Regulation of the *Sacchromyces cerevisiae MAL*-activator by the Hsp90/Hsp70 molecular chaperone machine

By

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ABSTRACT

REGULATION OF THE *SACCHROMYCES CEREVISIAE MAL*-ACTIVATOR BY THE HSP90/HSP70 MOLECULAR CHAPERONE MACHINE

By

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The Hsp90/Hsp70 chaperone machine is an essential regulator of cell growth and division. It is required for activation of select client proteins, chiefly protein kinases and transcription activators, and thus plays a major role in regulating intracellular signaling and gene expression. This report demonstrates, *in vivo*, the association of the *Saccharomyces cerevisiae* maltose-responsive transcription activator Mal63 (*MAL*-activator) with the yeast Hsp70 (Ssa1), Hsp90 (Hsp82), and Hop (Sti1) homologs using a collection of inducible, constitutive, and noninducible alleles. Each class of mutant activator forms a distinctly different stable multi-chaperone complex in the absence of maltose. Inducible Mal63p associates with Ssa1, Hsp82, and Sti1 and is released in the presence of maltose. Noninducible mal63 mutant proteins bind to Ssa1 alone and do not stably associate with Hsp82 or Sti1. Constitutive *MAL*-activators bind well to Hsp82 and poorly to Ssa1 and Sti1, but deletion of *STI1* restores Ssa1 binding. Taken together, Mal63p regulation requires the formation of Hsp90/Hsp70 sub-complexes comparable to yet distinct from those observed with previously characterized Hsp90 clients including glucocorticoid receptor and yeast Hap1p. Thus, comparative studies of different client proteins highlight functional diversity in the operation of the Hsp90/Hsp70 chaperone machine.

In this report, we also investigate the negative role played by Aha1 cochaperone in *MAL*-activator regulation. Deletion of *AHA1* increases the induced level of *MAL* gene expression approximately 2-fold indicating that it functions as a negative regulator of *MAL* gene induction. Genetic interaction is observed between *aha1Δ* and mutations in the C-terminal region of the *MAL*-activator suggesting that Aha1 cochaperone interacts with this region of the activator. Strains with *aha1Δ* exhibit enhanced binding of Mal63 *MAL*-activator to Hsp82 and Ssa1. Aha1p associates with noninducible mutant *MAL*-activators, and with inducible Mal63 in an *sti1Δ* strain, which are found in the "early complex", but not with constitutive *MAL*-activator or with Mal63 in a *STI1* strain. These results support a novel function for Aha1 cochaperone in *MAL*-activator regulation suggesting that it acts as a governor that reduces *MAL*-activator association with Hsp70 and Hsp90 and inhibits activator entry into the chaperone cycle.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Cell metabolism is under tight control largely by turning on/off the expression of specific genes. Yeast cells can respond to environmental changes such as temperature, nutrition, and carbon source by regulating the activity of specific transcription factors that regulate gene expression. Signals that are sensed from the environment are delivered by a variety of mechanisms to the cellular machinery to yield a response. Activated transcription activators bind to a specific DNA sequence along with other components of the transcription machinery to initiate expression of target genes and produce an appropriate cellular response.

Mal63 *MAL*-activator is the *Saccharomyces* transcription factor that responds to the level of maltose in the growth medium and governs the expression of the *MAL* structural genes (BARNETT and ENTAIN 2005; NEEDLEMAN 1991). Hsp90 (Heat-Shock Protein 90) is an abundant molecular chaperone that functions in the folding and stabilization of a select number of proteins termed "client proteins" consisting of transcription factors and kinases (reviewed in (CAPLAN *et al.* 2003); (PEARL and PRODROMOU 2006); (PICARD 2006a); (PRATT *et al.* 2004); (PRATT and TOFT 2003); (WEGELE *et al.* 2004)). As a client protein of Hsp90, the induction of the *MAL*-activator is regulated by the Mal63-Hsp90 interaction (BALI *et al.* 2003). Mammalian steroid receptors are Hsp90 client proteins and have been used extensively for studying the mechanism of molecular chaperone regulation (PRATT and TOFT 2003). In *Saccharomyces*, some Hsp90 client proteins including the Hap1, Gcn2 kinase, and Ste11 protein kinase appear to have a different regulation pathway from that of steroid receptors (ABBAS-TERKI *et al.* 2000; HON *et al.* 2001; LAN *et al.* 2004).

In this study, we investigated the role of the Hsp90/Hsp70 chaperone machine in the

regulation of the activity and stability of transcription factor Mal63 *MAL*-activator by analyzing their interaction with inducible Mal63 and its non-inducible, constitutive, and superinducible mutant alleles. Our results show that Mal63 activation follows the same basic pathway as glucocorticoid receptor maturation. We also explore the functional specificity of several known Hsp90/Hsp70 cochaperones with regard to the regulation of Mal63 client protein. A survey of different cochaperones deletion mutations indicate that Aha1 cochaperone is a negative regulator of Mal63 *MAL*-activator. The mechanism of this regulation is explored in detail.

PART I: MOLECULAR CHAPERONE AND COCHAPERONE PROTEINS

Chaperone proteins are considered as cellular developmental network hub capacitors (RUTHERFORD *et al.* 2007). They participate in a large variety of cellular processes that regulate cell growth and differentiation. These include such diverse processes as the stabilization of nascent proteins during translation, the transport of proteins across the mitochondrial membrane, the trafficking and modification of membrane proteins in the ER lumen and Golgi, the activation of signal transduction proteins, the facilitation of chromosome disassembly and telomerase activation, and protein quantity control in conjunction with the proteasome degradation of ubiquitin-conjugated proteins (CRAIG *et al.* 1993; HAMPTON 2002; HOCHSTRASSER 1996; KAUFMAN 1999; MAYER and BUKAU 1999; MAYER and BUKAU 2005). Molecular chaperones are a large group of unrelated protein families whose role is to assist other proteins in processes such as folding or re-folding and association with multi-protein complexes. Under conditions of environmental stress, cells express several stress proteins, some of which have been termed Heat Shock Proteins (HSP) due to their initial discovery in tissues exposed to elevated temperatures. Many, but not all,

stress proteins and heat shock proteins are molecular chaperones. Moreover, some heat shock proteins are expressed under normal growth temperatures conditions. According to their molecular mass, heat shock proteins are classified into specific families that include the small heat shock proteins (sHSPs)/alpha-crystalline, HSP10, HSP40, HSP60, HSP70, HSP90, HSP100, and HSP110.

Hsp90 and Hsp70 are two major categories of molecular chaperone proteins: The heat shock protein Hsp90 is expressed in all eukaryotic and prokaryotic cells and is 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 the most abundantly expressed protein (~2% of cytosolic proteins) in unstressed cells (WELCH and FERAMISCO 1982). Hsp90 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 the fact that Hsp90 is essential suggests a role in processes controlling cell growth and division 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 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 a key role in signal transduction network. Hsp90 is not required for the folding of most newly synthesized proteins (NATHAN *et al.* 1997). Although Hsp90 binds transiently to non-native proteins, Hsp90 normally associates only with nascent client

proteins after a considerable amount of secondary and tertiary structure have formed (JAKOB *et al.* 1995).

Hsp90 is a homodimer in which the C-terminal domains are in contact with each other (NEMOTO *et al.* 1995). It has a highly conserved N-terminal ATPase domain that is connected to a highly conserved C-terminal dimerization domain by a middle domain that is proposed to be involved client protein and cochaperone binding. A charged linker region of variable 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 the binding site for TPR domain containing cochaperones (CHEN *et al.* 1998; YOUNG *et al.* 1998). A TPR domain (tetratricopeptide repeat) is a degenerate 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.

The ATPase activity of Hsp90 is essential for chaperone function. Binding of substrate to Hsp90 requires ATP, but release of substrate from the complex requires ATP hydrolysis (OBERMANN *et al.* 1998; PANARETOU *et al.* 1998; PRODROMOU *et al.* 1997). The N-terminal ATP binding domain of Hsp90 is structurally related to the superfamily of homodimeric ATPase such as the DNA mismatch repair protein MutL, DNA gyrase, and type II topoisomerase (BAN *et al.* 1999; BECKER and CRAIG 1994; WIGLEY *et al.* 1991).

In *Saccharomyces*, there are two different genes encoding Hsp90, *HSP82* and *HSC82* (BERCOVICH *et al.* 1997; PRATT and TOFT 2003). 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 homologous 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.

The heat shock protein Hsp70 is another molecular chaperone that plays an important role in protecting cells from stress and in the folding and maturation of nascent proteins. Unlike Hsp90, Hsp70 binds to nascent, partially folded, or completely unfolded polypeptides directly, thereby preventing aggregation and facilitating correct folding.

