THE ROLE OF THE HSP90 MOLECULAR CHAPERONE COMPLEX IN THE REGULATION OF MAL63 *MAL*-ACTIVATOR PROTEIN IN *SACCHAROMYCES CEREVISIAE*

by

MEHTAP BALI

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy,

The City University of New York.

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ABSTRACT

The role of the Hsp90 molecular chaperone complex in the regulation of the activity of Mal63 *MAL*-activator protein in *Saccharomyces cerevisiae*

by

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We investigate the role of the Hsp90 molecular chaperone complex in the regulation of the *MAL*-activator. Analysis of mutant alleles of the *MAL*-activator led us to explore the possibility that *MAL*-activator protein could be a client protein of Hsp90 and thus may be involved in the maltose stimulation of the *MAL*-activator. We find that Hsp90 chaperone mutant strains are defective for maltase induction and exhibit significantly reduced growth rates on media containing a limited concentration of maltose (0.05%) suggesting a role for the Hsp90 chaperone in *MAL* gene induction. This growth defect is suppressed by providing maltose in excess. Overexpression of Mal63p in the *hsc82Δ hsp82-T101* and *hsc82Δ cpr7Δ* strains suppresses their Mal^{-} growth phenotype, suggesting that Mal63 protein levels are limiting for maltose utilization in strains with defective Hsp90 activity. Depletion of Hsp90 causes *MAL*-activator instability. Besides, a triple HA-tagged allele of Mal63 *MAL*-activator co-precipitates with Myc-tagged Hsp90 indicating that Mal63 *MAL*-activator protein is found in association with the Hsp90 chaperone complex. Moreover, triple HA-tagged allele of Mal63 *MAL*-activator co-immunoprecipitates with Myc-tagged Hsp70. So, Mal63 *MAL*-activator is a client protein of the Hsp90 chaperone complex.

The interaction between Mal63 *MAL*-activator with Hsp90 and Hsp70 is regulated by maltose. Growth in maltose increases the association of Mal63p with Hsp90 and decreases association with Hsp70. Moreover, maltase expression in constitutive *MAL*-activator mutants is dependent on Hsp90 chaperone complex.

Hsp90 chaperone complex is involved in the folding and stability of client proteins. We tested the hypothesis that the phenotype of various mutant *MAL*-activator proteins correlates with changes in their ability to interact with Hsp90 chaperone and/or their rate of degradation compared to wild type Mal63p. Several non-inducible mutant *MAL*-activator alleles exhibit variation in rate of degradation. A super-inducible Mal63/HA3p mutant showed a reduced rate of degradation. The turnover rate of the constitutive *MAL*-activators does not differ significantly from wild-type. The ability of the several non-inducible mutant *MAL*-activators to associate Hsp90 was comparable to wild-type. The relative amount of Hsp90 protein associated with the super-inducible and constitutive Mal63/HA3p mutant alleles increased compared to wild-type while interaction with non-inducible *MAL*-activator proteins is not affected.

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INTRODUCTION

Transcription factors are proteins that bind specific DNA sequences either directly or indirectly and activate or repress transcription of certain target genes. Yeast cells react to their environment by regulating the activities of these transcription factors. Signals that are received from the environment are integrated by the cellular machinery to yield a response. The final steps in these pathways that extend from the cell surface to the nucleus is the regulation of the synthesis and/or the activity of specific transcriptional regulators that in turn govern the expression of target genes to produce an appropriate cellular response. The transcription factor Mal63 *MAL*-activator is required for the expression of *MAL* structural genes and induction of the *MAL*-activator appears to involve a highly programmed refolding of the protein in response to inducer and complex protein-protein interactions. Hsp90 (heat-shock protein 90) is an abundant molecular chaperone that functions in the folding and stabilization of a select number of proteins involved in cell signaling and transcription regulation. Although little is known about Hsp90 chaperone client protein selection, about 100 substrates of Hsp90 have been identified. In *Saccharomyces*, the heme regulated transcription activator Hap1, Gcn2 kinase, and Ste11 protein kinases were identified as substrates of the Hsp90 molecular chaperone complex (Lee et al., 2002; Louvion et al., 1998).

In this study, we investigated the role of the Hsp90 molecular chaperone complex in the regulation of the activity and stability of transcription factor Mal63 *MAL*-activator. Our results show that the *Saccharomyces MAL* activator is a client of the Hsp90 chaperone complex (Bali et al., 2003). Hsp90 operates as a part of a large multiprotein complex in the cytosol. This complex includes Hsp90 and Hsp70. Our results show that

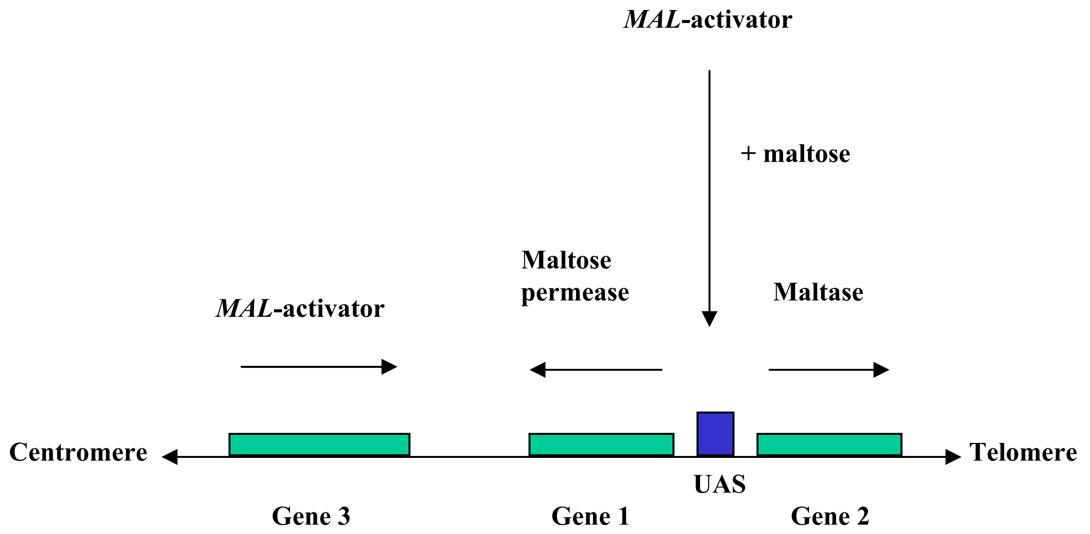
triple hemagglutinin-tagged Mal63 *MAL*-activator is found in association with Myc-tagged Hsp90 and Myc-tagged Hsp70 as demonstrated by co-immunoprecipitation analysis. We explore the role of this interaction in *MAL*-activator regulation and investigate the aspects of non-inducible, constitutive, and super-inducible mutations on this interaction and *MAL*-activator stability.

Part I. Maltose fermentation and *MAL* loci

Maltose fermentation in *Saccharomyces* requires the presence of one or more of five unlinked, telomere-associated *MAL* loci, *MAL1, MAL2, MAL3, MAL4,* and *MAL6*, which show extensive sequence and functional homology to each other throughout an approximately 9.0 kb region. The arrangement of a *MAL* locus is shown in Figure 1 and is reviewed in Needleman (1991). Each locus is a complex consisting of three genes essential for maltose fermentation. GENE 1 at each locus encodes maltose permease, a proton symporter that transports maltose across the plasma membrane. GENE 2 at each locus encodes maltase, an α-glucosidase that hydrolyzes maltose to two molecules of glucose. GENE 3 at each locus encodes the *MAL*-activator, a DNA binding transcription activator regulating GENE 1 and GENE 2. Transcription of GENE 1 and GENE2 is induced by maltose and repressed by glucose. The five *MAL* loci each map to different *Saccharomyces* chromosome, as follows: *MAL1*, chromosome VII; *MAL2*, chromosome III; *MAL3*, chromosome II; *MAL4*, chromosome XI; and *MAL6*, chromosome VIII. The very high degree of sequence homology of the telomere linked *MAL* loci suggests that they evolved by translocation from telomeric regions on different chromosomes (Charron et al., 1989).

Figure 1. The structure and organization of *MAL* locus

The *MAL* locus consists of three genes. GENE 1 encodes maltose permease, GENE 2 encodes maltase and GENE 3 encodes *MAL*-activator. The *MAL* structural genes are divergently transcribed from a common 874 bp promoter region. A 68 bp region of the *MAL61-MAL62* intergenic region called as the UAS_{MAL} is necessary for the maltose-induced expression of both *MAL61* and *MAL62*.


MAL-activator
+ maltose
Maltose
permease
Maltase
MAL-activator
Centromere
Telomere
UAS
Gene 3
Gene 1
Gene 2

Naturally occurring variant alleles of *MAL1* and *MAL3* are found in the common laboratory strains S288C, YPH500 (an S288C derivative) and W303 as well as strains collected from the wild. Of greatest interest are the *MAL1* and *MAL3* variants found in S288C, the strain whose sequence is available on the *Saccharomyces* Genome Database. Strain S288C contains only these *MAL1* and *MAL3* variant loci. The *MAL*-activator genes at both loci exhibit significant sequence variations compared to the inducible *MAL63* gene and are functionally inactive. Thus, strains carrying only these loci are unable to ferment maltose. In addition, the maltose permease gene at the S288C *MAL1* locus is distinct from *MAL61*, the well-characterized gene encoding maltose permease (see below).

The *MAL6* structural genes are coordinately and divergently transcribed from a common 874-bp promoter region (Figure1) (Levine et al., 1992). This region between *MAL61* and *MAL62* ORFs contain the binding site for the *MAL*-activator. A 68bp region of the *MAL61-MAL62* intergenic region referred as the UAS_{MAL} is necessary for the maltose-induced expression of both *MAL61* and *MAL62* and sufficient for the maltose-regulated expression of a *LacZ* reporter. The non-coding intergenic region also contains additional elements that regulate *MAL61* and *MAL62* expression, particularly two Mig1 repressor -binding sites at basepairs –273 to-288 and –578 to –592 adjacent to the UAS_{MAL} (Hu et al., 1995).

Maltose permease and maltase: The rate-limiting step in maltose fermentation is the transport of the maltose across the cell membrane, which is carried out by maltose

permease. Moreover, strains deleted for all maltose permease genes do not induce maltase expression indicating that maltose permease is essential for maltose induction (Charron et al., 1986). *MAL61* of the *MAL6* locus and its nearly identical homologs at the other *MAL* loci (*MAL11, MAL21, MAL31,* and *MAL41*) encode maltose permease, a high affinity maltose/proton symporter (Cheng and Michels1991). Maltose permease is a member of the 12 transmembrane domain superfamily of sugar transporters (Cheng and Michels 1989; Han et al., 1995). The predicted topology of Mal61p suggests that the N-terminal 109 residue, the C-terminal 65 residue, and the central region of 72 residues are each positioned on the cytoplasmic side of the plasma membrane.

Studies of the natural variants of *MAL1* locus found that the maltose permease encoded by the S288C *MAL1* locus is novel compared to the permease at *MAL6*. We refer to this maltose permease gene as *AGT1* (Han et al., 1995). Mal61p and Agt1p share 57% identity and 75% similarity and the homology is distributed throughout the length. The substrate specificities of Mal61p and Agt1p are overlapping but distinct. Agt1 transports a broader range of α-glucosides including maltotriose and isomaltose while Mal61p is able to transport only maltose and turanose. Maltose transport by maltose permease is independent of intracellular ATP levels but is coupled to the electrochemical gradient of protons, therefore Mal61p is a high-affinity proton symporter (Cheng and Michels, 1991; Han et al., 1995). Recent studies suggest that Mal61 permease from *MAL6* locus does not function as the maltose sensor. It serves only as a maltose transporter to accumulate intracellular concentrations of maltose to levels sufficient to induce *MAL* gene expression (Wang et al., 2002).

Maltase is an α-glucosidase that hydrolyzes maltose to two molecules of glucose which can enter glycolysis and are metabolized to CO_2, ethanol, and ATP. Synthesis of maltose permease and maltase is induced by maltose and repressed by glucose (Charron et al., 1986a; Hu et al., 1995; Needleman et al., 1984). Loss of maltase has no obvious effect on induction.

***MAL*-activator:** *MAL63* encodes an inducible allele of the *MAL*-activator at the *MAL6* locus (Needleman et al., 1984, Charron et al., 1989). The *MAL63* gene encodes a 470-residue protein with an N-terminal cysteine-rich zinc finger DNA-binding domain (Kim and Michels et al., 1988; Chang et al., 1988). Based on deletion analysis of *LexA-MAL63* gene fusions, the sequence-specific DNA-binding domain of Mal63p is contained in residues 1-100 and the single transactivation domain is contained in residues 60-250 (Hu et al., 1999). Mutations in these cysteine residues inhibit DNA binding. The C-terminal portion of the *MAL*-activator, approximately residues 200-470, contains residues essential for negative regulation of the *MAL*-activator. Multiple clusters of point mutations or deletion of this region cause loss of negative regulation and creates alleles with the constitutive phenotype (Gibson et al., 1997; Hu et al., 1999; Danzi et al., 2000).

Genetic analysis of the *MAL* loci identified constitutive and non-inducible mutant alleles of *MAL*-activator. *mal64* is a nonfunctional homologue of *MAL63* but can be activated to a constitutive *MAL*-activator by mutation (Dubin et al., 1988). These *MAL64-C* mutations are nonsense mutations at codons 282 and 307. Early studies had isolated constitutive mutations mapping to *MAL2* and *MAL4* locus. The sequence of the *MAL*-activator constitutive mutants *MAL23-C* and *MAL43-C* of the *MAL2* and *MAL4* loci, respectively, is reported in Gibson *et al*. (1997). Both mutant genes contain multiple

sequence alterations located largely in the C-terminal regulatory domain. Mal23p, the inducible *MAL*-activator allele encoded by *MAL2*, is 95% identical to Mal63p (Kim et al., 1988; Gibson et al., 1997). There is no inducible allele of *MAL43* available.

Danzi *et al*. (2000) used *in vitro* mutagenesis to define in greater detail the residues required for negative regulation of the *MAL*-activator. Analysis *MAL*-activator constitutive mutant alleles localized residues required for this negative regulatory function to three short domains (250-307, 343-359, and 419-461) and showed that alterations within any one of these domains are sufficient to produce the constitutive phenotype. The constitutive phenotype of these mutant alleles could be suppressed (that is, restored to an inducible phenotype) by secondary mutations at adjacent or more distant sites. These results suggest that complex and very specific protein-protein interactions are responsible for negative regulation of the *MAL*-activator regulation in the absence of maltose and that formation/maintenance of the inactive conformation is exquisitely sensitive to sequence alterations. Furthermore, unsuccessful efforts to identify interactions between different domains of Mal63p using two-hybrid analysis (Hu et al., 1999) point to the fact that the interactions regulating the *MAL*-activator activity are intermolecular rather than intramolecular. These results taken together with those showing that abundant overproduction of the Mal63p does not overcome the negative regulation of *MAL* gene expression indicate that no *MAL*- activator specific negative regulator (repressor) similar to Gal80p is involved in the maltose inducible regulation of the *MAL*-activator.

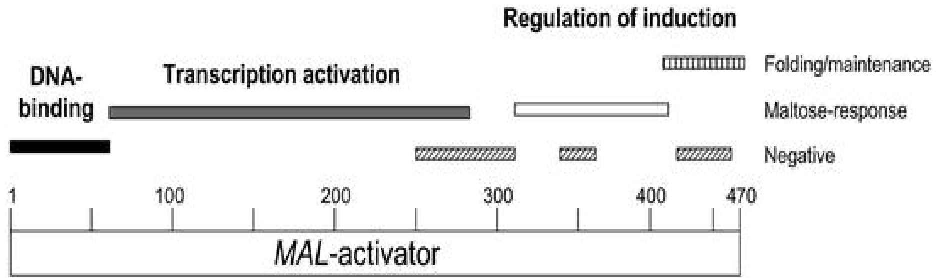
The C-terminal domain is also involved in positive regulatory functions, such as inducer responsiveness. Because Gibson *et al*. (1997), Hu *et al*., (1999), and Danzi *et al*.,

(2000) showed that short deletions or missense alterations within the C-terminal regulatory domain result in a non-inducible phenotype. Charged-cluster to alanine scanning mutagenesis of the regulatory domain of the inducible *MAL*-activator gene *MAL63* and the constitutive *MAL*-activator gene *MAL43-C* were undertaken in an effort to define distinct residues or regions involved in positive regulation (Danzi et al., 2003). These results suggest that approximately 90 residues (residues 331-423) are involved in maltose sensing or responding the presence of maltose and that 50 residues at the extreme C-terminus (residues 420-470) are involved in activation, that is, the formation and maintenance of the transcriptionally active state. Taken together, genetic analysis mutant *MAL*-activator alleles suggests that conformational changes involving complex protein-protein interactions regulate *MAL*-activator activity.

Figure 2. ***MAL*****-activator functional domains**.

Mal63p is 470 residues in length and contains a six-cycteine 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.

(Taken from Danzi et al., 2003)


Regulation of induction
DNA-
binding
Transcription activation
Folding/maintenance
Maltose-response
Negative
1
100
200
300
400
470
MAL-activator

Part II. Heat shock proteins and molecular chaperones

Molecular chaperones can be defined as proteins that promote proper folding of newly synthesized proteins and their refolding under conditions of denaturing stress like elevated temperature or other environmental stresses. The general function of molecular chaperones is to prevent misfolding and aggregation of misfolded proteins. Protein function is determined by its three dimensional structure which is determined by its amino acid sequence and most proteins are able to fold properly on their own. Chaperones do not determine the three dimensional structure of the proteins, but help non-native proteins find their stable and active conformation and prevent them from entering folding pathways that lead to inactive or unstable conformations (Hartl 1996). Many molecular chaperones were initially identified because of their marked increase in expression after stress conditions like exposure to high temperature and therefore are referred to as heat shock proteins. Most of the heat shock proteins, but not all, are molecular chaperones. Heat shock proteins are classified by their molecular weight. For example, Hsp70 is the 70kDa heat shock protein and Hsp90 is the 90kDa heat shock protein. Chaperones are ubiquitous and well-conserved proteins. This thesis will focus on one type of molecular chaperone, the Hsp90 chaperone complex. This complex consists of about 10 or more different proteins of which Hsp90 is the most important. Much of the general information on the Hsp90 chaperone complex presented in Part II of this Introduction is taken from the following reviews (Pearl and Prodromou 2000; Hohfeld et al., 2001; Frydman 2001; Young et al., 2001; Pratt and Toft 1997; Caplan et al., 2003; Pearl and Prodromou 2000; Johnson et al., 1997; Mayer at al., 1999; Picard et al., 2002). A few specific citations are also presented where appropriate.

Hsp90: The heat shock protein Hsp90 is expressed in all eukaryotic and prokaryotic cells and localized in both the cytoplasm and nucleus. It is essential for viability at all temperatures in yeast (Borkovich et al., 1989). Although it is called a heat-shock protein, it is most abundantly expressed protein (~2 % of cytosolic proteins) in unstressed cells (Welch et al., 1982). It has both stress-related and housekeeping functions. It appears to play a role in the maturation and activation of a specific set of client proteins and in the refolding and assembly of the misfolded proteins that accumulate in the cell following to heat shock and other stress. The fact that Hsp90 is essential suggests a role in processes controlling cell growth and differentiation in unstressed cells. More than 100 proteins have been identified as client proteins of the Hsp90 chaperone complex. Almost all client proteins of Hsp90 molecular chaperone are involved in signal transduction including transcription factors such as the steroid hormone receptors, glucocorticoid receptor and progesterone receptor and protein kinases, but a few enzymes have also been shown to require Hsp90 complex (Picard et al., 1990; Xu and Lindquist 1993). Because Hsp90 is indispensable for regulating the activity of certain signaling factors, it plays an essential role in signal transduction networks. Hsp90 is not required for the folding of the newly synthesized proteins (Nathan et al., 1997). Although, Hsp90 binds transiently to non-native proteins, Hsp90 normally only associates with its client proteins after a considerable amount of secondary and tertiary structure have formed (Jacob et al., 1995).

Hsp90 is a homodimer in which only the C-terminal domains are in contact with each other (Nemoto et al., 1995). It has a highly conserved NH_2-terminal ATPase domain that is connected to a highly conserved C-terminal dimerization domain by a middle

domain which is proposed to be involved client protein and co-chaperone binding. A charged linker which is variable in length and composition connects the N-terminal domain and middle region of Hsp90. The charged linker is not essential for Hsp90 function. The extreme C-terminus of Hsp90 contains the MEEVD motif which is binding site for TPR containing co-chaperones (Chen et al., 1998; Young et al., 1998). A TPR domain (tetratricopeptide repeat) is a degenerate three 34 amino acid, helix turn helix motif. Elevated temperatures, stress, or the addition of ATP promotes association of the N-termini. The N-terminal domain is the binding site for ATP and geldanamycin, a competitive inhibitor of Hsp90. Hsp90 has ATPase activity and this is essential for chaperone function. Binding of substrate to Hsp90 requires ATP, but release of substrate from the complex requires ATP hydrolysis and ATPase activity is essential for Hsp90 function (Prodromou et al., 1997, 2003; Obermann et al., 1998; Panaretou et al., 1998). The N-terminal ATP binding domain of Hsp90 is structurally related to a superfamily of homodimeric ATPases such as the DNA mismatch repair protein MutL, DNA gyrase, and a type II topoisomerase (Wigley et al., 1991, Ban et al., 1999).

