscopy after immunogold labeling with anti-Hsp104 antibody. At normal temperature (25°C), a small amount of Hsp104 was located in the cytoplasm and nucleus. On exposure to mild heat-shock at 40°C, protein aggregates appeared in the cytoplasm and nucleus, and Hsp104 increased around the aggregates with increasing time of the mild heat-shock treatment. Moreover, at lethal heat-shock temperature (51°C) for 20 min after mild heat treatment at 40°C, the intracellular localization of Hsp104 and intracellular structures were similar to those of the mild heat-shocked cells. However, in the lethally heat-shocked cells, certain intracellular structures were destroyed, and Hsp104 was not expressed. In the *hsp104* null mutant strain Δ*hsp104* which was treated at 40°C, Hsp104 was not localized around the aggregates. Additionally, in the Δ*hsp104* strain, even mild heat-shocked cells at 37°C or 40°C, showed destruction of intracellular structure compared to the wild-type strain. Our data suggest the following: (1) Hsp104 is associated closely with protein aggregates during heat-shock treatment, (2) Hsp104 is important for maintenance of the intracellular structure under lethal heat-shock conditions, (3) acquisition of thermotolerance depends on the amount of Hsp104 produced during mild heat-shock treatment.

INTRODUCTION

When organisms are exposed to some stress: physical stressors (e.g. high temperature, hydrostatic pressure (Iwahashi et al. 1993)), chemical stressors (e.g. ethanol, heavy metal ions, oxidation and other chemical agents) and biological stressors (e.g. microbial infection, starvation of glucose, arrest in the cell cycle including the stationary phase), the synthesis of heat-shock proteins (Hsps) is induced and stress tolerance is acquired (Nover 1991). This phenomenon is called the stress response and is highly conserved during evolution (Lindquist 1986). In the yeast *Saccharomyces cerevisiae*, Hsps have been studied extensively and Hsp104 has attracted much attention because of its role in thermotolerance and other stress conditions (Sanchez et al. 1992). However, the precise way Hsp104 performs its function is not understood. Hsp104 is a member of the 100 kD Hsps in yeast (Sanchez & Lindquist 1990), and it has characteristics different from those of other Hsps in that it is strongly induced in an emergency.

To elucidate the function of Hsp104, some previous morphological research on its intracellular localization during stress has been done. The association with the Hsp100 family and nucleoli in mammals has been reported (Subjeck et al. 1983). *Escherichia coli* clpB, which is a homolog of Hsp104 in yeast *S. cerevisiae*, has been reported to be localized in the cytoplasm (Park et al. 1993). In addition, intracellular localization of Hsp26 (Rossi and Lindquist 1989) and Hsp70 in the cell wall has been shown (López-Ribot and Chaffin 1996) using an immunofluorescence microscopic method. This method does not afford high enough resolution to investigate localization on an organelle scale.

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On the other hand, an immunoelectron microscopic method has a notable advantage for directly investigating the localization of specific proteins compared with the immunofluorescence microscopic method. Since the in vivo action of a protein may be revealed at an ultrastructural level, we attempted to localize Hsp104 in the *hsp104* null mutant (Δ*hsp104*) and the wild-type strain by immunoelectron microscopy using immunogold labeling with anti-Hsp104 antibody.

On exposure to mild heat, cells temporarily enhance the synthesis of Hsps and trehalose and then become resistant to secondary lethal heat stress, i.e. thermotolerance (Piper 1993). Hsp104 is required for the acquisition of thermotolerance (Sanchez and Lindquist 1990; Sanchez et al. 1992). On exposure to other stresses (e.g. ethanol, arsenite and copper), Hsp104 is strongly expressed and thermotolerance is acquired (Sanchez et al. 1992). Although thermotolerance is acquired due to these stresses even in the Δ*hsp104* mutant strain, thermotolerance in the wild-type strain is greater than in the mutant strain (Sanchez et al. 1992). The essential mechanism of the acquisition of thermotolerance by Hsp104 induction (including other factors) has not been explained. Therefore, a visual approach to the intracellular localization of Hsp104 may help in understanding this mechanism.