Saccharomyces cerevisiae encodes 14 members of the Hsp70 family of chaperones that are localized to the cytosol, mitochondrion, and endoplasmic reticulum (BOORSTEIN *et al.* 1994; CRAVEN *et al.* 1996; RASSOW *et al.* 1995). Nine of them are located in the cytosol and can be grouped into the subfamilies Ssa, Ssb, Sse, and Ssz. The Ssa subfamily has four members (Ssa1-Ssa4) and represents the predominant cytoplasmic Hsp70 homologues. Ssa1p and Ssa2p are expressed at normal growth temperatures while Ssa3p and Ssa4p are largely stress induced. Ssb1p and Ssb2p are approximately 99% identical and are found on ribosomes bound to nascent peptides and are believed to facilitate the translation process (BECKMANN *et al.* 1990; GAUTSCHI *et al.* 2002; NELSON *et al.* 1992). Ssa1p and Ssa2p exhibit about 60% sequence identity to Ssb1p and Ssb2p. Sse1 and Sse2 are members of the Hsp110 class , which are related to Hsp70 family (ANDREASSON *et al.* 2008; DRAGOVIC *et al.* 2006; LIU and HENDRICKSON 2007; SHANER *et al.* 2005). Sse1,2 interact with Ssa and Ssb and serve as nucleotide exchange factors for these Hsp70 working at the ribosome in nascent polypeptide synthesis.

Cochaperones and other Hsp90 Associated Proteins: Hsp90 functions in conjunction with more than 10 cochaperones that facilitate its chaperone activity. Many

of the Hsp90 associated protein are conserved both in structure and function from *Saccharomyces cerevisiae* to mammals (CAPLAN 2003; CHANG and LINDQUIST 1994). In yeast, the Hsp90 chaperone multi-complexes have been demonstrated to contain Hsp90, Hsp70, Hsp40, immunophilins (peptidyl-prolyl cis-trans isomerases), p60/Hop, p50/Cdc37, p23, Aha1p, and Hsp110. Genetic approaches used in *Saccharomyces* have identified other proposed cochaperones, such as Cns1, of unknown function. These cochaperones are involved in client protein selection, modulation of ATPase activity of Hsp90 and Hsp70, and client protein binding (LUCERO *et al.* 2002; MORISHIMA *et al.* 2003; PRATT and TOFT 1997; PRINCE and MATTS 2004).

Table 1 lists the more important Hsp90 and Hsp70 cochaperones but is by no means an exhaustive list. Cochaperones fall largely into two categories: the TPR-containing cochaperones and all others. The TPR-containing cochaperones include p60/Hop and the immunophilins Cpr6p and Cpr7p. The TPR domain of the cochaperones compete with each other for binding to the conserved C-terminal MEEVD sequence of Hsp90 (YOUNG *et al.* 2001). *STI1* encodes the *Saccharomyces* homologue of p60/Hop (ABBAS-TERKI *et al.* 2002; LEE *et al.* 2004; PRODROMOU and PEARL 2003; WEGELE *et al.* 2006). Hop functions as a scaffold protein that promotes the association of Hsp90 and Hsp70. It contains two TPR domains each of which binds the MEEVD sequences found in Hsp90 and Hsp70 to form a Hsp90 – Hsp70 – Hop complex. The other cochaperones bind to different regions of Hsp90 via special binding sequences. Cdc37/p50 functions

Table 1: Hsp90 and Hsp70 cochaperones

Human	Yeast homologue	Inter-action	Cellular function	Essential (yeast)	Hsp70 Co-chaperone	Hsp90 Co-chaperone
Hop	Sti1	TPR	Complex assembly	--	+	+
p23	Sba1	Direct	Substrate release	--	--	--
TTC4	Cns1	TPR	unknown	+	+	+
FKBP52		TPR	Prolyl-isomerase	n.d.	--	+
FKBP51		TPR	Prolyl-isomerase	n.d.	--	+
Cyp40	Cpr6,Cpr7	TPR	Prolyl-isomerase	--	--	+
Hsp40	Ydj1, Sis1 Zuo1	Direct	Chaperone ATPase activator	+	+	--
Bag1	Snl1, Mge1	Direct	Nucleotide release	+	+	--
CHIP		TPR	Ubiquitin ligase	--	+	+
PP5	Ppt1	TPR	Phosphatase	--	--	+
XAP2		TPR	Prolyl-isomerase-related protein	n.d.	--	+

Table 1 (Continued): Hsp90 and Hsp70 cochaperones

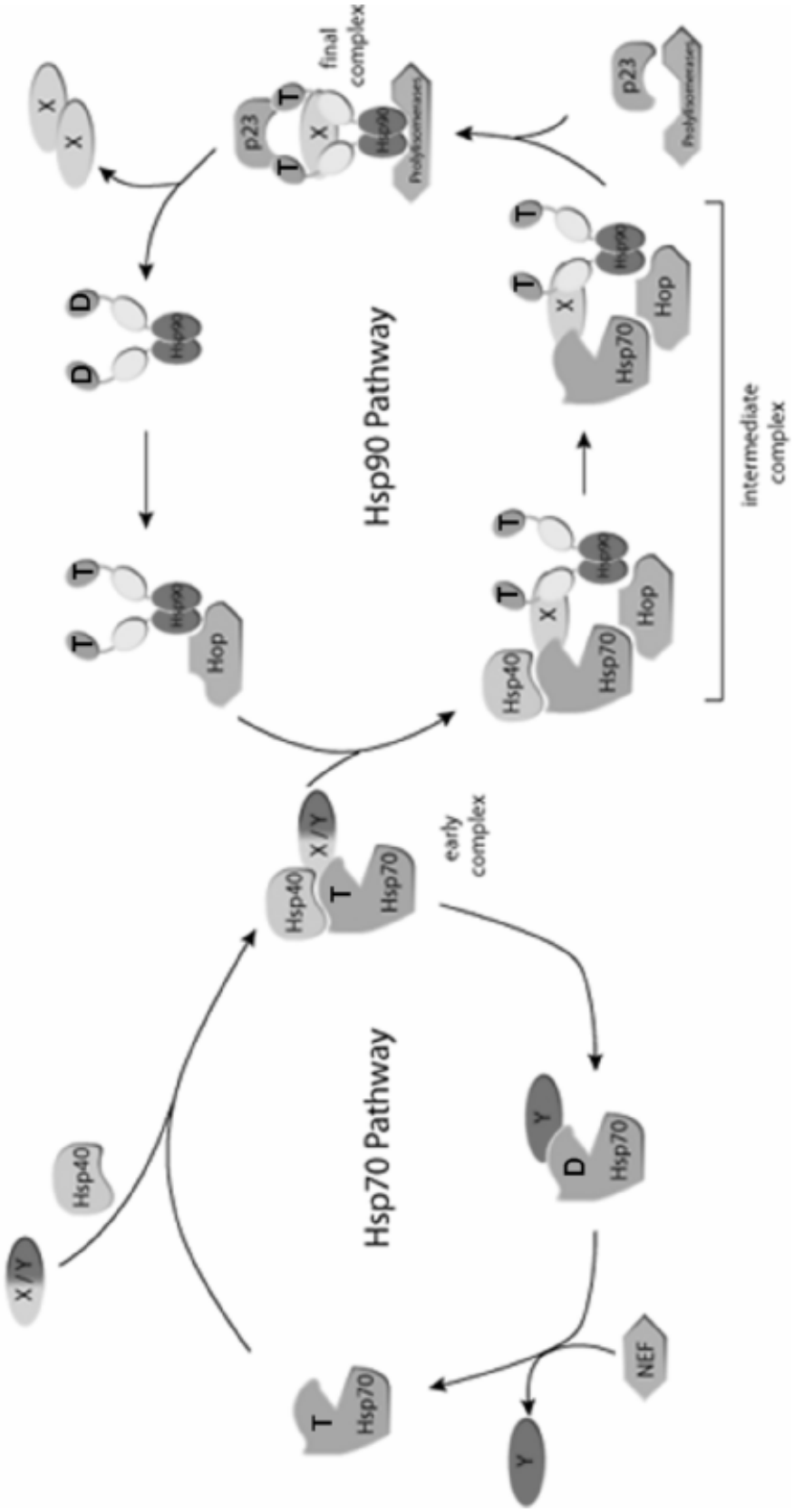
Cdc37/ p50	Cdc37	Direct	Kinase client binding	+	--	+
Aha1	Aha1/Hch1	Direct	Hsp90 ATPase regulator	--	--	+

almost exclusively for kinase clients of Hsp90 (ABBAS-TERKI *et al.* 2000; MACLEAN and PICARD 2003; PRODROMOU and PEARL 2003; VAUGHAN *et al.* 2006). It binds the kinase to the middle domain of Hsp90 and is essential for kinase activation. P23 binds near the N-terminal ATPase domain of Hsp90 and inhibits ATPase activity resulting in client protein retention (ALI *et al.* 2006; PICARD 2006b; PRATT *et al.* 2006). Aha1 cochaperone, which will be described in greater detail in Chapter 2, binds to the middle region of Hsp90 and activates Hsp90 ATPase activity (HARST *et al.* 2005; LOTZ *et al.* 2003; PANARETOU *et al.* 2002; PRODROMOU and PEARL 2003).