In *Saccharomyces*, there are two different genes encoding Hsp90, *HSP82* and *HSC82* (Pratt and Toft 2003; Borkovich et al., 1989). Loss of either gene has little phenotypic effect, but double deletion strains are not viable. Hsp82 and Hsc82 proteins share 97% identity at the amino acid level. The two homologs have both unique and overlapping functions. *HSC82* is expressed constitutively at high level and is moderately induced by heat shock. *HSP82* is expressed constitutively at low level but is induced strongly by heat shock.

Co-chaperones and other Hsp90-associated proteins. Hsp90 molecular chaperone cannot function by itself. It operates as a part of multichaperone machinery to accomplish its cellular functions, but it is the key component of this large protein complex (Frydman and Hohfeld 1997, Buchner 1999, Caplan 1999). Hsp90 interacts with 10 or more different co-chaperones, as well as Hsp70 and its co-chaperones. These co-chaperones are involved in client protein selection, modulation of ATPase activity of Hsp90, and client protein maturation and activation (Pratt and Toft 2003, Prince and Matts 2004, Morishima et al., 2003, Lotz et al., 2002). Many of the Hsp90 associated proteins are conserved both in structure and function from *Saccharomyces cerevisiae* to mammals (Chang et al., 1994). In yeast, the Hsp90 chaperone complex has been demonstrated to contain Hsp90, Hsp70, Hsp40, immunophilins (peptidyl-prolyl cis-trans isomerases), p60/Hop, p50, p23, Aha1p, and Hsp110. The co-chaperones by in large fall into two categories: the TPR-containing co-chaperones and all others. The TPR-containing co-chaperones include p60/Hop and the immunophilins Cpr6p and Cpr7p. The TPR domain of the co-chaperones competes with each other for binding to the conserved five C-terminal MEEVD residues of Hsp90 (Young et al., 1998). The other co-chaperones bind to different regions of Hsp90 via special binding sequences.

The following *Saccharomyces* genes encode Hsp90 co-chaperones: *STI1* and *CNS1* encode Hop/p60 protein (Chang et al., 1997), *SBA1* gene encodes acidic protein p23 (Bohen 1998; Fang et al., 1998), *CPR6* and *CPR7* encode immunophilin (Duina et al., 1996), *AHA1* and *HCH1* encode Aha1p (Panaretou et al., 2002), and *SSE1* and *SSE2* encode a yeast Hsp110 family member (Liu et al., 1999). *Saccharomyces* does not encode

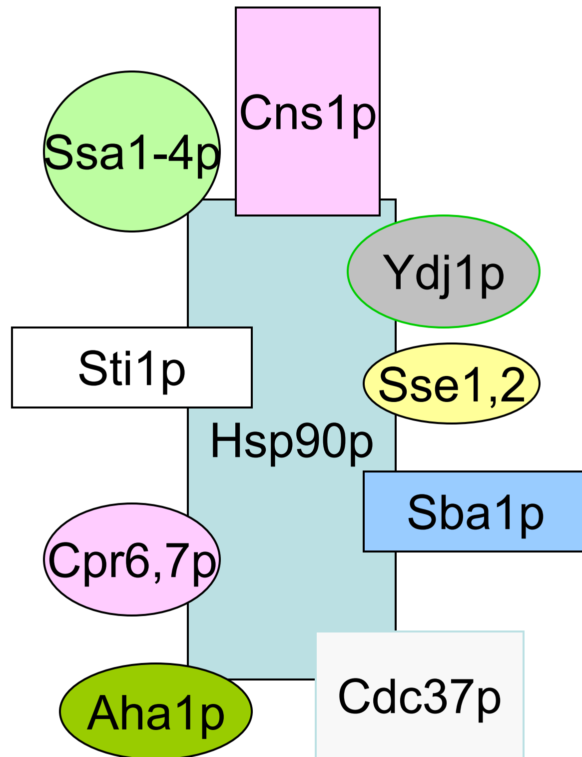
a homolog of Hip/p48. Of these, only *CNS1* is essential. Even the double null mutant *cpr6*Δ *cpr7*Δ is viable (Duina et al., 1996).

Like many other genes in *Saccharomyces*, some of the Hsp90 chaperone genes come in homologous pairs, including *HSP82* and *HSC82* and *CPR6* and *CPR7*. Often one partner is constitutively expressed and the other is induced by heat shock. For example, *CPR6* is stress-induced; *CPR7* is constitutively expressed cyclophilin. Expression of the constitutively expressed homolog is sufficient for functions under normal conditions but at elevated temperatures the higher level of protein expression is required. Expression levels of those homologous genes is often regulated differentially at the transcriptional level by heat shock transcription factor (HSF) that binds to heat shock elements (HSE) to induce expression of heat shock proteins. In yeast, there is a single HSF, encoded by *HSF1*, which possesses an amino-terminal and carboxyl-terminal transcription activation domain (CTA) (Sorger et al., 1990; Nieto-Soleto et al., 1990). The *HSP82* and *HSC82* genes, encoding Hsp90, and *SSE1*, encoding a member of the Hsp110 family of heat shock proteins, are targets of carboxyl-terminal trans-activation domain of HSF1 (Liu et al., 1999). Strains expressing the mutant allele *HSF(1-583)*, a truncated form of HSF lacking carboxyl-terminal trans-activation domain, are defective in expression of *HSP82* and *HSC82* and *SSE1*. The heat induced expression of Hsp70 family of genes *SSA1, SSA3* and *SSA4* is HSF (CTA)-independent (Tamai et al., 1994; Liu et al., 1996).

Figure 3. Components of Hsp90 chaperone complex

Hsp90 potentially interacts with 10 or more different co-chaperones, as well as Hsp70 and its co-chaperones. In yeast, the Hsp90 chaperone complex has been demonstrated to contain Hsp90, Hsp70, Hsp40, immunophilins (peptidyl-prolyl cis-trans isomerases), p60/Hop, p50, p23, Aha1p, and Hsp110.

Components of Hsp90 chaperone complex



Saccharomyces cerevisiae	Vertebrate	Function
Hsc82/Hsp82	Hsp90	Chaperone, ATPase activity
SSa1-4p	Hsp70	Client protein selection, ATPase
Ydj1p	Hsp40	Activates SSa ATPase activity
Cpr6,7	cyclophilin	Peptidypronyl isomerase
Cns1p	cyclophilin	Activates SSa ATPase activity
Sti1p	Hop	Mediates Hsp90-Hsp70 interaction
Sba1p	p23	Stabilize receptor-Hsp90 complexes
Cdc37p	p50	Client protein specificity
SSe1,2p	Hsp110	Function unknown
Aha1p		Stimulates Hsp90- ATPase activity

Hsp70 chaperones: Hsp70 proteins are important components of the Hsp90 molecular chaperone complex and are the second most abundantly expressed and highly conserved heat shock protein in eukaryotes. The functions of Hsp70 family are broader than those of Hsp90 and involve translocation of proteins into cellular compartments, folding of proteins in the cytosol, endoplasmic reticulum (ER) and mitochondria, and rescuing of cellular proteins from denaturation. The Hsp70 proteins have an amino-terminal ATPase domain and a carboxyl-terminal substrate-binding domain. They interact with substrates through the C-terminal substrate binding domain. The substrate binding and release cycle is driven by the switching of Hsp70 between the low affinity ATP bound state and the high affinity ADP bound state which is the substrate-bound state of Hsp70. Thus, ATP binding and hydrolysis are essential for the chaperone activity of Hsp70 proteins (Frydman and Hohfeld, 1997; Prodromou et al., 1999; Young and Hartl, 2000; Hartl et al., 1996).

The mode of action of Hsp70 is different from Hsp90. Hsp70 binds to proteins that are largely unfolded and have extended conformations and even binds to very short peptide fragments that essentially lack tertiary structure such as those exposed in a growing polypeptide chain (Jakob et al., 1995). In *Saccharomyces cerevisiae*, there are at least 14 different members of the Hsp70 family of chaperones that are localized to the cytosol, mitochondrion and endoplasmic reticulum. The cytosol of yeast *Saccharomyces* contains four Hsp70 homologous, called Ssa1, Ssa2, Ssa3, and Ssa4 and three ribosome associated Hsp70s, called Ssb1, Ssb2 and Pdr13 (or Ssz1) (Pfund et al., 2000). A strain containing only the *SSA1* gene is functional indicating that it can compensate for the

absence of the other members of the *SSA* family, but Ssb activity cannot be replaced by Ssa activity (Craig et al., 1994). Hsp70 and Hsp90 have been proved to work with the proteasome pathway (Schneider et al., 1996; Bercovich et al., 1997; Dul et al., 2001).

The ATPase activity of Hsp70 is regulated by co-chaperones. The Ssa family of Hsp70 proteins activated by Hsp40 encoded by *YDJ1* (Kimura et al., 1995). Other members of the Hsp70 family are associated with different co-chaperones, sometimes referred to as DNA-J proteins because of their homology to the *E. coli* dnaJ protein.

Formation of client protein-Hsp90 chaperone complex:

Of the more than 100 proteins are known client proteins regulated by Hsp90, glucocorticoid receptor (GR) is one of the most studied. Hsp90 has a dual role in the regulation of steroid receptor. It promotes repression in the absence of ligand by keeping GR in a hormone-binding competent state but DNA-binding incompetent state. It also promotes activation in the presence of ligand.

The minimal Hsp90 complex capable of regulating glucocorticoid receptor *in vivo* contains five proteins: Hsp90, Hsp70, Hsp40, Hop (Sti1), and p23 (Sba1). Hsp90 chaperone complex is formed by the sequential addition of these five proteins. Hsp70 and Hsp40 are first to bind the client protein glucocorticoid receptor. Then, Hsp70 and Hsp90 bind to Hop simultaneously through its two TPR domains. Hop binds to Hsp70 through its N-terminal TPR domain, and to Hsp90 through its central TPR domain. So, binding of Hop brings Hsp90 in contact with client protein. The resulting complex is referred as the intermediate complex. Then p23 joins and Hsp70 and Hop dissociate from the complex to form mature complex in which GR is held in hormone-binding competent state. Hormone

binding releases GR from the complex and GR can bind to DNA (Pratt et al., 1998; Pratt and Dittmar et al., 1998). In the case of other client proteins, release from proteins of the complex may not occur. Hsp90 may stay bound to the progesterone receptor (Pratt et al., 1997) and both Hsp90 and Hsp70, as well as other chaperone components, stay bound to Hap1p (Lan et al., 2004).

Summary of thesis goals

The genetic analyses of *MAL*-activator mutant alleles carried out by Gibson *et al.* (1997) and particularly Danzi *et al.* (2000, 2003) suggest that the *MAL*-activator undergoes a highly programmed protein folding in response to the inducer maltose. Molecular chaperones are required for certain proteins to fold correctly, and folding is sometimes regulated. For this reason I set out to explore the role of Hsp90 molecular chaperone in the regulation of the *MAL*-activator. My results, described in Chapter 1, indicate that the *Saccharomyces MAL*-activator Mal63p is a client protein of the Hsp90 molecular chaperone complex.

My second goal was to explore possible mechanisms by which maltose and the Hsp90 chaperone complex regulate Mal63 *MAL*-activator functions. I tested whether Mal63 protein turnover rates varied in different carbon sources. The relative association of Mal63p with Hsp90 and Hsp70 in the presence and the absence of maltose were determined. A model of *MAL*-activator regulation by Hsp90 molecular chaperone complex and maltose is presented. These results are reported in Chapter 2.

Many of the non-inducible *MAL*-activator mutant alleles isolated by Danzi *et al* (2003) could be suppressed by overexpression, suggesting that the defect could be the result of increased protein turnover. Chapter 3 examines the role of Hsp90 chaperone complex in regulating constitutive *MAL*-activator mutants. Additionally, the stability of non-inducible, constitutive, and super-inducible Mal63 mutant proteins and association of these mutant proteins with Hsp90 is compared. I determined whether mutant protein stability or Hsp90 association could be correlated with phenotype. I also investigated

whether constitutive *MAL*-activator mutants remain dependent on Hsp90 chaperone complex. These results are reported in Chapter 3.

MATERIAL AND METHODS

Yeast strains and plasmids: The *Saccharomyces* strains used in this study are listed in Table 1. Strain W303 *(MATa leu2-3,112 ura3-1 trp1-1 his3-11,15 can1-100 GAL SUC2)* carries naturally occurring defective copies of the *MAL1* and *MAL3* loci (Han et al.,1995). 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 *MAL32*, 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. Therefore, strain W303 requires a plasmid-borne copy of the *MAL*-activator gene for the expression of the *MAL* structural genes. Strain JN516 (*MATa ura3-52 leu2-3,112 his3-11 trp1Δ1 lys2 ssa2::LEU2 ssa3::TRP1 ssa4::LYS2 hem1-Δ100*) (from Elizabeth Craig) contains deletions in *SSA2*, *SSA3*, and *SSA4* providing *SSA1* as only source of Hsp70 gene.

Construction of YCp50 MAL63: Plasmid pMAL63 was constructed by subcloning the *Bam*H1-*Sal*I fragment carrying *MAL63* on its native promoter into the *E. coli* yeast/shuttle vector YCp50 as described in Gibson *et al*. (1997).

Construction of p414GPD-MAL63/FLAG: The following series of plasmids were constructed using vectors described in Mumberg *et al*. (1995). These vectors were designed for the convenient cloning of genes and their controlled expression at different levels. 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

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*	A. Duina
CMY1200	Isogenic to *hsc82Δ* except *HSP82/Myc*	This study
S153	*MATa leu2-3,112 ura3-1 trp1-1 ade2-1 can1-100 GAL SUC* hsc82Δ*::LEU2 hsp82Δ::LEU2 pGPD-hsp82-T1011*	D. Nathan
cpr7Δ	Isogenic to W303 except *cpr7Δ::HIS3*	A. Duina
hsc82Δ cpr7Δ	Isogenic to W303 except *hsc82Δ::LEU2 cpr7Δ::TRP1*	A. Duina
5CG2	*MATα ura3-52 lys2-801 ade2-101 trp1-63 his3-200 leu2-1 hsc82::URA3 hsp82::GAL1-HSP82::LEU2*	S. Lindquist
JN516	*MATa ura3-52 leu2-3,112 his3-11 trp1Δ1 lys2 ssa2::LEU2 ssa3::TRP1 ssa4::LYS2 hem1-Δ100)*	Elizabeth Craig
CMY1300	Isogenic to JN516 except *SSA1/Myc*	This study

dehydrogenase, which is a highly expressed constitutive promoter. It also provides six to nine unique restriction sites that allows for the cloning of the genes. The FLAG epitope which consists of eight amino acids (DYKDDDDK) has been inserted at the N-terminus of the coding region of *MAL63* gene. The *MAL63* coding region was amplified by PCR using an upstream primer that inserts a *Bam*HI site and the sequence encoding the FLAG epitope at the 5' end of the *MAL63* ORF (5'-GGG**GGATCC**ATGGATTATAAGGATGACGATGACAAGGGTATTGCG AAACAGTCTTGC-3') and a downstream primer that inserts a *Sal*I site immediately following the *MAL63* termination codon (5'-GGGG**GTCGAC**AACGGCGTGAACAATAAA-3'). The *Bam*HI cutting site in upstream primer and *Sal*I cutting site in downstream primer are in bold type. The sequence encoding the FLAG epitope is underlined. The 1.5 kb *Bam*HI – *Sal*I fragment containing FLAG tag and entire *MAL63* coding region was amplified from the template plasmid YCp50 MAL63 and inserted into the *Bam*HI and *Sal*I sites in the multiple cloning sequence of p414GPD creating a GPDpromoter-*MAL63/FLAG* fusion gene and plasmid p414GPD-MAL63/FLAG. The 1.5 kb *Bam*HI – *Sal*I fragment containing FLAG tag and entire *MAL63* coding region was also inserted into the *Bam*HI and *Sal*I sites in the multiple cloning sequence of p416GPD creating a GPDpromoter-*MAL63/FLAG* fusion gene and plasmid p416GPD-MAL63/FLAG.

Construction of p414GPD-MAL63/HA3: The FLAG-tag sequence in plasmid p414GPD-MAL63/FLAG was replaced with a triple HA-tag sequence to create plasmid p414GPD-MAL63/HA3. The HA tag which is derived from viral hemagglutinin (HA) protein consists of nine amino acids. To construct p414GPD-MAL63/HA3, the fragment

encoding the 5' half of the *MAL63* ORF was amplified using a 5' primer (5'-GG**GGATCC**AAAATGG GCGGCCGCATTGCGAAACAGTCTTGC-3') that inserts a *Not*I site between codons 2 and 3 of *MAL63* and a 3' primer complementary to a sequence just downstream of the *Eco*RI site at codons 215/216 of *MAL63*. The 3' primer is just beyond natural *Eco*RI site at 676. The *Bam*HI cutting site in upstream primer and *Eco*RI cutting site in downstream primer are in bold type and *Not*I site is underlined. The triple-A sequence before ATG was inserted in order to help translation. The reading frame was corrected by adding a single nucleotide G just after start codon ATG. This amplified N-terminal fragment from 1-676 was digested with *Bam*HI and *Eco*RI and used to replace the *Bam*HI – *Eco*RI fragment containing the 5' end of the tagged *MAL63* gene in p414GPD-MAL63/FLAG thereby removing the FLAG sequence. A 115 base pair *Not*I fragment containing 3 copies of the sequences encoding the HA epitope (YPYDVPDYA) was inserted into the *Not*I site in the proper orientation creating a GPDpromoter*MAL63/HA3* fusion gene and plasmid p414GPD-MAL63/HA3. The resulting plasmid was sequenced to confirm the construction. Furthermore, the functional activity of the *MAL63* gene in new construct was confirmed by maltose fermentation test and measurement of maltase activities after transforming into strain W303. Maltase expression is maltose inducible to normal levels in these transformants and they ferment maltose in 1 day.

Construction of p416GPD-MAL63/HA3: Plasmid p416GPD from the Mumberg *et al*. (1995) series contains *URA3* as the selectable marker but is otherwise the same as p414. The 2kb *Sac*I – *Kpn*I fragment containing the entire GPDpromoter-*MAL63/HA3* tagged fusion gene was released from p414GPD-MAL63/HA3 by digestion with *Sac*I and

*Kpn*I and inserted into *Sac*I – *Kpn*I digested plasmid p416 to create plasmid p416GPD-MAL63/HA3. W303 transformants carrying these plasmid induced maltase expression to normal levels and ferment maltose in 1 day.

Construction of p416TEF-MAL63/HA3: Vector plasmid p416TEF is another from the Mumberg *et al*. (1995) 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* ORF was amplified by PCR using primer (5' GGGGATCCAAAATGG<u>GCGGCCGC</u>GGTATTGCG AAACAGTCTTGC 3') as the 5' primer, which inserts a *Not*I site between codons 2 and 3 of *MAL63*, and a 3' primer (5'-GGGG**GTCGAC**AACGGCGTGAACAATAAA**-**3') complementary to the sequence just downstream of the natural *MAL63* termination codon. The *Bam*HI cutting site in the upstream primer and the *Sal*I cutting site in the downstream primer are in bold type and the *Not*I site is underlined**.** The amplified product was digested with *Bam*HI and *Sal*I and inserted into *Bam*HI and *Sal*I digested plasmid p416TEF to create a *TEF1promoterMAL63* fusion gene. A 115 basepair *Not*I fragment containing three copies of the sequences encoding the HA epitope was amplified using upstream primer (5' CCAAAATGG**GCGGCCGC**ATCTTTTACC 3') and downstream primer (5' CGCAATACC**GCGGCCGC**ACTGAGCAG 3') from p414GPD-MAL63/HA3 and inserted into the *Not*I site of the *TEF1promoterMAL63* fusion gene in the proper orientation producing a *TEFpromoterMAL63/HA3* fusion gene and plasmid pTEF**-**MAL63/HA3. The resulting plasmid was sequenced to confirm the construction. The functional activity of the *MAL63* gene in the new construct was

confirmed by a maltose fermentation test and measurement of induced maltase activity of W303 transformants.

Construction of a triple HA-tagged mutant MAL-activators: A PCR-based method described above was used to construct triple HA tagged versions of *MAL63* mutant alleles expressed from GPD promoter. The fragment encoding the mutant 3' half of the *MAL63* ORF was amplified using a 5' primer (5' GGG**GAATTC**CTTCCCTTCGGTGAACAA3'), which anneals to *Eco*RI site at the codon 215/216 and downstream primer (5'GGG**GTCGAC**CCCGGGATCGATGTGAACAATAAA 3'), that inserts sequences recognized by enzymes *Cla*I and *Sma*I immediately following the *MAL63* termination codon and just before the *Sal*I cutting site, respectively. The *Eco*RI cutting site in upstream primer and *Sal*I site in downstream primer are in bold type. The amplified 0.7 kb product containing the desired changes was digested with *Eco*RI and *Sal*I and used to replace the 0.7 kb *Eco*RI and *Sal*I fragment containing the 3' end of the wild type *MAL63* gene in plasmid p416GPD-MAL63/HA3. Since the size of the released fragment and replaced fragment are the same, the *Cla*I and *Sma*I (underlined) sites were inserted for the purpose of diagnostic digestion. The constructs were confirmed by the presence of a diagnostic *Cla*I and *Sma*I site and by sequencing the full open reading frame of each construct to verify that only intended changes were present (Rockefeller University Sequencing Center, NY and Korean Sequencing Center, Korea). Furthermore, the resulting plasmids were transformed into the strain W303, transformants were selected on synthetic minimal media lacking uracil, and the ability of the triple HA-tagged mutant *MAL*-activators expressed from the GPD promotor to ferment maltose was determined.