Recently, one possible mechanism of heat-shock protection, which is mediated by Hsp104, has been reported. On exposure to heat stress, unfolded proteins appear and then aggregate in the entire cell (Bentley et al. 1992; Parsell and Lindquist 1993; Webster and Watson 1993). Hsp104 may work as molecular chaperone to rescue these insolubilized proteins (Parsell et al. 1994; Chernoff et al. 1995; Patino et al. 1996). This suggestion is based on the following analysis using an *hsp104* null mutant strain. In the wild-type strain, protein aggregates which were formed during heat-shock treatment (44°C) disappeared during recovery at 25°C. On the other hand, in the *hsp104* null mutant strain, the protein aggregates remained (Parsell et al. 1994). To advance this relationship between Hsp104 and protein disaggregation, we decided to study the intracellular action of Hsp104 against aggregates by investigating Hsp104 localization by immunoelectron microscopy under heat-shock conditions. We show that Hsp104 closely associates with aggregates formed during heat-shock in the cytoplasm and nucleus. Thermotolerance data are presented to support our microscopic observation.

MATERIALS AND METHODS

Yeast strain and growth conditions

S. cerevisiae W303aLEU2+ (*MATa can1-100 ade2-1 his3-11,15 leu2-3, 12 trp1-1 ura3-1*+) and the *hsp104* null mutant strain; W303aΔhsp104-LEU+ (*MATa can1-100 ade2-1 his3-11, 15 leu2-3, 12 trp1-1 ura3-1 Δhsp104::LEU2*+; Δ*hsp104*) were grown overnight at 25°C in 10 ml YPD medium (1% yeast extract, 2% polypeptone and 2% glucose) to the stationary phase (OD660 > 2.5). Of these cultures 1 ml was transferred to fresh 200 ml YPD medium and incubated at 25°C for 12 h to the exponential phase (OD660 = 1.0) at 25°C.

Cells were collected and inoculated in fresh YPD medium, then heat-treated. Mild heat-shock treatment was carried out at 37°C and 40°C. For the thermotolerance experiment, heat-treated cells were shifted to 51°C.

Cell viability measurement

To determine the survival under lethal heat-shock conditions, we measured colony-forming units (CFU) on a YPD-agar plate (YPD medium and 2% agar). The survival rate was evaluated as the CFU (%) of mild heat-treated cells relative to that of untreated control cells.

Western blot analysis

The protein samples were extracted and separated on 7.5% SDS-polyacrylamide gel with 4% stacking gel (Laemmli 1970). Gels were transferred to a nitrocellulose membrane (ATTO) and immunoassayed with anti-Hsp104 antibodies (Affinity Bioreagents, Inc.). For visualizing, membranes were incubated with peroxidase (DAB substrate kit, Vector Lab., Inc.) conjugated to anti-rabbit IgG (Vecterstain ABC kit, Vector Lab., Inc.).

Immunoelectron microscopy

Immunoelectron microscopy was done according to Kamasawa et al. (1992) with some modification. Various treated cells were collected, washed with distilled water, and were then fixed with a mixture of 3% paraformaldehyde and 0.5% glutaraldehyde in 0.1M phosphate-buffered saline (0.1M PBS: 0.1M phosphate buffer, pH 7.2, 0.85% NaCl) at 4°C for 2 h. Cells were washed with 50 mM PBS and were then treated with 1% sodium metaperiodate in distilled water for 15 min and 50 mM ammonium chloride in 50 mM Tris-buffered saline (50 mM TBS: 50 mM Tris-HCl buffer, 0.8% NaCl) at 4°C for 30 min. Cells were washed with 50 mM TBS and then embedded in 2% agarose. Blocks were dehydrated with a graduated ethanol series and were embedded in LR White resin (London Resin Co., Ltd).

For immunolabeling, ultrathin sections were mounted on a nickel grid, incubated with 1% BSA in 50 mM TBS (1% BSA/TBS) for 30 min, and then incubated with anti-HSP104 antibody (Affinity Bioreagents, Inc., 1: 2000 dilution) in 0.1%BSA/TBS at room temperature for 1 h.

Sections were washed with 0.1% BSA/TBS and then incubated with goat anti-rabbit IgG conjugated to 10 nm colloidal gold (British BioCell International Technical Services Department, 1: 40 dilution) at room temperature for 1 h. After washing with 0.1% BSA/TBS, sections were stained with uranyl acetate, and then observed with a transmission electron microscope (Hitachi H-7000) at 75 kV.

Conventional electron microscopy

Conventional electron microscopy was performed according to Sato et al. (1996) with some modification. Treated cells were collected, washed with distilled water, and then fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, at 4°C for 2 h. Cells were washed with same buffer and then postfixed with 3% potassium permanganate in distilled water at room temperature for 90 min. After washing with distilled water, cells were embedded in 2% agarose, and then block staining was carried out with 2% uranyl acetate in distilled water at 4°C for over 30 min. Blocks were dehydrated with a graduated acetone series and were embedded in Quetol 653 mixture. Ultrathin sections were stained with uranyl acetate and lead citrate and then were observed with a transmission electron microscope (Hitachi H-7000) at 75 kV.

