Cytosolic Hsp60 Can Modulate Proteasome Activity in Yeast*

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Background: Hsp60 is a mitochondrial molecular chaperone conserved from yeast to human.

Results: Hsp60 in the yeast cytosol binds the proteasome and inhibits proteasome activity.

Conclusion: Localization of Hsp60 to the cytosol may modulate proteasome activity according to the cell needs.

Significance: Proteasome inhibition by Hsp60 reveals a novel aspect of cell regulation and may present a target for therapeutics.

Hsp60, an essential oligomeric molecular mitochondrial chaperone, has been subject to rigorous basic and clinical research. With yeast as a model system, we provide evidence for the ability of cytosolic yHsp60 to inhibit the yeast proteasome. (i) Following biological turnover of murine Bax (a proteasome substrate), we show that co-expression of cytosolic yHsp60 stabilizes Bax, enhances its association with mitochondria, and enhances its killing capacity. (ii) Expression of yHsp60 in the yeast cytosol (yHsp60c) inhibits degradation of a cytosolic protein ΔMTS-Aco1 tagged with the degron SL17 (a ubiquitin-proteasome substrate). (iii) Conditions under which Hsp60 accumulates in the cytosol (elevated Hsp60c or growth at 37 °C) correlate with reduced 20 S peptidase activity in proteasomes purified from cell extracts. (iv) Elevated yHsp60 in the cytosol correlate with accumulation of polyubiquitinated proteins. (v) According to 20 S proteasome pulldown experiments, Hsp60 is physically associated with proteasomes in extracts of cells expressing Hsp60c or grown at 37 °C. Even mutant Hsp60 proteins, lacking chaperone activity, were still capable of proteasome inhibition. The results support the hypothesis that localization of Hsp60 to the cytosol may modulate proteasome activity according to cell need.

Yeast Hsp60 has been referred to as an exclusive mitochondrial protein and in fact has been used as a mitochondrial marker in numerous publications. Karniely and colleagues (1, 2) detected cytosolic Hsp60 presence in a genome-wide screen for dual targeting of proteins using an α-complementation assay. One concern at that time was that fusion of the α peptide to the Hsp60 C terminus impaired import into mitochondria. However, the possibility that yeast Hsp60 has a small cytosolic subpopulation could not be ruled out. Based on observations in mammalian systems (see below), this was the starting point for the current study with the question, whether we can detect Hsp60 protein, activity, and function in the yeast cytosol. We initially took an unusual approach to identify the presence and activity of Hsp60, which involved expression of the mammalian apoptotic protein Bax in yeast, and our findings have surprisingly revealed an interaction between Hsp60 and the proteasome.

Although Hsp60 was initially considered to be an exclusive mitochondrial chaperone, in mammalian cells about 15–20% of Hsp60 protein can be detected, under certain conditions, outside the organelle (3), implying that it may function in other cellular compartments. Hsp60 appears to have both pro-survival and pro-death effects in different cells that have been suggested to be mediated by its interaction with specific proteins in the cytosol such as caspases, Bax, Survivin, p53, β-catenin, and IKK (I κ B kinase) (4–8). Hsp60 has also been detected on the surface of normal and tumor cells (9–11), in the extracellular space (12), and even in peripheral blood of healthy subjects (13). The vast amount of information regarding extramitochondrial activities of Hsp60 is confusing especially considering the lack of knowledge on the basic mechanisms of its dual localization and the specific molecular functions outside the organelle.

Hsp60 and the proteasome are fascinating multisubunit particles that form cavities capable of encapsulating protein substrates thereby determining their fate by folding, unfolding, or degradation. Hsp60 is a molecular chaperone initially discovered in bacteria and eukaryotic organelles such as mitochondria, which are evolutionary derivatives of bacteria. In mitochondria, Hsp60 functions as a barrel-shaped oligomeric molecular chaperone that together with Hsp10, engulfs protein substrates and catalyzes their folding. Formation of the complex and its dissociation depends on the binding and hydrolysis of ATP by the Hsp60 subunits (14). The 26 S proteasome is a multicatalytic complex composed of a 20 S catalytic core and one or two 19 S regulatory caps attached at one or both ends. The 20 S catalytic core contains four stacked rings that form a barrel-shaped particle with a central cavity. These stacked rings include two non-catalytic outer rings called α-rings and two catalytic inner rings called β-rings. At least three primary proteolytic activities are confined to the β-subunits, chymotrypsin-like, caspase-like, and trypsin-like activities, rendering the 20 S complex, a broad specificity protease. The 19 S regulatory cap is involved in binding and unfolding of ubiquitinated proteins, and opening the α ring gated channel of the 20 S allowing entry of substrates into the catalytic core. The proteasome subunits and proteasome activity have been detected in the eukary-
otic cytosol and nucleus but not inside the mitochondrial matrix (15–17).

An important protein linking the ubiquitin-proteasome system with mitochondria, is Bax, a member of the Bcl-2 protein family, which is degraded by the ubiquitin-proteasome system (18). Bax is localized to the cytosol or is loosely associated with mitochondrial membranes, prior to death signaling (19–21). Following a death signal, Bax undergoes a conformational change that enables it to target and integrate into mitochondrial outer membrane (22, 23), leading to the release of apoptotic factors followed by caspase activation and cell death. The yeast genome, however, does not contain genes encoding recognizable members of the Bcl-2 protein family. Nevertheless, aspects of Bax-induced apoptosis are conserved in yeast and in fact expression of murine Bax in yeast facilitates cell death (24–26). Murine Bax localizes to yeast mitochondria and functional mutants of Bax, incapable of inducing apoptosis in mammalian cells, are also nontoxic in yeast. The anti-apoptotic Bcl-xL also localizes to mitochondria and allows yeast cell survival in response to Bax. Because Bax is a small heterologous cytosolic protein with a distinct phenotype in yeast (induction of cell death), we decided to examine if it could be used to probe cytosolic yeast Hsp60 activity and/or function; asking whether Hsp60 facilitates or inhibits Bax-induced cell death.