Construction of a triple HA-tagged MAL63 (1-283): The upstream primer (5' GGG**GAATTC**CTTCCCTTCGGTGAACAA3') that contains *Eco*RI (in bold) site at codons 215/216 of *MAL6 3* and the downstream primer (5'-CCCC**GTCGAC**TTACTTTCCTGGTATAGTGAA3'), which anneals to codons 278-283 of *MAL63* but creates translation stop codon at 284 (underlined) followed by a *Sal*I site (in bold) were used to amplify 200 base pair fragment. The amplified PCR product was digested with *Eco*RI and *Sal*I and the product was cloned into the vector fragment of p416GPD-MAL63/HA3 digested with *Eco*RI and *Sal*I to produce p416GPD-MAL63 (1-283)/HA3.

Addition of a Myc epitope tag to HSP82 and SSA1: The Myc epitope consists of ten amino acids (EQKLISEEDL) derived from the protein sequence of the human proto-oncogen p62-Myc. The sequence encoding one copy of the Myc tag was inserted at the 3'end of the ORF of the genomic copy of *HSP82* and *SSA1* creating a C-terminal Myc-tagged allele expressed from the native promoter. The Myc-tagged strains do not exhibit any growth defects indicating that tags do not alter the function of the tagged gene. Western blotting and PCR were used to confirm the constructs.

A PCR-based one-step replacement method was used to insert the Myc tag at the C-terminus of each gene. The PCR product consists of the following sequences in order: 45 bp upstream of the stop codon of *HSP82* gene, one copy of the sequence encoding the Myc epitope, a stop codon, the kan^R gene cassette, and 45 bp downstream of the *HSP82* stop codon. This PCR product was synthesized as follows. Plasmid pFA2-kanMX2 was used as a template to amplify the kanamycin (G418) resistance gene (Wach et al., 1994) using the following primers. The downstream primer contains the following sequences in

the order presented: 45bp upstream of the TAA stop codon of *HSP82*, 2 glycine codons, the 30 bp of sequence encoding the Myc epitope, a stop codon, and an 18bp sequence complementary to the 5' end of *kan*^{R} (5'-CCGGTTGAAGAGGTTCCAGCTGACACCGAAATGGAAGAGGTAGATGGGGGGAACAAAAACTTATTTCTGAAGAAGATCTGTAG**CAGCTGAAGCTTCGTACG**-3'). The sequence encoding the Myc tag is underlined; bases homologous to the template are in bold. The downstream primer contains 45 bp from the 3' end of *HSP82* followed by 18bp of sequence from the 3' end of *kan*^{R} . (5'CATTGTAATGTTTTACCCAGTTATTTCCATGCAGATGCCCTATTTAC**GCATAGGCCACTAGTGGA**-3'). Bases homologous to the template are in bold. The PCR product was transformed directly into strain W303 *hsc82*Δ *HSP82* and transformants were selected on YPD supplemented with 50mg/l geneticin (G418 sulfate from Life Technologies, Gaithersburg, MD) (Guldener et al., 1996). Epitope tagging of *HSP82* was confirmed by PCR and Western blotting.

The *SSA1* gene was Myc-tagged by a similar procedure. The primer sequences used for tagging *SSA1* are as follows. The upstream primer contains the following sequences in the order genes: 45bp upstream of the TAA stop codon of *SSA1*, 2 glycine codons, and 30bp encoding the Myc sequence, and 18bp 5' sequence of *kan*^{R}, (5'TCCAGCTCCAGAGGCTGAAGGTCCAACCGTTGAAGAAGTTGATGGGGGGAACAAAAACTTATTTCTGAAGAAGATCTGTAA**CAGCTGAAGCTTCGTACG**-3'). The sequence encoding the Myc tag is underlined; bases homologous to the template are in bold. The downstream primer contains the following sequence in the order genes: 45 bp from *SSA1* 3'sequence, and 18bp of 3' sequence *kan*^{R}

(5'TTCCTCATTATACCCAGATCATTAAAAGACATTTTCGTTATTATCAATTGC**G CATAGGCCACTAGTGGA**-3'). Bases homologous to the template are in bold. The PCR product was transformed directly into strain JN516 (from Elizabeth Craig) that contains deletions of *SSA2*, *SSA3*, and *SSA4* leaving *SSA1* as the only Hsp70 gene and transformants were selected on YPD supplemented with 50mg/l geneticin. The construct was confirmed using PCR and Western analysis.

Measurement of maltase activity and maltose fermentation test: Cells were grown to mid-log phase (optical density at 600nm = 0.3-0.5) in SM media containing the indicated carbon source and lacking appropriate nutrients for plasmid selection, harvested by centrifugation, re-suspended in 0.5 ml of potassium phosphate buffer, and an equal volume of glass beads was added. Extracts were prepared by votexing the cell suspension 3 times for 1 minute each, keeping the mixture cooled on ice. Maltase activity was measured in whole cell extracts as described in Dubin *et al.* (1985). Activity is expressed as nmoles of PNPG (p-nitrophenol-α-glucopyranoside) hydrolyzed per minute per mg of protein. Protein concentration of the cell extracts was measured using the BioRad Protein Assay Dye Reagent. Assay values are the average of results from three independent transformants assayed in duplicate. Variation is approximately 20%. Fermentation was determined by the number of days required for initial gas bubbles to form in Durham tubes after inoculation of approximately 10^5 cells into YP plus 2% (w/v) maltose.

Bacterial growth and transformation: E. coli strain DH5α was used for plasmid amplification. Competent DH5α were purchased from Gibco/BRL. Cells were transformed according to manufacturer's protocol and transformants were selected as amplicilin resistant colonies on LB plates containing amplicilin. Transformants were

screened by polymerase chain reaction (PCR) and /or restriction analysis of purified plasmid.

Yeast growth and transformation: Yeast cells were grown at 30 ^{0}C on rich media, YPD (1%yeast extract, 2% peptone, 2% dextrose), or synthetic minimal media, SM (Bio 101 Inc) containing appropriate carbon source but lacking relevant nutrients for plasmid selection. Yeast cells were made competent by using lithium acetate as described by Ito et al. (1983). Transformants were selected on synthetic minimal media lacking appropriate nutrient. Plasmid transformation was confirmed based on the ability of the transformants to lose the plasmid following growth under non-selective conditions.

Preparation of cell extracts and Western analysis: Strains were grown in the appropriate selective minimal medium to mid-logarithmic phase (OD_{600} of 0.2-0.5). An aliquot of the culture containing about 15 OD units of cells 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 minutes. The frozen cells were defrosted and resuspended in 1 ml of 50 mM HEPES buffer pH 7.4 containing a cocktail of protease inhibitors (Roche, complete, mini, EDTA-free Protease Inhibitor tablets inhibiting a broad spectrum of serine and cysteine proteases (Cat # 1836170) plus Sigma Yeast Protease Inhibitor Cocktail specific for the inhibition of serine, cysteine, aspartic, metalloproteases (Cat # P8215), and a toothpick tip-full of sodium bisulfite pelleted by centrifugation, and re-suspended in 300 µl of SB buffer. SB buffer is prepared by dissolving one tablet of Roche Protease Inhibitor, 8 µl Sigma Yeast Protease Inhibitor Cocktail, 0.1 g 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 $4^{o}C$ for 20 minutes. The samples were placed in a $37^{o}C$ water bath for 20 minutes, after which an additional 50 µl of SB buffer was added to each sample, and the samples were vortexed again for 2 minutes at $4^{o}C$. The glass beads were separated from the extract by centrifugation at $4^{o}C$ and the supernatant removed. The samples were boiled for 4 minutes and stored at $-80^{o}C$. 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 detected using the 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 approximately 5 logs. M2 anti-FLAG antibody was obtained from Sigma. The anti-Myc antibody was obtained from Roche Applied Science. PGK (phosphoglycerol kinase) 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 cells grown in selective media. Cells were harvested, resuspended in a non-denaturing extraction buffer containing 50 mM sodium molybdate, 20 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 1mM EDTA and extensive protease inhibitors and flash-frozen in liquid nitrogen. Protein extracts were made via glass bead lysis. An equal volume of glass beads was added to the cell suspension and the samples were vortexed at maximum speed at $4^{o}C$ for four times for 1 minute each, and centrifuged 2 minutes at $4^{o}C$ to separate the

extract from the glass beads. The samples were boiled and protein concentration of the cell extract was determined by the Lowry assay method. Approximately 400 µl of lysate was then combined with 6-8 µl of anti-HA agarose slurry (Pierce ProFound™ HA Tag IP/Co-IP Kit and Application Set) and incubated with gentle end-over-end mixing overnight. Slurry is poured into a disposable tube provided by Pierce and were washed by BupH Tris buffered saline (25 mM Tris, 0.15 M NaCI, pH 7.2), plus 0.05%Tween –20 (TBS-T). Remaining protein was eluted by adding 25 µl 2X non-reducing sample buffer [prepared from immunopure lane marker non-reducing sample buffer (5X)] to the anti-HA agarose, heated at 95-100 ºC on a heat block for 5 minutes, and pulse centrifuged for 10 seconds. The supernatant was analyzed by Western blotting. 25µl of the eluted proteins were separated by 10% SDS-PAGE, and transferred to nitrocellulose membrane, and probed with anti-HA antibody and anti-Myc antibody (Roche). Anti-ECF was used as a secondary antibody and protein levels were visualized by using a Storm 860 PhosphoImage analyzer (Molecular Dynamics) and the signal quantified using the manufacturer provided software. The HA-tagged Positive Control that is provided by the HA-tag IP/Co-IP Kit assisted in verifying whether the anti-HA agarose can successfully capture the HA-tagged protein.

CHAPTER 1

The role of the Hsp90 molecular chaperone complex in the regulation of the activity of Mal63 *MAL*-activator protein in *Saccharomyces cerevisiae*

Chapter 1 of this thesis was published in the Journal of Biological Chemistry largely as presented here. The full reference is: Bali, M., Zhang, B., Morano, A.K., and Michels, C. A., 2003. The Hsp90 Molecular Chaperone Complex Regulates Maltose Induction and Stability of the *Saccharomyces MAL* Gene Transcription Activator Mal63p. Journal of Biological Chemistry 278, 47441-47448. My contributions are as follows. I constructed plasmids p416GPD-MAL63/HA3 and p416TEF-MAL63/HA3. I am responsible for the results shown in Figures 5, 6, 8, 9A, and 10. The remainder of the work was done by Bin Zhang or Marcelo Taborga (both technicians in the Michel's lab) or by our collaborator Kevin A. Morano, University of Texas-Houston Medical School, Houston, Texas.

Introduction

Our genetic studies of the *MAL*-activator by Gibson et al (1997) and Danzi *et al.* (2000, 2003) suggest that protein folding and stability play important roles in *MAL*-activator regulation. (Danzi et al., 2000) used *in vitro* mutagenesis to localize the residues in the *MAL*-activator that are important for negative regulation. Analysis *MAL*-activator constitutive mutant alleles localized those residues required for this negative regulatory function to three short domains (250-307, 343-359, and 419-461) and showed that alterations within any one of these domains are sufficient to produce the constitutive phenotype. Besides, the constitutive phenotype of those mutant alleles could be suppressed by intragenic missense mutations at closely linked or more distant sites to restore the inducible phenotype. Gibson *et al.* (1997) carried out a deletion analysis of the *LexA-MAL63* fusion gene to create N-terminal, C-terminal and internal deletions in *MAL63* in order to define the negative regulatory domains. Deletions of C-terminal residues 302-349, 352-399, 402-449 and 457-470 did not cause a constitutive phenotype but instead those deletion mutations caused a non-inducible phenotype. Additionally, there is no *MAL*-specific repressor similar to Gal80p involved in the negative regulation of the *MAL*-activator. Since, although overexppression of Gal4p inhibits the negative effect of Gal80p, the increase in the concentration of Mal63p does not overcome the negative regulation of *MAL*-gene expression (Hu et al., 1999). These results suggest that complex and very specific protein-protein interactions are responsible for negative regulation of the *MAL*-activator regulation in absence of maltose and that formation/maintenance of the inactive conformation is exquisitely sensitive to sequence alterations. 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.

Hsp90 is a molecular chaperone that is involved in folding of its substrate proteins. Hsp90 is an ATPase and its ATPase activity is essential for its chaperone function (Prodromou et al., 1997; Obermann et al., 1998; Panaretou et al., 1998; Pratt et al., 2003). Several of the components of the Hsp90 molecular chaperone complex, including Hsp70, Hsp40, Hip/p48, and Hop/p60, are involved in client protein selection and the assembly of the chaperone complex. Others such as p23, $p50^{Cdc37}$, and Aha1 (activator of Hsp90 ATPase) stimulate the intrinsic ATPase activity of the Hsp90 molecular chaperone (Lotz et al., 2003). The immunophilins, which include cyclophilins and FBPK proteins, have peptidyl proline isomerase activity and tetratricopeptide repeat (TPR) 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, while much important information has been obtained, it would be valuable to identify and characterize endogenous yeast substrates. In *Saccharomyces*, only a few client proteins have been identified. The heme regulated transcription activator Hap1, Gcn2, Swe1 and Ste11 protein kinases, the catalytic subunit of calcineurin Cna1p are among the client proteins identified as substrates of the Hsp90 molecular chaperone complex (Lee et al., 2002, Louvion et al., 1998). Therefore, we investigated the role of

Hsp90 molecular chaperone complex in *MAL* gene regulation. We report here on strains carrying mutations in components of the Hsp90 complex that 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, triple HA3 Mal63 *MAL*-activator immunoprecipitates with Myc-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 non-stress cellular metabolism.

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 (Nathan et al., 1995). In strains expressing only *hsp82-T101I* no growth is observed on media containing glucose as the sole carbon source at temperatures above approximately 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*. (Duina et al., 1996)) found that growth on glucose-containing media at 30ºC was slowed in the *cpr7*Δ strain, unaffected in the *hsc82*Δ strain, but reduced in the *cpr7*Δ *hsc82*Δ double null strain.

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°C or 35°C. Maltase activity was assayed at time zero and at four hours after addition of maltose. The results are shown in Figure 4.

The wild-type strain is able to induce to similar levels at both 24ºC and 35ºC. Loss of *HSC82* alone causes a modest decrease in the rate of induction at 24°C, approximately 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 to the effect of the *hsc82Δ* 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*. (Duina et al., 1998).

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. Figure 5 compares the growth rate of wild-type and Hsp90 chaperone mutant strains on 0.05% maltose and 0.05% glucose at RT and 35ºC. 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 (Figure 6). 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 Hap1 (Fang et al., 1998; Hon et al., 2001). The results reported in Figures 4-6 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.

Figure 4. Maltase induction in Hsp90 chaperone mutant strains. The isogenic strains W303 (*HSC82 HSP82 CPR7*), hsc82Δ (*hsc82Δ HSP82 CPR7*), S153 (*hsc82Δ hsp82-T101I CPR7*), cpr7Δ (*hsc82Δ HSP82 CPR7*), and hsc82Δ cpr7Δ (*hsc82Δ HSP82 cpr7Δ*) were transformed with plasmid pMAL63. Transformants were grown in selective minimal medium containing 2% glycerol plus 3% lactic acid (vol/vol) at 24ºC. Maltose was added to 2% (wt/vol) to induce the expression of maltase following 4 hours of induction at either 24ºC or 35ºC. Maltase activity was determined as described in Dubin *et al*. (Dubin et al., 1985) and is expressed as nmoles PNPG produced per mg protein per minute. Assays were carried out in duplicate on at least three independent transformants.

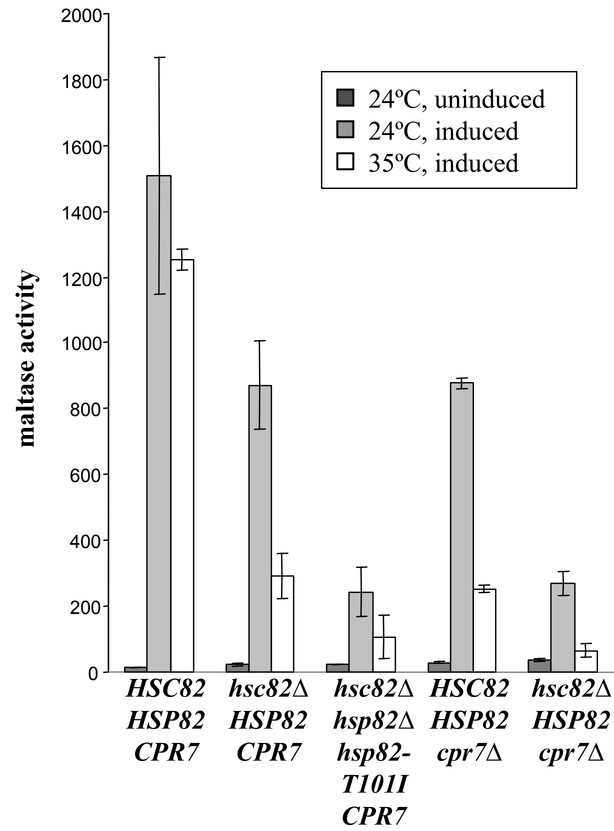

2000
1800
1600
1400
1200
1000
800
600
400
200
0
maltase activity
24ºC, uninduced
24ºC, induced
35ºC, induced
HSC82 HSP82 CPR7
hsc82Δ HSP82 CPR7
hsc82Δ hsp82Δ hsp82-T101I CPR7
HSC82 HSP82 cpr7Δ
hsc82Δ HSP82 cpr7Δ

Figure 5. 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 hsc82Δ cpr7Δ (*hsc82Δ HSP82 cpr7Δ*) transformed with plasmid pMAL63 was determined by serial dilution on selective minimal media containing either 0.05% maltose or 0.05% glucose. Plates were grown at room temperature (approximately 21ºC) or 35ºC for 3 days.

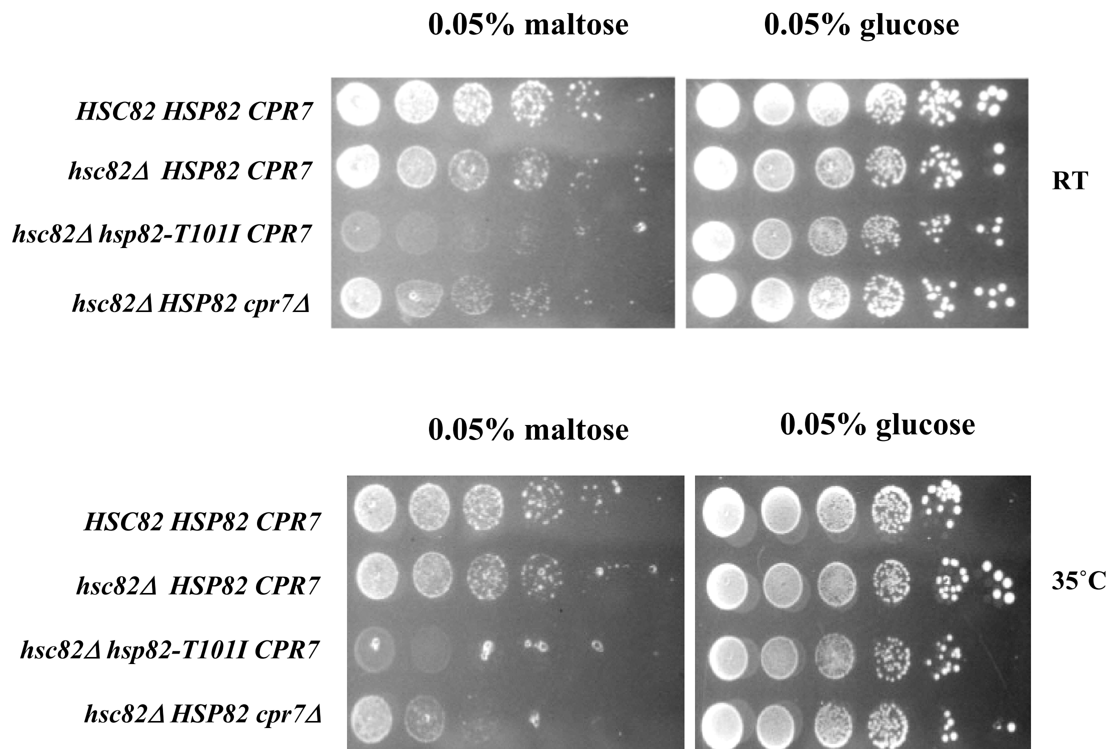
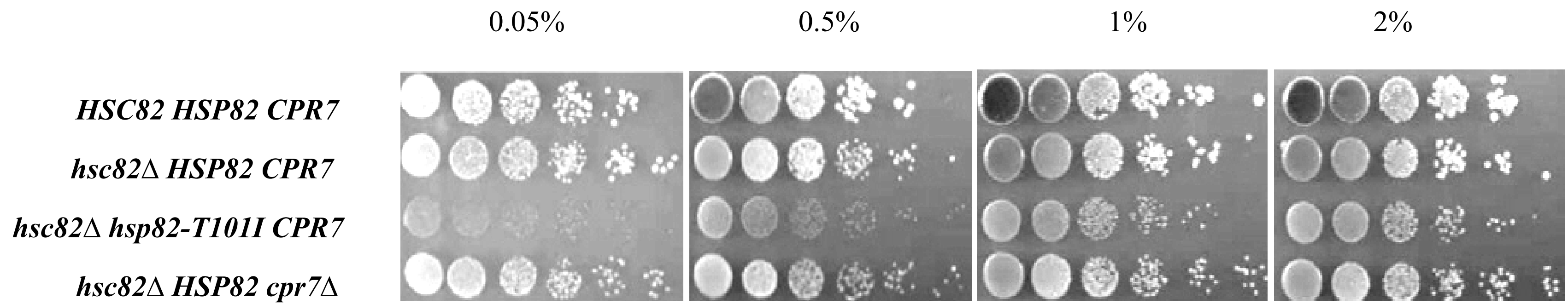

0.05% maltose
0.05% glucose
HSC82 HSP82 CPR7
hsc82Δ HSP82 CPR7
hsc82Δ hsp82-T101I CPR7
hsc82Δ HSP82 cpr7Δ
RT
0.05% maltose
0.05% glucose
HSC82 HSP82 CPR7
hsc82Δ HSP82 CPR7
hsc82Δ hsp82-T101I CPR7
hsc82Δ HSP82 cpr7Δ
35˚C

Figure 6. Growth of Hsp90 chaperone mutant strains on different concentrations of maltose. Growth of the isogenic strains W303 (*HSC82 HSP82 CPR7*), hsc82Δ (*hsc82*Δ *HSP82 CPR7*), S153 (*hsc82*Δ *hsp82-T101I CPR7*), and hsc82Δ cpr7Δ (*hsc82*Δ *HSP82 cpr7*Δ) transformed with plasmid pMAL63 was compared by a dilution on selective minimal medium containing increasing concentrations of maltose, as indicated. Plates were grown at room temperature (approximately 21ºC) for 3 days.