RESULTS

Hsp104 induction as a function of the time of mild heat-shock treatment

To determine the intracellular localization of Hsp104 by immunoelectron microscopy, first, we examined Hsp104 induction as a function of the time of mild heat-shock treatment at 37°C and 40°C by Western blot analysis using the antibody against Hsp104 (Fig. 1a). Figure 1B shows the amount of Hsp104 induced relative to the untreated control (25°C, 0 min) in the wild-type strain. On exposure to mild heat conditions at 40°C for 15 min, Hsp104 induction was found to be approximately 2.2-fold relative to the untreated control. The Hsp104 induction gradually increased with longer times of mild heat-shock treatment at 40°C. The relative amount of Hsp104 increased approximately 5.4-fold. Although Hsp104 levels also gradually increased at 37°C, it was less than at 40°C. In contrast, Hsp104 induction in the *Δhsp104* strain was not detected (Fig. 1a).

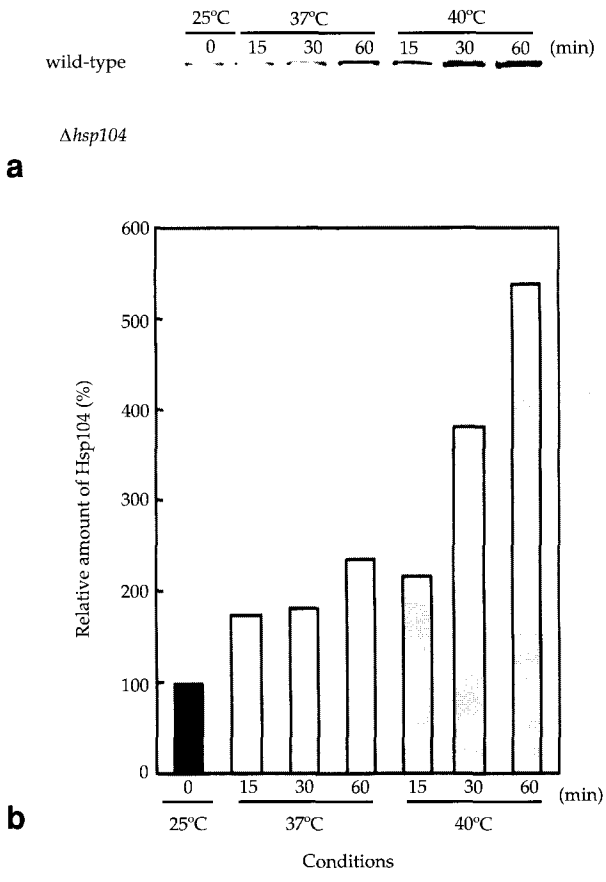

Fig. 1 Hsp104 induction according to the time of mild heat-shock treatment. Cells were incubated in YPD medium at 25°C and 37°C for 15, 30, and 60 min. Western blot analysis of Hsp104 was determined using the anti-Hsp104 antibody. (a) The results of Western blot analysis of Hsp104. (b) The amount of induced Hsp104 relative to the untreated control at 25°C for 0 min in the wild-type strain.

Immunoelectron microscopic analysis for the intracellular localization of Hsp104 under mild heat conditions

We performed immunoelectron microscopic analysis to obtain direct evidence for the intracellular localization of Hsp104. At the same time, morphological changes associated with mild heat-shock treatment at 40°C were investigated by conventional electron microscopy. Figure 2 shows the electron micrographs of control cells at 25°C for both 0 min (a1 and b1) and 60 min (a2 and b2). A few gold particles which showed the intracellular localization of Hsp104 were found to be located in the nucleus and cytoplasm (Fig. 2A). On the other hand, after incubation at 40°C, protein aggregates which are electron-dense materials were found in the cytoplasm and nucleus and Hsp104 was localized around these aggregates (Fig. 3). In mild heat-shocked cells at 40°C for 15 min, the aggregates appeared in the cytoplasm and nucleus, and were surrounded with Hsp104 (Fig. 3a1). In mild heat-shocked cells at 40°C for 30 min, the intracellular protein aggregates increased (Fig. 3 a2 and b2), and Hsp104 was localized around these aggregates (Fig. 3a2). Furthermore, in mild heat-shocked cells at 40°C for 60 min, the aggregates continued to increase (Fig. 3 a3 and b3), and Hsp104 was similarly localized around the