The goals of the present study were to (i) develop an assay for the cytosolic activity of Hsp60 in yeast, (ii) examine conditions in which Hsp60 may appear in the yeast cytosol, and (iii) reveal a possible cytosolic molecular function for Hsp60.

MATERIALS AND METHODS

Strains and Plasmids

Strains—Saccharomyces cerevisiae strains used were: YPH499(7A) (Mat a; ade2–101; lys2–801; ura3–52; trp1–Δ63; his3–Δ200; leu2Δ1, BY471 (Mato; His3Δ1;leu2Δ0;met15Δ0; ura3Δ0), KY698 (MATa; ura3–1;can1–100 GAL + leu2–3,112; trp1–1;ade2–1;his3–11,15 pdrΔ5::hisG), W303 (Mat a ade2–1; can1–100; ura3–1; his3–11,15; trp1–1;leu2–3, 112 can1–100/112), and m875 (α1-TAP 20 S subunit, his3o1; Leu2ko0, met15ko0; ura3o0, YGL011c-TAP::KIURA) expressing α1 subunit tagged by TAP, was kindly provided by Michael Glickman, Technion, Haifa, Israel. Ub (SUB288/H9004), and H10Ub (+Pact T317) yeast strains were kindly provided by Norbert Lehming, NUS, Singapore. The Ub (SUB288/H9004) strain the same vector encodes his3-1 trp1–1;ade2–1;his3–11,15,1 pdr5 and tagged with a degron, and pFT2 encoding fumarase have been described previously (29).

Plasmids—YPE51-Hsp60, the yeast Hsp60 gene (YLR259C) cloned into the Yep51 vector, was under the Gal10 promoter, p424-ΔMTS-yHsp60, the DNA sequence of yHsp60 (Hsp60 lacking the MTS encoding DNA) was cloned into plasmid p424 under the Gal10 promoter. The Hsp60c (D108E) point mutation was planned on the basis of homology region with the bacterial GroEL (D87E) point mutation. The yHsp60c (D420A) point mutation was planned on the basis of the homology region with the human Hsp60 point mutation (D423A). The mutations were constructed using KAPAHiFi™ DNA Polymerase (Kapa Biosystems) and DpnI (Kapa Biosystems). Plasmids pMB272-Bax encoding murine (m)Bax-HA, pMB272-Bax(G67R) encoding mBax(G67R)-HA (a nonfunctional Bax mutation), and pBF339-Bcl-xL encoding constitutive expression of Bcl-xL (28) were kindly provided by Atan Gross, Weizmann Institute, Rehovot, Israel. ΔMTS-Aco-SL17 encoding aconitate lacking a mitochondrial targeting sequence (MTS)3 and tagged with a degron, and pFT2 encoding fumarase were described previously (29).

Growth Conditions

Wild type (WT) strains, and strains harboring the appropriate plasmids were grown overnight at 30 °C in synthetic depleted (SD) medium containing 0.67% (w/v) yeast nitrogen base, 2% (w/v) glucose medium, supplemented with the appropriate amino acids (50 µg/ml). The next day the cells were transferred to medium with the same composition, but with 2% galactose, for the induction, and the cells were grown at 30, 37, or 40 °C as indicated.

Pulse-Chase

Yeast grown in galactose medium were labeled with 140 mCi of [35S]methionine and further incubated for 15 min at 30, 37, or 40 °C. Labeling and translation were stopped by addition of excess cold 0.003% methionine, 0.004% cysteine, and 0.001% cycloheximide. Samples of 900 µl were withdrawn at the times indicated and placed on ice with sodium azide to a final concentration of 10 mM (29). Cell lysates were analyzed by immunoprecipitation and SDS-PAGE followed by visualization with the BAS2000 image analyzing system (Fuji Corp., Tokyo, Japan) and autoradiography.

Subcellular Fractionation

Yeast cultures were grown to A600 of 1.5. Mitochondria were isolated as described previously (30). Spheroplasts were prepared in the presence of Zymolase 20T (MP Biomedicals, Irvine, CA). Each of the subcellular fractionation experiments was assayed for cross-contaminations using Aco1, as a mitochondrial matrix marker and αHks1 as a cytosolic marker. In some cases αTom-40 was used as an outer membrane marker and αAac1 as an inner membrane marker.

Proteasome Pulldown

α1 TAP strains harboring the appropriate plasmids were grown at the indicated temperature in SD media containing the relevant amino acids and glucose. To induce the expression of yHsp60c or fumarase, cells were washed and incubated in 2% galactose selection medium for 48 h. Following harvesting, the pellet was resuspended in 3 pellet volumes of “binding buffer” (10% glycerol, 50 mM Tris, pH 7.4, 150 mM NaCl, 2 mM CaCl2, 1 mM ATP, and 1 mM DTT) and lysed by agitation with glass beads at 4 °C for 7 min. The lysates were centrifuged for 30 min at 17,000 × g at 4 °C and the protein concentration of the clarified lysates was determined using the Bradford assay (Thermo scientific). Identical amounts (200–300 µg) of total protein extracts were loaded on calmodulin bead columns (GE Health-
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care). Each sample was adjusted to a final volume of 1 ml with the above mentioned binding buffer, and rolled at 4 °C for 2 h. The flow-through was collected and the beads were washed extensively 4 times, each with 2 column volumes of binding buffer. The bound 26 S proteasome was then eluted with 4 column volumes of elution buffer (binding buffer, containing 2 mM EGTA instead of 2 mM CaCl$_2$). The elutes were stored at −80 °C and thawed only once.

