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The Hsp90 Molecular Chaperone Complex Regulates Maltose Induction and Stability of the *Saccharomyces MAL* Gene Transcription Activator Mal63p*

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Induction of the *Saccharomyces MAL* structural genes encoding maltose permease and maltase requires the *MAL* activator, a DNA-binding transcription activator. Genetic analysis of *MAL* activator mutations suggested that protein folding and stability play an important role in *MAL* activator regulation and led us to explore the role of the Hsp90 molecular chaperone complex in the regulation of the *MAL* activator. Strains carrying mutations in genes encoding components of the Hsp90 chaperone complex, *hsc82*Δ *hsp82-T101I* and *hsc82*Δ *cpr7*Δ, are defective for maltase induction and exhibit significantly reduced growth rates on media containing a limiting concentration of maltose (0.05%). This growth defect is suppressed by providing maltose in excess. Using epitope-tagged alleles of the *MAL63 MAL* activator, we showed that Mal63p levels are drastically reduced following depletion of cellular Hsp90. Overexpression (~3-fold) of Mal63p in the *hsc82*Δ *hsp82-T101I* and *hsc82*Δ *cpr7*Δ strains suppresses their Mal$^-$ growth phenotype, suggesting that Mal63p levels are limiting for maltose utilization in strains with abrogated Hsp90 activity. Consistent with this, the half-life of Mal63p is significantly shorter in the *hsc82*Δ *cpr7*Δ strain (reduced about 6-fold) and modestly affected in the Hsp90-ts strain (reduced about 2-fold). Most importantly, triple hemagglutinin-tagged Mal63p protein is found in association with Hsp90 as demonstrated by co-immunoprecipitation. Taken together, these results identify the inducible *MAL* activator as a client protein of the Hsp90 molecular chaperone complex and point to a critical role for chaperone function in alternate carbon source utilization in *Saccharomyces cerevisiae*.

In *Saccharomyces cerevisiae*, maltose fermentation requires maltose permease, a proton symporter that transports maltose across the plasma membrane; maltase, an α-glucosidase that hydrolyzes maltose to produce glucose; and the *MAL* activator, a DNA-binding transcription activator (reviewed in Refs. 1 and 2). Maltose-induced expression of maltase and maltose permease requires the *MAL* activator and maltose permease, and strains lacking either gene are noninducible (3). Wang *et al.* (4) showed that intracellular maltose is sufficient to stimulate induction, thereby demonstrating that the role of the permease in induction is simply to provide sufficient intracellular inducer to activate the maltose sensor. The maltose sensor has not been identified, but the *MAL* activator is a candidate.

The genes encoding maltose permease, maltase, and the *MAL* activator are clustered in a complex *MAL* locus. *S. cerevisiae* yeast strains can carry anywhere from one to five unlinked copies of a *MAL* locus, named *MAL1–4* and *MAL6*, which map to sites near the telomere of different chromosomes (5). The presence of any one of these five loci is sufficient for maltose fermentation. All of the five *MAL* loci are highly homologous both structurally and functionally. The genes encoding maltose permease and maltase share a bi-directional promoter that contains the DNA-binding site of the *MAL* activator, thereby providing for the coordinate expression of these maltose utilization enzymes (2, 6).

MAL63 encodes an inducible allele of the *MAL* activator at *MAL6* (3, 5). Mal63p is 470 residues in length and contains a six-cysteine zinc finger DNA-binding domain in the N-terminal ~60–100 residues, a single transcription activation domain in approximately residues 60–250, and a C-terminal regulatory domain in approximately residues 250–470 (2, 7). Mal23p, the inducible *MAL* activator allele encoded by *MAL2*, is 95% identical to Mal63p (7, 8).

Our genetic studies of the *MAL* activator suggest that protein folding and stability play important roles in *MAL* activator regulation (2, 8, 9, 10). Characterization of constitutive alleles of the *MAL* activator localized the maltose-responsive regulatory domain to the C-terminal ~200 residues and demonstrated that this region is a negative regulator of *MAL* activator function. *mal64* is a nonfunctional homologue of *MAL63* but can be activated to a constitutive *MAL* activator by mutation (11). These *MAL64-C* mutations are nonsense mutations at codons 282 and 307 (8). The sequence of the *MAL* activator constitutive mutants *MAL23-C* and *MAL43-C* of the *MAL2* and *MAL4* loci, respectively, reported in Gibson *et al.* (8) contain multiple sequence alterations located largely in the C-terminal regulatory domain. Danzi *et al.* (9) used *in vitro* mutagenesis to localize those residues in the *MAL* activator that are important for negative regulation. They identified clustered alterations in three regions (residues 250–307, 343–357, and 419–461), and the introduction of multiple alterations in any one of these

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TABLE I
List of strains

Strain	Genotype	Source
W303	*MATa leu2–3,112 ura3–1 trp1–1 his3–11,15 can1–100 GAL SUC2*	S. Lindquist
hsc82Δ	Isogenic to W303 except *hsc82Δ::LEU2*	Ref. 26
S153	*MATa leu2–3,112 ura3–1 trp1–1 ade2–1 can1–100 GAL SUC hsc82Δ::LEU2 hsp82Δ::LEU2 pGPD-hsp82-T1011*	Ref. 36
cpr7Δ	Isogenic to W303 except *cpr7Δ::HIS3*	Ref. 26
hsc82Δ cpr7Δ	Isogenic to W303 except *hsc82Δ::LEU2 cpr7Δ::TRP1*	Ref. 26
5CG2	*MATα ura3–52 lys2–801 ade2–101 trp1–63 his3–200 leu2–1 hsc82::URA3 hsp82::GAL1-HSP82::LEU2*	S. Lindquist

regions fully relieves negative regulation by this C-terminal domain. They also found that other sites throughout the protein could modulate the constitutive phenotype of mutations in these regions. But the C-terminal region also plays a positive role in *MAL* activator induction. Charged cluster to alanine scanning mutagenesis of this C-terminal negative regulatory domain of Mal63p produced a series of noninducible alleles that alter residues in regions adjacent to or overlapping two of the three negative regulatory regions defined by Danzi *et al.* (9, 10). Taken together, this genetic analysis suggests that conformational changes involving complex protein-protein interactions regulate *MAL* activator activity. All efforts to identify interactions between different domains of Mal63p using two-hybrid analysis were unsuccessful, and Hu *et al.* (2) proposed that the interactions were likely to be intermolecular. The well documented role for the Hsp90 molecular chaperone complex in the inducer binding and regulation of other transcription activators such as the steroid hormone receptors raised the possibility that the Hsp90 chaperone complex could be a candidate for this *MAL* activator-interacting protein(s) and thus may be involved in the maltose stimulation of the *MAL* activator. The results reported here explore this possibility.