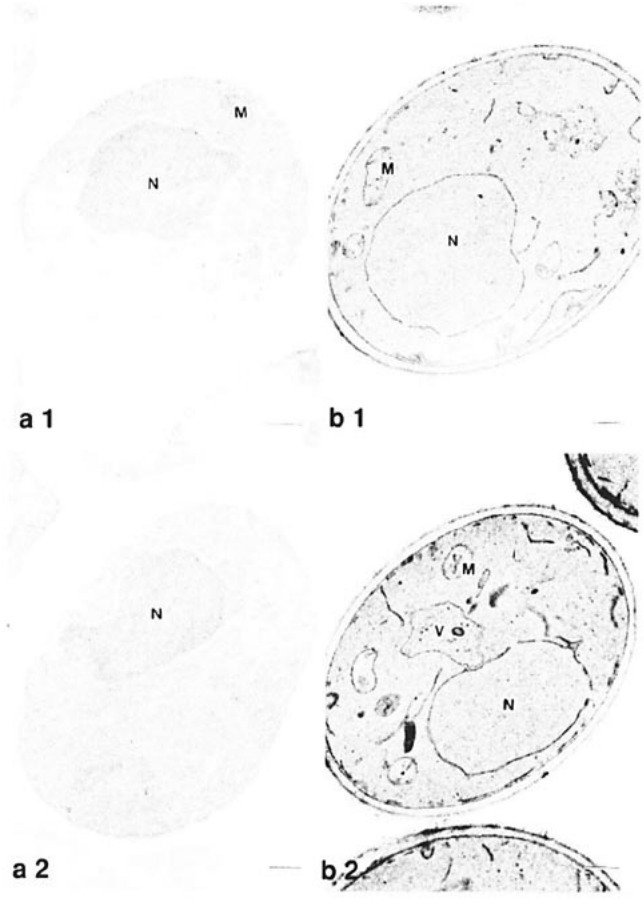

Fig. 2 Electron micrographs of *S. cerevisiae* W303a control cells (25°C). (a) Immunogold labeling of Hsp104. Ultrathin sections were incubated with the anti-Hsp104 antibody and labeled with colloidal gold conjugating anti-rabbit IgG. (b) Morphological image of the cells fixed by 2.5% glutaraldehyde and 3% potassium permanganate. Ultrathin sections were stained with uranyl acetate and lead citrate. Cells were incubated at 25°C for 0 min (1), and 60 min (2). N, nucleus; M, mitochondria; V, vacuole. Bars, 500 nm.

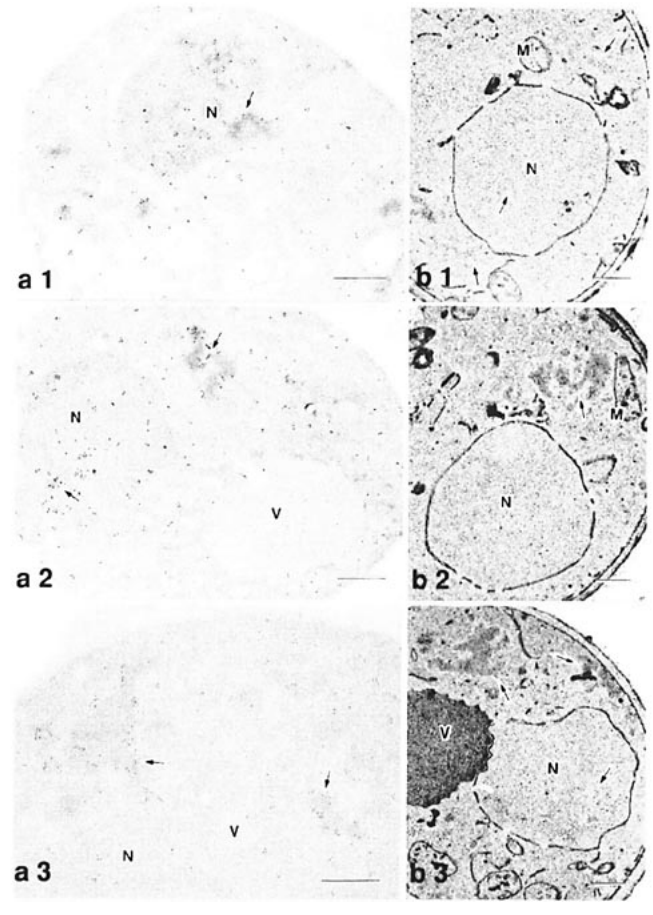

Fig. 3 Electron micrographs of *S. cerevisiae* W303a mild heat-shocked cells (40°C). (a) Immunogold labeling of Hsp104. (b) Morphological image of the cells. The aggregates appeared in the cells (arrows). Cells were incubated at 40°C for 15 min (1), 30 min (2), and 60 min (3). N, nucleus; M, 'mitochondria; V, vacuole. Bars, 500 nm.