**Proteasome Activity Assay**—Similar amounts of purified proteasome (elute) from each group were subjected to the 20 S peptidase activity assay. The assay included incubation of the isolated proteasome sample in the presence of its peptide substrate Suc-LLVY-AMC. The reaction was followed by excitation at 390 nm and recording the emission at 450 nm. The assay was conducted using a Flusstar fluorometer (Fluostar Galaxy, BMG Labtechnologies, Germany), in Nunc clear 96-well plates. Each well contained 5–20 µl of appropriate elute, 100 µM Suc-LLVY-AMC (Bachem, Bubendorf, Switzerland), in buffer A (25 mM Tris, pH 7.4, 10 mM MgCl$_2$, 10% glycerol, 1 mM ATP, 1 mM DTT, and 0.02% SDS) in a final volume of 120 µl. The assay was performed at 37 °C in a plate reader at 10-min intervals, during 2 h. The specificity of the proteasomal assay was confirmed with 100 µM MG-132 (Sigma), and each elute was assayed at three concentrations in duplicate.

**Blue Native Gels**

Blue Native gel electrophoresis was performed according to Schagger (31). Detergents were omitted from solutions. Culture extracts were prepared with glass beads in buffer (0.5 M e-caproic acid, 20 mM Bistris, pH 7.0, 2 mM EDTA, and 10% glycerol, 1 mM PMSF) and centrifuged for 15 min at 10,000 × g at 4 °C. The recovered supernatants were incubated at 4 °C for 1 h with native loading buffer, separated on Blue Native gel gradient (4–16.5%), and blotted.

**Ubiquitin-histidine Pulldown Assay**

*S. cerevisiae* strains Ub(SUB288Δwl) and F10Ub(+Pact T317), harboring a plasmid encoding ΔMTS-yHsp60 (under the GAL promoter), were grown in galactose SD medium, to mid-log phase ($A_{600} = 1$). Yeast cell extracts were prepared by the addition of 1 ml of buffer A containing: 6 M guanidine, 0.1 M Na$_2$HPO$_4$, and 0.01 M Tris, and glass beads to cell pellet, and rolled at 4 °C for 30 min. Samples were centrifuged at 13,800 × g for 30 min at 4 °C. Aliquots of 250 µl of nickel-nitrilotriacetic acid HisBind resin (Novagen) were pre-equilibrated with 750 µl of buffer A. Equal protein amounts of each extract were transferred to vials containing the equilibrated nickel beads, mixed gently for 1 h at 4 °C, and transferred to Bio-spin disposable chromatography columns (Bio-Rad). The columns were washed with 0.5 ml of buffer A, followed by 0.5 ml of buffer B containing: 8 M urea, Na$_2$HPO$_4$, and 0.01 M Tris, pH 8, 0.5 ml of buffer B, pH 6.3, and 0.5 ml of buffer B, pH 4.5. The elutes were discarded and the ubiquitinated proteins were eluted with 200 µl of buffer B, pH 6.3, containing 250 mM imidazole. The final elutes of each column were mixed with sample buffer at a 1:4 ratio, respectively (without heating), and subjected to a 4–20% gradient, SDS-PAGE.

**Primary Antibodies**

Polyclonal antibodies were rabbit serum against yHsp60, Aconitase, fumarase, HxK, Tom40, Aac1, and monoclonal antibody against HA (from a mouse hybridoma), which have been described previously.

**Densitometric Analysis**

The intensity of individual bands from Western blots and phosphorimages (Fujifilm Bas-2500) was determined by using TINA 2.09 software.

**Statistical Analysis**

When more than two groups were compared, statistical analysis was performed by one-way repeated measure analysis of variance with Tukey test. When only two groups were compared, significance was analyzed by paired t test.

**RESULTS**

Hsp60 Can Stabilize Bax in Yeast and Facilitate Bax-dependent Cell Death—Cytosolic accumulation of Hsp60 in mammalian cells has been correlated with either pro-apoptotic or pro-survival functions and has been shown to involve different interactions with the apoptotic apparatus (4). The first goal of the current study was to establish an assay for detection of Hsp60 activity in the yeast cytosol. Our initial attempt focused on Bax, because it is a cytosolic protein, a component of the apoptotic apparatus that may interact with Hsp60 (5) and it has been shown to affect yeast viability under certain conditions (28). To this end, the effect of Hsp60 on Bax-induced cell death was first evaluated. The yeast strain YPH499 was transformed with plasmids encoding yeast Hsp60 (yHsp60), murine Bax tagged with HA (referred to from here on as Bax), Bax-G67R-HA (a non-functional mutant of murine-Bax), Bcl-xL (an anti-apoptotic protein belonging to the Bcl-2 family of proteins), and various combinations. The effects of the various expressed proteins on cell viability were assessed by growing a series of 10-fold dilution of the yeast strains on the appropriate agar media. As shown in Fig. 1A (left panel) all the transformed yeast strains exhibited similar growth on noninducing dextrose media. However, upon induced expression from the galactose promoter (galactose medium, Fig. 1A, right panel), we detected that co-expression of Hsp60 and Bax facilitates cell death, whereas co-expression of Hsp60 with the Bax non-functional mutant, G67R, had no observable affect. Consistent with previous studies (28) the anti-apoptotic protein Bcl-xL did not facilitate Bax-induced cell death. These results demonstrate that overexpression of yHsp60 facilitates cell death induced by a functional Bax.