Hsp90 is a highly conserved, abundantly expressed, essential protein in eukaryotes that is localized to the cytoplasm and nucleus (specific references may be found in Refs. 12–20). Hsp90 is the key component of a large complex of proteins, many of them molecular chaperones, that function together assisting certain proteins to achieve an activated conformation in response to external or physiological signals. Despite its abundance, Hsp90 is unlikely to be required for *de novo* folding of the bulk of newly synthesized proteins. Instead it appears to play a role in the maturation of a specific set of newly synthesized proteins, so-called client or substrate proteins, and in the refolding and assembly of misfolded proteins that accumulate in cells following exposure to mild heat shock or other stresses. Therefore, Hsp90 has both stress-related and housekeeping functions and, as such, plays an essential role in processes controlling cell growth and differentiation in unstressed cells.

Many of the components of the Hsp90 chaperone complex are conserved both in structure and function from *S. cerevisiae* to mammals. In *Saccharomyces* this includes the following genes: *HSC82* and *HSP82* encode Hsp90; the *SSA* and *SSB* family of genes encode Hsp70 isoforms (21, 22); *YDJ1* encodes Hsp40 (23); *STI1* encodes Hop/p60 protein (24); *SBA1* encodes p23 (22, 25); *CPR6* and *CPR7* encode cyclophilins (26); *CDC37* encodes $p50^{Cdc37}$ (27); *AHA1* and *HCH1* encode hAha1 (28); and *SSE1* encodes a yeast Hsp110 family member (29). *Saccharomyces* does not encode a homologue of Hip/p48. *HSC82* and *CPR7* are constitutively expressed, whereas their homologues, *HSP82* and *CPR6*, respectively, are stress-induced. Thus, although the different isoforms are functionally overlapping, they are differentially expressed and probably play distinct cellular roles.

Hsp90 is an ATPase, and its ATPase activity is essential for its chaperone function (reviewed in Ref. 20). Several of the components listed above, including Hsp70, Hsp40, Hip/p48, and Hop/p60, are involved in client protein selection and the assembly of the chaperone complex (reviewed in Ref. 20). Others such as p23, $p50^{Cdc37}$, and Aha1 modulate the ATPase activity of Hsp90. The immunophilins, which include cyclophilins and FBPK proteins, have peptidyl proline isomerase activity and tetratricopeptide repeat domains and work to modulate client protein maturation and activation. *Saccharomyces* has been used for the study of mammalian steroid hormone receptor activation (particularly glucocorticoid and androgen receptors) and Src protein kinase maturation. Neither of these proteins is a natural substrate of the yeast Hsp90 chaperone complex, and although much important information has been obtained, it would be valuable to identify and characterize endogenous yeast substrates. To date the only *Saccharomyces* proteins identified as substrates of the Hsp90 chaperone complex are Ste11 protein kinase, Gcn2 kinase, and the heme-regulated transcription activator HapI (30–32). Here we investigate the role of Hsp90 chaperone complex in *MAL* gene regulation. We find that in strains carrying mutations in components of the Hsp90 complex maltose-dependent *MAL* gene induction is defective. Depletion of Hsp90 causes the rapid loss of Mal63p *MAL* activator protein, and in Hsp90 chaperone mutant strains, Mal63p half-life is reduced up to 5-fold. Most significantly, Mal63 *MAL* activator immunoprecipitates with His-tagged Hsp90 from native cell extracts. Thus, the *Saccharomyces MAL* activator is shown to be a novel signal transducing protein client of the Hsp90 chaperone complex, further demonstrating the integration of chaperone function into nonstress cellular metabolism.

MATERIALS AND METHODS

Yeast Strains and Plasmids—The *Saccharomyces* strains used in this study are listed in Table I. Strain W303 carries naturally occurring defective copies of the *MAL1* and *MAL3* loci (33). Both loci contain functional maltose permease and maltase genes, referred to as *MAL11* (also known as *AGT1*) and *MAL12*, respectively, at *MAL1*, and *MAL31* and *MAL33*, respectively, at *MAL3*. Sequences homologous to the *MAL63 MAL* activator gene are found at both *MAL1* and *MAL3*, referred to as *mal13* and *mal33*, respectively, but these genes are nonfunctional. Thus, strain W303 does not ferment maltose. Plasmid-borne copies of *MAL63* complement the defective chromosomal copies of the *MAL* activator genes. Plasmid pMAL63 was constructed by subcloning the BamHI-SalI fragment carrying *MAL63* on its native promoter into the *Escherichia coli* yeast/shuttle vector YCp50 as described by Gibson *et al.* (8).

The following series of plasmids were constructed using vectors described by Mumberg *et al.* (34). Plasmid p414GPD of this series was used to construct p414GPD-MAL63/FLAG. It is a CEN vector containing the *TRP1* selection marker and the promoter of *TDH1*, encoding glyceraldehyde-3-phosphate dehydrogenase, which is a highly expressed constitutive promoter. The *MAL63* coding region was amplified by PCR using an upstream primer that inserts a BamHI site and the sequence encoding the FLAG epitope at the 5′ end of the *MAL63* open reading frame (primer B11; Table II) and a downstream primer that inserts a SalI site immediately following the *MAL63* termination codon (primer B5; Table II). The amplified BamHI-SalI fragment was inserted into the BamHI and SalI sites in the multiple cloning sequence of p414GPD creating a GPD promoter-*MAL63/FLAG* fusion gene and plasmid p414GPD-MAL63/FLAG.

The FLAG tag sequence in plasmid p414GPD-MAL63/FLAG was

TABLE II
List of primers

The BamHI site in primer B11 is in bold type, and the sequence encoding the FLAG epitope tag is underlined. The SalI site in primer B5 is in bold type. The BamHI site in primer KM-N is in bold type, and the NotI site is underlined. The NotI sites in primers MB-4 and MB-5 are underlined.