aggregates (Fig. 3 a3, arrows). In this way, Hsp104 increased along with the increasing amount of aggregates (Fig. 3a). Although on exposure to mild heat-shock at 37°C the intracellular aggregates and Hsp104 increased, the ratio of increase was less than at 40°C (data not shown). The Hsp104 induction as shown in Figure 1 corroborates the results of these immunoelectron micrographs. Nevertheless, except for aggregation, remarkable morphological changes with mild-heat shock treatment were not observed (Fig. 3b).

We performed the same analysis in the *hsp104* mutant strain (Δ*hsp104*) to confirm whether the appearance of the gold particles showed Hsp104 induction and intracellular localization (Fig. 4). In mild heat-shocked cells at 40°C for 60 min, although the intracellular aggregation was observed as in the wild-type strain (Fig. 4), few gold particles were found and were not specifically localized around aggregates (Fig. 4a). There were a small number of gold particles in the Δ*hsp104* mutant strain probably caused by non-specific binding of the anti-Hsp104 antibody; however, the difference between wild type and Δ*hsp104* mutant mild heat-shocked cells is clear from the immunoelectron micrographs (Figs 3 and 4). Based on these comparisons of immunoelectron micrographs of both wild-type and Δ*hsp104* mutant strains, it was concluded that the gold particles indicate Hsp104 induction and intracellular localization.

Effect of Hsp104 on the acquisition of thermotolerance

We studied the role of Hsp104 in the acquisition of thermotolerance by immunoelectron microscopy under lethal heat-shock conditions at 51°C and compared it with cell viability data. Both *hsp104* null mutant (Δ*hsp104*) and

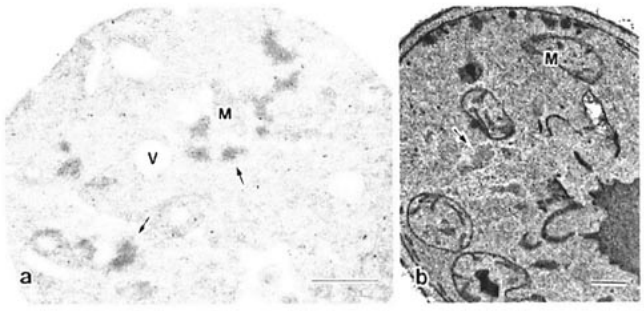

Fig. 4 Electron micrographs of *S. cerevisiae* W303a *hsp104* null mutant (Δ*hsp104*) mild heat-shocked cells (40°C). (a) Immunogold labeling of Hsp104. (b) Morphological image of the cells. The aggregates appeared in the cells (arrows). Cells were incubated at 40°C for 60 min. M, mitochondria; V, vacuole. Bars, 500 nm.

wild-type cells were treated at 37°C and 40°C for 60 min and then shifted to 51°C for 20 min. As a control, cells were incubated at 25°C and were shifted directly to 51°C. Figure 5 shows the cell viability under lethal heat conditions at 51°C. In both Δ*hsp104* mutant and wild-type strains, the relative CFU of control cells immediately decreased under the lethal heat conditions at 51°C; however, the Δ*hsp104* mutant strain was less thermotolerant than the wild-type strain (60-fold). In the wild-type strain, mild heat-shocked cells at 40°C were more thermotolerant compared to mild heat-shocked cells at 37°C for 20 min (2.3-fold). In the Δ*hsp104* mutant strain, mild heat-shocked cells at 40°C were less thermotolerant than the wild-type strain which was heat-shocked at both 37°C (4.5-fold) and 40°C (10.5-fold). Especially, in the mild heat-shocked cells at 37°C, on exposure to 51°C for 20 min, the relative CFU was decreased almost to the level of the wild-type control cells.