Current study on Hsp90 and Hsp70 relay team and two well studied client proteins: Hsp70 and Hsp90 are essential molecular chaperones that work together as a "relay team" in the activation, folding, subcellular localization, and maturation of selected, so-called client proteins. This process is referred to as the Hsp70/Hsp90 chaperone machine (reviewed in (BUKAU *et al.* 2006); (CAPLAN *et al.* 2007); (PICARD 2006a); (PRATT and TOFT 1997); (PRATT and TOFT 2003); (WEGELE *et al.* 2004)). Hsp90 is unique in that it acts on nascent, partially folded client proteins that are recruited by the sequential activity of Hsp40 and Hsp70. Hsp70/Hsp90 chaperones function in the cytosol, endoplasmic reticulum, and mitochondrion.

Figure 1 diagrams this process for two types of proteins: Y proteins, which utilize only Hsp70, and X proteins, which are non-kinase clients of Hsp90 and require both Hsp70 and Hsp90 for proper activation. Figure 1 is taken from WEGELE *et al.* (2004). Many of the cochaperones known to be required for this process are not included and major questions in the field relate to the roles of the cochaperones in the process and variations in these roles with different client proteins. Nucleotide exchange factors are

Figure 1: Connecting the Hsp90 and Hsp70 pathways. The diagram in Figure 1 is taken from (WEGELE *et al.* 2004). The client proteins are labeled as (X/Y). The ATP-bound form of Hsp70 or Hsp90 is indicated with a (T). The ADP-bound form of Hsp70 or Hsp90 is indicated with a (D).


Hsp70 Pathway
Hsp90 Pathway
X / Y
Hsp40
Hsp70
T
D
Y
NEF
early complex
Hop
Hsp90
p23
Polyisomerases
final complex
intermediate complex

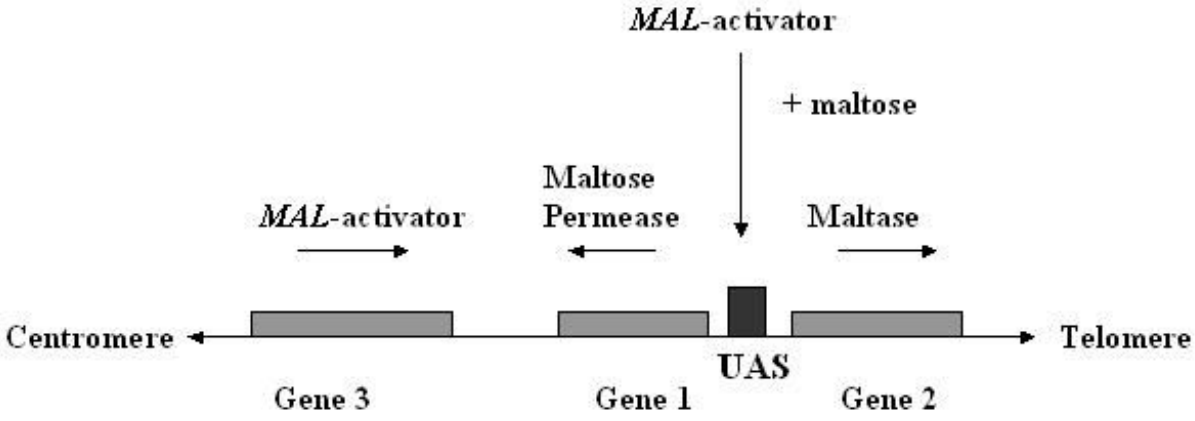
not shown but the ATP-bound versus ADP-bound state of Hsp70 and Hsp90 is indicated with a T or a D.

Approximately 10% of newly synthesized proteins in eukaryotic cells require Hsp70 for folding. These proteins are delivered to the Hsp70 complex via Hsp40 as nascent polypeptides to form the "early complex". Substrate (Y) is an example of a protein that can be processed by Hsp70 alone. Following ATP hydrolysis, a nucleotide exchange factor releases the substrate and Hsp70 reenters the cycle. Approximately 100 to 200 substrates also require Hsp90 for proper folding or activation. These are shown as (X) and are called Hsp90 client proteins. The characteristics of these substrates that allow them to be selected as Hsp90 client proteins are poorly understood and probably involves input from both the chaperones and select cochaperones. Transfer to Hsp90 occurs with the formation of the "intermediate complex", which is facilitated by the scaffold protein Hop that brings together Hsp70 and Hsp90 via binding to its TPR domains. Following transfer of the client protein to Hsp90, Hsp70 dissociates and p23 and prolylisomerases enter the complex to form the "final complex". The client is released from this "final complex" in an activated form. In the case of client proteins whose activation is regulated by a particular signal, such as an inducer, the client protein is maintained in an inactive form in one of these chaperone complexes until the signal is presented.

PART II: MALTOSE FERMENTATION AND THE *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 2 and

Figure 2: The structure and organization of a *Saccharomyces MAL* locus. Each *MAL* locus consists of three genes. Gene 1 encodes maltose permease, Gene 2 encodes maltase, and Gene 3 encodes the *MAL*-activator. The *MAL* structural genes are divergently transcribed from a common 874bp promoter region. A 68 bp region of the *MAL61*-*MAL*62 intergenic region called as the UASMAL is necessary for the maltose-induced expression of both *MAL61* and *MAL62*.


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

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. Gene2 at each locus encodes maltase, an α-glucosidase that hydrolyzes maltose to two molecules of glucose. Gene3 at each locus encodes the *MAL*-activator, a DNA binding transcription activator that regulates the expression of Gene1 and Gene2. Transcription of Gene1 and Gene2 is induced by maltose and repressed by glucose. The five *MAL* loci each map to different *Saccharomyces* chromosomes, 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).

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 most well-characterized gene encoding maltose permease and is also referred to as *AGT1* (HAN *et al.* 1995).

The *MAL6* structural genes are coordinately and divergently transcribed from a

common 874-bp promoter region (Figure 2) (LEVINE *et al.* 1992). This region between the *MAL61* and *MAL62* open reading frames contains 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 and –288 and –578 and –592 adjacent to the UAS_{MAL} (HU *et al.* 1995; WANG and MICHELS 2004).

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; WANG *et al.* 2002). *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 MICHELS 1991). 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 1989; HAN *et al.* 1995). Recent studies suggest that Mal61 permease from *MAL6 l*ocus does not function as the maltose sensor. It serves only as a maltose transporter to accumulate intracellular concentration of maltose to levels sufficient to induce *MAL* gene expression (WANG *et al.* 2002). While it has yet to be demonstrated experimentally, it is suggested that binding of maltose to the *MAL*-activator is the mechanism of maltose sensing.

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.* 1986; HU *et al.* 1995; NEEDLEMAN *et al.* 1984). Loss of maltase has no obvious effect on induction.

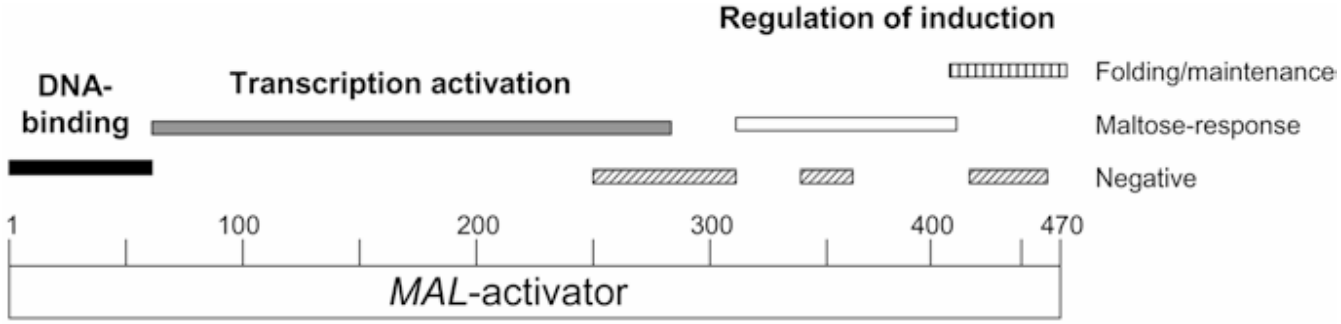
The *MAL*-activator: *MAL63* encodes an inducible allele of the *MAL*-activator at the *MAL6* locus (CHARRON *et al.* 1989; NEEDLEMAN *et al.* 1984). The *MAL63* gene encodes a 470-residue protein with an N-terminal cysteine-rich zinc finger DNA-binding domain (CHANG *et al.* 1988; KIM and MICHELS 1988). 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 (DANZI *et al.* 2000; GIBSON *et al.* 1997; HU *et al.* 1999) (Figure 3).