Primer	Primer sequence
B11	5′-GGGGGATCCATGGATTATAAGGATGACGATGACAAGGGTATTGCGAAACAGTCTTGC-3′
B5	5′-GGGGGTCGACAACGGCGTGAACAATAAA-3′
KM-N	5′-GGGGATCCAAAATGGGCGGCCGCATTGCGAAACAGTCTTGC-3′
MB-4	5′-CCAAAATGGGCGGCCGCATCTTTTACC-3′
MB-5	5′-CGCAATACCGCGGCCGCACTGAGCAG-3′

replaced with a triple HA[1] tag sequence to create plasmid p414GPD-MAL63/HA3 as follows. The fragment encoding the 5′ half of the *MAL63* open reading frame was amplified using a 5′ primer (KM-N; Table II) that inserts a NotI site between codons 2 and 3 of *MAL63* and a 3′ primer complementary to a sequence just downstream of the EcoRI site at codons 215/216 of *MAL63*. This amplified product was digested with BamHI and EcoRI and used to replace the BamHI-EcoRI fragment containing the 5′ end of the tagged *MAL63* gene in p414GPD-MAL63/FLAG, thereby removing the FLAG sequence. A 115-base pair NotI fragment containing three copies of the sequences encoding the HA epitope was inserted into the NotI site in the proper orientation, creating a GPD promoter-*MAL63*/*HA3* fusion gene and plasmid p414GPD-MAL63/HA3.

Plasmid p416GPD from the Mumberg *et al.* (34) series contains *URA3* as the selectable marker but is otherwise the same as p414. The SacI-KpnI fragment containing the entire GPD promoter-*MAL63/HA3* tagged fusion gene was released from p414GPD-MAL63/HA3 by digestion with SacI and KpnI and inserted into SacI-KpnI-digested plasmid p416 to create plasmid p416GPD-MAL63/HA3.

Vector plasmid p416TEF is another from the Mumberg *et al.* (34) series. It is a CEN plasmid and contains *URA3* as the selectable marker gene and the promoter of the *TEF1* gene, a lower level constitutive promoter. The full *MAL63* open reading frame was amplified by PCR using primer KM-N (Table II) as the 5′ primer, which inserts a NotI site between codons 2 and 3 of *MAL63*, and a 3′ primer complementary to the sequence just downstream of the natural *MAL63* termination codon (B5; Table II). The amplified product was digested with BamHI and SalI and inserted into BamHI- and SalI-digested plasmid p416TEF to create a TEF1 promoter-*MAL63* fusion gene. A 115-base pair NotI fragment containing three copies of the sequences encoding the HA epitope was amplified using primers MB-4 and MB-5 (Table II) from p414GPD-MAL63/HA3 and inserted into the NotI site of the TEF1 promoter-*MAL63* fusion gene in the proper orientation producing a TEF1 promoter-*MAL63*/*HA3* fusion gene and plasmid pTEF-MAL63/HA3.

Preparation of Cell Extracts and Immunoblot Analysis—The strains were grown in the appropriate selective minimal medium to mid-log phase (A_{600} of 0.2–0.5). An aliquot of the culture, volume in milliliters approximately equal to 15 divided by A_{600}, was harvested by filtration, washed with 50 mM KPO_4 buffer, pH 7.4, plus 2% sodium azide, and frozen while still on the filter paper at −80 °C for at least 20 min. The frozen cells were defrosted and resuspended in 1 ml of 50 mM HEPES buffer, pH 7.5, containing a mixture of protease inhibitors (Roche Applied Science; complete, mini, EDTA-free protease Inhibitor tablets (catalogue number 1836170) plus Sigma yeast protease inhibitor mixture (catalogue number P8215)), pelleted by centrifugation, and resuspended in 300 μl of SB buffer. SB buffer is prepared by dissolving one tablet of Roche Applied Science protease inhibitor, 8 μl of Sigma yeast protease inhibitor mixture, 0.1 g of SDS, and a toothpick tip-full of sodium bisulfite in 2 ml of H_2O. An equal volume of glass beads was added to the cell suspension, and the samples were vortexed at a medium speed at 4 °C for 20 min. The samples were placed in a 37 °C water bath for 20 min, after which an additional 50 μl of SB buffer was added to each sample, and the samples were vortexed again for 2 min at 4 °C. The glass beads were separated from the extract by centrifugation at 4 °C, and the supernatant was removed. The samples were boiled for 4 min and stored at −80 °C. The protein concentration of the cell extract was determined by the Lowry assay method.

Western blot analysis was carried out using standard methods, and the proteins were detected using an Amersham Vistra ECF kit in which the secondary antibody is conjugated to a fluorescent dye. The signal was visualized using a Molecular Dynamics Storm 860 and quantitated using software provided by the manufacturer. This method allows relatively accurate quantitation of the signal that is linear over ~5 logs. M2 anti-FLAG antibody was obtained from Sigma. The anti-Hsp90 antibody was a gift from Susan Lindquist. Phosphoglycerol kinase (PGK) was detected by anti-PGK antibody from Molecular Probes. PGK levels are relatively constant at different growth conditions, and thus PGK levels were used as a control to adjust for loading variations in those experiments in which accurate quantitation was needed.

Co-immunoprecipitation—The tagged proteins were co-expressed in wild-type cells and grown in selective media. The cells were harvested, resuspended in a nondenaturing buffer containing 50 mM sodium molybdate and extensive protease inhibitors, and flash frozen in liquid nitrogen. The protein extracts were made via glass bead lysis at 4 °C and quantitated by Lowry assay. 50 μg of extract was mixed with 2× sample loading buffer and boiled for 3 min. Approximately 1 mg of total cell extract was then combined with 100 μl of a 50% slurry of Ni^{2+}-nitrilotriacetic acid-agarose (Qiagen) and incubated on ice for 15 min. The resin was washed twice with lysis buffer containing 5 mM imidazole and twice again with lysis buffer containing 10 mM imidazole. The remaining protein was eluted by boiling in sample buffer for 3 min. All of the protein samples were electrophoresed using 10% SDS-PAGE and electroblotted to nitrocellulose, and specific proteins were detected using the indicated antibodies and enhanced chemiluminescence.