To examine Bax and Hsp60 protein levels in the yeast cell, extracts were analyzed by Western blot using anti-Hsp60 and anti-HA antibodies. As shown in Fig. 1B, when grown un-induced on dextrose, no Bax could be detected in yeast cultures (bottom panels) and no increase in yHsp60 levels could be detected (bottom panels, dextrose). However, when induced with galactose (GAL), detectable amounts of Bax (lane 3, top left panel) and large amounts yHsp60 (lane 2, middle row, top left panel) were detected in the respective cell lysates. Co-expres-
sion of Bax or Bax-G67R together with yHsp60 induced a large increase in Bax protein levels (lane 4, top row of left panel, and lane 7, right panel). Co-expression of Bcl-xL and Bax also resulted in an increased Bax protein level (lane 9, top row of right panels). This increase was expected due to binding of Bcl-xL to Bax, neutralization of Bax activity by its sequestration, which probably also prevents its degradation.

To rule out the possibility that overexpression of any protein can affect Bax-induced cell death by protein stabilization; we compared the effect of yHsp60 overexpression to the effect of yeast fumarase overexpression, both in the mitochondria and cytosol. Fig. 1D shows that, whereas co-expression of yHsp60 and Bax results in Bax stabilization (lane 3) and cell death (Fig. 1C), co-expression of fumarase and Bax does not (Fig. 1D). Taken together, these results bring us to the conclusion that Hsp60 specifically induces stabilization of Bax in its active form, leading to increased cell death.

Oligomeric Cytosolic Hsp60 Lacking Chaperone Activity, Can Induce Bax Protein Stabilization and Bax-dependent Cell Death in Yeast—Because overexpression of Hsp60 results in increased levels of Hsp60, both in the mitochondria but also in the cytosol (Fig. 2B, top panel, lanes 5 and 6), we could not tell which fraction of Hsp60 (the cytosolic or the mitochondrial) is responsible for Bax-induced cell death and Bax protein stabilization. We constructed and overexpressed Hsp60 without its mitochondrial targeting sequence, which was indeed detected in the cytosol, and not in mitochondria (Fig. 2B, top panel, compare lanes 8 and 9). This cytosolic Hsp60 (termed from here on Hsp60c/H11005/H9004/mTS-yHsp60) induces Bax-dependent cell death (Fig. 2A), stabilizes the Bax protein, and facilitates Bax association with mitochondria (Fig. 2B, second row).

It was important to determine whether the cytosolic Hsp60 is in an oligomeric or monomeric form. Yeast cells expressing cytosolic yHsp60 were extracted under native extraction conditions and subjected to a 4–16% gradient Blue Native gel electrophoresis. The results in Fig. 2C indicate that cytosolic yHsp60 is in an oligomeric state, composed of 7 to 14 oligomers. When SDS was added to the native extract to a final concentration of 2% and subjected to 10% SDS denaturing PAGE, as expected only 60-kDa monomers could be resolved (Fig. 2D).

Is the chaperone activity of Hsp60 required for the stabilization of Bax in its active form? Two mutant proteins, each harboring a single amino acid mutation (known to be critical for the chaperone activity of Hsp60), were expressed in the yeast cytosol (mutations were created within Hsp60c = ΔMTS-yHsp60). The missense mutation D108E (corresponding to D87E of GroEL) and the missense mutation D420A (corre-

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**FIGURE 1.** Overexpression of Bax with yHsp60 stabilizes active Bax. A, co-expression of Bax with Hsp60 facilitates Bax-induced cell death. Yeast harboring plasmids expressing the indicated proteins were grown on dextrose medium, serially diluted, and spotted on dextrose (left panel) or galactose (right panel) plates. B, co-expression of Bax with Hsp60 results in Bax protein stabilization. Yeast strains expressing the indicated proteins were grown on dextrose (DEX) or galactose (GAL) media. Total cell extracts were prepared and subjected to SDS-PAGE and Western blotting, using the indicated antibodies. C, co-expression of Bax with a plasmid encoding the fumarase protein has no effect on Bax-induced cell viability. Yeast harboring plasmids expressing the indicated proteins were grown on dextrose or galactose media, serially diluted, and spotted on dextrose and galactose plates. D, overexpression of fumarase has no effect on Bax protein stabilization. Yeast lysates of the indicated strains, grown on galactose medium, were subjected to SDS-PAGE and Western blot analysis, using the specified antibodies. The data presented in Fig. 1, in each case, represent the results of three similar experiments.
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FIGURE 2. Overexpression of Bax with cytosolic yHsp60 (in its active oligomeric form) stabilizes Bax. A, co-expression of Bax with Hsp60c facilitates Bax-induced cell death. Yeast harboring plasmids expressing the indicated proteins were grown as described in the legend to Fig. 1A, serially diluted, and spotted on dextrose (left panel) or galactose (right panel) plates. B, co-expression of Bax with Hsp60c results in stabilization of Bax and its association with the mitochondria. Yeast harboring plasmids expressing the indicated proteins were grown on galactose media and subjected to subcellular fractionation. Equivalent portions of the total (T), cytosolic (C) fractions, and a 4-fold concentrated mitochondrial (M) fraction, for better detection of Bax, were subjected to Western blot analysis, using the antibodies against the indicated proteins; yHsp60, HA (Bax levels), Aco1 (mitochondrial matrix marker), and hexokinase-1 (cytosolic marker). C, yHsp60c is present in the cytosol as an oligomer of 7 to 14 monomers. Yeast expressing Hsp60c (or not, control) were grown in galactose medium, lysed with glass beads without detergent, and run under conditions of Blue Native gel electrophoresis (gradient 4–16%). Arrows on the right show positions of the oligomeric complex containing 7 and 14 monomers; indications on the left refer to molecular weight markers. D, the Hsp60 complexes are composed of monomeric Hsp60. SDS was added to the natively prepared extracts in C, which were then subjected to denaturing 10% SDS-PAGE. Molecular weight markers are indicated on the right. In each case, the figures represent the results of at least three similar experiments.