Mutations in the *MAL* loci affecting *MAL* structural gene expression were isolated by many workers over the last 50 or more years including both noninducible and constitutive mutations (reviewed in (BARNETT and ENTAIN 2005)). GIBSON *et al.* (1997) described two classes of constitutive *MAL*-activator mutant alleles, those carrying nonsense mutations at codons 283 or 307 of this 470-residue protein and those containing multiple sequence alterations affecting residues in the region from 300 to 461. *MAL23-C* and *MAL43-C* encode the *MAL*-activator at *MAL2* and *MAL4*, respectively, and are members of this second class of constitutive mutants. DANZI *et al.* (2003) carried out a charged-cluster to alanine scanning mutagenesis and a random mutagenesis of the C-terminal regulatory domain of the inducible *MAL*-activator encoded by *MAL63*. Almost all of these mutations caused a noninducible phenotype. Two mutations, *MAL63-460* and *MAL63-462*, were exceptional in that they increased the induced level of *MAL* structural gene expression and created a super-inducible *MAL*-activator.

Mal63p is an Hsp90 client protein (BALI *et al.* 2003). Strains carrying mutant alleles of components of the Hsp90 chaperone complex are defective for induction of the *MAL* structural genes and grow poorly on limiting concentrations of maltose, the inducer. On the other hand, high inducer concentrations suppress this growth defect. Mal63 protein is sensitive to degradation in cells depleted of Hsp90 and Mal63 protein binds to Hsp90

Figure 3: *MAL*-activator functional domains. Mal63p is 470 residues in length and contains a six-cysteine zinc finger DNA-binding domain in the N-terminal ~60-100 residues, a single transcription activation domain in approximately residues 60-250, and a C-terminal regulatory domain in approximately residues 250-470. (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

in vivo, both hallmarks of Hsp90 chaperone client proteins.

The goal of my thesis project is to use the *Saccharomyces MAL*-activator to explore the Hsp70/Hsp90 chaperone cycle *in vivo*. The large number of *MAL*-activator mutant alleles provides a rich resource for this study. I was particularly interested in determining the roles of Hsp70 and Hsp90 in the regulation of *MAL*-activator induction and in possible variability of cochaperone utilization for this particular client protein compared to other more well-studied client proteins such as the mammalian glucocorticoid receptor.

MATERIALS AND METHODS

Yeast strains and plasmids: Table 2 lists the *Saccharomyces* strains used in this study. 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 and its derivatives do not ferment maltose and require 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 and as modified by Li Zhang) contains deletions in *SSA2*, *SSA3*, and *SSA4* providing *SSA1* as the sole source of this class of Hsp70 (BECKER *et al.* 1996; LAN *et al.* 2004). Strain 5B6 (*MATα ura3-52 leu2-3,112 his3-11 trp1Δ1 lys2 ssa1:: HIS3 ssa2::LEU2 SSA3 ssa4::LYS2 hem1-Δ100 pGAL1-SSA1*) (from Elizabeth Craig and as modified by Li Zhang) contains deletions in *SSA1*, *SSA2*, and *SSA4* providing *GAL1*-dependent *SSA1* expression from a *URA3* plasmid vector (BECKER *et al.* 1996; LAN *et al.* 2004). The *SSA3* gene of strain 5B6 by itself is not capable of supporting growth and this strain is viable only on galactose medium in the absence of glucose. As described above for W303, strains JN516 and 5B6 carry only nonfunctional *MAL*-activator genes, *mal13* and *mal33*, and a plasmid-borne copy of the *MAL*-activator gene is required for the expression of the *MAL* structural genes.

Table 2
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
CMY8015	Isogenic to W303 except *STI1/Myc*	This study
CMY8301	Isogenic to W303 except *AHA1/Myc3*	This study
CMY1200	Isogenic to *hsc82Δ* except *HSP82/Myc*	This study
CMY8002	Isogenic to CMY1200 except *sti1Δ::Hyg^R*	This study
CMY8005	Isogenic to CMY1200 except *sse11Δ::Hyg^R*	This study
CMY8007	Isogenic to CMY1200 except *sba1Δ::Hyg^R*	This study
CMY8009	Isogenic to CMY1200 except *sro9Δ::Hyg^R*	This study
CMY8010	Isogenic to CMY1200 except *aha1Δ::Hyg^R*	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
cdc37^{ts}	Isogenic to W303 except *cdc37-S14A:URA3*	A. J. Caplan

Table 2 (Continued)
List of strains

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 SSA1ssa2::LEU2 ssa3::TRP1 ssa4::LYS2 hem1-Δ100*	E. Craig, L. Zhang
CMY1300	Isogenic to JN516 except *SSA1/Myc*	This study
CMY8003	Isogenic to CMY1300except *aha11Δ::Hyg^R*	This study
CMY8004	Isogenic to CMY1300except *sro9Δ::Hyg^R*	This study
CMY8006	Isogenic to CMY1300except *sba1Δ::Hyg^R*	This study
CMY8008	Isogenic to CMY1300 except *sse1Δ::Hyg^R*	This study
CMY8001	Isogenic to CMY1300 except *sti1Δ::Hyg^R*	This study
CMY8020	Isogenic to CMY1300 except *sse2Δ::Hyg^R*	This study
CMY8031	Isogenic to CMY1300 except *hch1Δ::Hyg^R*	This study
CMY8301	Isogenic to W303 except *AHA1/Myc3*	This study
CMY8305	Isogenic to CMY8301 except *sti1Δ::Hyg^R*	This study
5B6	*MATa ura3-52 leu2-3,112 his3-11 trp1Δ1 lys2 ssa1::HIS3 ssa2::LEU2 SSA3 ssa4::LYS2 hem1-Δ100 pGAL1-SSA1*	E. Craig, L. Zhang
BY4741	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Invitrogen
3012	Isogenic to BY4741 except *ydj1Δ::KanMX*	A. J. Caplan

Plasmids YCp50-MAL63 and YCp50-MAL43-C carry the inducible *MAL63* and constitutive *MAL*-activator gene *MAL43-C*, respectively, in vector YCp50 (GIBSON *et al.* 1997). Plasmid p416GPD from the MUMBERG *et al*. (1995) series contains *URA3* as the selectable marker but is otherwise the same as p414 (BALI *et al.* 2003). The 2kb *Sac*I – *Kpn*I fragment containing the entire GPDpromoter-*MAL63/HA3* tagged fusion gene was subcloned into plasmid p416 to create plasmid p416GPD-MAL63/HA3. An untagged version, referred to as p416GPD-MAL63, was created by an in-frame deletion of the *Not*I fragment encoding the sequence encoding triple-HA. W303 transformants carrying this plasmid induced maltase expression to normal levels and ferment maltose in 1 day.

Construction of *cdc37-ts* mutant: Plasmid pRS314.cdc37U (a gift from A. J. Caplan) carrying the Ser14Ala temperature sensitive alleles of *CDC37* was digested with *Bam*HI, *Hind*III and *Kpn*I and the digest used to transform strain W303. Ura+ transformants were tested for growth at 30°C and 37°C. Those colonies that failed to grow at 37°C were considered to be *cdc37-ts* (MANDAL *et al.* 2007).

Construction of the triple HA-tagged mutant *MAL*-activators: A PCR-based method 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 an upstream primer (5' GGG**GAATTC**CTTCCCTTCGGTGA ACAA3'), which anneals to *Eco*RI site at the codon 215/216 and downstream primer (5'GGG**GTCGAC**CCCGGGATCGATGTGAACAATAAA 3'), that inserts *Cla*I and *Sma*I sites immediately following the *mal63* termination codon and just before the *Sal*I cutting site, respectively. The *Eco*RI cutting site in the upstream primer and the *Sal*I site in the downstream primer are in bold type. The amplified 0.7 kb product 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. The *Cla*I and *Sma*I (underlined) sites were inserted for the purpose of diagnostic digestion. The constructs were confirmed by sequencing the full open reading frame.

Construction of a triple HA-tagged *MAL63(1-283)-T247A*: The upstream primer (5' GGG**GAATTC**CTTCCCTTCGGTGAACAA3') that contains an *Eco*RI site (in bold) at codons 215/216 of *MAL63* and the downstream primer (5'-CCCC**GTCGA C**TTACTTTCCTGGTATAGTGAA 3'), 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 a 200 base pair fragment using the constitutive mutant *MAL63-NS284-C* (GIBSON *et al.* 1997). The amplified product was digested with *Eco*RI and *Sal*I and inserted into the vector fragment of *Eco*RI and *Sal*I digested p416GPD-MAL63/HA3 to produce p416GPD-MAL63(1-283)-T247A/HA3.