Maltase Assay—The cells were grown to mid-log, harvested by centrifugation, and resuspended in 0.5 ml of potassium phosphate buffer, and an equal volume of glass beads was added. The extracts were prepared by votexing the cell suspension three times for 1 min each, keeping the mixture cooled on ice. Maltase activity was measured in whole cell extracts as described in Dubin *et al.* (35). Activity is expressed as nmol of *p*-nitrophenol-α-glucopyranoside hydrolyzed per min/mg of protein. Protein concentration of the cell extracts was measured using the Bio-Rad protein assay dye reagent. The assay values are the averages of results from three independent transformants assayed in duplicate. Variation is ~20%.

RESULTS

Maltase Induction and Maltose Utilization Are Defective in Strains Carrying Mutations in Components of the Hsp90 Chaperone Complex—We investigated the effects of Hsp90 chaperone mutations using an isogenic strain series derived from strain W303 carrying mutations in the genes encoding the two differentially expressed Hsp90 isoforms, *HSC82* and *HSP82*, or in the gene encoding the constitutively expressed cyclophilin isoform, *CPR7*. Strain hsc82Δ (*hsc82Δ HSP82*) lacks the gene encoding the constitutive Hsp90 isoform, *HSC82*. Strain S153 (*hsc82Δ hsp82-T101I*) contains a null mutation in both *HSC82* and *HSP82* but carries a plasmid-borne copy of the temperature-sensitive allele *hsp82-T101I* expressed from the high level constitutive glyceraldehyde-3-phosphate dehydrogenase gene promoter. The *hsp82-T101I* product exhibits reduced activity even at the permissive temperature (24 °C) but is inactivated further at higher temperatures (36). In strains expressing only *hsp82-T101I*, no growth is observed on media containing glucose as the sole carbon source at temperatures above ~35 °C, indicating full loss of Hsp90 activity. Strains carrying a null mutation in *CPR7* and null mutations in both *CPR7* and *HSC82* were also studied. Duina *et al.* (26) found that growth on glucose-containing medium at 30 °C was slowed in the *cpr7Δ* strain, unaffected in the *hsc82Δ* strain, but reduced in the *cpr7Δ hsc82Δ* double null strain.

[1] The abbreviations used are: HA, hemagglutinin; PGK, phosphoglycerol kinase.

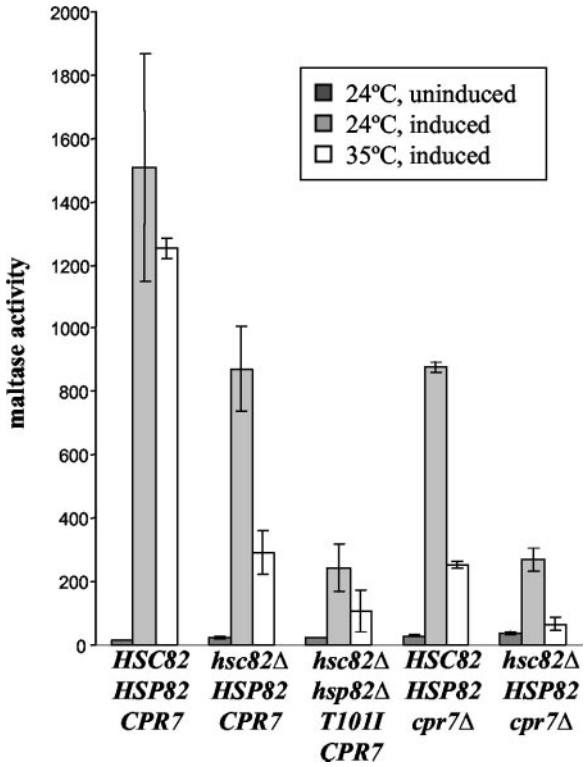

FIG. 1. **Maltase induction in Hsp90 chaperone mutant strains.** The isogenic strains W303 (*HSC82 HSP82 CPR7*), hsc82Δ (*hsc82*Δ *HSP82 CPR7*), S153 (*hsc82*Δ *hsp82-T101I CPR7*), cpr7Δ (*hsc82*Δ *HSP82 CPR7*), and hsc82Δ cpr7Δ (*hsc82*Δ *HSP82 cpr7*Δ) were transformed with plasmid pMAL63. Transformants were grown in selective minimal medium containing 2% glycerol plus 3% lactic acid (v/v) at 24 °C. Maltose was added to 2% (w/v) to induce the expression of maltase following 4 h of induction at either 24 or 35 °C. Maltase activity was determined as described by Dubin *et al.* (35) and is expressed as nmol of *p*-nitrophenol-α-glucopyranoside produced per mg protein/min. The assays were carried out in duplicate on at least three independent transformants.

Strain W303 carries two copies of the genes encoding maltose permease (*MAL11*, also called *AGT1*, and *MAL31*) and maltase (*MAL12* and *MAL32*) but does not ferment maltose because it lacks a functional *MAL* activator gene. To study maltose utilization in this strain series *MAL63*, the *MAL* activator gene from the *MAL6* locus was introduced into the Hsp90 mutant strains by transformation with the *CEN* plasmid pMAL63. The ability of these strains to induce maltase expression was determined. Transformants were grown in selective medium under uninduced conditions at 24 °C to mid-log phase and induced with maltose at either 24 or 35 °C. Maltase activity was assayed at time 0 and at 4 h after the addition of maltose. The results are shown in Fig. 1.

The wild-type strain is able to induce to similar levels at both 24 and 35 °C. Loss of *HSC82* alone causes a modest decrease in the rate of induction at 24 °C, ~2-fold, but induction at 35 °C is reduced to about 25% of the wild-type strain. Maltase induction is significantly decreased in the Hsp90 temperature-sensitive strain (*hsc82*Δ *hsp82-T101I*) at both the permissive and the nonpermissive temperatures. Deletion of one of the two immunophilin genes, *cpr7,* slightly decreases maltase induction, about 2-fold at 24 °C and about 4-fold at and 35 °C. This is comparable with the effect of the *hsc82*Δ null mutation alone. Double deletion of both *cpr7* and *hsc82* enhances the maltose induction defect seen in the single deletion strains and decreases induction even further, about 6-fold at 24 °C and 12-fold at 35 °C. This synergistic effect between the *hsc82*Δ and *cpr7*Δ deletions is consistent with the previously reported derepression of the yeast heat shock factor HSF observed by Duina *et al.* (37).