Maltose

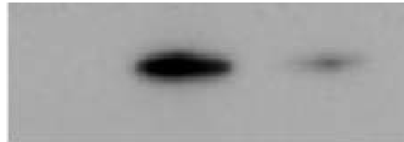


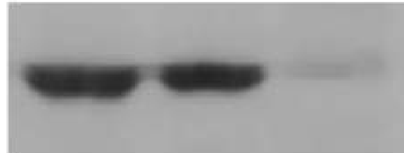
***Mal63 MAL-activator is destabilized in Hsp90 depleted cells*:**

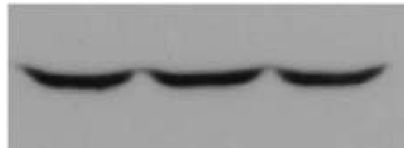
One hallmark of Hsp90 chaperone client proteins is that they become very sensitive to degradation under conditions that interfere with chaperone complex formation or interaction. We used strain 5CG2, which expresses *HSP82* from the *GAL1* promoter as the sole source Hsp90, to deplete Hsp90 and monitor the effect of loss of Hsp90 protein on Mal63p levels. The Hsp90 expression level in 5CG2 strain was controlled using media containing glucose 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 (Figure 7). Following 7.5 hours of growth on glucose medium, which blocks the continued expression of *GAL1promoter-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.

Figure 7. Stability of Mal63 *MAL*-activator in cells depleted of Hsp90. Strain 5CG2 containing an integrated *GAL1promoter-HSP82* gene was transformed with plasmid p414GPD-MAL63/FLAG or the empty vector p414GPD (Mumberg et al., 1995) 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 hours in either galactose- or glucose-containing medium, as indicated. Cells were harvested, flash-frozen, and protein extracts prepared for Western analysis with the indicated antibodies. PGK, phosphoglycerate kinase, was used as a loading control.

GAL1-HSP82
carbon source: gal gal glc
FLAG-Mal63p: – + +
α-FLAG
α-Hsp90
α-PGK







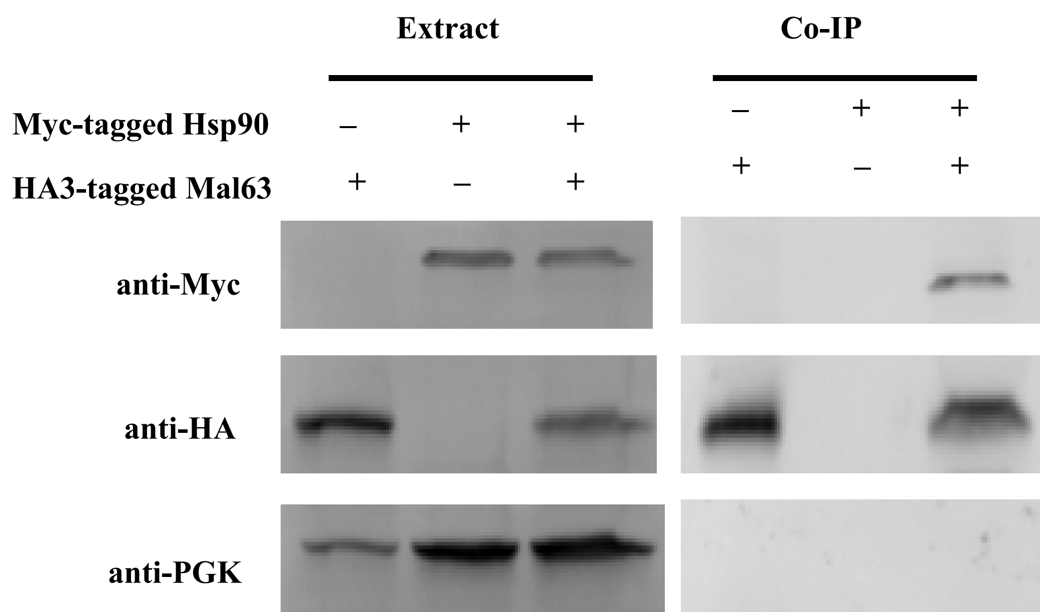
Mal63 MAL-activator binds to Hsp90 in vivo

Hsp90 client proteins can be found bound to the Hsp90 chaperone complex. In *Saccharomyces*, Ste11 protein kinase, Gcn2 kinase, and the heme-regulated transcription activator Hap1 proteins were identified as substrates of the Hsp90 chaperone complex and co-purify with Hsp90 (Louvion et al., 1998; Donze et al., 1999; Lee et al., 2002). To determine if Mal63p *MAL*-activator binds to the Hsp90 chaperone complex, plasmid-borne triple HA-tagged Mal63 protein (pGPD-MAL63/HA3) was expressed in a strain in which the genomic *HSP82* gene is Myc-tagged at its C-terminus. The Myc-tag was inserted by a PCR-based one-step replacement method as described in Materials and Methods into strain hsc82Δ (*hsc82*Δ *HSP82 CPR7*) creating a genomic C-terminal tagged allele of *HSP82* expressed from the native *HSP82* promoter. Addition of this tag has no detectable effect on growth indicating that the Myc-tag does not significantly alter the function of the tagged *HSP82* gene.

Triple HA-tagged Mal63 protein was purified from native cell extracts by immunoprecipitation with anti-HA antibody as described in Materials and Methods and the purified proteins analyzed by Western blotting. All protein samples were size-separated using 10% SDS-PAGE, electroblotted to nitrocellulose, and Mal63/HA and Hsp82-Myc proteins were detected using anti-HA and anti-Myc antibodies, respectively. Figure 8 shows that Myc-tagged Hsp82 is found in association with triple hemagglutinin-tagged Mal63 protein. Isolation of Myc-tagged Hsp90 is completely dependent on the presence of triple HA-tagged Mal63 protein since Hsp82-Myc is detected only in the immunoprecipitate from cells that co-express both proteins. Strain *hsc82*Δ *HSP82/Myc* transformed with plasmid p414GPD-FLAG/MAL63 serves as a negative control.

Analysis of the co-immunoprecipitation (Co-IP) samples and the total cell extracts from which they were prepared demonstrates that Myc-tagged Hsp82 could not be detected in the precipitated samples in the absence of HA-tagged Mal63p. Strain hsc82Δ (*hsc82*Δ *HSP82 CPR7*) lacking the Myc-tagged *HSP82* transformed with plasmid p416GPD-MAL63/HA3 serves as a control demonstrating that no other proteins are detected with the Myc antibody.

Figure 8. Co-precipitation of Hsp90 and transcription factor Mal63 *MAL*-activator. Nondenaturing protein extracts were prepared as described in Materials and Methods. A strain in which the genomic *HSP82* gene is Myc-tagged was transformed with plasmid p416GPD-MAL63/HA3 or plasmid p414GPD-FLAG/MAL63. Strain hsc82Δ lacking the Myc-tag (*hsc82Δ HSP82 CPR7*) was transformed with plasmid p416GPD-MAL63/HA3 expressing triple-HA-tagged Mal63p. Co-IP samples and the total cell extracts from which they were prepared were analyzed by Western blotting using the indicated antibodies. Phosphoglycerate kinase (PGK) is shown as a control.


Extract
Co-IP
Myc-tagged Hsp90
HA3-tagged Mal63
–
+
+
+
–
+
anti-Myc
anti-HA
anti-PGK

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. 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 Figure 7 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 might be destabilized, and, if so, we should expect to find that over-production of Mal63 protein should relieve the defects observed in strains with mutant Hsp90 chaperone complex. We tested this as follows. The ORF 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*. (Mumberg et al., 1995). Plasmids pTEF-MAL63/HA3 and pGPD-MAL63/HA3 were transformed into strains W303 (*HSC82 HSP82 CPR7*), S153 (*hsc82Δ hsp82-T101I CPR7*), and the double disruption strain hsc82Δ cpr7Δ (*hsc82Δ HSP82 cpr7*Δ) for characterization.

The results in Figure 9A are consistent with our hypothesis. Over-production 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 then the *hsc82*Δ *cpr7*Δ double null.

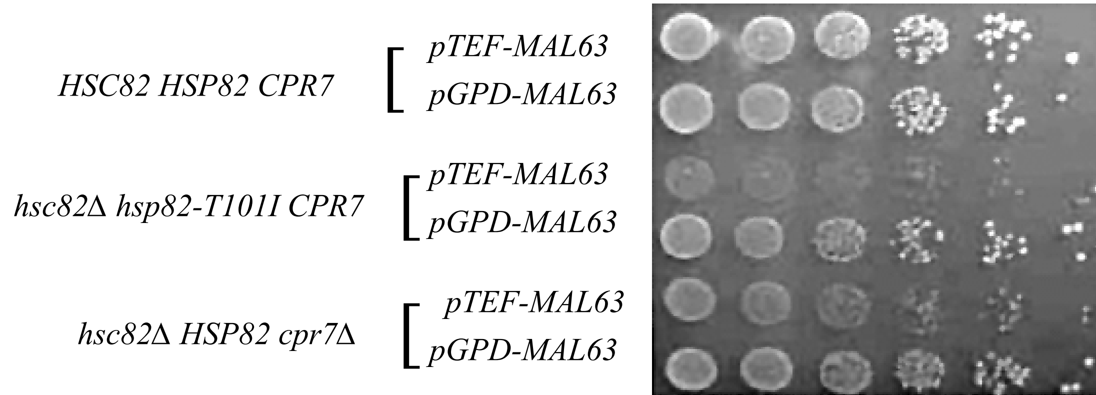
This is also observed in Figures 5 and 6 in which *MAL63* is expressed from its native promoter. Total cell extracts were prepared from the transformant strains shown in Figure 9A and Western blot analysis carried out to determine the relative level of Mal63/HA3 protein expression in each strain. The level of Mal63/HA3 protein was quantitated using the ECF detection system as described in Materials and Methods and normalized to the level of the PGK signal in the same lane. Figure 9B confirms that the expression levels of Mal63/HA3 from the GPD promoter are approximately 2.5 to 3-fold higher in each of the strains tested compared to 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 *hsp82*Δ *cpr7*Δ double null mutant strains carrying a plasmid-borne GPDpromoter-*MAL63/HA3* gene were cultured in selective media under uninduced conditions (3% glycerol + 2% lactic acid) at 21°C to mid log. Cycloheximide was added to the culture to stop further protein synthesis and total cell extracts prepared from culture samples taken at time zero and at one, three, five, and six hours after the addition of cycloheximide. Western blot analysis of the samples is shown in Figure 10. The relative level of Mal63/HA3 protein was quantitated as described in 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 Figure 10). The half-life of Mal63/HA3 protein in the Hsp90-ts mutant strain is approximately half that of the wild-type but in the *hsc82*Δ *cpr7*Δ double null strain Mal63/HA3p is significantly shorter, approximately 1/5 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.

Figure 9. Overproduction Mal63 *MAL*-activator suppresses the maltose growth defect 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. Plates were grown for 3 days at room temperature (approximately 21ºC). B.) The transformed strains were grown to early log phase at room temperature (approximately 21ºC) 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 in 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.

A



B

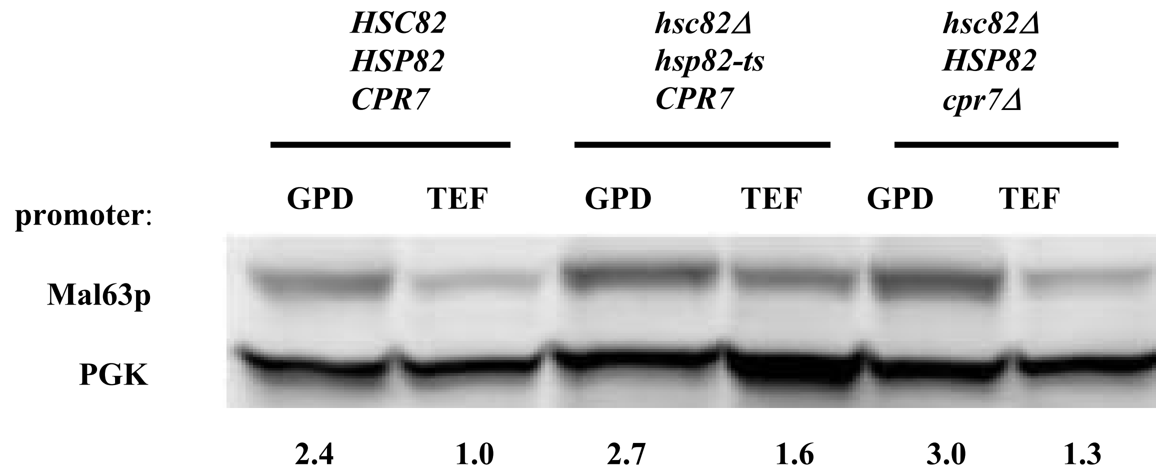
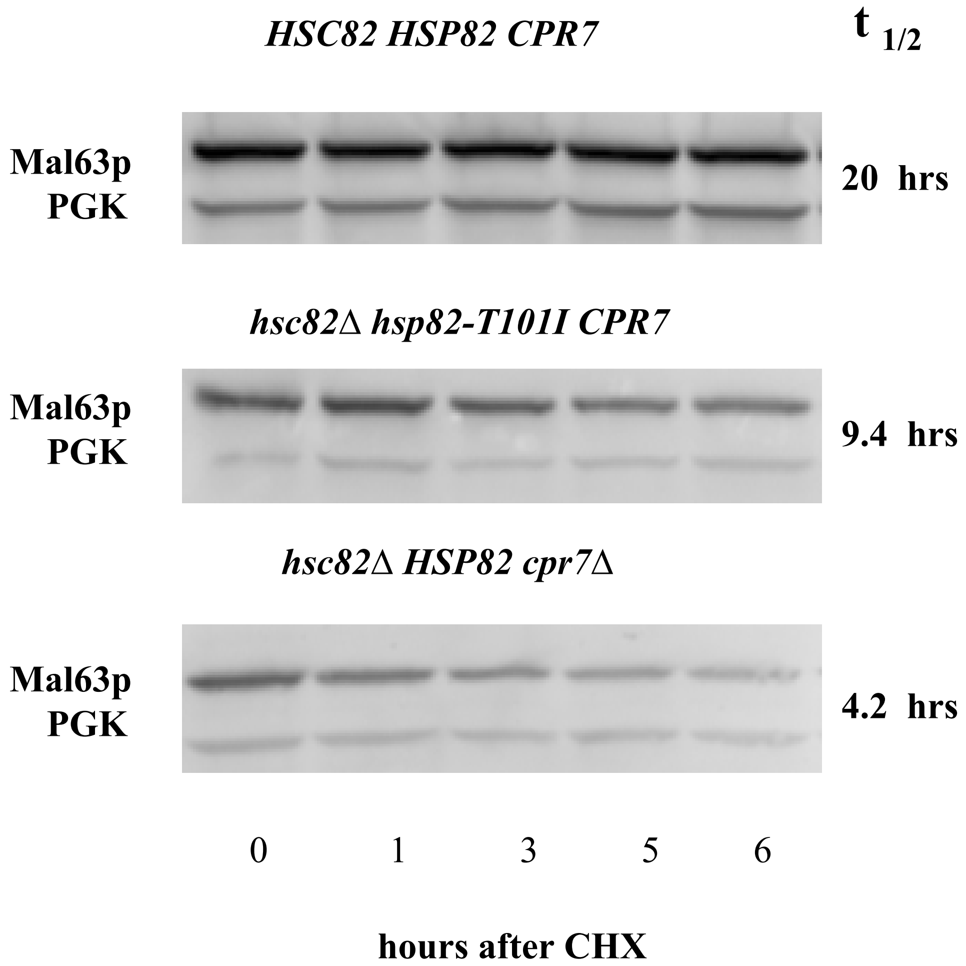


Figure 10. The half-life of Mal63/HA3 *MAL*-activator in the Hsp90 chaperone mutant strains. W303 (*HSC82 HSP82 CPR7*), S153 (*hsc82*Δ *hsp82-T101I CPR7*), and hsc82Δ cpr7Δ (*hsc82*Δ *HSP82 cpr7*Δ) were transformed with p416GPD-MAL63/HA3. Transformant strains were grown to early log phase in selective minimal medium containing glycerol/lactate at room temperature (approximately 21ºC). At time zero, cycloheximide was added to a final concentration of 50 µg/ml and total cell extracts prepared at time zero and at the indicated time points for six hours. Western analysis was carried out using anti-HA antibody. The relative level of Mal63/HA3p at each time point was quantified as described in Materials and Methods and normalized to the level of PGK. The Mal63/HA3p half-life was calculated from at least three independent transformants and average values are presented.


HSC82 HSP82 CPR7
t 1/2
Mal63p
PGK
20 hrs
hsc82Δ hsp82-T101I CPR7
Mal63p
PGK
9.4 hrs
hsc82Δ HSP82 cpr7Δ
Mal63p
PGK
4.2 hrs
0
1
3
5
6
hours after CHX

Discussion

The Hsp90 molecular chaperone complex regulates maltose induction.

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 (Pratt et al., 1997, 2003, Xu et al*.,* 1993, 1999). Genetic analysis of *MAL*-activator constitutive mutations demonstrated that complex folding patterns and intramolecular protein-protein interactions regulate *MAL*-activator activity (Danzi et al., 2000). Additionally, overproduction of some of these noninducible *mal63* mutant proteins suppresses the mutant phenotype and restores maltose inducibility. In Figure 9 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. This possibility is explored in Chapter 3 of this thesis.

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.

Wang *et al*. (Wang et al. 2002) 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*. (2002) 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*. (2003) 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*. (2000) 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 (Pratt et al., 1997; Pratt et al., 2003). 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 Hap1, the *Saccharomyces* heme-regulated transcription activator. Lee *et al.* (2002) demonstrated that the Hap1 activator is a substrate of the Hsp90 chaperone complex. Heme induction of Hap1 is dependent on Hsp90 chaperone

complex binding and requires one of seven putative heme-binding sites, HRM7, (heme-responsive motif seven) (Zhang et al., 1998). Surprisingly, Hon *et al*. (2001) found that in strains with reduced Hsp70 levels Hap1p is constitutively active suggesting that Hsp70/Ydj1 mediates repression of Hap1 activator and that the Hsp90 chaperone complex functions differently in Hap1 regulation compared to its role in steroid receptor activation. Chapter 2 explores 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. 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.

CHAPTER 2

The role of Hsp90 and Hsp70 chaperones in Mal63 *MAL*-activator regulation by maltose

The results described in Chapter 2 of this thesis were carried out entirely by me.

Introduction

Chapter 1 shows that Mal63 *MAL*-activator is a client protein of the Hsp90 chaperone complex. In this chapter we investigate mechanism whereby maltose regulates this Hsp90 chaperone complex client protein. Hsp90 chaperone complex has been suggested to regulate client protein stability and to facilitate folding. Based on this, we explored the stability and chaperone-binding of the *MAL*-activator in different carbon sources: maltose, glucose, and glycerol/lactate.