Construction of 6His-tagged *SSA1* plasmids: The upstream primer (5' GGG **GGATCC**ATGCATCATCATCATCATCATGGGGGGTCAAAAGCTGTCGGTATTG AT 3') contains a *Bam*HI site (in bold) and anneals to the 5' end of the *SSA1* open reading frame. It places six histidine codons (underlined) and two glycine codons at the 5' end of *SSA1* creating a 6His tag at the N-terminal end of Ssa1 protein. The downstream primer (5' GGGCTCGAGTTAATCAACTTCTTCAACGGT 3') contains an *Xho*I site (in bold) and anneals to the 3'end of the *SSA1* open reading frame. The stop codon is underlined. This primer pair was used to amplify the *SSA1* open reading frame from the pGAL1-SSA1 plasmid carried by strain 5B6. The amplified product was digested with *Bam*HI and *Xho*I and subcloned into *Bam*HI and *Xho*I digested

p414GPD and p414TEF, both CEN vectors that contain *TRP1*, to create p414GPD-SSA1/6His and p414TEF-SSA1/6His (MUMBERG *et al.* 1995).

A 6His-tagged allele of *SSA1* expressed from its native promoter was constructed as follows. Upstream primer (5' GGG**GGATCC**ATGCATCATC ATCATCATCATGGGGGGTCAAAAGCTGTCGGTATTGAT 3') contains a BamHI site (in bold) and anneals to the 5' end of the *SSA1* open reading frame. It places six histidine codons (underlined) and two glycine codons in frame at the 5' end of *SSA1* creating a 6His tag at the N-terminal end of Ssa1 protein. The downstream primer (5' GG**GCATGC**GTTAGCGATAATCAAGAAGTG 3') contains a *Sph*I site (in bold) and anneals to the 3'end of the *SSA1* open reading frame. The stop codon is underlined. The *SSA1* promoter was amplified from the pGAL1-SSA1 plasmid carried by strain 5B6 using the following primer pair. Primer (5' GGG**GAGCTC**CAAAGGCTCGGTTG TCGACAA 3') contains a *Sac*I site (in bold) and anneals to a site approximately 600 basepairs upstream of the *SSA1* open reading frame. Primer (5' GGG**GGATCC**ATT ATCTGTTATTTACTTGAA 3') contains a *Bam*HI site (in bold) and anneals to a site immediately upstream of the *SSA1* open reading frame. The *SSA1* promoter was amplified using this PRO primer pair from the genome of S288C. The ORF amplified product was digested with *Bam*HI and *Sph*I and the PRO amplified product was digested with *Bam*HI and *Sac*I and the two digested products cloned into *Sph*I – *Sac*I digested pUN30 (ELLEDGE and DAVIS 1988). This created plasmid pUN30-SSA1/6His, a *TRP1* CEN plasmid.

Addition of a Myc epitope tag to *HSP82*, *SSA1*, and *STI1*: The Myc epitope consists of ten amino acids (EQKLISEEDL) derived from the protein sequence of the

human proto-oncogene 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*, *SSA1*, and *STI1* by a PCR-based technique described below thereby creating a C-terminal Myc-tagged allele expressed from the native promoter. Despite the fact that each gene is the sole copy of the chaperone or cochaperone present in the strain, the Myc-tagged strains do not exhibit any growth defects or defects in maltase expression (see data below) indicating that tags do not significantly alter the function of the tagged gene.

To construct *HSP82/Myc*, the *kan*^{R} gene cassette was amplified using the following primers. The upstream primer contains the following sequences in the order presented: 45bp upstream of the TAA stop codon of *HSP82*, 2 glycine codons, 30 bp of sequence encoding the Myc epitope, a stop codon, and an 18bp sequence complementary to the 5' end of the *kan*^{R} cassette (5' CCGGTTGAAGAGGTTCCAGCTGACACCGAAA TG GAAGAGGTAGATGGGGGGAACAAAAACTTATTTCTGAAGAAGATCTGTA G**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 the *kan*^{R} cassette (5'CATTGTAATGTTTTACCCAGTTATTTCCATGCAGATGCCCTATTT AC**GCATAGGCCACTAGTGGA** 3'). Bases homologous to the template are in bold. Plasmid pFA6-kanMX2 was used as a template to amplify the kanamycin (G418) resistance gene (WACH *et al.* 1994). The PCR product was transformed directly into strain W303 *hsc82*Δ *HSP82* and transformants were selected on YPD supplemented with 200 μg/ml geneticin (Cat. #10131-035 Invitrogen Life Technologies) (GULDENER *et al.* 1996). Epitope tagging of *HSP82* was confirmed by PCR and Western blotting. When

transformed with pUN30-MAL63, maltose-induced expression of maltase in the tagged strain, CMY1200, was not significantly different from that in the untagged parental strain (368 ± 38 versus 367 ± 57 units, respectively).

SSA1 and *STI1* were Myc-tagged by a similar procedure. For *SSA1*, the upstream primer (5'TCCAGCTCCAGAGGCTGAAGGTCCAACCGTTGAAGAAGTTGATGGG GGGGAACAAAAACTTATTTCTGAAGAAGATCTGTAA**CAGCTGAAGCTTCGT ACG** 3') and downstream primer (5'TTCCTCATTATACCCAGATCATTAAAAGACA TTTTCGTTATTATCAATTGC**GCATAGGCCACTAGTGGA**-3') were used to amplify the *kan*^{R} cassette. 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. When transformed with pUN30-MAL63, maltose-induced expression of maltase in the tagged strain, CMY1300, was not significantly different from that in the untagged parental strain (1201 ± 177 versus 1313 ± 203 units, respectively).

For *STI1*, the upstream primer (5'CAGACGTTGATCGCTGCTGGTATCA TCCGGACTGGCCGCGGGGGGGAACAAAAACTTATTATTTCTGAAGAAGATCT GTAA**CAGCTGAAGCTTCGTACG** -3') and downstream primer (5'TTTCCCTC GCCCAAGAAACTTATTATCTTCAAGTTCCGATTTCTCAAA**GCATAGG CCACTAGTGGA** -3') were used to amplify the *kan*^{R} cassette. The PCR product was transformed directly into strain W303. When transformed with pUN30-MAL63, maltose-induced expression of maltase in the tagged strain, CMY8015, was not significantly different from that in the untagged parental strain (1215 ± 190 versus 1240 ± 239 units, respectively).

Construction of the modified *kan*^{R} cassette containing a triple-Myc tag insert: A DNA fragment encoding three copies of the Myc-tag flanked at both ends by *Sal*I sites was inserted at the 5' end of the *kan*^{R}-containing cassette pFA6-KanMX2 as follows. Two partially overlapping and complementary primers were synthesized. Primer 1 (5'GGG **GTCGAC** GGG GGG GGG GAG CAG AAA CTC ATC TCT GAA GAA GAT CTG GAA CAA AAG TTG ATT TCA GAA GAA GAT CTG) consists of: a *Sal*I site (in bold) and three glycine codons followed by the sequence encoding two copies of the 10 residue Myc epitope tag, Myc-1 (dotted underline) and Myc-2 (solid underlined). Primer 2 (5' GGG **GTCGAC** *TTAATTAATTAA* CAG ATC TTC CTC AGA GAT GAG CTTCTG TTC CAG ATC TTC TTC TGA AAT CAA CTT TTG TTC 3') is complementary to and anneals to the Myc-2 sequence of Primer 1. Primer 2 consists of a *Sal*I site (in bold) and four stop codons (italics) followed by sequences encoding Myc-2 (solid underline) and Myc-3 (double underline). Primers 1 and 2 were annealed to one another and the 3' ends extended by one round of DNA synthesis in the absence of added template DNA. The double-stranded product was then amplified using primers that anneal to the ends of the fragment (5' GGG GTCGACGGGGGGGGGGAGCAGAAA 3' and 5' GGGGTCGACTTAATTAATTAA CAGATCTTC3'). The amplified product was digested with *Sal*I and cloned into *Sal*I digested pFA6-KanMX2 upstream of the *kan*^{R} gene (WACH *et al.* 1994). The sequence of the Myc insert region of the Myc3-Stop-*kan*^{R} cassette was confirmed by sequencing. The modified plasmid is referred to as pFA6-Myc3-Stop-kanMX2.