The maltose induction defect in the Hsp90 chaperone mutant strains can also be observed as a reduced ability to grow on a low concentration (0.05%) of maltose. Fig. 2 compares the growth rate of wild-type and Hsp90 chaperone mutant strains on 0.05% maltose and 0.05% glucose at 30 °C. Growth on limiting glucose was not affected for any of the strains, but the Hsp90-ts and the *hsc82*Δ *cpr7*Δ double null strain exhibited a significant growth defect on 0.05% maltose. We also found that these maltose growth defects were suppressed by increasing the maltose concentration in the medium (Fig. 3). Increased inducer concentration has similarly been found to improve the activation of other known Hsp90 chaperone client proteins including the human androgen receptor and the *Saccharomyces* heme-responsive transcription activator HapI (25, 38). The results reported in Figs. 1–3 indicate genetically that the Hsp90 molecular chaperone complex is involved in *MAL* activator-dependent *MAL* gene induction and maltose utilization but do not identify the specific client protein.

Mal63 MAL Activator Is Destabilized in Hsp90 Depleted Cells—One hallmark of Hsp90 chaperone client proteins is that they become very sensitive to degradation under conditions that interfere with chaperone complex formation or interaction. We used strain 5CG2, which expresses *HSP82* from the *GAL1* promoter as the sole source of Hsp90, to deplete Hsp90 and monitor the effect of loss of Hsp90 protein on Mal63p levels. Plasmid p414GPD-MAL63/FLAG carries *MAL63* tagged with an N-terminal FLAG epitope tag expressed from the high level glyceraldehyde-3-phosphate dehydrogenase promoter. Mal63/FLAG *MAL* activator is functional and able to induce wild-type levels of the *MAL* structural genes (data not shown). Plasmid p414GPD-MAL63/FLAG was transformed into strain 5CG2. When grown on galactose both Hsp90 (Hsp82p) and the FLAG-tagged *MAL* activator Mal63/FLAG protein are easily detected (Fig. 4). Following 7.5 h of growth on glucose medium, which blocks the continued expression of GAL1 promoter-*HSP82*, Hsp90 is nearly fully depleted, and Mal63/FLAG protein levels are barely detectable. In contrast, PGK levels are apparently unaffected. These results strongly suggest that Mal63/FLAG *MAL* activator may depend on the Hsp90 chaperone for stability and function.

Mal63 MAL Activator Binds to Hsp90 in Vivo—Hsp90 client proteins can be found bound to the Hsp90 chaperone complex. To determine whether Mal63p *MAL* activator binds to the Hsp90 chaperone complex, His_6-Hsp82 and triple hemagglutinin-tagged Mal63 were co-expressed in the wild-type strain W303. His_6-Hsp82 was purified from native cell extracts using Ni^{2+} resin, and the bound proteins were analyzed by Western blotting. A low level of triple hemagglutinin-tagged Mal63 protein is observed in the control strain lacking His_6-Hsp82 (Fig. 5), likely because of weak binding of the cysteine-rich DNA-binding domain of Mal63 *MAL* activator to the resin. Nevertheless, Fig. 5 shows a significant enhancement of the amount of Mal63/HA3 protein that co-purifies with His_6-Hsp82 when both proteins are co-expressed. We attempted the reciprocal experiment, *i.e.* immunoprecipitating triple hemagglutinin-tagged Mal63p and testing for co-isolation of Hsp90, but we were unable to effectively purify Mal63/HA3 protein.

Overproduction of Mal63 MAL Activator Suppresses the Maltose Growth Defect in Hsp90 Mutant Strains—Hsp90 chaperone complex reportedly plays various roles in regulation of client proteins. It is proposed to stabilize client proteins, protecting them from degradation by the proteasome pathway. It also is suggested to maintain client proteins in an activation-competent state poised to respond to the regulating signal.

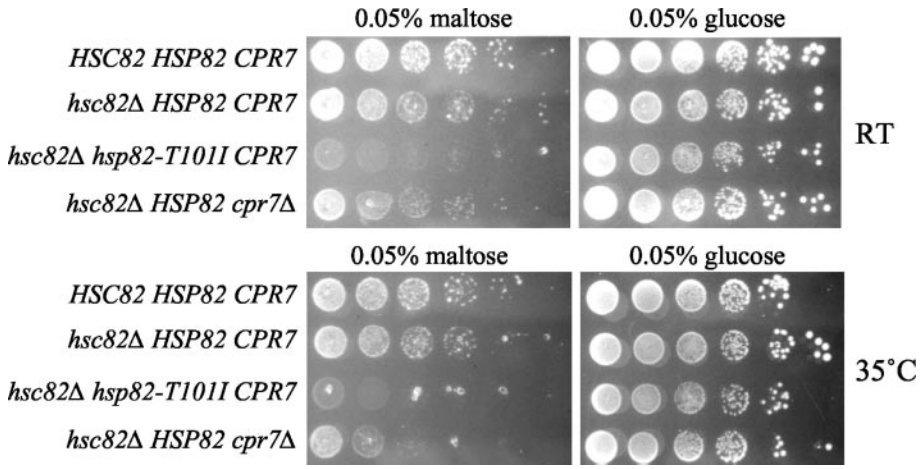

FIG. 2. **Growth on low concentrations of maltose or glucose of Hsp90 chaperone mutant strains.** Growth of the isogenic strains W303 (*HSC82 HSP82 CPR7*), hsc82Δ (*hsc82Δ HSP82 CPR7*), S153 (*hsc82Δ hsp82-T101I CPR7*), and hsc82Δcpr7Δ (*hsc82ΔHSP82 cpr7Δ*) transformed with plasmid pMAL63 was determined by serial dilution on selective minimal medium containing either 0.05% maltose or 0.05% glucose. The plates were grown at room temperature (*RT*, ~21 °C) or 35 °C for 3 days.