The *Saccharomyces* transcription factor Gcn4, the general regulator of the genes encoding several amino acid synthetic pathways, is regulated by the amount of available amino acids at the levels of both protein synthesis and stability. When amino acids are abundant Gcn4p is degraded very rapidly but, under the condition of amino acid starvation, it is stabilized (Kornitzer et al., 1994). The activity of Gcn4 is controlled at the level of protein synthesis by Gcn2 kinase and at the level of protein stability by the cyclin-dependent kinase Pho85. Phosphorylation of Gcn4 at a specific residue by cyclin-dependent kinase Pho85 promotes degradation of Gcn4 (Shemer et al., 2002; Meimoun 2000). Degradation of Gcn4 occurs via the proteosome and the ubiquitin-conjugating pathway.

The yeast heme activator protein and steroid receptors such as glucocorticoid receptor and progesterone receptor are substrates of the Hsp90 and Hsp70 molecular chaperones. In both heme signaling and steroid signaling, Hap1 and the receptor interact first with Hsp70 and then Hsp90 molecular chaperone. Hsp90 and Hsp70 may stay bound to substrate or exit from the complex after ligand binding. In the case of glucocorticoid receptor, Hsp90 along with the Hsp70 is bound to glucocorticoid receptor in the absence

of steroid hormone and is released from the complex when hormone binds to receptor. In the case of progesterone receptor, they are not released after hormone binding instead; they remain bound to the substrate protein (Prat and Toft 1997; Pratt et al., 2003).

Li Zhang and co-workers isolated two multi-chaperone–Hap1 complexes (Lan et al., 2004). One complex contains Hap1p, Hsp70 (Ssa protein), the Hsp70 co-chaperone Ydj1p and Sro9p and RNA-binding protein associated with ribosome (Sobel and Wolin 1999). The second complex contains all these proteins and Hsp90. That is, Hsp70 (Ssa) and its co-chaperones are found associated with Hap1p continuously. But Hsp90 associated with Hap1p transiently and only small amount of Hap1p, Ssa1p, Ydj1p and Sro9p are associated with Hsp90p. The continuous association of Hsp70 with Hap1p is the fundamental difference between heme signaling and steroid (GR) signaling. Moreover, addition of heme does not affect association of Hap1p with Ssa1, Ydj1 and Sro9 but heme enhances the interaction of Hap1p with Hsp90 protein.

For these reasons, we explored the turnover rate Mal63/HA3 in induced (maltose), uninduced (glycerol/lactate), and repressed (glucose) conditions and compared the association of Mal63/HA3 protein with Hsp90 and Hsp70 in glycerol/lactate and maltose. A model for *MAL*-activator regulation based on these results is presented.

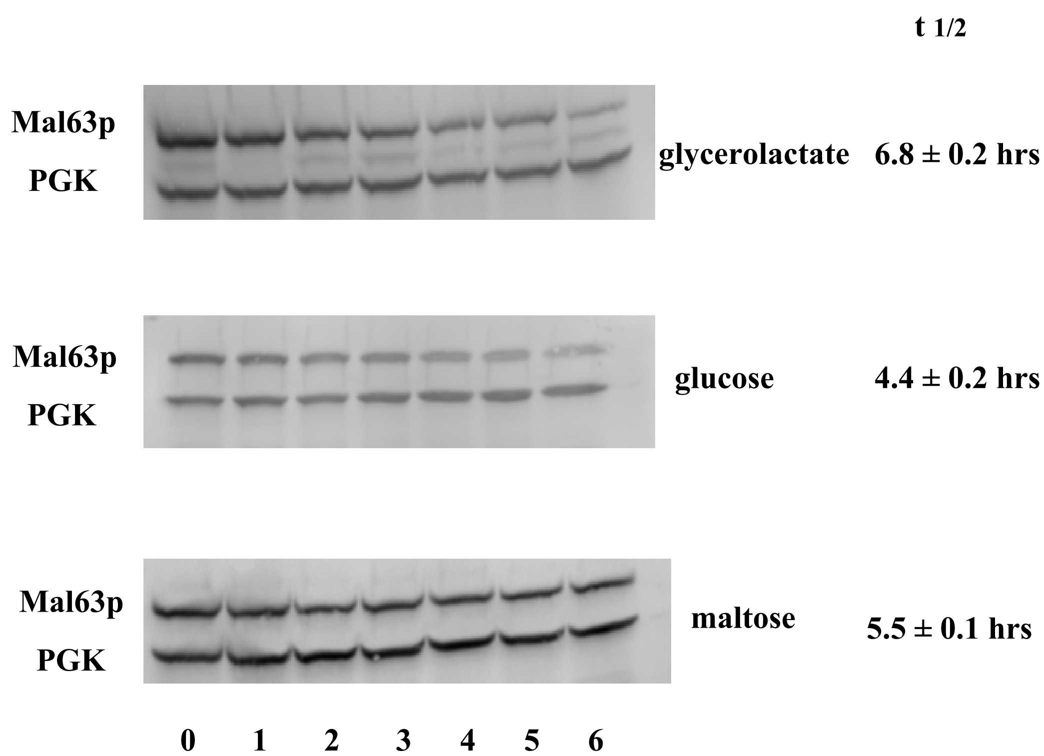
Results

Mal63 MAL-activator turnover rate is moderately regulated by the carbon source

We investigated whether the rate of degradation of wild-type Mal63p is dependent on carbon source. The half-life of Mal63/HA3 protein was determined in cells grown in glycerol/lactate (uninduced condition), maltose (induced condition), and glucose (repressed condition) at 30 ºC. The plasmid p416-MAL63/HA3, which carries the wild-type *MAL*-activator under the control of the GPD promoter was introduced into strain W303 (*HSC82 HSP82*) and transformants were cultured in selective media at 30 ºC to mid-logarithmic phase. Protein synthesis was stopped by the addition cycloheximide to a final concentration of 50 mg/ml and total cell extracts prepared from culture samples taken at one hour intervals for six hours after the addition of cycloheximide. Western blot analysis of total cell extracts probed with anti-HA antibody is shown in Figure 11. The relative level of Mal63/HA3 protein was quantified as described in Methods and Materials and normalized to the level of PGK. The relative level of Mal63/HA3protein was plotted and used to calculate the half-life of Mal63/HA3 protein.

The half-life of Mal63/HA3 *MAL*-activator is approximately 6.8 hours in cells grown in glycerol/lactate. In maltose grown cells, the half-life of Mal63/HA3 *MAL*-activator is reduced to 5.5 hours, and in glucose grown cells the half-life of *MAL*-activator is further reduced to 4.4 hours. These results show that the stability of Mal63p is moderately regulated by carbon source availability but these differences, while significant, are not sufficient to provide the basis of maltose induction or glucose repression of *MAL*-gene expression.

Figure 11. ***MAL63 MAL*-activator turnover rate is moderately regulated by the carbon source.** Strains W303 (*HSC82 HSP82*) was transformed with plasmid p416GPD-MAL63/HA3. Transformants were grown at 30 ºC in selective media lacking uracil in uninduced (3% glycerol and 2% lactate), induced (2% maltose), and repressed (2% glucose) conditions. Cycloheximide was added at time zero and total cell extracts prepared as described in Materials and Methods from culture samples taken at time zero and at one, two, three, four, five and six hours after the addition of cycloheximide. The level of Mal63/HA3p was determined by Western blot analysis of total cell extracts separated on a 10% SDS-PAGE gel and probed with anti-HA antibody. Western blotting of PGK is shown as the loading control. The Mal63/HA3p half-life was calculated based on the band intensities in at least three independent transformants. The average values are presented.


t 1/2
Mal63p
PGK
glycerolactate
6.8 ± 0.2 hrs
Mal63p
PGK
glucose
4.4 ± 0.2 hrs
Mal63p
PGK
maltose
5.5 ± 0.1 hrs
0 1 2 3 4 5 6

Mal63 MAL-activator binds to Hsp70 in vivo

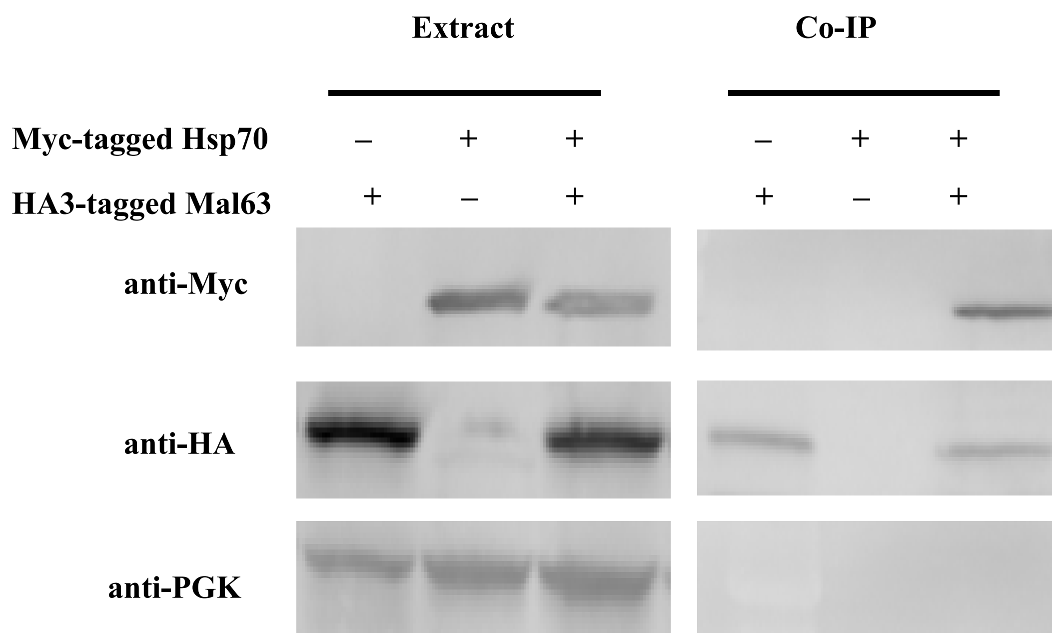
Hsp70 protein is central to the formation of the Hsp90 molecular chaperone complex but also functions independently as chaperone. Hsp70 and Hsp40 are reported to be the first chaperones to bind Hsp90 complex client proteins. In *Saccharomyces cerevisiae*, there are four Hsp70 homologs namely, *SSA1*, *SSA2*, *SSA3*, and *SSA4* (stress seventy A) (Slater et al., 1989; Young et al., 1993). We explored the role of Hsp70 protein in *MAL*-activator regulation by investigating Hsp70 protein association with Mal63 protein in induced and uninduced condition. For this we used a strain that has all members of the *SSA* family of Hsp70 proteins deleted except *SSA1* (*ssa2::LEU2 ssa3::TRP1 ssa4::LYS2*). The strain containing only the *SSA1* gene is viable indicating that expression of only *SSA1* is sufficient to compensate for the absence of the other members of the *SSA* family (Werner et al., 1987, Becker et al., 1996).

To determine whether Mal63p *MAL*-activator binds to the Hsp70, triple HA-tagged Mal63 protein was expressed in a strain in which the genomic copy of *SSA1* is Myc-tagged. Strain JN516 (from Elizabeth Craig) was used to Myc-tag the genomic *SSA1* gene using a PCR-based one-step replacement method described in the Materials and Methods. This procedure places the sequence encoding a single copy of the Myc epitope at the C-terminus of the *SSA1* open reading frame creating a Myc-tagged fusion gene expressed from the native promoter. Addition of this tag has no detectable effect indicating that the Myc-tag does not alter the function of the tagged *SSA1* gene. The strain is referred as CMY1300.

Strains CMY1300 and JN516 were transformed with either the plasmid harboring the HA-tagged MAL63/HA *MAL*-activator gene (p416GPD-MAL63/HA3) or the FLAG-

tagged *MAL*-activator gene (p416GPD-MAL63/FLAG). Mal63 protein was purified from native cell extracts using the immunoprecipitation procedure described in the Materials and Methods with the anti-HA antibody. Bound proteins were analyzed by Western blotting. All protein samples were size-separated by 10% SDS-PAGE, electroblotted to nitrocellulose, and Mal63/HA3 and Ssa1/Myc proteins were detected using anti-HA and anti-Myc antibodies, respectively. The results are shown in Figure 12. Clearly, immunoprecipitation of Ssa1/Myc is dependent on the co-expression of Mal63/HA3 protein. These results provide evidence for a stable interaction between Mal63p and Hsp70p.

Figure 12. Co-precipitation of Hsp70 and transcription factor Mal63 *MAL*-activator. Strain JN516 (*SSA1* ssa2,3,4Δ) and CMY1300 (*SSA1/Myc ssa2, 3, 4*Δ) in which the genomic *SSA1* gene is Myc-tagged were transformed with either plasmid p416GPD-MAL63/HA3 or plasmid p416GPD-FLAG/MAL63. Transformants were grown to mid-log in glycerol/lactate at 30 ºC. Nondenaturing protein extracts were prepared as described in Materials and Methods. Co-IP samples and the total cell extracts from which they were prepared were size-separated by 10% SDS-PAGE, electroblotted to nitrocellulose, and Mal63/HA and Ssa1/Myc protein detected using anti-HA and anti-Myc antibodies, respectively. Phosphoglycerate kinase (PGK) is used as a loading control.


Extract
Co-IP
Myc-tagged Hsp70
–
+
+
–
+
+
HA3-tagged Mal63
+
–
+
+
–
+
anti-Myc
anti-HA
anti-PGK

The effect of maltose on association of Hsp90 and Hsp70 with Mal63 MAL-activator protein

Heme enhances the binding of the heme-responsive transcription factor Hap1 to an Hsp90-containing chaperone complex but is found in association with Hsp70 under all conditions (Lan et al., 2004). We hypothesized that maltose might have similar effect on association of Hsp90 with Mal63 *MAL*-activator protein. To reveal how maltose affects the association of Hsp90 and Hsp70 with Mal63 *MAL*-activator protein, strains containing Myc-tagged alleles of the genomic *HSP82* and *SSA1* genes were transformed with the plasmid harboring *MAL63/HA3* and the relative level of Myc-tagged chaperone protein co-immunoprecipitated with Mal63/HA3 protein determined. We used a strain in which genomic copy of *HSP82* gene is Myc-tagged in order to rule out the possibility that variation in Hsp82p (Hsp90) protein was not caused by different expression levels of Hsp90 protein.

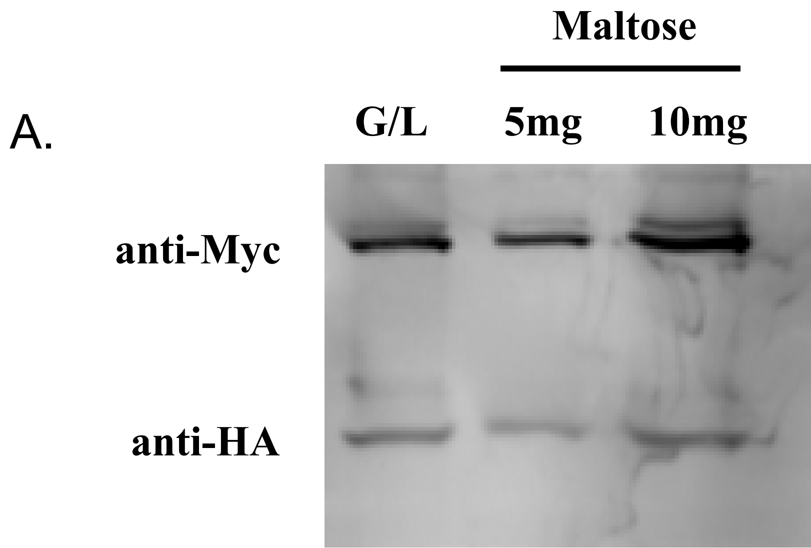
Strains CMY1200 and CMY1300 were transformed with plasmid p416GPD-MAL63/HA3. Transformants were grown in selective minimal media lacking uracil at 30 ºC to mid-logarithmic phase (O.D 0.3-0.5) in glycerol/lactate (3% glycerol and 2% lactic acid) or 2% maltose and total cell protein extracts prepared under nondenaturing conditions. Extracts from cells grown in glycerol/lactate were incubated with or without 5µg/ml or 10µg/ml of maltose for 60 minutes after which the Mal63/HA3p containing complexes were purified by immunoprecipitation with anti-HA antibody as described in Materials and Methods. The bound proteins were eluted from the beads, size-separated by 10% SDS-PAGE, and analyzed by Western blotting. Membranes were probed with both anti-HA antibody and anti-Myc antibody and the relative abundance of Hsp90 and Hsp70

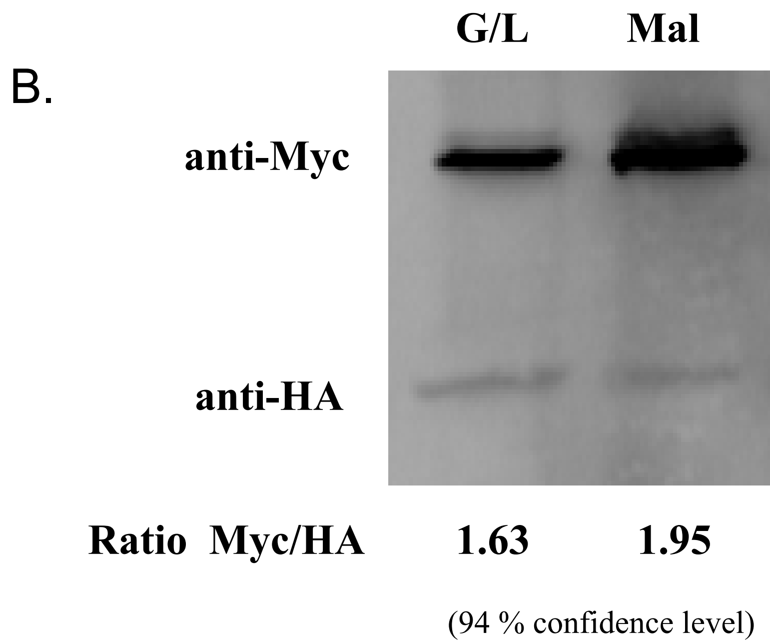
was quantified. The results, shown in Figure 13A suggest that treatment of the extract with maltose slightly increases the association of Hsp90 with Mal63 *MAL*-activator protein.

Figure 13B compares the relative level of Myc-tagged Hsp82 found in association with Mal63/HA protein in cells grown to mid-log in 2% glycerol/ 3% lactate or 2% maltose. Clearly, one can observe a significant increase in the amount of Hsp82-Myc complexed with Mal63/HA3 protein in cells grown in maltose. This increase is statistically significant at the 94 % confidence level.

Quite the opposite is seen for Hsp70 association. Strain CMY1300 (*SSA1/Myc ssa2, 3, 4*Δ) was transformed with plasmid-borne *MAL63/HA3* (pGPD416-MAL63/HA3). Transformants were grown in 2% glycerol/ 3% lactate (uninduced) or 2% maltose (induced) conditions. Total cell extract were prepared and Mal63/HA3 containing complexes purified by immunoprecipitation with anti-HA antibody as described in Materials and Methods. The purified proteins were eluted from the beads, size-separated by 10% PAGE, and analyzed by Western blotting. The membranes were probed with both anti-HA and anti-Myc antibody and the bands quantified as described in Materials and Methods. The results shown in Figure 14 indicate that binding of Hsp70 to Mal63p is significantly decreased by growth in maltose, by approximately one-third. This decrease is statistically significant at the 97 % confidence level.

Figure 13. Effect of maltose on association of Hsp90 with Mal63 *MAL*-activator protein. Strain CMY1200, in which genomic *HSP82* gene is Myc-tagged, was transformed with plasmid pGPD416-MAL63/HA3. **A)** Transformants were grown to mid-log in glycerol/lactate at 30 ºC. Nondenaturing protein extracts were prepared as described in Materials and Methods. Extracts were incubated with or without 5mg/ml or 10mg/ml of maltose for 60 minutes. Co-IP samples were analyzed by Western blotting using anti-HA and anti-Myc antibodies. **B)** Transformants were grown to mid-log in 2%glycerol / 3%lactate or 2% maltose at 30 ºC. Total cell extracts were prepared in nondenaturing conditions. Mal63/HA3 was immunoprecipitated as described in Materials and Methods and the samples were analyzed by Western blotting using anti-HA and anti-Myc antibodies. Quantification of protein expression levels was carried out by using ECF detection system as described in Material and Methods.