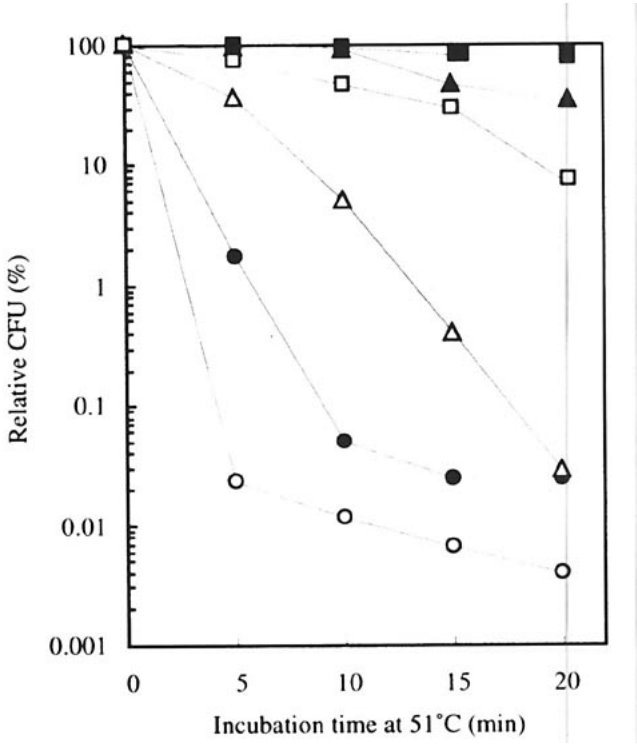

Fig. 5 Acquired thermotolerance after mild heat-shock treatment. Cells were incubated for 60 min at 25°C and 40°C, and the treated cells were then shifted to 51°C. After heat-shock treatment, the cells were incubated on a YPD-agar plate. Cell viability was evaluated by the CFU (%) of the treated cells relative to that of non-treated cells. Symbols: •, 25°C (wild-type); ▲, 37°C (wild-type); ■, 40°C (wild-type); ○, 25°C (Δ*hsp104*); △, 37°C (Δ*hsp104*); □, 40°C (Δ*hsp104*).

Intracellular structure and localization of Hsp104 under lethal heat conditions

To investigate the change in intracellular structures and localization of Hsp104 after the transfer from mild heat temperature to lethal heat temperature, we performed immunoelectron microscopic analysis under lethal heat conditions at 51°C. Based on the result of cell viability under lethal heat conditions, immunoelectron microscopic analysis was carried out at 51°C. Cells were treated at 37°C or 40°C for 60 min and then were shifted to lethal heat conditions at 51°C for 20 min. Control cells were incubated at 25°C and were shifted directly to 51°C. Figure 6 shows the electron micrographs under lethal heat-shock treatment at 51°C. On exposure to 51°C for 20 min, in the wild-type strain, the intracellular structure of mild heat-shocked cells at 40°C was almost the same as only mildly heat-shocked cells (Fig. 6a1). The aggregates remained in the cytoplasm and nucleus (Fig. 6a1, arrows). In the mild heat-shocked cells at 37°C, although the aggregates increased (Fig. 6a2, arrows), the intracellular structure was largely maintained (Fig. 6a2). In contrast, the wild-type control cells (25°C) and all conditions (25°C, 37°C and 40°C) of the Δ*hsp104* mutant strain were damaged by heat (Fig. 6 a3 and b1–3, small arrow and double arrow). In 37°C mild heat-shocked Δ*hsp104* mutant cells, the intracellular damage is the same as in the wild-type untreated control cells at 25°C. The membranes were damaged by heat, and it was difficult to distinguish the organelles (Fig. 6b2). In untreated Δ*hsp104* mutant and wild-type cells and 37°C mild heat-shocked Δ*hsp104* mutant cells, the cells were filled with aggregates, and the intracellular structure was further destroyed (Fig. 6 a3 and b2–3).

Figure 7 shows the immunoelectron micrographs of wild-type cells with mild heat-shock treatment at 40°C under lethal heat conditions at 51°C for 20 min, Hsp104 was localized around the aggregates similarly to that with only mild heat-shocked cells (Fig. 7). On the other hand, when untreated controls (25°C) were heated to 51°C, the intracellular structure was destroyed and the aggregates increased further; however, Hsp104 was not increased (data not shown). In addition, in wild-type cells,