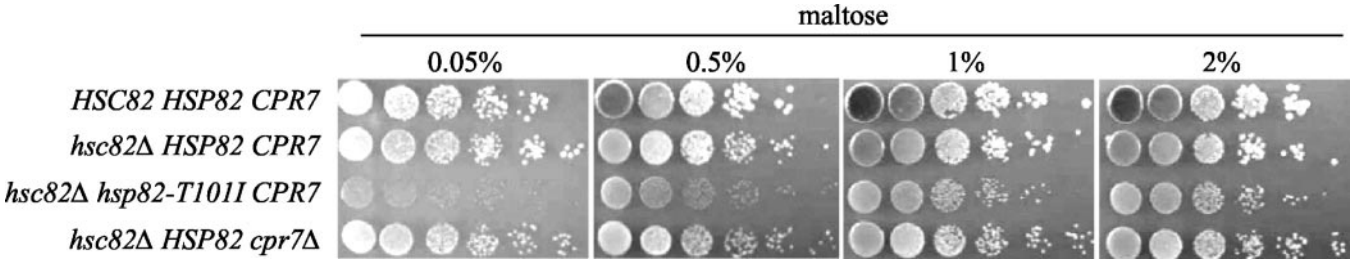

FIG. 3. **Growth of Hsp90 chaperone mutant strains on different concentrations of maltose.** Growth of the isogenic strains W303 (*HSC82 HSP82 CPR7*), hsc82Δ (*hsc82Δ HSP82 CPR7*), S153 (*hsc82Δ hsp82-T101I CPR7*), and hsc82Δ cpr7Δ (*hsc82Δ HSP82 cpr7Δ*) transformed with plasmid pMAL63 was compared by a dilution on selective minimal medium containing increasing concentrations of maltose, as indicated. The plates were grown at room temperature (~21 °C) for 3 days.

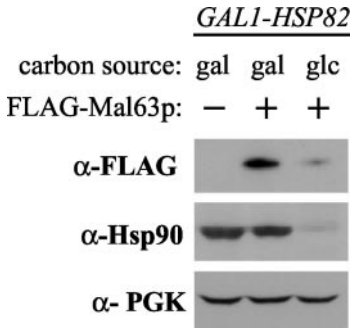

FIG. 4. **Stability of Mal63 *MAL* activator in cells depleted of Hsp90.** Strain 5CG2 containing an integrated GAL1 promoter-*HSP82* gene was transformed with plasmid p414GPD-MAL63/FLAG or the empty vector p414GPD (34) and grown to mid-logarithmic phase in selective minimal galactose-containing medium. The cells bearing the FLAG-tagged *MAL63* plasmid were harvested, washed, and grown for an additional 7.5 h in either galactose-containing (*gal*) or glucose-containing (*glc*) medium, as indicated. The cells were harvested and flash frozen, and the protein extracts were prepared for Western analysis with the indicated antibodies. PGK was used as a loading control.

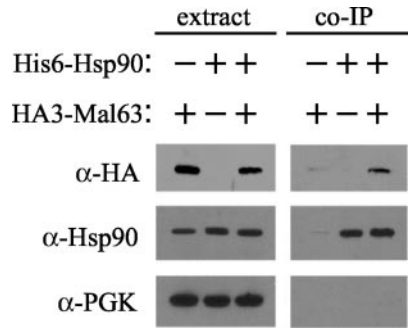

FIG. 5. **Co-precipitation of Hsp90 and Mal63 *MAL* activator.** Nondenaturing protein extracts were prepared as described under "Materials and Methods" from strain W303 co-transformed with plasmids expressing triple-HA-tagged Mal63p (p414GPD-MAL63/HA3), His_6-tagged Hsp82, or the untagged alleles as indicated. His-tagged Hsp82 was isolated from the native cell extracts using Ni^{2+}-nitrilotriacetic acid-agarose (Qiagen) according to the procedure outlined under "Materials and Methods." Co-immunoprecipitation (*co-IP*) samples, and the total cell extracts from which they were prepared were analyzed by Western blot using the indicated antibodies.

Finally, in response to the appropriate signal, client proteins are released from the Hsp90 chaperone complex and proper folding stimulated by the chaperone to enable the client protein to achieve the activated conformation. The results in Fig. 4 indicate that Hsp90 stabilizes the *MAL* activator protein and protects it from degradation. We postulated that in strains with defective Hsp90 chaperone complex *MAL* activator protein may be destabilized, and if so, we should expect to find that overproduction of Mal63 protein should relieve the defects observed in strains with mutant Hsp90 chaperone complex. We tested this as follows. The open reading frame of the triple HA-tagged allele of *MAL63/HA3* was fused to the high strength GPD promoter or the lower strength *TEF1* promoter using the plasmid vector series developed by Mumberg *et al.* (34). Plasmids pTEF-MAL63/HA3 and pGPD-MAL63/HA3 were transformed into strains W303 (*HSC82 HSP82 CPR7*), S135 (*hsc82Δ hsp82-T101I CPR7*), and the double disruption strain hsc82Δ cpr7Δ (*hsc82Δ HSP82 cpr7Δ*) for characterization.

The results in Fig. 6*A* are consistent with our hypothesis. Overproduction of Mal63/HA3 suppresses the maltose growth phenotype observed in the mutant strains but has no noticeable effect on the wild-type strain. It should be noted that the Hsp90-ts strain exhibits a significantly slower growth rate on maltose than the *hsc82Δ cpr7Δ* double null. This is also observed in Figs. 2 and 3 in which *MAL63* is expressed from its native promoter. Total cell extracts were prepared from the transformant strains shown in Fig. 6*A*, and Western blot analysis was carried out to determine the relative level of Mal63/