We exploited a previously defined protein, ΔMTS-Aco1-SL17, a ubiquitin-proteasome system substrate. This hybrid protein includes SL17, a specific degradation signal (termed a degron), for ubiquitination by Ubc6/Ubc7 (34). The SL17 degron is fused to the C terminus of yeast aconitase (Aco1), which lacks the MTS and therefore resides only in the cytosol (29). Yeast strains expressing ΔMTS-Aco1-SL17 were subjected to [35S]methionine pulse labeling followed by a chase with cold methionine and cycloheximide. As shown in Fig. 4B, ΔMTS-Aco1-SL17 is rapidly degraded (upper arrow, top panel) when compared with endogenous wild type aconitase (lower arrow). 30 min after stopping the protein synthesis and chase, a significant reduction in labeled ΔMTS-Aco1-SL17 could be detected, whereas the lower band corresponding to wild type Aco1 (Fig. 4B, left panel) did not diminish during 90 min. This is expected because the ubiquitinated SL17 degron targets the whole ΔMTS-Aco1-SL17 protein for rapid proteasomal degradation. We anticipated that if Hsp60c induces Bax protein stabilization due to inhibition of proteasomal degradation, then co-expression of ΔMTS-Aco1-SL17 and Hsp60c should attenuate the degradation of ΔMTS-Aco1-SL17. Indeed, as demonstrated in Fig. 4B (middle panel, compare with the upper panel), in the presence of Hsp60c, ΔMTS-Aco1-SL17 (upper arrow) remained stable, indicating that Hsp60c inhibited ΔMTS-Aco1-SL17 ubiquitin-proteasome degradation. As a control, overexpression of fuma-
sponding to D423A of human Hsp60), both impair binding and/or hydrolysis of ATP by Hsp60 (8, 32). Fig. 3, A and B, demonstrate that Hsp60c harboring the mutations can, nevertheless, induce Hsp60-dependent cell death (Fig. 3A, fifth and seventh rows, respectively) and can induce Bax stabilization (Fig. 3B, top panel, lanes S and 7). Based on these results we conclude that the chaperone activity of Hsp60c is not required for Bax stabilization or Bax-induced cell death.

**Hsp60c Can Inhibit Protein Degradation by the Ubiquitin-Proteasome System**—In mammalian cells, Bax is degraded by the ubiquitin-proteasome system (18) so we examined whether this holds true for Bax in yeast. We used the yeast strain KY698, which is mutated for the multidrug resistance channels Pdr5 and the C-24 sterol methyltransferase Erg6, rendering the strain sensitive to the proteasome inhibitor MG-132 (33). The KY698 strain, transformed with the Bax expressing plasmid, was induced in galactose medium for 4 h and MG-132, or dimethyl sulfoxide (DMSO) (its vehicle control) were added for an additional 2 h. As shown in Fig. 4A, incubation of cells with MG-132 resulted in more than a 3-fold increase in Bax protein (lane 4) as compared with the control (lane 2), suggesting that Bax in yeast is also degraded by the ubiquitin-proteasome system.

To further investigate whether Hsp60-induced Bax stabilization reflects attenuation of ubiquitin-proteasome degradatio, we explored a previously defined protein, ΔMTS-Aco1-SL17, a ubiquitin-proteasome system substrate. This hybrid protein includes SL17, a specific degradation signal (termed a degron), for ubiquitination by Ubc6/Ubc7 (34). The SL17 degron is fused to the C terminus of yeast aconitase (Aco1), which lacks the MTS and therefore resides only in the cytosol (29). Yeast strains expressing ΔMTS-Aco1-SL17 were subjected to [35S]methionine pulse labeling followed by a chase with cold methionine and cycloheximide. As shown in Fig. 4B, ΔMTS-Aco1-SL17 is rapidly degraded (upper arrow, top panel) when compared with endogenous wild type aconitase (lower arrow). 30 min after stopping the protein synthesis and chase, a significant reduction in labeled ΔMTS-Aco1-SL17 could be detected, whereas the lower band corresponding to wild type Aco1 (Fig. 4B, left panel) did not diminish during 90 min. This is expected because the ubiquitinated SL17 degron targets the whole ΔMTS-Aco1-SL17 protein for rapid proteasomal degradation. We anticipated that if Hsp60c induces Bax protein stabilization due to inhibition of proteasomal degradation, then co-expression of ΔMTS-Aco1-SL17 and Hsp60c should attenuate the degradation of ΔMTS-Aco1-SL17. Indeed, as demonstrated in Fig. 4B (middle panel, compare with the upper panel), in the presence of Hsp60c, ΔMTS-Aco1-SL17 (upper arrow) remained stable, indicating that Hsp60c inhibited ΔMTS-Aco1-SL17 ubiquitin-proteasome degradation. As a control, overexpression of fuma-
FIGURE 3. The chaperone activity of cytosolic Hsp60 is not involved in the stabilization of Bax in its active form. A, the chaperone activity of Hsp60 is not involved in Bax-induced cell death. Yeast strains harboring the indicated plasmids encoding mutant yHsp60 (cytosolic yeast ΔMTS-Hsp60) lacking chaperon activity were serially diluted and spotted on dextrose or galactose plates. B, the chaperon activity of Hsp60 is not involved in Bax protein stabilization. Alternatively, total cell lysates were subjected to protein extraction, SDS-PAGE, and Western blot analysis. The figures represent the results of three similar experiments.