Addition of a triple-Myc epitope tag to genomic *AHA1*: A PCR-based one-step replacement method was used to insert the triple-Myc epitope tag at the C-terminus of

each gene. Plasmid pFA6-Myc3-Stop-kanMX2 was used as a template to amplify the *kan*^{R} gene with the upstream primer (5' CGTTCTATTAAATTGACATTCGGCTTTG GTGCCGTATTA**GGGGGGGGGGAGCAGAAACTC** 3') and downstream primer (5'TGGTAAATTCCGGGGAGGCCGTCGGCGCCGGGCCGCAAAAGT**AGGGCA TAGGCCACTAGTGGA** 3'). The upstream primer contains 38 bases upstream of but not including the TAA stop codon of *AHA1* and a 21 bases sequence complementary to the 5' end of the modified triple Myc-Stop-*kan*^{R} cassette. The downstream primer contains 40 basepairs from the 3' end of *AHA1* followed by 18 basepairs of sequence from the 3' end of the *kan*^{R} cassette. Bases homologous to the template are in bold. The PCR product was transformed directly into strain W303 and transformants were selected on YPD supplemented with 200mg/L geneticin (Cat. #10131-035 Invitrogen Life Technologies) (GULDENER *et al.* 1996). Epitope tagging of *AHA1* was confirmed by PCR and Western blotting analysis.

Construction of high-copy overexpression plasmid p423GPD-AHA1/Myc3: A two-step PCR procedure was used to amplify the open reading frame of *AHA1/Myc3* from strain CMY8301. In the first round, the upstream primer (5'CTTACTTTCG TTATTCC TTTCAGTCTTATT3') annealing to the *AHA1* promoter region and downstream primer (5' CGGATTCAGTCGTCACTCAT 3') annealing to a site 500 basepairs downstream of the genomic *AHA1/Myc3* of CMY8301. This PCR product was then used as the template in a second round of PCR to amplify the complete triple-Myc tagged ORF of *AHA1/Myc3* gene with the upstream primer (5'GGG**GGATCC**AT GGTCGTGAATAACCCA 3') containing a *Bam*HI site (in bold) and the downstream primer (5' GG**ATCGAT**TTAATTAATTAACAGATCTTC-3') containing a *Cla*I site (in

bold). The second PCR product was digested with *Bam*HI and *Cla*I and cloned into *Bam*HI and *Cla*I digested vector p423GPD. The construction was confirmed by sequencing and Western Blot analysis.

Construction of *sti1Δ*, *aha1Δ*, *sba1Δ*, *sse1Δ*, *sse2Δ*, *sro9Δ*, and *hch1Δ* by one-step gene replacement with *Hyg^R*: Deletion of *STI1* in strains CMY1200 and CMY1300 carrying Myc-tagged *HSP82/Myc* and *SSA1/Myc*, respectively, was complicated by the presence of the *kan*^{R} cassette at each of these tagged genes. One-step gene disruption to replace *STI1* with the hygromycin resistance gene *hyg^R* was accomplished by replacing the open reading frame of *STI1* with the coding region of *hyg^R* without including homologous sequences derived from the *kan*^{R} cassette. The upstream primer(5'GCCCAA AAGTCTGCTCCCAAATTCCTCACTGTAGCTACTAAAACAACCTATA CGCAA GAAAG**ATGGGTAAAAAGCCTGAACTCA** 3') consists of 62 bases of the *STI1* promoter sequence immediately upstream of the start codon (ATG) of *STI1* followed by 22 bases of the *hyg^R* open reading frame (boldface). The downstream primer (5' ATGTATGAAAAAGCAGTAAAAAAAGAATTCAAGATAATAAAGTTAT ATTTCGTATTATTT**TTATTCCTTTGCCCTCGGACG** 3') consists of 60 bases of sequence homologous to the region immediately downstream of *STI1* and 21 bases homologous to the 3' end of the *hyg^R* open reading frame (boldface) including the stop codon. This primer pair was used to amplify the *hyg^R* open reading frame from the template plasmid pAG32 (GOLDSTEIN and MCCUSKER 1999). The PCR product was transformed directly into strains CMY1200 and CMY1300 and hygromycin resistant transformants were selected on YPD supplemented with 200 μg/ml hygromycin (Cat.# 10687-010 Invitrogen Life Technologies). Constructs were confirmed by PCR and

Western blotting. The other deletion strains derived from CMY 1200 and CMY130 were constructed by the similar procedure using the primer pairs listed in the Table 3.

Maltase activity: Extracts were prepared and maltase activity was measured in whole cell extracts as described in HU *et al.* (1999) and DUBIN *et al.* (1985). Activity is expressed as nmoles of PNPG (p-nitrophenol-α-glucopyranoside) hydrolyzed per minute per mg of protein.

β-Galactosidase assay: β-Galactosidase was assayed in strains bearing both the rat glucocorticoid receptor-containing plasmid p413GPDGR and the *lacZ* reporter gene plasmid pYRP-G2lacZ (MORANO *et al.* 1999). Cells were grown in selective minimal medium to mid-logarithmic phase (OD600 of 0.2-0.5) and glucocorticoid receptor expression induced by the addition of DOC (21-hydroxyprogesterone, Sigma Cat. # D6875) to a final concentration of 20 μM. After a one-hour induction, cells were collected by filtration and total extracts prepared for β-galactosidase assay as described in HU *et al* (1999).

Preparation of cell extracts, immunoblot analysis, and coimmunoprecipitation: Strains were grown in the appropriate selective minimal medium to mid-logarithmic phase (OD_{600} of 0.2-0.5). Denaturing cell extracts were prepared as described in BALI *et al*. (2003). 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 quantified using software provided by the manufacturer. Anti-Myc antibody was obtained from Roche Applied Science. PGK (phosphoglycerol kinase) was detected by anti-PGK antibody from Molecular Probes.

Table 3
Primers used for construction of *Hyg^R* deletion mutations

Gene deletion	Primer sequence (5' to 3')
aha1Δ::HygR	**Upstream**: 5'CTTACTTTCGTTATTCCTTTCAGTCTTATTCTTAATCGTTTATAGTAGCAACAATATATCAAT**ATGGGTAAAAAGCCTGAACTA** **Downstream**: 5'TATGCCAATAGTGGTATGTAAATATTTACGCATACTTTTATTGAAACATGAGAACAATATATC**TTATTCCTTTGCCCTCGGACG**
sba1Δ::HygR	**Upstream**: 5'GAAACCTAGTGGACTCAATTCATTACAACAACAAGTTCCCAAGATCATCGATTCATAATAGTC**ATGGGTAAAAAGCCTGAACTCA** **Downstream**: 5'CATATATTCGTCTTTTATTCTAAGTTACTCATTCTAGCACTCCAGGTTGATTTGCTCCTCCTTT**TATTCCTTTGCCCTCGGACG**
sse1Δ::HygR	**Upstream**: 5' CTTATTTTTAACCTCGATAGCCATAAGCAAAAAGTACATTGACAAACAACATTTCTTTAAAAG**ATGGGTAAAAAGCCTGAACTCA** **Downstream**: 5'GTGAGTTAAAAAAATCGGAAAAAACAATAAAGATCCTTTTCTAGTTACTTTGCTGCATTAACA**TTATTCCTTTGCCCTCGGACG**
sse2Δ::HygR	**Upstream**: 5'TAAAACTTCCTTTTTTTTTTTTTTTACCTGTAACAGACGTAACCAAAGGATATAATATA**ATGGGTAAAAAGCCTGAACTCA** **Downstream**: 5'ACTAGTGGTTCATTTAAGAAAGAATAAAGAGGGAACAATCCAAATAGACAAAAATTCCGA**TTATTCCTTTGCCCTCGGACG**
sro9Δ::HygR	**Upstream**: 5'GAACATCAGGATTACTCTGGACCTGCTAACGTGTTTCATCTTCTTTTCACTTCTCTGCCCACG**ATGGGTAAAAAGCCTGAACTCA** **Downstream**: 5'ATGGTTTTTTGTGTAAAATGCAATGAACGAATCCATTTTTTATTTTGCAAGTGTGAGAGGCCT**TTATTCCTTTGCCCTCGGACG**