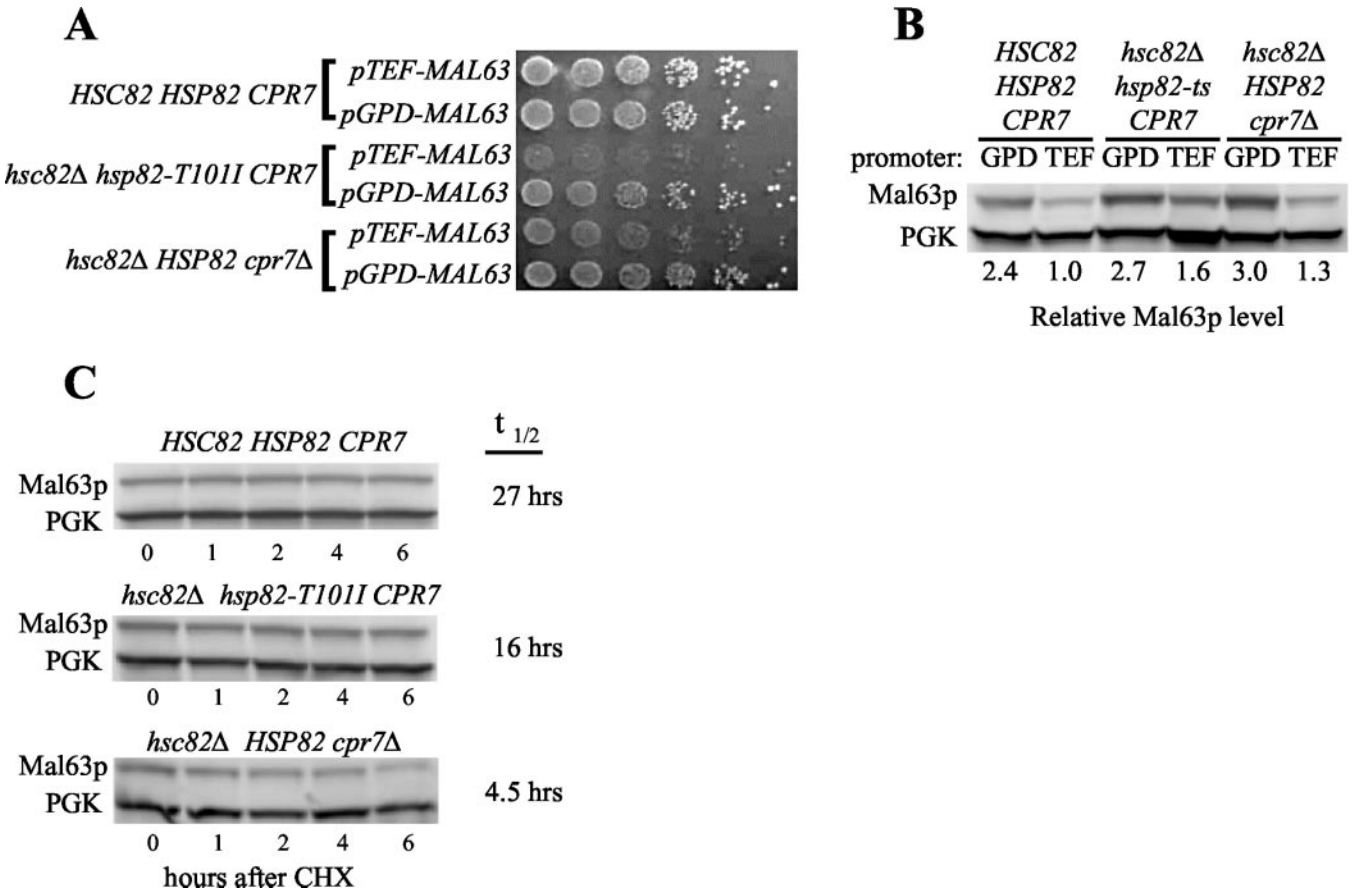

FIG. 6. **Stability of Mal63 *MAL* activator in Hsp90 chaperone mutant strains.** Strains W303 (*HSC82 HSP82 CPR7*), hsc82Δ (*hsc82Δ HSP82 CPR7*), S153 (*hsc82Δ hsp82-T101I CPR7*), and hsc82Δ cpr7Δ (*hsc82Δ HSP82 cpr7Δ*) were transformed with either p416GPD-MAL63/HA3 or p416TEF-MAL63/HA3. These transformants were characterized as follows. *A*, growth of the transformant strains on 0.05% maltose was compared using serial dilution. The plates were grown for 3 days at room temperature (~21 °C). *B*, the transformed strains were grown to early log phase at room temperature (~21 °C) in selective minimal medium containing 2% maltose. Total cell extracts were prepared for Western analysis using anti-HA antibody. Quantitation of Mal63/HA3p expression levels was carried out as described under "Materials and Methods" using PGK levels to normalize for variation in loading. The relative level of Mal63/HA3p was determined in at least three independent transformants. The results presented are the average of at least three experiments. *C*, transformant strains were grown to early log phase in selective minimal medium containing 2% maltose at room temperature (~21 °C). At time 0, cycloheximide (*CHX*) was added to a final concentration of 30 μg/ml, and the total cell extracts were prepared at time 0 and at the indicated time points for 6 h. Western analysis was carried out using anti-HA antibody. The relative level of Mal63/HA3p at each time point was quantified as described under "Materials and Methods" and normalized to the level of PGK. The Mal63/HA3p half-life was calculated from at least three independent transformants, and the average values are presented.

HA3 protein expression in each strain. The level of Mal63/HA3 protein was quantitated using the ECF detection system as described under "Materials and Methods" and normalized to the level of the PGK signal in the same lane. *Fig. 6B* confirms that the expression levels of Mal63/HA3 from the GPD promoter are ~2.5–3-fold higher in each of the strains tested compared with expression from the *TEF1* promoter.

The half-life of Mal63/HA3 *MAL* activator in the Hsp90 chaperone mutant strains was determined. Transformants of the wild-type, Hsp90-ts, and *hsp82Δ cpr7Δ* double null mutant strains carrying a plasmid-borne GPD promoter-*MAL63/HA3* gene were cultured in selective media under uninduced conditions (3% glycerol with 2% lactic acid) at 30 °C to mid-log. Cycloheximide was added to the culture to stop further protein synthesis, and total cell extracts were prepared from culture samples taken at time 0 and at 1, 2, 4, and 6 h after the addition of cycloheximide. Western blot analysis of the samples is shown in Fig. 6*C*. The relative level of Mal63/HA3 protein was quantitated as described under "Methods and Materials" and normalized to the level of PGK. The results were plotted, and rate of Mal63p loss used to calculate the half-life of Mal63/HA3 protein (presented in Fig. 6*C*). The half-life of Mal63/HA3 protein in the Hsp90-ts mutant strain is approximately half that of the wild-type strain but in the *hsc82Δ cpr7Δ* double null strain Mal63/HA3p is significantly shorter, ~⅙ of that observed in the wild-type strain. Thus, the half-life of the *MAL* activator is shortened in Hsp90 chaperone mutant strains, leading to reduced ability to activate the necessary *MAL* gene products required for maximal maltose utilization

DISCUSSION

The results reported here demonstrate that the *Saccharomyces MAL* activator Mal63p is a client protein of the Hsp90 molecular chaperone complex. We show that the growth rate on low concentrations of maltose (0.05%) is significantly slowed in strains carrying Hsp90 chaperone mutations and that induction of the *MAL* structural gene encoding maltase is defective. Mal63p levels are drastically reduced in strains depleted of Hsp90, and the rate of Mal63/HA3p degradation is significantly higher in strains carrying mutations in Hsp90 chaperone complex components. Most significantly, Mal63/HA3p binds to the Hsp90 complex *in vivo*.