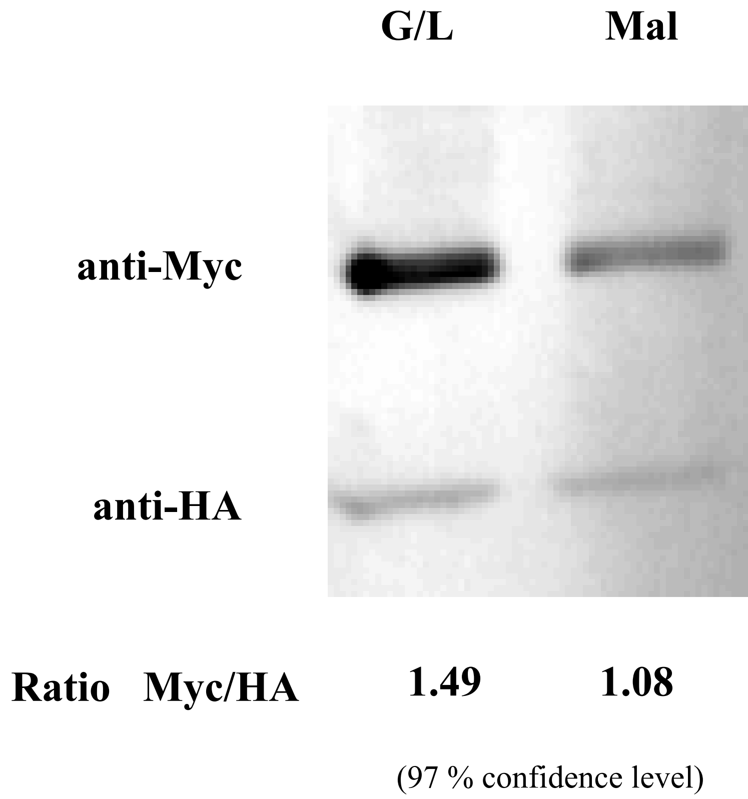




Ratio Myc/HA	1.63	1.95

(94 % confidence level)

Figure 14. Effect of maltose on association of Hsp70 with Mal63 *MAL*-activator protein. Strain CMY1300 (SSA/Myc ssa2, 3, 4Δ) was transformed with plasmid pGPD416-MAL63/HA3. Transformants were grown to mid-log in 2%glycerol / 3%lactate or 2% maltose at 30 ºC. Nondenaturing protein extracts were prepared as described in Materials and Methods. Co-IP samples were analyzed by Western blotting using anti-HA and anti-Myc antibodies. Quantification of protein expression levels was carried out by using ECF detection system as described in Material and Methods.


G/L
Mal
anti-Myc
anti-HA
Ratio Myc/HA
1.49
1.08
(97 % confidence level)

Discussion

The results reported here suggest that the *MAL*-activator binds to Hsp70 and Hsp90 in both induced and uninduced growth conditions but that interaction with each of the chaperones is differentially regulated. Binding of Mal63/HA3p to Hsp90 is increased slightly by growth in maltose. In contrast, binding to Hsp70 is dramatically decreased by growth on maltose, although some association with Hsp70 persists.

We propose the model for *MAL*-activator interaction with Hsp70 and Hsp90 shown in Figure 15. In uninduced conditions, *MAL*-activator is bound to Hsp70 and Hsp90 with the complex containing all three proteins the predominate complex. It is likely that the presence of Hsp70 acts to repress *MAL*-activator function despite Hsp90 binding and may also act to facilitate Hsp90 binding. Addition of maltose promotes the release of Hsp70 from the complex and drives the reaction to the right, which favors Hsp90 association. Whether these different complexes are in equilibrium with each other or if complex formation occurs in irreversible steps, as shown, can not be determined by our study.

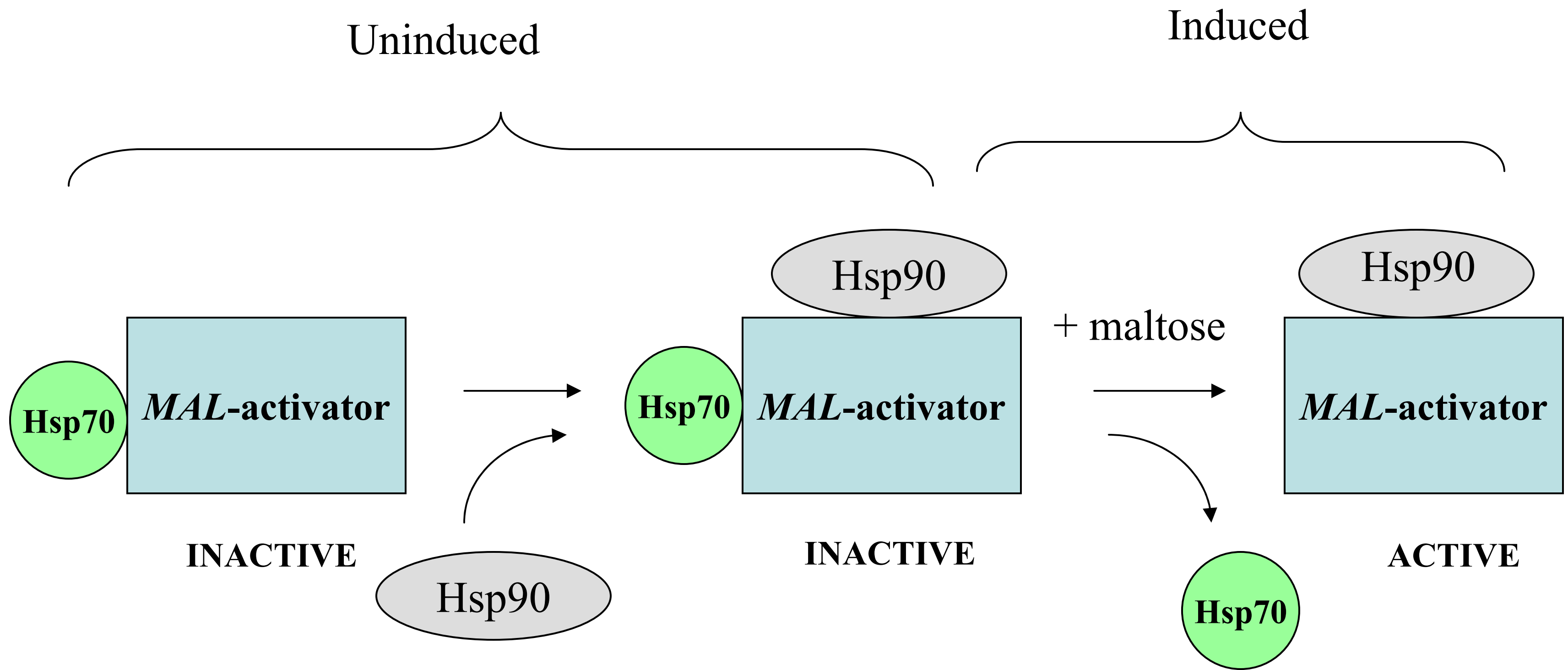
Thus, we suggest that negative regulation of the *MAL*-activator is achieved in the absence of inducer by the strong association with Hsp70 despite the presence of Hsp90 bound to this complex. Addition of maltose promotes the release of Hsp70 and enhances association with Hsp90. The strengthening of the interaction between Hsp90 and *MAL*-activator may lead to a conformational change or re-folding of the *MAL*-activator into an active DNA-binding competent state. We can not be certain at this point whether Hsp90-bound *MAL*-activator binds to the UAS_{MAL} , as is proposed for Hap1p (Lan et al., 2004) or if the *MAL*-activator is released from the Hsp90 complex prior to DNA binding, as is

the case for glucocorticoid receptor (Pratt et al., 1998; Ditmar et al., 1998). Based on our findings shown in Figure 13 A and B that maltose enhances Hsp90 interaction; we suggest that *MAL*-activator DNA-binding occurs while still bound to Hsp90 and perhaps other chaperone complex components also.

In the case of several other transcription factors that are clients of the Hsp90 chaperone complex, the inducer molecule binds directly to the transcription factor. This has been demonstrated for glucocorticoid receptor (Pratt et al., 1998; Ditmar et al., 1998), androgen receptor (Fang et al., 1996) and the heme-responsive activator Hap1p (Zhang et al., 1998). In such cases, increased inducer concentration has been found to improve activation by the transcription factor (Zhang et al., 1998; Hon et al., 2001). Chapter 1 demonstrates that this is also true for the Mal63/HA *MAL*-activator. Thus, we propose that maltose binds directly to the *MAL*-activator while it is in a complex with Hsp70 and Hsp90. Moreover, this binding causes the release of Hsp70, enhanced binding of Hsp90, and refolding to an active DNA-binding competent state.

Figure 15: Model of *MAL*-activator regulation by Hsp70, Hsp90 and maltose.

Model for *MAL*-Activator Regulation



CHAPTER 3

The role of Hsp90 chaperone protein in the regulation of non-inducible, super-inducible, and constitutive *MAL*-activator mutant alleles

The results described in Chapter 3 of this thesis were carried out entirely by me.

Introduction

Mutations in the *MAL* loci affecting maltose utilization and maltose expression have been isolated by many workers over the last 50 or more years (reviewed in Barnett et al., 2005). Because in most strains the *MAL*-structural genes are repeated (Charron et al., 1989) the *MAL* loci are a repeated family of genes, all of these mutations were later shown to be alterations in the genes encoding the *MAL*-activator (Charron and Michels 1983; Gibson et al., 1997). A detailed structural function analysis of the *MAL*-activator has been a focus of several studies in our laboratory. Chapter 3 of this thesis uses several of these mutant *MAL*-activator alleles to investigate the molecular basis of their mutant phenotype.

According to the studies results presented by Hu *et al*. 1999 and Gibson *et al*. 1997, the C-terminal region of *MAL*-activator is responsible for negative regulation. Genetic analysis of several mutant *MAL*-activator alleles showed that the C-terminal region of the *MAL*-activator from residues 250 to 470 contains a maltose-responsive regulatory domain and extensive mutations or deletions within that C-terminal region creates a constitutive *MAL*-activator. Gibson *et al.* (1997) also reported that, the constitutive *MAL*-activator mutations fell into two classes: truncation mutations at codon 283 and multiple sequence alterations that alter residues in the region from 300 to 470. Transformants carrying constitutive *MAL*-activators shows 50 to 150 % increase in maltase expression in induced conditions, although wild-type *MAL*-activator is able to induce maltase expression 50-fold at most when fully induced. Besides, a constitutive activator does not need induction to be active. Danzi *et al*. (2000) used *in vitro* mutagenesis to identify residues responsible for the negative regulation of the *MAL*-

activator and localized three negative regulatory domains (250-307, 343-359, and 419-461) at the C-terminus of the *MAL*-activator. Alterations in any of these domains result in loss of negative regulation and a constitutive phenotype.

Danzi *et al.* (2003) used charged-cluster to alanine scanning mutagenesis and random mutagenesis of the regulatory domain of the inducible *MAL*-activator gene *MAL63* and the constitutive *MAL*-activator gene *MAL43-C* in order to define residues involved in positive regulation of the *MAL*-activator. The mutations that affect the activity of *MAL63* fall into three classes: those that decrease activator activity, those that have no effect on activity of *MAL63* and those that improve the activator activity. Almost all of the mutations in the region of 331 to 470 decrease the activity of *MAL63* and cause a non-inducible phenotype with two exceptions. Two mutations, mal63-460 and mal63-462 increase the activity of *MAL63* and create super-inducible *MAL*-activator. Transformants carrying these mutant *MAL*-activator alleles ferment maltose more rapidly than the wild-type. Transformants carrying super-inducible *MAL*-activator mutant allele, *MAL63-460*, shows 100 % higher maltase activity than wild-type in cells grown in induced (2% maltose, 3% glycerol, 2% lactate) conditions. But in uninduced conditions (3% glycerol, 2% lactate), transformants carrying *MAL63-460* do not show increased levels of maltase activity (Danzi et al., 2003). Several of these non-inducible mutant *MAL*-activator alleles could be suppressed by overexpression, suggesting the possibility that the non-inducible mutant *MAL*-activator may be unstable.

Danzi *et al.* (2003) further characterized the non-inducible mutants. Transformants carrying non-inducible *MAL*-activator mutant alleles shows considerably reduced maltase expression in induced (2% maltose, 3% glycerol, 2% lactate) and

uninduced (3% glycerol, 2% lactate) growth conditions (Danzi et al., 2003). Mutations within the region 331-423 resulted in non-inducible phenotype in the inducible *MAL*-activator gene *MAL63*. When the same mutations were introduced in to the constitutive *MAL*-activator gene *MAL43-C*, there was no change in phenotype, pointing out the fact that the region spanning 331 to 423 is required for inducible activation of the *MAL*-activator, that is either to sense or respond the presence of maltose. Mutations within the region 438-470 also resulted in non-inducible phenotype in the inducible *MAL*-activator gene *MAL63*, but, when the same mutations were introduced in to the constitutive *MAL*-activator gene *MAL43-C*, the expression was dramatically reduced but still constitutive. So, the region spanning 438 to 470 is required for the formation and/or maintenance of the transcriptionally active state.

Chapter 3 of this thesis carries out a detailed characterization of the *MAL*-activator mutant proteins encoded by various non-inducible, constitutive, and super-inducible *MAL63* mutants with the goal of understanding the basis of the mutant phenotype. We determined their turnover rate and Hsp90 binding. Finally, based on the model of *MAL*-activator regulation presented in Figure 15, we postulate the molecular basis of the mutant phenotype.

Results

MAL63 mutant alleles utilized in this study

Gibson *et al.* (1997) and Danzi *et al.* (2000, 2003) describe variety of non-inducible, constitutive and super-inducible *MAL*-activator mutant alleles. We made use of several of these for the analysis described in this chapter. The mutant alleles are listed in Table 2 which also presents the specific altered residues in each. The alterations in each mutant allele were introduced into the triple HA-tagged *MAL63/HA3* coding sequence expressed under the control of the high-level constitutive GPD promoter in vector p416GPD as described in the Materials and Methods. These plasmid-borne mutant constructs were introduced into strain W303 and the ability of the transformants to ferment maltose determined (Table 3).

Three constitutive *MAL63* alleles were studied. *MAL63-block2* was isolated by Danzi *et al*. (2000). It contains 10 clustered alterations in codons 343-359 derived from the sequence of *MAL23-C*. The *MAL63-block 2* alterations include eight amino acid substitutions and two deletions (L343F, H344Q, K346N, M348T, R349K, M350Δ, N351Δ, L353F, T356A, and T359A). W303 over-expressing *MAL63-block2* (p416GPD-MAL63-block2/HA3) ferments maltose in one day and significantly more rapidly than comparably over-expressed wild-type *MAL63/HA* (Table 3).

MAL63/43-C is a hybrid *MAL*-activator containing the first 215 codons of *MAL63* fused to codons 216-470 of *MAL43-C.* The transformants carrying the native *MAL63/43-C* express comparably high levels of maltase in the absence of inducer maltose. W303 transformed with p416GPD-MAL63/43-C/HA3 ferments maltose more rapidly than wild-type *MAL63* (Table 3).

Table 2.

Mutant MAL-activator alleles used in this study

Mutant allele	Altered residues	Reference
MAL63/43-C	F238Y, A251T, I280V, R307W, W320Y, F327Y, H344N, M345K, G347D, L353F, N358A, R367K, G371Q, F374L, K378I, N379D, I388V, N404S, V417I, Y419C, N433H, D437Q, G445S, T449D, K454R, Q457R, N461D	Gibson et al. 1997
MAL63/23-SVI	S392A, V395I, I402V	Gibson et al. 1997
mal63-283	K283A, D287A	Danzi et al. 2003
mal63-331	R331A, R335A	Danzi et al. 2003
mal63-364	E364A, R367A	Danzi et al. 2003
mal63-391	K391A, E394A	Danzi et al. 2003
mal63-401	D401A, K405A	Danzi et al. 2003
mal63-460	D460A, K463A	Danzi et al. 2003
mal63-467A9N	D467A, I469N	Danzi et al. 2003
mal63-467S9V	D467S, I469V	Danzi et al. 2003
MAL63-block2	L343F, H344Q, K346N, M348T R449K, M350Δ, N351Δ, L353F, T356A, T359A	Danzi et al. 2003
mal63-block3	P352L, R367K, G371D, T373I, K378N	Danzi et al. 2003
MAL63(1-282)-T247A	Stop codon at 283 and T247A	Danzi et al. 2003
mal63-(1-445)	Stop codon at 445	This study

MAL63(1-282)-T247A was obtained by random mutagenesis of the truncated noninducible *MAL63(1-283*) mutant (Gibson et al., 1997). It contains a single base change, threonine 247 to alanine. Strain W303 carrying p416GPD-mal63(1-283)-T247A/HA3 ferments maltose more rapidly than comparably over-expressed wild-type *MAL63/HA* (Table 3).

The MAL63/23- S392A, V395I, I402V/HA3 is constitutive and was isolated by Danzi *et al*. (2000). It contains the first 215 codons of *MAL63* fused to codons 216-470 of *MAL23* with the following three substitutions S392A, V395I and, I402V. These substitutions are based on differences between inducible Mal63p and Mal23p but create a constitutive allele. W303 expressing this allele (p416GPD-MAL63/23- *S392A, V395I, I402V* /HA3) ferments maltose in two days compared to one day for the comparable construct expressing wild-type *MAL63/HA3* (Table 3).

The *mal63-460* (D460A, K463A) is a super-inducible allele that exhibits significantly increased, inducible maltase expression and ferments maltose faster than wild-type (Danzi et al., 2003). Strain W303 expressing the wild-type allele (p416GPD-MAL63/HA3) ferments maltose in three days but, W303 expressing the super-inducible allele (p416GPD-mal63-460/HA3) ferments maltose in one day.

The following non-inducible *MAL*-activator alleles were isolated by Danzi *et al.* (2003) by charged-cluster to alanine mutagenesis: *mal63-331* (R331A, R335A), *mal63-364* (E364A, R367A), *mal63-391* (K391A, E394A), *mal63-401* (D401A, K405A), *mal63-283* (K283A, D287A) and *mal63-467A9N* (D467A, I469N). Danzi *et al*. (2003) introduced these alterations into the constitutive *MAL43-C* and found that *mal63/43C-283, mal63/43C-331, mal63/43C*-401, and *mal63/43C-467A9N* were now constitutive

suggesting that these could affect maltose binding. (see Table 3). Additionally, the overproduction of some restored inducibility and maltose utilization (See Table 3). We show similar results in Table 3.

Non- inducible *mal63-467S9V* (D467S, I469V) was obtained by random mutagenesis (Danzi et al., 2003). The altered residues in *mal63-467S9V* are listed in Table 2 and are also found at the carboxyl terminus of the nonfunctional Mal63p homologue Mal64p. W303 over-expressing mutant *mal63-467S9V* (p416GPD- mal63-467S9V/HA3) ferments maltose in three days, which is comparable to wild-type *MAL63/HA*. *Mal63-block3* encodes a non-inducible activator (Danzi et al., 2003). It contains alterations of five residues within codons 362 and 378 (P362L, R367K, G371D, T373I, K378N). These changes were based on a subset of alterations found in the constitutive *MAL23-C* allele. W303 expressing mal*63-block3* (p416GPD-mal63-block3/HA3) does ferment maltose even after 5 days (Table 3).

The noninducible *mal63-(1445)* is a truncation mutation at codon 445 created during the construction of the triple-HA tagged mutant *mal63* allele. W303 expressing this allele (p416GPD-mal63-380/HA3) does not ferment maltose even after five days (Table 3).

Table 3. Maltose fermentation rate and phenotype of mutant *MAL*-activator alleles. The triple HA-tagged mutant *MAL*-activator alleles carried on the vector p416 and expressed under the control of the high-level constitutive GPD promoter were introduced into strain W303 (*HSC82 HSP82*) and the ability of the transformants to ferment maltose determined in medium containing 2% maltose. Maltose fermentation is monitored by the production of CO_2 gas as described in Materials and Methods. The phenotype of the mutant genes expressed from the native promoter, except *mal63(1-445)* as reported in Gibson *et al*. (1997) and Danzi *et al.* (2000, 2003) is presented and indicated with “n”. The phenotype of the mutant genes expressed from the GPD promoter is based on maltose fermentation rate (observed up to 5 days) and maltose induction. Those noninducible mutants shown by Danzi *et al*. (2003) to be suppressed by overproduction are indicated by an asterisks (*) and those believed to be defective in maltose binding are indicated with an “m”.

Table 3.

Fermentation rate of mutant MAL-activator alleles

***MAL63*-mutant allele**	**Maltose Fermentation**			**Phenotype from native promoter [n]**
	day1	day3	day5	
Vector	-	-	-	
MAL63	+	+++	++++	wild-type
mal63-283 [*]	-	++	++	non-inducible
mal63-331	-	++	+++	non-inducible
mal63-364 [* m]	-	++	++	non-inducible
mal63-391 [m]	-	-	+	non-inducible
mal63-401 [*]	-	+	+	non-inducible
mal63-460	++++++	+++++	+++++	super-inducible
mal63-467A9N [*]	-	++	++	non-inducible
mal63-467S9V [*]	-	++	+++	non-inducible
MAL63-block3	-	-	-	non-inducible
MAL63/43-C	++	+++++	+++++	constitutive
MAL63/23- S392A, V395I, I402V	++	+++++	+++++	constitutive
MAL63-block2	+++++	+++++	+++++	constitutive
MAL63(1-283)-T247A	+++	+++++	+++++	constitutive
mal63(1-445)	-	-	-	non-inducible

Activity of the constitutive MAL-activators is dependent on interaction with the Hsp90 chaperone complex

Given the model the *MAL*-activator regulation proposed in Chapter 2, one might propose that the constitutive *MAL*-activators are able to fold to the active conformation without the assistance of the Hsp90 chaperone complex. We set out to test this using the constitutive alleles *MAL63/43-C, MAL63/23- S392A, V395I, I402V* and *MAL63-block2* described in Gibson *et al*. (1997) and Danzi *et al*. (2000). CEN plasmid constructs carrying these three mutant genes expressed from the native *MAL63* promoter were introduced into the strains W303 (*HSC82 HSP82*), hsc82Δ (*hsc82Δ HSP82 CPR7*), and hsc82Δ cpr7Δ (*hsc82Δ HSP82 cpr7Δ*). All three constitutive mutant *MAL*-activator alleles carry multiple point mutations in the C-terminal regulatory domain. A hybrid *MAL*-activator gene *MAL63/43-C* has been constructed by fusing approximately 200 base pairs of the promoter and the N-terminal end of *MAL63* gene encoding residues 1-215 to C-terminal residues 215 –470 of constitutive *MAL43-C MAL*-activator gene. The C-terminal region of Mal43-Cp from residues 215-470 contains 27 altered residues compared to Mal63p (Danzi et al., 2003). *MAL63/23-S392A, V395I, I402V* was constructed by fusing the promoter and the first 215 codons of *MAL63* to codons 216-470 of *MAL23,* then site-directed mutagenesis was converted three residues in the C-terminal Mal23p sequence to those found in Mal63p. *MAL63-Block2* contains eight amino acid substitutions and two deletions (L343F, H344Q, K346N, M348T, R349K, M350Δ, N351Δ, L353F, T356A, and T359A) within codons 343 and 359. Transformants were grown to early mid-logarithmic phase at 24ºC in selective synthetic medium containing 3% glycerol and 2% lactate as a carbon source (uninduced condition) lacking tryptophan

for plasmid selection. Maltase activity was assayed as described in Materials and Methods. The results are shown in Figure 16.