FIGURE 4. Hsp60c, either overexpressed or temperature induced, can inhibit protein degradation by the proteasome system. A, Bax is degraded by the proteasome system in yeast. The yeast strain KY698 (sensitive to MG-132) expressing HA-tagged Bax, under the galactose promoter, was transferred from dextrose into galactose media. The cultures were grown on galactose for 4 h before MG-132 (Sigma), at a final concentration of 250 μM, or dimethyl sulfoxide (DMSO) was added for an additional 2 h. Aliquots taken at the indicated times were subjected to protein extraction, SDS-PAGE, and Western blot analysis, using the indicated antibodies. B, cytosolic Hsp60c can inhibit degradation of ΔMTS-Aco1-SL17 (a substrate of the ubiquitin-proteasome system). Yeast expressing the indicated proteins were induced in galactose medium and pulse labeled with [35S]methionine for 15 min, followed by a chase (addition of cold methionine and cycloheximide) for the times indicated. Total cell extracts were immunoprecipitated with Aco1 antiserum and analyzed by SDS-PAGE and autoradiography. ΔMTS-Aco1-SL17 is indicated by the upper arrow and Aco1 is indicated by the lower arrow. The relative band intensity ratio of ΔMTS-Aco1-SL17 (upper band) to Aco1 (lower band) for the strains overexpressing fumarase (bottom left panel) or no protein (top left panel, empty plasmid), significantly decreased. In contrast, in strains overexpressing Hsp60c (middle left panel), ΔMTS-Aco1-SL17 was relatively stable. The lower panel shows densitometric analysis of the first three time points of strains harboring an empty plasmid (tubulin), overexpressing Hsp60c (ΔMTS-Hsp60c), or fumarase (ΔMTS-fumarase), as a function of time. The results present the mean ± S.E. of 3 independent experiments and * indicates a significant difference, p < 0.05. C, the cytosolic level of Hsp60c increases at 37 and 40 °C. Yeast strains were grown to midlog phase in galactose medium at 30, 37, or 40 °C and cells were subjected to subcellular fractionation. Total (T), cytosol (C), and mitochondrial (M) fractions were analyzed by Western blotting (upper panel), using the indicated antibodies: anti-yHsp60, anti-Hxk1 (cytosol), anti-Aco1 (mitochondrial matrix), and anti-Tom-40 (mitochondrial outer membrane). The lower panel shows a histogram of cytosolic to mitochondrial (C/M) ratios of Hsp60 band intensities at 30 versus 37 °C (significant difference, *, p < 0.05, of three independent experiments). D, ΔMTS-Aco1-SL17 degradation is slower at 37 than at 30 °C. Yeast expressing ΔMTS-Aco1-SL17 were grown in galactose medium at 30 or 37 °C, and pulse labeled with [35S]methionine for 15 min, followed by a chase for the times indicated. Total cell extracts were prepared, immunoprecipitated with Aco1 antiserum, and analyzed by SDS-PAGE and autoradiography. ΔMTS-Aco1-SL17 and Aco1 are indicated by the upper lower arrows. The lower panel shows densitometric analysis of the respective bands, indicating the ratios of ΔMTS-Aco1-SL17 to Aco1 band intensities at 30 °C (ΔMTS-Aco1-SL17) and 37 °C (ΔMTS-Aco1), as a function of time. The results present the mean ± S.E. of 3 independent experiments and *, indicates a significant difference, p < 0.05.
employed yeast strains expressing the proteasome 20 S subunits and is physically associated with the proteasome—ubiquitin-proteasome system. Cytosolic Hsp60 presence at 37 °C and the inhibition of the proteasome. Importantly this was accompanied by a specific increase in the total Hsp60 protein level at the higher temperatures, as expected from a temperature-regulated heat shock induction. Hsp60 was detected in the yeast cytosol. Whereas various stress and growth conditions were examined, we discovered that only growth at higher temperatures was sufficient to cause a significant presence of chromosomaly encoded wild type Hsp60 in the cytosol. The yeast strain W303 was grown at 30, 37, and 40 °C for 24 h and as shown in Fig. 4C we detected an increase in the total Hsp60 protein level at the higher temperatures, as expected from a temperature-regulated heat shock protein. Importantly, this was accompanied by a specific increase in cytosolic Hsp60 as indicated by a significant increase in the ratio of cytosolic to mitochondrial band intensity at 37 °C (from 0.14 to 0.37, Fig. 4C, right panel). Accordingly, the degradation rate of ΔMTS-Aco1-SL17 was lower (Fig. 4D); the relative intensity of ΔMTS-Aco1-SL17 (top/bottom bands) at 30 °C decreased significantly faster than at 37 °C (Fig. 4D, right panel). This points to a causal relationship between increased cytosolic Hsp60 presence at 37 °C and the inhibition of the ubiquitin-proteasome system. Cytosolic Hsp60 Is Correlated with Decreased Proteolytic Activity and Is Physically Associated with the Proteasome—How does Hsp60c affect the ubiquitin-proteasome system? One possibility is that Hsp60c reduces proteasome activity. To examine this we employed yeast strains expressing the proteasome 20 S α1 tagged with TAP, which allows isolation of proteasome particles from cell lysates on calmodulin-binding protein (CBP) columns. Strains either overexpressing, or not, yHsp60c or yeast fumarase were grown in galactose medium for 24 h at 30 or 37 °C. Cell extracts were loaded onto CBP columns and eluted. Proteasome activity was measured using LLVY-AMC as a fluoroscentic substrate (Fig. 5, A and C). In parallel aliquots from extracts and elutes were subjected to denaturing SDS-PAGE, followed by Western blot analysis (Fig. 5, B and D). The pulled down proteasomes from yeast expressing cytosolic Hsp60 (Fig. 5A) exhibit about 40% decreased peptidase activity when compared with pulled down proteasomes from control or fumarase expressing cells. Yeast grown at 37 °C (Fig. 5C) exhibited although a smaller (~20%) but yet significant inhibition of proteasome activity as compared with yeast grown at 30 °C. This reduction in proteasome peptidase activity could not be attributed to a decrease in levels of the 20 S complex because levels, detected by Western blot, were similar in all cases (Fig. 5, A and C, lower panels). These results indicate that cytosolic Hsp60, whether due to external expression, or naturally occurring at 37 °C, is correlated with reduction in 20 S peptidase activity.