In the case of steroid hormone receptors, stabilization and hormone binding competence are achieved by association with Hsp90. The Hsp90 chaperone complex appears to play a similar role in stabilizing the *MAL* activator protein. Similar decreases in protein stability are obtained with other known Hsp90 complex client proteins, like human glucocorticoid receptor and Src

kinase (12, 20, 39, 40). Genetic analysis of *MAL* activator constitutive mutations demonstrated that complex folding patterns and intramolecular protein-protein interactions regulate *MAL* activator activity (9). Additionally, overproduction of some of these noninducible *mal63* mutant proteins suppresses the mutant phenotype and restores maltose inducibility. In Fig. 6 we demonstrate that overexpression of the *MAL* activator can overcome the maltose utilization defects in Hsp90 chaperone mutant strains. This suggests the possibility that these previously isolated Mal63 mutant proteins may be reduced in their ability to interact with the Hsp90 chaperone complex and thus might exhibit higher rates of degradation than wild-type Mal63p. We are currently exploring this possibility.

Two types of defective Hsp90 chaperone strains were tested, one expressing a temperature-sensitive Hsp90 allele and another lacking both the constitutively expressed *HSC82* and *CPR7* genes. Maltose utilization and maltase induction are defective in both types of mutants, indicating that not only is Hsp90 required for maltase induction but that other components of the Hsp90 chaperone complex are also required, specifically the Cpr7 cyclophilin. Moreover, it is interesting to note that although the Hsp90-ts strain exhibited a more significant growth defect than the *cpr7*Δ *hsc82*Δ double null strain, we found that Mal63/HA3 *MAL* activator degraded much more rapidly in the double null strain. This result suggests that Hsp90 itself functions to optimize induction and that this is in addition to its role in stabilizing the *MAL* activator as a component of the Hsp90 chaperone complex. This function could be to maintain the *MAL* activator in a maltose-binding competent conformation and/or stimulate the conformational changes required for maltose induction. We are currently testing other Hsp90 temperature-sensitive alleles to determine the severity of the maltose growth defect and the half-life of Mal63 *MAL* activator.

Wang *et al.* (4) reported that constitutive expression of maltose permease, the maltose transport protein, suppresses the ability of strain YPH500 to ferment maltose and enables it to activate *MAL* gene expression in response to maltose. YPH500 carries the same *mal1* and *mal3* loci as W303, including the defective alleles of *mal13* and *mal33*. Wang *et al.* (4) showed that this suppression is dependent on the defective *mal13* allele that results in constitutive expression of maltose permease and suggest that the very high concentration of intracellular maltose achieved in this strain overcomes the defect in the *mal13* gene product. We suggest that the mutant *mal13 MAL* activator may be defective in its ability to respond to maltose, and in light of the findings reported here, this could indicate a defect in either Hsp90 chaperone complex interaction, a defect in maltose binding capacity of Mal13 mutant *MAL* activator, or a defect in the Hsp90 chaperone-dependent maltose-induced activation. Some of the noninducible *mal63* mutants isolated by Danzi *et al.* (10) are similarly suppressed by constitutive maltose permease. Analysis suggests that these alterations are in a region of Mal63p involved in maltose sensing/binding. Thus, we suggest that the Hsp90 chaperone complex may also be involved in maintaining the *MAL* activator in a conformation that is induction-competent.

Danzi *et al.* (9) identified three regions in the C-terminal regulatory domain of the *MAL* activator involved in negative regulation. Multiple point mutations clustered in these regions produced a constitutive mutation. Moreover, alteration of only one of these sites is sufficient for the constitutive phenotype. The findings reported here might indicate that these regions could represent interaction sites with some component of the Hsp90 chaperone complex. The glucocorticoid receptor is dependent on Hsp90 chaperone complex for hormone-dependent induction and does not bind hormone with high affinity unless it is also bound to the chaperone (reviewed in Refs. 12 and 20). Additionally, binding of the glucocorticoid receptor to Hsp90 chaperone complex inhibits receptor activation in the absence of hormone. These results suggest that Hsp90 functions as both a positive and negative regulator of glucocorticoid receptor. Somewhat different findings are reported for HapI, the *Saccharomyces* heme-regulated transcription activator. Lee *et al.* (32) demonstrated that the HapI activator is a substrate of the Hsp90 chaperone complex. Heme induction of HapI is dependent on Hsp90 chaperone complex binding and requires one of seven putative heme-binding sites, HRM7 (heme-responsive motif 7) (41). Surprisingly, Hon *et al.* (38) found that in strains with reduced Hsp70 levels Hap1p is constitutively active, suggesting that Hsp70/Ydj1 mediates repression of HapI activator and that the Hsp90 chaperone complex functions differently in HapI regulation compared with its role in steroid receptor activation. We are currently exploring the role of Hsp70 in *MAL* activator regulation.

Taken together, these results are consistent with the following model of *MAL* activator regulation. We propose that, in the absence of maltose, *MAL* activator protein is bound to Hsp90 chaperone complex and is protected from degradation. The addition of maltose stimulates the release of *MAL* activator from the complex in an active conformation, allowing it to bind to *MAL* gene promoters and activate transcription. The Hsp90 chaperone complex is clearly involved in the stabilization of the *MAL* activator. A role in maintaining an induction-competent state and in the process of achieving the activated conformation is suggested but has not been demonstrated.

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