All three constitutive mutant *MAL*-activator alleles cause high constitutive levels of maltase expression but to varying levels in the wild type strain W303 (*HSC82 HSP82*). Uninduced maltase expression from constitutive *MAL*-activator allele *MAL63/43* was higher than that the constitutive *MAL*-activator alleles *MAL63/23-S392A, V395I, I402V* and *MAL63-block2*. On the other hand, although loss of *HSC82* alone causes no significant change in maltase expression in strain expressing constitutive *MAL*-activator allele *MAL63/43-C*, maltase expression is reduced about 50% in strain hsc82Δ expressing constitutive *MAL*-activator alleles *MAL63/23*-S392A, V395I, I402V and *MAL63-block2*. Furthermore, maltase expression is severely decreased in the double deletion strain hsc82Δ cpr7Δ expressing the constitutive mutant *MAL*-activator alleles. These results indicate that the Hsp90 chaperone complex is required for the functional activation of the constitutive *MAL*-activator mutant proteins. Thus, constitutive *MAL*-activator alleles do not by-pass the need for the Hsp90 molecular chaperone complex to be activated. Instead, these results suggest that activation, which probably involves a folding to the active configuration, does not require maltose stimulation but instead occurs spontaneously.

To further explore the dependency of the constitutive *MAL*-activators on Hsp90 chaperone complex, we investigated the stability of *MAL63/43-C, MAL63-block2,* and *MAL63/23- S392A, V395I, I402V* proteins. We expected that constitutive *MAL*-activator proteins should have a longer half-life than that of the wild-type Mal63 protein. For this purpose, we used the triple-HA tagged the constitutive mutant constructs expressed from

the GPD promoter described above and in Table 2. These were transformed into W303 and isogenic hsc82Δ cpr7Δ and the half-life of the constitutive *MAL*-activator proteins determined. The results shown in Figure 17 and summarized in Table 4 demonstrate that contrary to our expectations, the half-life of these constitutive *MAL*-activator proteins is not significantly different from wild-type.

If, as is suggested by the results in Figure 16 and the constitutive *MAL*-activators are dependent on Hsp90 chaperone complex, we would expect that their stability should be decreased in an Hsp90 chaperone defective strain. To test this, the triple HA-tagged plasmid constructs expressing *MAL63/43-C* and *MAL63/23-* S392A, V395I, I402V were introduced into hsc82Δ cpr7Δ and the half-life of these constitutive activator proteins determined. The half-life of *MAL63/43-C* protein is decreased from 6.5 hours to 3.2 hours and the half-life of constitutive *MAL63/23-* S392A, V395I, I402V decreased from 4.5 hours to 2.8 hours, again supporting our conclusion that the constitutive activator interacts with Hsp90 chaperone complex.

Figure 16. Maltase expresssion of constitutive *MAL*-activators is dependent on the Hsp90 chaperone complex. Strains W303 (*HSC82 HSP82*), hsc82Δ (*hsc82Δ HSP82 CPR7*) and hsc82Δ cpr7Δ (*hsc82Δ HSP82 cpr7Δ*) were transformed with plasmids pUN30-*MAL63/43-C*, pUN30- MAL63*/23-S392A, V395I, I402V* and pUN30-*MAL63/Block2*. Transformants were grown at 24ºC in selective synthetic medium containing 3% glycerol and 2% lactate (vol/vol) lacking tryptophan for plasmid selection. Maltase activity was assayed as described Material and Methods and is expressed as nmoles PNPG (p-nitrophenol-α-glucopyranoside) produced per mg protein per minute. The assays were carried out on at least three independent transformants. The error bars indicate standard deviation from three independent experiments done in duplicate.

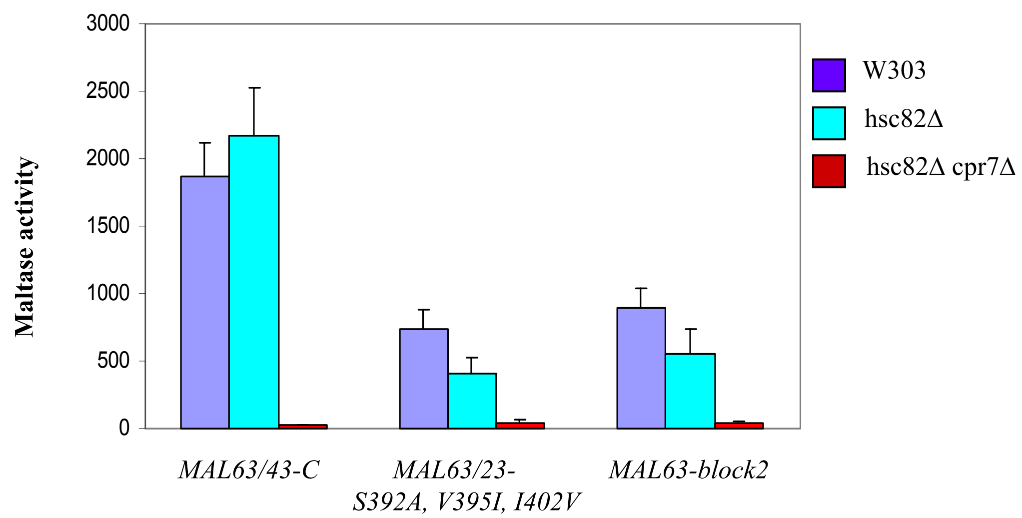

3000
2500
2000
1500
1000
500
0
Maltase activity
W303
hsc82Δ
hsc82Δ cpr7Δ
MAL63/43-C
MAL63/23-
S392A, V395I, I402V
MAL63-block2

Figure 17. Turnover rate of constitutive *MAL*-activator mutant alleles in strain W303. Strain W303 (*HSC82 HSP82*) was transformed with plasmid harboring triple HA-tagged constitutive *MAL*-activator mutant alleles expressed under the control of the high-level constitutive GPD promoter, p416GPD-MAL63-block2**,** p416GPD-MAL63/43-C and p416GPD-MAL63/23-S392A, V395I, I402V**.** Transformants were grown at 30ºC in selective media lacking uracil in uninduced condition (3% glycerol and 2% lactate). Cycloheximide was added to the culture to the final concentration of 50 mg/ml and total cell extracts prepared from culture samples taken at time zero and at one, two, three, four, five and six hours after the addition of cycloheximide. The level of constitutive *MAL*-activator protein was determined by Western blotting of total cell extracts size-separated on a 10% SDS-PAGE gel and probed with anti-HA antibody. Western blotting of PGK is shown as the loading control. The half-life of constitutive *MAL*-activator mutant proteins was calculated based on the band intensities in at least three independent transformants. The average values are presented.

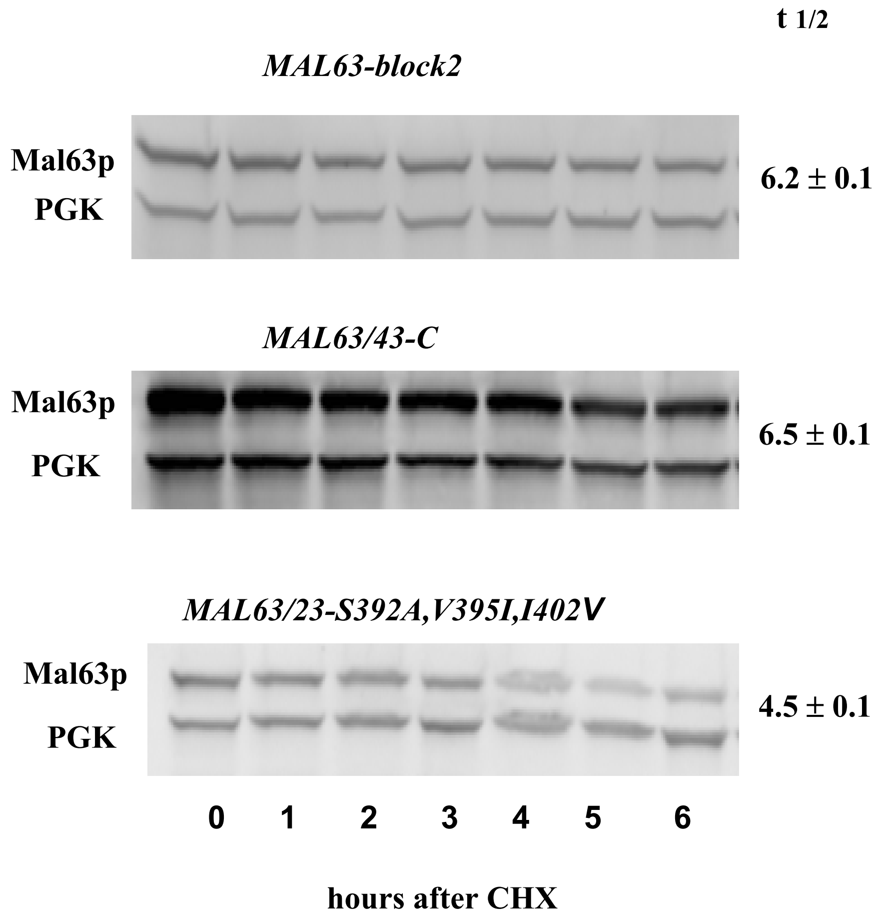
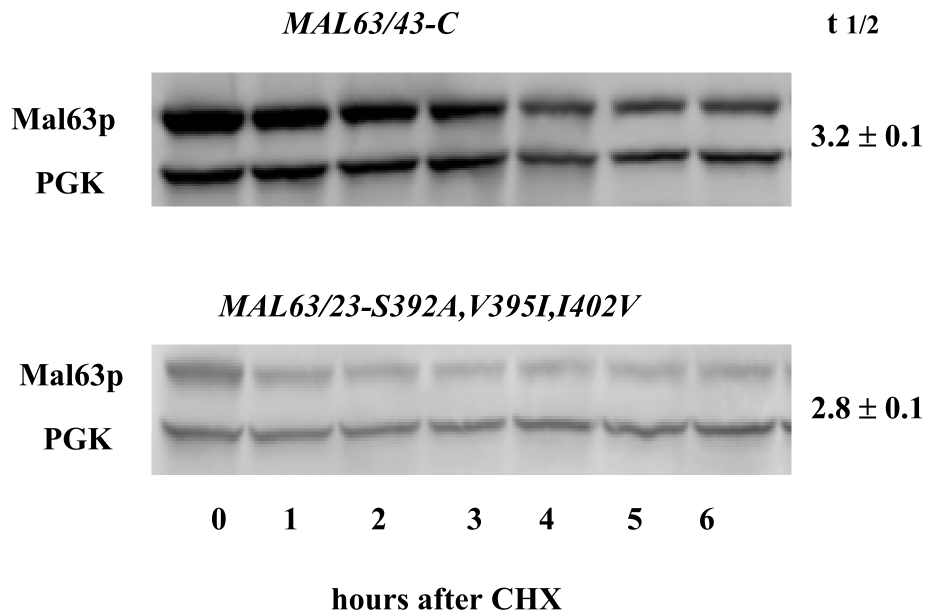

t 1/2
MAL63-block2
Mal63p
PGK
6.2 ± 0.1
MAL63/43-C
Mal63p
PGK
6.5 ± 0.1
MAL63/23-S392A,V395I,I402V
Mal63p
PGK
4.5 ± 0.1
0 1 2 3 4 5 6
hours after CHX

Table 4.

Half-lives of mutant MAL-activator proteins

***MAL63* allele**	**Mal63p half-life**
MAL63	6.8 ± 0.2 hrs
Constitutive	
MAL63/43-C	6.5 ± 0.1 hrs
MAL63/23-S392A, V395I, I402V	4.5 ± 0.1 hrs
MAL63-block2	6.2 ± 0.1 hrs
Super-inducible	
MAL63-460	16.0 ± 0.1 hrs
Non-inducible	
mal63-283	13.7 ± 0.1 hrs
mal63-331	4.5 ± 0.1 hrs
mal63-364	4.2 ± 0.1 hrs
mal63-391	7.8 ± 0.1 hrs
mal63-401	9.6 ± 0.2 hrs
mal63-467A9N	3.9 ± 0.1 hrs
mal63-467S9V	4.5 ± 0.2 hrs
mal63-block3	11.3 ± 0.1 hrs

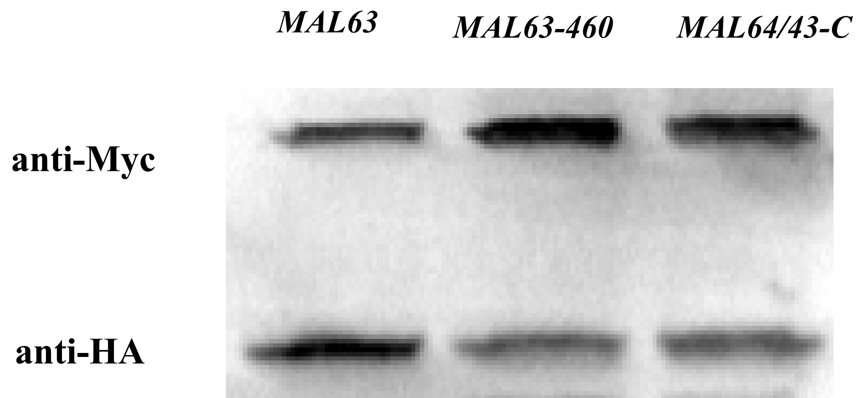
Figure 18. Turnover rate of constitutive *MAL*-activator mutant alleles in hsc82Δ cpr7Δ strain. Strain hsc82Δ cpr7Δ (*hsc82Δ HSP82 cpr7Δ*) was transformed with plasmids, p416GPD-MAL63/43-C and p416GPD-MAL63/23-S392A, V395I, I402V**.** Transformants were grown at 30ºC in selective media lacking uracil in uninduced condition (3% glycerol and 2% lactate). Turnover rate was determined as described in Figure 16.


MAL63/43-C
t 1/2
Mal63p
PGK
3.2 ± 0.1
MAL63/23-S392A,V395I,I402V
Mal63p
PGK
2.8 ± 0.1
0 1 2 3 4 5 6
hours after CHX

Association of constitutive MAL-activator mutant proteins with Hsp90

We investigated the relative level of Hsp90 molecular chaperone binding to the constitutive *MAL*-activator mutant protein to determine if the constitutive activator proteins differ compared to the inducible Mal63p in their ability to bind to the Hsp90 molecular chaperone. Strain CMY1200 in which the genomic *HSP82* gene is Myc tagged, was transformed with the plasmid harboring the triple HA-tagged constitutive *MAL*-activator mutant allele (p416GPD-MAL63/43-C). Transformants were grown in selective minimal media lacking uracil at 30 ºC to mid-logarithmic (O.D 0.3-0.5) in glycerol/lactate (3% glycerol and 2% lactic acid) and total cell protein extracts prepared under nondenaturing conditions. Extracts from cells grown in glycerol/lactate were purified by immunoprecipitation as described in Materials and Methods. The bound proteins were eluted, size-separated by 10% SDS-PAGE, and analyzed by Western blotting. Membranes were probed with both anti-HA antibody and anti-Myc antibody and the relative abundance of Hsp90 and mutant *MAL*-activator protein was quantified. The results, shown in Figure 19 shows that relative level of Myc-tagged Hsp82 found in association with constitutive *MAL*-activator mutant protein MAL63/43-C is enhanced compared to wild-type. The intensity of the Myc band is approximately the same as or slightly less than that of the HA band. In contrast, when the constitutive Mal63/43-C/ HA3 activator was precipitated, the intensity of the Myc band is significantly greater than the HA band indicating that the constitutive protein is complexed with Hsp90 to a much greater extent.

Figure 19. Association of constitutive and super-inducible *MAL*-activator mutant proteins with Hsp90. Strain CMY1200 (*HSP82/Myc hsc82*Δ) was transformed with the plasmid p416GPD-MAL63/43-C harboring the triple HA-tagged constitutive *MAL*-activator mutant gene *MAL63/43-C* or plasmid p416GPD-MAL63-460 carrying the super-inducible *MAL*-activator mutant gene *MAL63-460*. Transformants were grown to mid-log in glycerol/lactate at 30 ^{0}C. Nondenaturing protein extracts were prepared as described in Materials and Methods. Co-IP samples were size-separated by 10% SDS-PAGE, electroblotted to nitrocellulose, and Mal63/43-C/HA3 and Hsp82/Myc protein detected using anti-HA and anti-Myc antibodies, respectively.


MAL63
MAL63-460
MAL64/43-C
anti-Myc
anti-HA

Turnover rate and association of super-inducible MAL-activator mutant allele with Hsp90

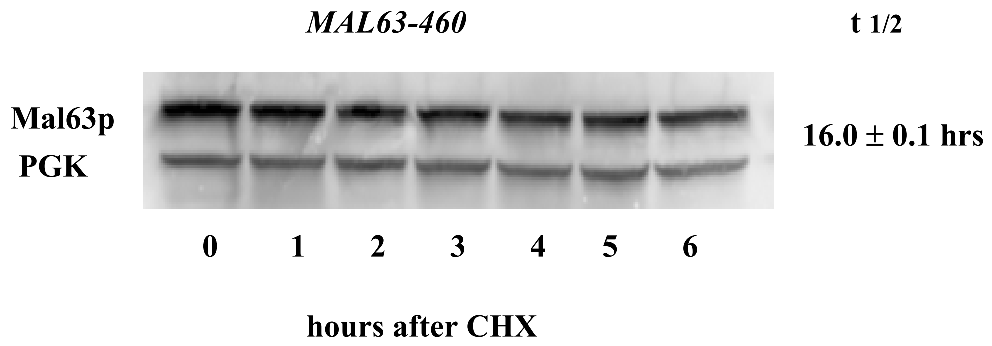
The super-inducible mutant *MAL*-activator allele, *MAL63-460* was obtained by charged-cluster to alanine mutagenesis of the regulatory domain of *MAL63* (Danzi et al., 2003). Almost all mutations in the C-terminus of the protein decreased activity of the *MAL*-activator. But, two mutations near the extreme C-terminus of protein enhanced the function of the *MAL*-activator. Transformants carrying those two super-inducible *MAL*-activator alleles, *MAL63-460* (D460A, K463A) and *MAL63-462* (S462A) ferment maltose more rapidly than the wild-type (Table 3) and show increased maltase activity. In this study we used the triple-HA tagged constructs carrying the super-inducible *MAL*-activator mutant allele, *MAL63-460* expressed from the GPD promoter.

Strain W303 was transformed with plasmid pGPD-MAL63-460/HA3 carrying the triple HA-tagged *MAL63-460* and half-life of mutant Mal63-460/HA3 protein determined in cells grown in glycerol/lactate (uninduced condition) at 30 ºC as described in Figure 17. The relative level of mutant Mal63-460/HA3 protein at each time point was quantified as described in Methods and Materials and normalized to the level of PGK. The relative level of Mal63-460/HA3 protein was plotted and used to calculate the half-life of Mal63-460/HA3 protein. Figure 20 shows that super-inducible mutant *MAL*-activator protein exhibits significantly longer half-life compared to the wild-type.

The association of super-inducible *MAL*-activator mutant protein with Hsp90 was also determined. Plasmid pGPD-MAL63-460/HA3 was transformed into strain CMY1200 containing the Myc-tagged *HSP82*. Total cell extract was prepared using non-denaturing conditions from cells grown in glycerol/lactate at 30 ºC. The HA-tagged

mal63-460 protein was immunopurified, the bound proteins size-separated by SDS-PAGE, and western blot analysis was carried out using anti-HA and anti-Myc antibodies. Figure 19 shows that the ability of super-inducible mutant *MAL*-activator allele to bind Hsp90 is also enhanced compared to wild-type. The Myc band intensity is significantly greater than that of the HA-band indicating that comparatively more Hsp82/Myc is associated with this super-inducible *MAL*-activator than with inducible Mal63p.

Figure 20. Turnover rate of super-inducible *MAL*-activator mutant allele. Strain W303 was transformed with the plasmid harboring the triple HA-tagged super-inducible *MAL*-activator mutant allele (p416GPD-MAL63-460). Transformants were grown to mid-log in glycerolactate at 30 ^{0}C. At time zero, cycloheximide was added to a final concentration of 50 μg/ml and total cell extracts prepared from cells harvested at time zero and at the indicated time points for six hours. Western analysis was carried out using anti-HA antibody. The relative level of Mal63-460/HA3p at each time point was quantified as described in Materials and Methods and normalized to the level of PGK. The Mal63-460/HA3p half-life was calculated from at least three independent transformants and average values are presented.