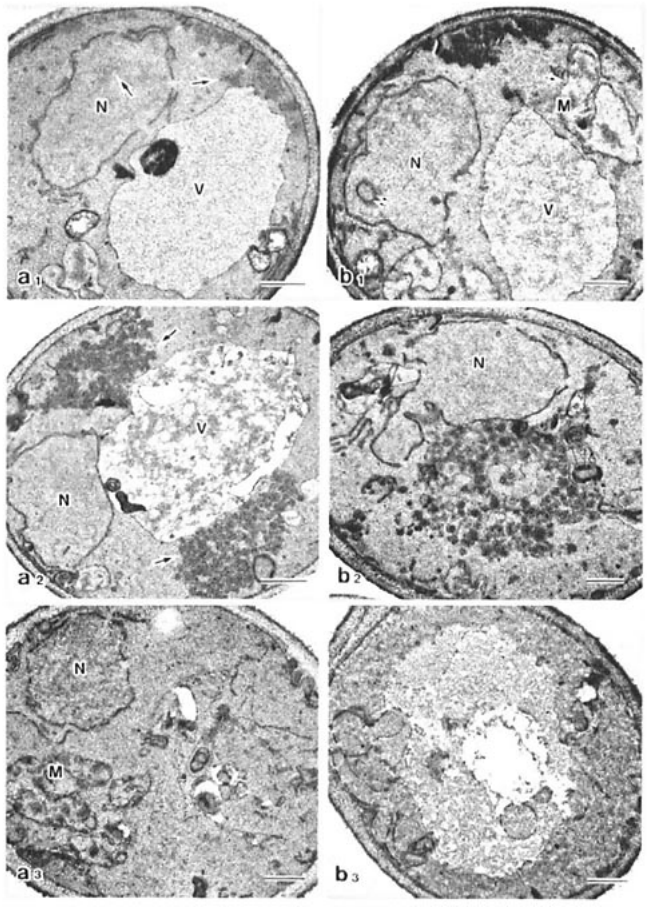

Fig. 6 Electron micrographs of *S. cerevisiae* W303a lethal heat-treated cells (51°C) after mild heat-shock treatment. (a) The wild-type strain. (b) *hsp104* null mutant (Δ*hsp104*). The aggregates remained in the cells (arrows), and small and double arrows indicate the intracellular damage by lethal heat. Cells were incubated at 40°C (1), 37°C (2), and 25°C (3) for 60 min, and the treated cells were then shifted to 51°C for 20 min. N, nucleus; M, mitochondria; V, vacuole. Bars, 500 nm.

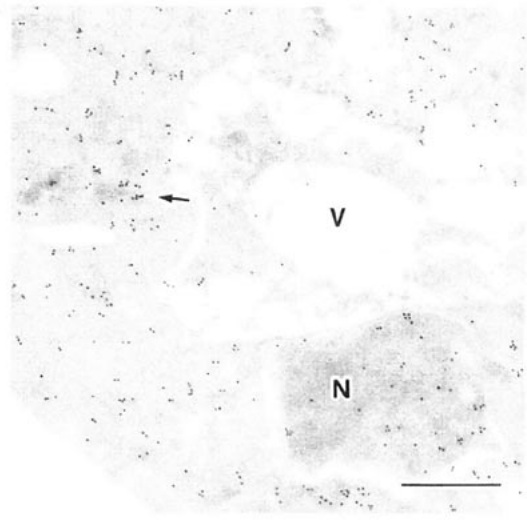

Fig. 7 Electron micrographs of *S. cerevisiae* W303a lethal heat-treated cells (51°C) after mild heat-shock treatment. Cells underwent immunogold labeling of Hsp104. The aggregates appeared in the cells (arrows). Cells were incubated at 40°C for 60 min, and the treated cells were then shifted to 51°C for 20 min. N, nucleus; V, vacuole. Bars, 500 nm.

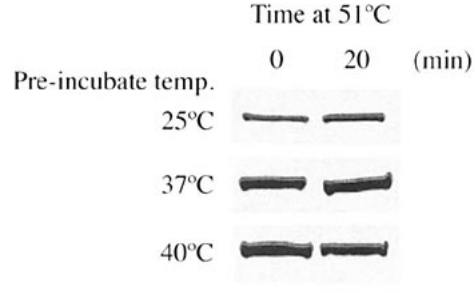

Fig. 8 Hsp104 induction of lethal heat-treated cells (51°C) after mild heat-shock treatment. Cells were incubated in YPD medium at 25°C and 37°C for 15, 30, and 60 min, and the treated cells were then shifted to 51°C for 20 min. Western blot analysis of Hsp104 was determined using the anti-Hsp104 antibody.

the Hsp104 levels did not increase after transfer from 25°C, 37°C and 40°C to 51°C (Fig. 8).