The same cell extracts and CBP column elutes were analyzed by Western blot using anti-yHsp60 antibodies. As expected, Hsp60 could be detected in whole cell extracts according to the level of induction (Fig. 5, B and D). Intriguingly, as shown in Fig. 5B (right panel), Hsp60 was detected in the proteasome-eluted fractions of cells overexpressing Hsp60c. This Hsp60 was detected at levels significantly higher than those observed in control or fumarase overexpressing cells. Accordingly, in cells grown at 37 °C (Fig. 5D, right panel), Hsp60 associated with the eluted proteasome was significantly higher than in cells grown at 30 °C (Fig. 5D, right panel). Taken together, these results strongly support our notion of direct interaction between the Hsp60 oligomer and the proteasome by which the activity of the latter is inhibited. Other control proteins such as mitochondrial acoenitase (Fig. 5, B and D) or cytosolic and mitochondrial fumarase (Fig. 5B) do not appear to associate with the proteasome. Reduced Protein Degradation Is Accompanied by Increased Levels of Ubiquitinated Proteins—A priori, the reduced protein degradation that we described in vivo could theoretically stem from reduced proteasome activity as we suggest above, or alternatively, from decreased ubiquitination. When protein degradation is decreased due to reduced ubiquitination, total protein ubiquitination will decrease. However, when proteasomal degradation is inhibited, it should be accompanied by accumulation of ubiquitinated protein conjugates, given that ubiquitinated

![FIGURE 5. Cytosolic yHsp60 inhibits 20 S proteasome activity.](image-url)
proteins are not degraded. We measured total protein ubiquitination levels in extracts of untransformed cells (Fig. 6, A and B, *lanes 1 and 2*), Hsp60c transformed cells (Fig. 6A, *lane 3*) and yeast grown at 37 °C (Fig. 6B, *lane 3*). The experimental system involved yeast strains in which all chromosomal ubiquitin genes were deleted and yet harbor a plasmid encoding unmutated ubiquitin (Fig. 6, A and B, *lanes 1*) or ubiquitin tagged with 10 histidines (Fig. 6, A and B, *lanes 2 and 3*) as described in Ref. 27). To avoid nonspecific reactions between the anti-ubiquitin antibody and nonubiquitinated proteins, cell extracts were eluted from nickel bead columns, and the eluted proteins were analyzed by SDS-PAGE followed by Western blotting with anti-ubiquitin antibodies. Untreated cell extracts were analyzed by Western blot analysis with anti-Hsp60 and anti-Hxk1 (*lower panels of A and B*). Arrowheads indicate positions on the gel of molecular mass markers (MW, arrows from top to bottom in kDa: 130, 70, 51, 29, and 14). The *right panels* show histograms indicating the accumulative relative intensity of ubiquitin-protein bands in cells overexpressing cytosolic Hsp60 or grown at 37 °C compared with the respective controls (*lanes 3 versus lanes 2 in A and B, respectively*). The results in A and B are representatives of 5 and 2 independent experiments, respectively.

**DISCUSSION**

Hsp60 an oligomeric molecular chaperone of mitochondrial/prokaryotic origin has been subject to rigorous basic research for many years. In the present study, using yeast as a model system, we provide evidence for the ability of Hsp60 to inhibit the yeast proteasome in the cytosol. (i) Using murine Bax (a proteasome substrate) as a tool, we show that coexpression of yHsp60c with Bax stabilizes the latter, enhances its association with mitochondria, and enhances its killing capacity. (ii) Expression of Hsp60 in the yeast cytosol inhibits the degradation of the cytosolic protein ΔMTS-Aco1 tagged with the degron SL17 (a ubiquitin-proteasome degradation signal). (iii) Conditions under which Hsp60 accumulates in the cytosol (cells expressing Hsp60c or grown at 37 °C) correlate with reduced 20 S peptidase activity in cell extracts. (iv) Conditions under which yHsp60 is expressed in the cytosol correlates with increased protein ubiquitination. (v) In 20 S proteasome pulldown experiments, Hsp60 was found to physically associate with the proteasome in extracts of cells expressing Hsp60c or grown at 37 °C.