MAL63-460
t 1/2
Mal63p
PGK
16.0 ± 0.1 hrs
0
1
2
3
4
5
6
hours after CHX

Turnover rate of non-inducible Mal63 mutant MAL-activators

Since several of the non-inducible *MAL63* alleles could be suppressed by overexpression, suggesting that they may be unstable, we determined the rate of turnover of eight non-inducible mutant *MAL*-activator alleles isolated by Danzi *et al*. (2003). We hypothesized that these non-inducible mutant *MAL*-activator proteins may have reduced ability to interact with the Hsp90 chaperone complex and thus might have higher rates of degradation compared to wild-type inducible *MAL*-activator. For this we constructed triple-HA tagged version of these non-inducible *MAL*-activator mutant alleles and expressed them from the GPD promoter in vector p416, as described in Materials and Methods. Strain W303 was transformed with a plasmid harboring each triple HA-tagged non-inducible *MAL*-activator mutant gene and the half-life of the mutant protein was determined as described in Figure 17. The results are shown in Figure 21.

The rate of degradation of non-inducible *MAL*-activators varies over about 3-fold from a half-life of 3.9 hours to 13.7 hours. Of the eight non-inducible alleles tested, four, *mal63-331*, *mal63-364*, *mal63-467A9N,* and *mal63-467S9V* have a shorter half-life than that of inducible Mal63p. The half-life of the other four non-inducible mutants, *mal63-block3*, *mal63-401*, *mal63-391*, and *mal63-283* is considerably longer than that of inducible Mal63p. Since we could not correlate the mutant phenotype of these non-inducible *MAL*-activator alleles with their turnover rate, we investigated their dependency to Hsp90 chaperone complex in order to find out the basis of mutant phenotype of these non-inducible mutant *MAL*-activator alleles. We questioned whether these long half-life proteins were still dependent on Hsp90 chaperone complex or if their non-inducible phenotype resulted from the formation of a stable but aggregated protein that is unable to

associate with the chaperone. To test this, we determined the half-life of two of the long-lived non-inducible mutant activator proteins in an Hsp90 chaperone defective strain. The triple HA-tagged plasmid constructs expressing long-lived mutant *MAL*-activator alleles *mal63-401* and *mal63-block3* were introduced into the double null strain hsc82Δ cpr7Δ and the half-life of these non-inducible activator proteins determined. The results, shown in Figure 22, show that the stable *MAL*-activator mutant proteins are de-stabilized in Hsp90 chaperone mutant strain. The half-life of non-inducible mal63-401 protein decreased from 9.6 hours to 3.8 hours while the half-life of non-inducible mal63-block3 protein decreased from 11.3 hours to 3.9 hours. The shorter half-life indicates that these mutant proteins are still dependent on Hsp90 for stability and suggests that these mutant proteins should still be able to form a complex with Hsp90.

The ability of non-inducible MAL-activator mutant alleles to interact with Hsp90

The results above are consistent with the suggestion that the non-inducible *MAL*-activator proteins are able to form complexes with Hsp90 but raise the possibility that the affinity for the complex may be altered. We hypothesized that the non-inducible *MAL*-activator proteins should have decreased ability (varying degrees) to interact with Hsp90 chaperone complex. To test this hypothesis, the triple HA-tagged mutant Mal63 proteins were expressed in strain CMY1200 in which the genomic *HSP82* gene is Myc-tagged. Since the level of Hsp82 expression is constant in this strain, we should be able to detect differences in affinity between *MAL*-activator and the Hsp90 chaperone complex in the ratio of co-precipitated Myc epitope and HA epitope. CMY1200 was transformed with the plasmid harboring the triple HA-tagged non-inducible mutant *MAL*-activator alleles

and transformants were grown in selective minimal media in glycerol/lactate and total cell protein extract was prepared under nondenaturing conditions. Extracts from cells grown in glycerol/lactate were purified by immunoprecipitations described in Materials and Methods. The bound proteins were eluted, size-separated by 10% SDS-PAGE, and analyzed by Western blotting. Membranes were probed with both anti-HA antibody and anti-Myc antibody and the relative abundance of Hsp90 and mutant *MAL*-activator protein were quantified.

The results, shown in Figure 23 show no significant difference in the relative level of Myc-tagged Hsp82 associated with the non-inducible *MAL*-activator mutant proteins compared to inducible Mal63p as opposed to our hypothesis. Thus, the non-inducible *MAL*-activator mutant proteins bind Hsp90 efficiently. In addition, it should be noted that the long-lived mutant proteins, such as mal63-401 and mal63-block3, do not exhibit enhanced Hsp90 binding. The results suggest that the non-inducible *MAL*-activator mutant proteins form complexes with Hsp90 chaperone but are maintained in the complex in an inactive conformation and addition of maltose is unable to drive the complex into the active conformation.

Figure 21. Turnover rate of non-inducible Mal63 mutant *MAL*-activators Strains W303 (*HSC82 HSP82*) was transformed with plasmids p416GPD-mal63-283, p416GPD-mal63-331, p416GPD-mal63-364, p416GPD-mal63-391, p416GPD-mal63-401, p416GPD-mal63-467A9N, p416GPD-mal63-467S9V, p416GPD-mal63-block3 carrying triple HA-tagged non-inducible mutant *MAL*-activator genes expressed from the GPD promoter. Transformants were grown at 30 ºC in selective media lacking uracil in uninduced (3% glycerol and 2% lactate) medium. The half-life of the mutant proteins was determined as described in Figure 17.

Turnover rate of non-inducible *MAL*-activator mutant alleles

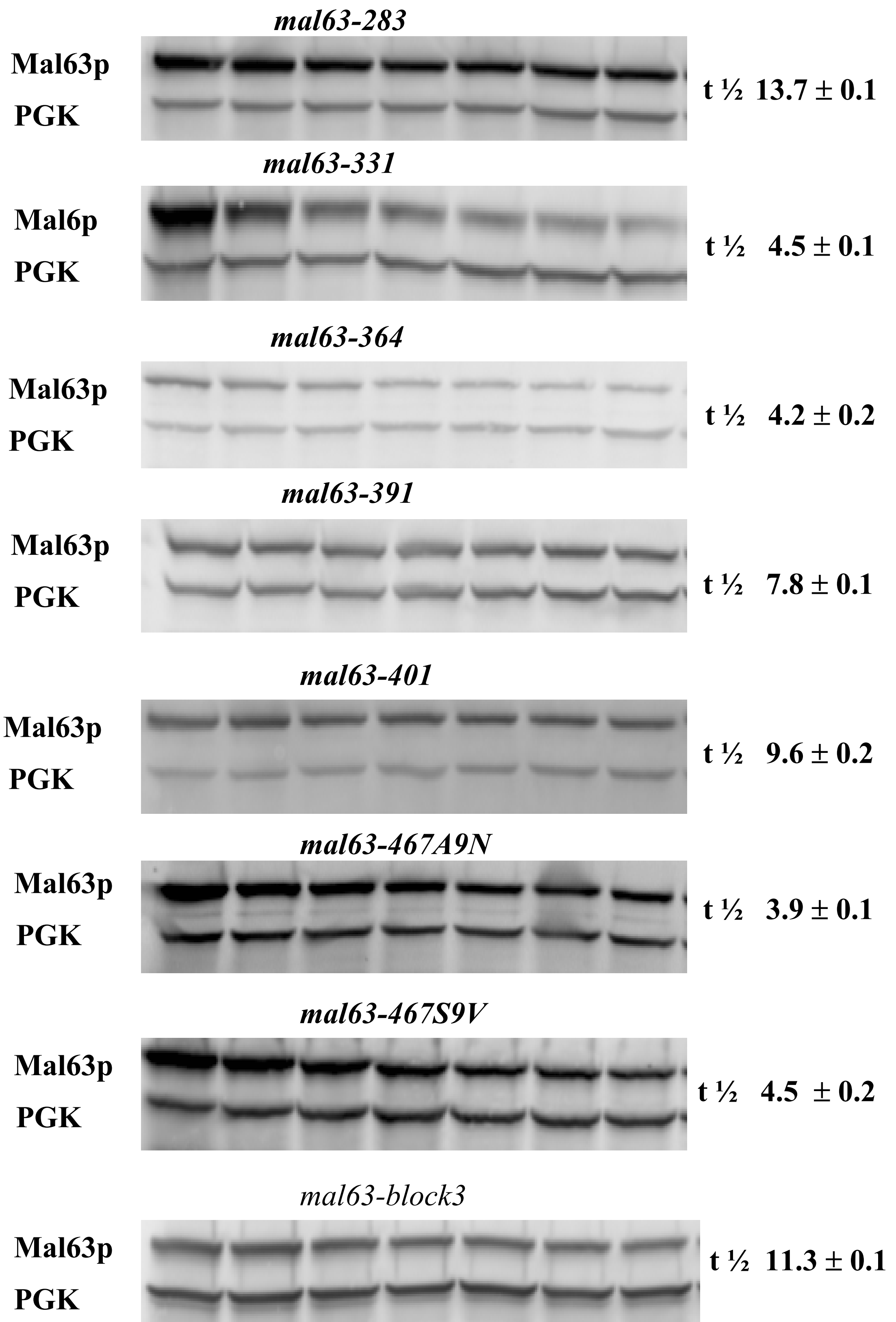


Figure 22 Turnover rates of long-lived *mal63-401 and mal63-block3 MAL*-activator mutant alleles in strain hsc82Δ cpr7Δ. Strain hsc82Δ cpr7Δ (*hsc82*Δ *HSP82 cpr7*Δ) was transformed with plasmids p416GPD-mal63-401 and p416GPD-mal63-block3. Transformants were grown at 30 ºC in selective media lacking uracil in uninduced (3% glycerol and 2% lactate) conditions. The half-life of the mutant protein was determined as described in Figure 17.

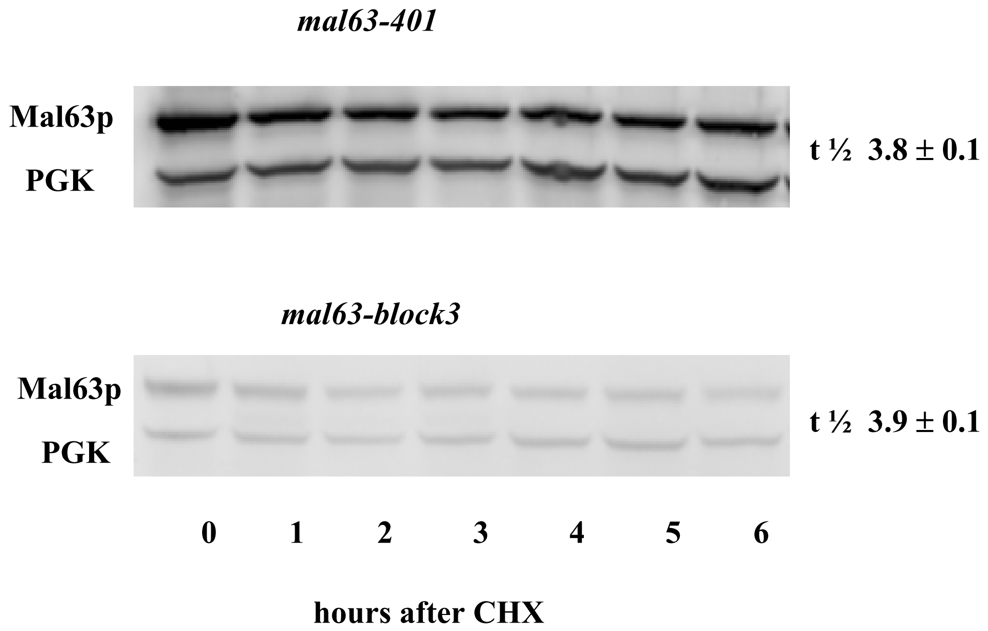
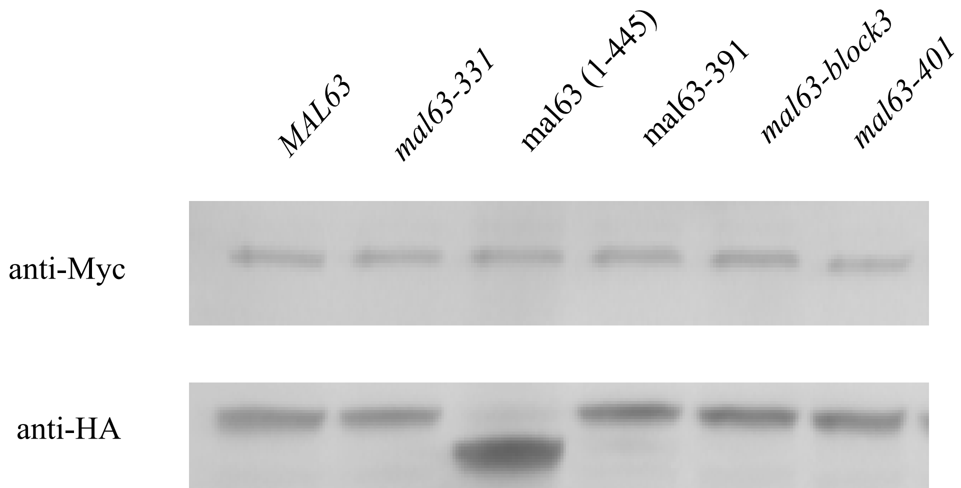

mal63-401
Mal63p
PGK
t ½ 3.8 ± 0.1
mal63-block3
Mal63p
PGK
t ½ 3.9 ± 0.1
0
1
2
3
4
5
6
hours after CHX

Figure 23. The association of non-inducible *MAL*-activator mutant alleles with Hsp90. Strain CMY1200 (*HSP82/Myc*) was transformed with the plasmids p416GPD-mal63-331, p416GPD-mal63-391, p416GPD-mal63-block3, and p416GPD-mal63-401 harboring the triple HA-tagged non-inducible *MAL*-activator mutant genes. Transformants were grown to mid-log in glycerol/lactate at 30 ^{0}C. Nondenaturing protein extracts were prepared and HA-tagged protein imminoprecipitated as described in Materials and Methods. The Co-IP samples were size-separated by 10% SDS-PAGE, electroblotted to nitrocellulose, and mutant mal63/HA proteins and Hsp82/Myc protein detected using anti-HA and anti-Myc antibodies, respectively.

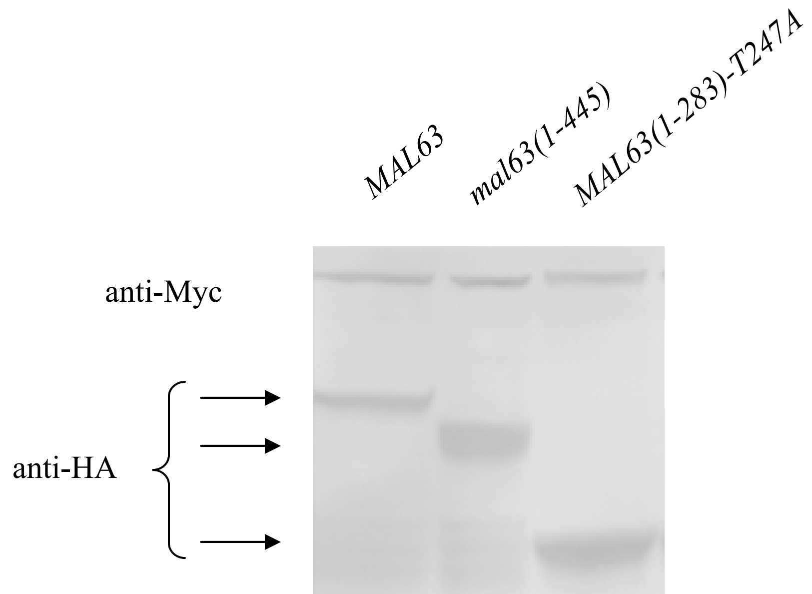

MAL63
mal63-331
mal63 (1-445)
mal63-391
mal63-block3
mal63-401
anti-Myc
anti-HA

Association of truncated mutant MAL-activator alleles with Hsp90

Mutation analysis of constitutive *MAL*-activator mutations carried out by Gibson *et al.* (1997) and Danzi *et al.* (2000) indicated that the C-terminal approximately 200 residues of the *MAL*-activator has both negative and positive regulatory function given the finding that Hsp90 complex is essential to *MAL*-activator regulation we wondered whether binding to the chaperone would be affected in truncated *MAL*-activator mutants. To test this, we investigated the ability of two truncated mutant *MAL*-activator proteins to interact with Hsp90 chaperone, the constitutive *MAL63(1-282)-T247A* and the non-inducible *mal63(1-445).* Strain CMY1200 in which genomic *HSP82* gene is Myc-tagged was transformed with plasmids harboring the truncated mutant *MAL*-activator mutant genes. Transformants were grown to mid-log in glycerol/lactate at 30 ºC. Nondenaturing protein extracts were prepared and the *MAL*-activator proteins immunoprecipitated. Co-IP samples were analyzed by Western blotting using anti-HA and anti-Myc antibodies. The results are shown in Figure 24. Clearly, the activator encoded by the constitutive truncated allele *MAL63(1-282)-T247A* and non-inducible mutant *MAL*-activator encoded by *mal63(1-445)*, containing stop codon at position 445, co-immuniprecipitated with Myc-tagged Hsp90. Loss of the C-terminal domain even as much as 187 residues of the C-terminal domain, does not significantly decrease association with Hsp90 molecular chaperone suggesting that the N-terminal or other regions of *MAL*-activator also interacts with Hsp90 molecular chaperone. The hypothesis of multiple sites of interaction with Hsp90 molecular chaperone or co-chaperone proteins has also been suggested with Danzi *et al*. (Danzi et al., 2000). They found that second site intragenic mutations in the constitutive *MAL*-activator mutant gene suppressed the constitutive phenotype and

restore inducibility. The results in Figure 24 are consistent with that observation and suggest that the chaperone binds to sites throughout the length of the *MAL*-activator protein.

Figure 24. Association of truncated mutant *MAL*-activator alleles with Hsp90. Strain CMY1200 was transformed with plasmids p416GPD-MAL63(1-282*)-T247A,* p416GPD-mal63(1-445) harboring triple HA-tagged truncated mutant *MAL*-activator alleles. Transformants were grown to mid-log in 2%glycerol / 3%lactate at 30 ºC. Nondenaturing protein extracts were prepared as described in Materials and Methods. Co-IP samples were analyzed by Western blotting using anti-HA and anti-Myc antibodies


MAL63
mal63(1-445)
MAL63(1-283)-T247A
anti-Myc
anti-HA

Discussion

Genetic analysis of the *MAL*-activator isolated constitutive, non-inducible, and super-inducible alleles of Mal63 *MAL*-activator is reported in Danzi *et al*. (2003). We tried to correlate the mutant phenotype of these isolated constitutive, non-inducible and super-inducible alleles *MAL*-activators with their turnover rates and their association with Hsp90 molecular chaperone in an effort to reveal the molecular basis of their phenotype. Our results show that the constitutive mutant *MAL*-activator proteins do not show significant increase or decrease in the rate of degradation compared to the wild type but the one super-inducible Mal63 *MAL*-activator tested showed a reduced rate of degradation. The non-inducible Mal63 *MAL*-activator mutant alleles showed great variation in half-life compared to inducible Mal63p.

Since the mutant phenotype is not consistently related to changes in the turnover rate of the *MAL*-activator, we investigated the ability of those mutant *MAL*-activators to interact with Hsp90 molecular chaperone complex. The results reported here demonstrate that all mutant *MAL*-activator proteins form complexes with Hsp90 molecular chaperone. We found that binding of the five non-inducible mutant *MAL*-activator proteins we tested to Hsp90 was comparable to the binding affinity of inducible Mal63p to Hsp90. We suggest that addition of inducer maltose is unable to drive the mutant *MAL*-activator-chaperone complex to the active conformation. This could result from an inability to bind maltose but we do not believe that this is the case. Danzi *et al*. (2003) proposed that the region from residues 331 to 423 bind maltose. Alterations in this region that cause a non-inducible phenotype in Mal63p have little effect when introduced into constitutive Mal63/43-C protein. The non-inducible mutants analyzed here map to this region of the

MAL-activator except for *mal63-467A9N* and *mal63-467S9V* which are in the region proposed to function in the formation and/or maintenance of the active conformation (see Figure 2). In view of this, we propose that the non-inducible activator studied here are unable to form the active DNA-binding conformation either because they are unable to respond to maltose (mutations *mal63-283, mal63-331, mal63-364, mal63-391, mal63-401* and *mal63-block3*) or because they are defective in the formation and/or maintenance of the active state (*mal63-467A9N* and *mal63-467S9V*). We found that the relative level of Hsp90 chaperone found in association with constitutive mutant protein was enhanced.

We found that, constitutive mutant *MAL*-activator proteins still needs Hsp90 molecular chaperone and that in chaperone-defective strains constitutive *MAL*-activator are unstable and inactive. Thus, we propose that the constitutive *MAL*-activator proteins still require Hsp90 chaperone to form the active configuration but that they undergo this activation spontaneously and in the absence of inducer maltose. The super-inducible *MAL*-activator appears to bind Hsp90 complex effectively but still require maltose to form the active conformation.

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