DISCUSSION

Earlier, yeast Hsp104 was speculated to be localized in the cytoplasm and nucleus (nucleolus) in response to heat-shock treatment (Parsell and Lindquist 1993; Mager and Ferreira 1993). In this work, we show direct evidence that Hsp104 is present in the cytoplasm and nucleus under all conditions of normal (25°C), mild heat (37°C and 40°C) and lethal heat (51°C) (Figs 2a, 3a and 7a) by immunoelectron microscopy. In mild heat-shocked cells, the gold particles increased compared with that in untreated control cells (Figs 2a and 3a), and this is coincident with the result of Western blot analysis (Fig. 1). Our data show that the electron dense material gradually appeared in the cytoplasm and nucleus with increasing time of the mild heat-shock treatment and were surrounded with gold particle (Fig. 3). The gold particles were not localized in similar electron dense material formed in the *hsp104* null mutant strain (Δ*hsp104*) (Fig. 4a). This visually proved that Hsp104 induction increased with mild heat-shock treatments (Fig. 3a). This assures us that the appearance of gold particles in the immunoelectron micrographs shows the intracellular localization of Hsp104. Electron dense material appeared in the cytoplasm and nucleus of heat-shocked cells. This kind of material has been found as PAB (protein aggregate body) by gene transfer of *Candida tropicalis* mutant isocitrate lyase into *S. cerevisiae* (Kamasawa et al. 1996). Therefore, the electron dense material can be regarded as protein aggregates (Figs 3, 4, 6 and 7).

In this research, the immunoelectron micrographs showed that intracellular aggregates produced by mild heat-shock treatment were surrounded by Hsp104, and this suggests that Hsp104 is associated closely with protein aggregates. It has been reported that Hsp104 mediates protein disaggregation under recovery conditions after mild heat-shock treatment (Parsell et al. 1994). It was shown that Δ*hsp104* mutant cells can not reduce the intracellular aggregates under recovery conditions. If Hsp104 continues to surround the aggregates during recovery, then Hsp104 is likely to function in protein disaggregation during recovery.

Next, to show the relationship between Hsp104 and the acquisition of thermotolerance, we performed a visual analysis by immuno- and conventional electron microscopy under lethal heat conditions after mild heat-shock treatment. Using this approach, we reached the following conclusions.

Transfer of the 40°C mild heat-shocked cells to 51°C showed almost the same intracellular structures as only mild heat-shocked cells (Fig. 6a1), and aggregates were surrounded by Hsp104 (Fig. 7). On the other hand, in the untreated control cells, intracellular structure was destroyed (Fig. 6a3) and Hsp104 was not increased in the cells (data not shown) at 51°C. In wild-type cells under lethal heat conditions, the Hsp104 levels were not increased compared to that before transfer to 51°C from 25°C, 37°C and 40°C (Fig. 8). Furthermore, in mild heat-shocked Δ*hsp104* mutant cells, the intracellular damage during 51°C is much more than wild-type (Fig. 6b1-2). In the previous studies, it was shown that high levels of Hsp104 expression from a heterologous promoter can provide thermotolerance in the absence of a conditioning pre-treatment, and this thermotolerance is likely to result from a direct effect of Hsp104 on heat sensitive targets (Lindquist and Kim 1996). In the present research, our data imply that Hsp104 which is induced during mild heat-shock treatment has a role in maintaining the intracellular structures under lethal heat condition. Moreover, this suggests that the acquisition of thermotolerance is largely dependent upon the amount of Hsp104 induction before lethal heat-shock treatment. We speculate that Hsp104 surrounds the protein aggregates and prevents them from causing further damages leading to the cell death.

However, the intracellular structures of 40°C mild heat-shocked Δ*hsp104* mutant cells were a little resistant compared with that of 37°C mild heat-shocked cells (Fig. 6b1–2). In 40°C heat-shocked Δ*hsp104* mutant cells, the amount of other thermotolerance factor (e.g. other hsp, trehalose) may be more than in 37°C heat-shocked Δ*hsp104* mutant cells. These may be related not only to Hsp104 but also to the other factors. In the future, observing these factors at the same time using immunoelectron microscopy may reveal the interaction among these factors in the acquisition of thermotolerance.

In the present research, the determination of the intracellular localization of Hsp104 by immunoelectron microscopic analysis not only revealed the function of Hsp104 concerning the acquisition of thermotolerance, but also advanced the possibility that Hsp104 possesses a protein disaggregation activity. This represents considerable progress in Hsp104 study. For example, it has been reported that Hsp104 is induced by various other stresses (Sanchez et al. 1992). It is possible that Hsp104 may be differentially localized in response to these stresses. To investigate this possibility, we intend to carry out visual analysis by immunoelectron microscopy during future stress response studies in yeast.

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