Does Hsp60 have a role in the yeast cytosol? One possibility, that we favor, is that yeast localize Hsp60 in the cytosol as a way of modulating proteasome activity. In this sense Hsp60 could be one of many mitochondrial proteins that when released or expressed in the cytosol regulate extra mitochondrial cellular activities. The best known example is cytochrome c, which is associated with the electron transport chain of the inner mitochondrial membrane, and when released into the cytosol, becomes an important regulator of apoptosis. The “classical” chaperone activity of Hsp60 does not appear to be required for proteasome inhibition. Thus, one can conclude based on our pulldown experiments that binding of Hsp60 by the proteasome is part of the mechanism by which Hsp60 exerts its affect, rather than an ATP consuming chaperone-catalyzed conformational change of the proteasome. The ubiquitin-proteasome system is active in the cytosol and the nucleus of all eukaryotic cells. This essential system is involved in the control of a variety of cell processes especially quality control of synthesized proteins and determination of protein half-life, by degradation of defective or old proteins. It is also involved in the control of cell cycle, gene expression, transduction pathways, apoptosis, and immune responses. During the last few years additional roles have been revealed in processes such as the cell response to stress, DNA damage repair, and receptor modulation (16, 35). Interestingly Hsp60c appears to be an oligomer yet its chaperone function is not required for its ability to inhibit proteasome activity because mutant Hsp60 proteins that have impaired binding or hydrolysis of ATP can inhibit the proteasome. This agrees with the finding that we achieved proteasome inhibition by overexpressing Hsp60 in the cytosol without a correspond-
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ing overexpression of Hsp10, its co-chaperone that is required for Hsp60 chaperone activity.

A question that has been difficult to approach is how Hsp60 is naturally localized in the yeast cytosol at higher temperatures? The main problem is the relatively small amounts of naturally occurring yeast cytosolic Hsp60 (for example, at 37 °C). There are theoretically a number of possible sources for naturally occurring cytosolic Hsp60. 1) The Hsp60 mRNA is translated from the single relevant AUG codon on cytosolic ribosomes and then the product is retained in the cytosol. This would predict a precursor sized Hsp60 containing its MTS because the mitochondrial processing peptidase (MPP) resides within the mitochondrial matrix. Because we can only detect mature Hsp60 (MTS removed) in cell extracts at 37 °C or on purified 20 S proteasomes, this is very unlikely. 2) A second possible source for cytosolic Hsp60 is the protein that has been imported into mitochondria, thereby allowing processing by MPP. In this case the processed Hsp60 has to be either exported or released from breached mitochondria in a currently unknown process in yeast. In this regard, Hsp60 release from mitochondria to the cytosol has been claimed to occur in cultured mammalian cells following induction of stress by the apoptotic material BMD188 (4). In that study, analysis of the cytosolic fraction revealed that cytosolic Hsp60 was processed, again suggesting that it was derived from a mitochondrial source. 3) An alternative mechanism involves partially translocated Hsp60 (amino terminus only), which is processed and then reversed translocated back into the cytosol in a process that has been described for a number of proteins including fumarase and aconitase in yeast (36–38). 4) A subset of Hsp60 is translocated by an unknown process into the ER and is then distributed to various compartments of the cell via the endomembrane system, a surprising mechanism that has been suggested very recently (39). This issue regarding Hsp60 targeting and localization in the cytosol remains to be resolved not only in yeast but also in higher eukaryotes.

Although in yeast cells Hsp60 is mainly located in the mitochondria, in mammalian cells 10–20% Hsp60 is found in the cytosol under certain conditions (3). The role of Hsp60 in the cytosol of mammalian cell lines is not completely clear. As mentioned in the Introduction it has been suggested that prosurvival or prodeath activities of Hsp60 are mediated via its interaction with caspases, Bax, Survivin, p53, β-catenin, and IKK (4–8). Moreover, Hsp60 is up-regulated in several cancers including: prostate (40), colorectal (41), cervical cancer (42), Hodgkin lymphoma (43) as well as in cisplatin-resistant A431 carcinoma cells (44). This up-regulation correlates with a bad prognosis for the patient; a poor response to chemotherapy. However, there are other tumors like esophageal squamous cell carcinoma (45) as well as ovarian cancer (46) in which high Hsp60 expression correlates with a good prognosis for the patient; a positive response to chemotherapy. It is also well documented that proteasomal inhibition induces growth arrest and apoptosis (35). Based on these observations there is increasing interest in developing specific proteasome inhibitors, which could be used for anticancer treatment, by inducing growth arrest and apoptosis in tumors (35). It is possible that in these latter tumors, the increase in Hsp60c inhibits the proteasome, making the cells more susceptible to apoptosis induced by chemotherapy. An excellent recent example for the use of a proteasome inhibitor is Bortezomb, which can restore Msh2 protein levels and its mismatch repair function in low-level variants and thereby reverse the resistance to cisplatin, a common chemotherapeutic (47). Fascinating future questions to be approached are whether Hsp60 inhibits proteasome activity in mammalian cells and in which disease processes it is involved.

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