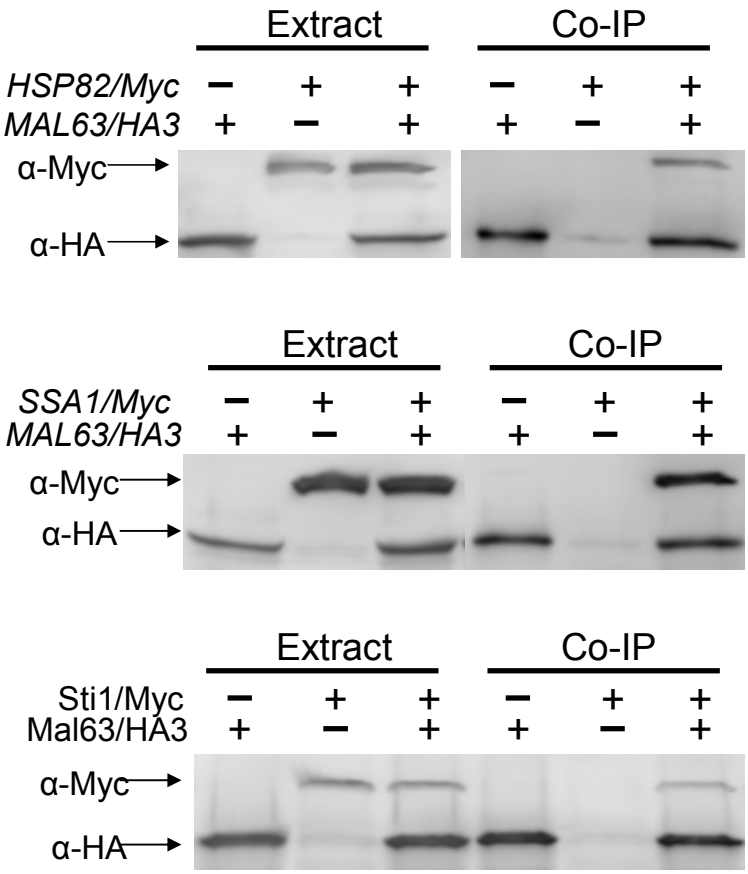
Table 3 (Continued)
Primers used for construction of *Hyg*^{R} deletion mutations

sti1Δ::HygR	**Upstream**: 5’GCCCAAAAGTCTGCTCCCAAATTCCTCACTGTAGCTACTA AAACAACCTATACGCAAGAAAG**ATGGGTAAAAAGCCTGA** **ACTCA** **Downstream**: 5’ATGTATGAAAAAGCAGTAAAAAAAGAATTCAAGATAAT AAAGTTATATT TCGTATTATTT**TTATTCCTTTGCCCTCGGACG**
hch1Δ::HygR	**Upstream**: 5’CATCAAACTACTACTAAAGTTTGAAACGATCGGAAAGTT ACAACACATTTACGATAAAAT**ATGGGTAAAAAGCCTGAAC** **TCA** **Downstream**: 5’AAAATTAAAAGGGGCGGTGGTCTATCTATGCA ACGCTCCCCTTTTCGTTACATGAACACA**TTATTCCTTTGCCC** **TCGGACG**

Nondenaturing total cell extracts were prepared as follows. An aliquot of the culture containing about 20 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^{o}C for at least 20 minutes. Cells were resuspended in 1 ml of a non-denaturing extraction buffer containing 50 mM sodium molybdate, 20 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 1mM EDTA plus a cocktail of protease inhibitors (Roche, complete, mini, EDTA-free Protease Inhibitor tablets (Cat # 1836170), Sigma Yeast Protease Inhibitor Cocktail (Cat # P8215), and a toothpick tip-full of sodium bisulfite), and flash-frozen in liquid nitrogen. Protein extracts were made via glass bead lysis. Protein concentration of the cell extract was determined by the Lowry assay method and extraction buffer added to equalize protein concentration and volume. A sample was removed for Western blot analysis (referred to as Extract) as described above.

Immunoprecipitation was carried out as follows. Approximately 200 µl of lysate was then combined with 30-40 µl of anti-HA agarose slurry (Roche Anti-HA Affinity Matrix, Cat.# 11 815 01 6001) and incubated with gentle mixing at 4°C overnight. The beads were washed 3 times with 300 µl of cold extraction buffer, 20 µl of cold extraction buffer and 40 µl of 2X sample buffer were added to the beads, and the suspension incubated at 75°C in a heating block for 15 minutes to extract the bead-bound protein. Samples were analyzed by Western blot analysis probed with anti-HA antibody and anti-Myc antibody (Roche) and the signals quantified as described above. The results in Figure 4 demonstrate that the Myc-tagged proteins encoded by *SSA1/Myc*, *HSP82/Myc*, and *STI1/Myc* do not bind to the anti-HA agarose beads in the absence of HA-tagged *MAL*-activator.

Figure 4: Myc-tagged Hsp82, Ssa1, and Sti1 do not immunoprecipitate in the absence of HA-tagged Mal63. Strains carrying the genomic Myc-tagged alleles and the parental strains lacking the Myc-tagged *HSP82* (strains hsc82Δ and CMY1200), *SSA1* (strains JN516 and CMY1300); and *STI1* (strains W303 and CMY8015) were transformed with plasmid p416GPD-MAL63/3HA carrying the triple-HA tagged *MAL63* or p416GPD-MAL63 carrying the untagged *MAL63*. Transformants were grown to mid-log at 30ºC in selective synthetic medium lacking uracil and containing 3% glycerol, 2% lactate. Nondenaturing protein extracts were prepared and incubated with anti-HA bound agarose beads as described in Materials and Methods. Co-immunoprecipitation (Co-IP) samples and the total cell extracts from which they were prepared were analyzed by Western blotting using anti-HA and anti-Myc antibodies. Experiments were done at least in triplicate and representative blots are shown.


Extract
Co-IP
HSP82/Myc
MAL63/HA3
α-Myc
α-HA
Extract
Co-IP
SSA1/Myc
MAL63/HA3
α-Myc
α-HA
Extract
Co-IP
Sti1/Myc
Mal63/HA3
α-Myc
α-HA

Chapter 1

Hsp90/Hsp70 chaperone machine regulation of the *Saccharomyces MAL*-activator as determined *in vivo* using noninducible and constitutive mutant alleles

(Chapter 1 was published in the May 2008 issue of *Genetics* largely as presented here. The full reference is: Ran, F., M. Bali and C. A. Michels, 2008 Hsp90/Hsp70 chaperone machine regulation of the *Saccharomyces MAL*-activator as determined *in vivo* using noninducible and constitutive mutant alleles. Genetics **178:** In press. The work in chapter 1 was carried out by me except for the following. Mehtap Bali constructed the plasmids carrying the triple-HA tagged alleles of *MAL63* and the various *MAL*-activator mutant alleles.)

INTRODUCTION

Hsp70 and Hsp90 are essential molecular chaperones that work together as a "relay team" in the activation, folding, subcellular localization, and maturation of selected, so-called client proteins. This process is referred to as the Hsp70/Hsp90 chaperone machine (reviewed in (BUKAU *et al.* 2006); (CAPLAN *et al.* 2007); (PICARD 2006a); (PRATT and TOFT 1997); (PRATT and TOFT 2003); (WEGELE *et al.* 2004)). Hsp90 client proteins fall chiefly into two categories, kinases and DNA-binding transcription activators. Thus, Hsp90 plays a major role in regulating intracellular signaling and gene expression. The chaperone activity of Hsp90 is unique in that it acts on nascent, partially folded client proteins that are recruited by the sequential activity of Hsp40 and Hsp70. Hsp70/Hsp90 chaperones function in the cytosol, endoplasmic reticulum, and mitochondrion. Compartment specific isoforms of Hsp70 and Hsp40 have been characterized in eukaryote species and, in *Saccharomyces*, fourteen genes encode various members of the Hsp70 family. In addition to Hsp70 and Hsp90, a number of cochaperones facilitate aspects of the process including stimulation of chaperone ATPase activity, nucleotide exchange, or client protein binding. Genetic methods have been particularly useful in identifying Hsp90 cochaperones and, while the function of some cochaperones is well understood, the role of others remains unclear. The results presented here lay the groundwork for studies of cochaperone function *in vivo* using a native client protein.

Perhaps the most extensively studied Hsp90 client is the glucocorticoid receptor, a DNA-binding transcription activator and member of the steroid receptor family (reviewed in (PRATT *et al.* 2004); (PRATT *et al.* 1996); (PRATT *et al.* 2006); (PRATT and TOFT 2003);

(WEGELE *et al.* 2006)). Minimal requirements for the sequential Hsp70/Hsp90 chaperone activation of glucocorticoid receptor have been defined, largely based on *in vitro* studies with purified proteins, but significant insights have come from studies of other steroid receptors. Nascent receptor is first recognized by Hsp40, which mediates receptor association with Hsp70 to form the "early complex". Transfer to Hsp90 occurs in the "intermediate complex" consisting of receptor, Hsp90, Hsp70, Hsp40, and Hop, a scaffold protein that facilitates intermediate complex formation by binding both Hsp90 and Hsp70. Lastly, p23 and an immunophilin replace Hop and Hsp70 to form the so-called "final complex". Glucocorticoid receptor is held in the final complex in an inactive conformation capable of binding hormone with high affinity. Hormone binding causes release of the activated receptor and dissociation of the final complex components. Thus, Hsp90 is both required for and a repressor of glucocorticoid receptor activation. *In vivo* studies of glucocorticoid receptor and other mammalian clients such as v-Src are frequently carried out in *Saccharomyces*, a heterologous host that may exhibit significant differences compared to the native mammalian host cell, such as diversity in the classes and function of the encoded cochaperones.

In vivo studies of Hsp90/Hsp70 chaperone regulation of Hap1p, the *Saccharomyces* heme-responsive transcription activator, revealed significant differences compared to the glucocorticoid receptor. Hap1 associates with Hsp70 in cells grown in both the presence and absence of the inducer heme while Hsp90 binding is transient and only enhanced in the presence of heme (LAN *et al.* 2004). Hsp70, as opposed to Hsp90, is the negative regulator of Hap1p. Moreover, Hap1p binds DNA while still bound to Hsp70 (LAN *et al.* 2004; XIN *et al.* 2007). When compared with the studies of the

glucocorticoid receptor, these finding reveal an unexpected plasticity in the fundamental regulatory roles of the chaperones with regard to these two transcription activators and underscores the importance of extending studies of the Hsp90/Hsp70 chaperone machine to other transcription activator clients *in vivo* in the native host cell. To this end, this report investigates the pathway by which Mal63p, a maltose inducible *Saccharomyces MAL*-activator, is regulated by the Hsp90/Hsp70 chaperone machine.

MAL63 encodes the *MAL*-activator from *MAL6* (HU *et al.* 1999). Mal63p is a DNA-binding transcription activator required for the maltose-dependent induction of the *MAL* structural genes encoding maltose permease and maltase. Mal63 protein is an Hsp90 client protein (BALI *et al.* 2003). Mal63p is sensitive to degradation in cells depleted of Hsp90 and binds to Hsp90 *in vivo*, both hallmarks of Hsp90 chaperone client proteins. A wide variety of mutant *MAL*-activator alleles is available with different regulatory phenotypes including inducible, noninducible, super-inducible, and constitutive *MAL*-activator alleles. This situation is unique among Hsp90 client proteins and provides a rich resource with which to explore the mechanism by which Hsp90/Hsp70 chaperone regulates another member of the transcription activator class of client proteins in the native host.

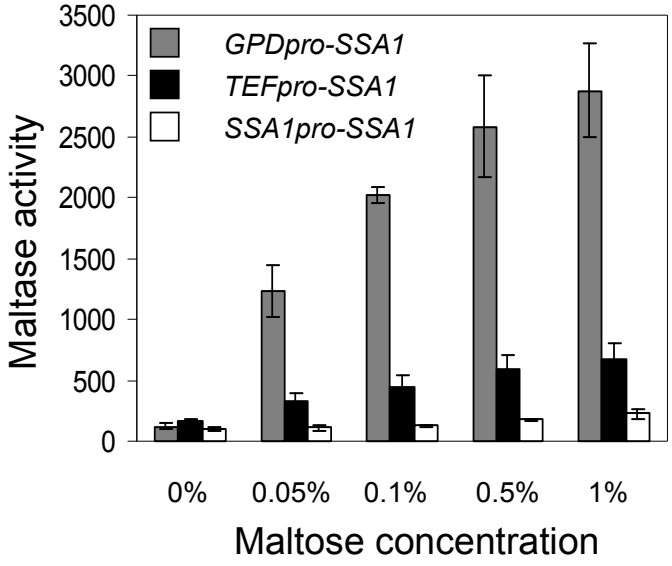
The analysis reported here is similar to a substrate-dependent epistasis analysis except that the various alleles of the *MAL*-activator substrate progress differently through the Hsp90 chaperone pathway forming distinct chaperone complexes. These findings allow us to propose the pathway for inducible *MAL*-activator activation via the Hsp90/Hsp70 chaperone cycle. Activation of the *MAL*-activator appears to parallel that observed *in vitro* for the glucocorticoid receptor, at least with regard to the major

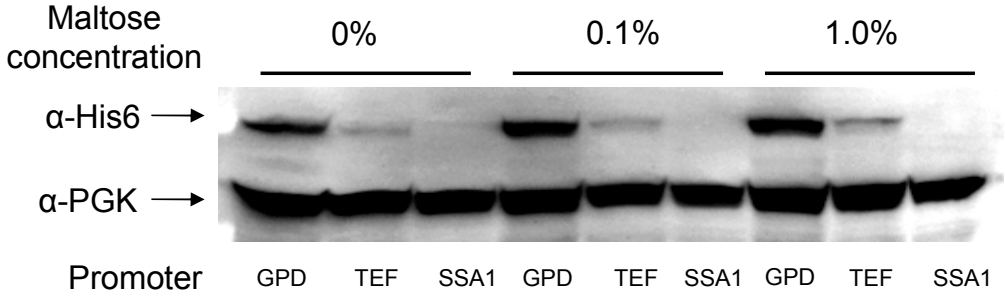
Saccharomyces chaperone components Ssa1 (cytosol Hsp70 homologue), Sti1 (Hop homologue), and Hsp82 (Hsp90 homologue). In the absence of inducer maltose, inducible Mal63p forms a stable complex with Ssa1, Hsp82, and Sti1. Results reported here and in BALI *et al*. (2003) show that optimal maltose induction is dependent on all three chaperone complex components indicating that all play a positive role in Mal63p induction. However, we find that *MAL*-activator constitutivity is correlated with a loss of Ssa1 binding and reduced dependency on Ssa1 suggesting that, in contrast to glucocorticoid receptor but similar to Hap1p, Ssa1 has a second role as the negative regulator of inducible Mal63 *MAL*-activator. This study highlights the need to study a variety of different transcription activator clients of the Hsp90/Hsp70 chaperone machine. Moreover, it lays the foundation for future *in vivo* analyses of the component composition of different client protein-containing complexes and provides the reagents needed to explore the role of the cochaperones in client protein activation *in vivo*.

RESULTS

Strains with reduced Hsp70 expression exhibit defects in maltase expression: Bali *et al.* (2003) reported that very low concentrations of maltose restricted growth of strains defective for Hsp90 molecular chaperone complex, including the temperature sensitive strain *hsc82Δ hsp82-T101I* and the double null strain *hsc82Δ cpr7Δ*. Induction of maltase expression in these strains requires higher maltose concentrations and does not achieve wild-type induced levels (data not shown). Figure 5 explores the role of Ssa1 in *MAL*-activator regulation. *SSA1-4* encoding *Saccharomyces* Hsp70 are essential (BECKER *et al.* 1996). A series of strains expressing varying levels of Ssa1 protein were

Figure 5: Maltase expression is dependent on Ssa1 chaperone. Plasmid series p414GPD-SSA1/6His, p414TEF-SSA1/6His, and pUN30-SSA1/6His, expressing 6His-tagged *SSA1/6His* from the GPD, TEF, and native *SSA1* promoters were used to replace the pGAL1-SSA1 plasmid of strain 5B6 (*ssa1::HIS3 ssa2::LEU2 SSA3 ssa4::LYS2 pGAL1-SSA1*). This strain series was transformed with plasmid pUN90-MAL63/HA. Transformants were grown at 30ºC in selective synthetic medium lacking uracil and tryptophan and containing 3% glycerol and 2% lactate (vol/vol) plus the indicated concentration of maltose. 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 (upper panel). Assays were carried out on at least three independent transformants. The error bars indicate standard deviation from three independent experiments done in duplicate. Western blot analysis was carried out as described in Methods and Materials on total cell extracts prepared from transformants grown at the indicated maltose concentration (lower panel). Blots were probed with anti-6His antibody and anti-PGK antibody was used as a loading control. Analysis was carried out on three independent transformants; a representative blot is shown.


3500
3000
2500
2000
1500
1000
500
0
Maltase activity
GPDpro-SSA1
TEFpro-SSA1
SSA1pro-SSA1
0%
0.05%
0.1%
0.5%
1%
Maltose concentration


Maltose concentration
0%
0.1%
1.0%
α-His6
α-PGK
Promoter
GPD
TEF
SSA1
GPD
TEF
SSA1
GPD
TEF
SSA1

constructed that express a plasmid-borne N-terminal tagged *SSA1/6His* gene fused to the GPD, TEF, and native *SSA1* promoters in a strain lacking genomic Ssa1,2,4 expression (*ssa1Δ ssa2Δ SSA3 ssa4Δ*). The impact of reduced Ssa1 levels on *MAL63*-dependent maltase expression was assayed (Figure 5). The turnover rate and expression level of Mal63/HA3p was determined in the strains carrying the GPD- and TEF-expressed *SSA1* and no effect of the reduced level of Hsp70 observed (data not shown).

Maximal *SSA1* expression is observed from the GPD promoter and expression from the *SSA1* promoter is the lowest of the three constructs (lower panel), although growth rates are not particularly affected (data not shown). Decreased expression of Ssa1p severely impacts maltase expression at all inducer concentrations and maltase activity correlates with the level of Ssa1p expression (upper panel). While *SSA3* expression may be up regulated in response to the extremely low levels of *SSA1* expression from the construct with its native promoter, it clearly is not sufficient to complement the defect in *SSA1* expression, a finding consistent with the fact that strains carrying only *SSA3* are inviable (BECKER *et al.* 1996). We conclude that Ssa1 is required for *MAL*-activator activation and plays a positive role in Mal63 *MAL*-activator activation.

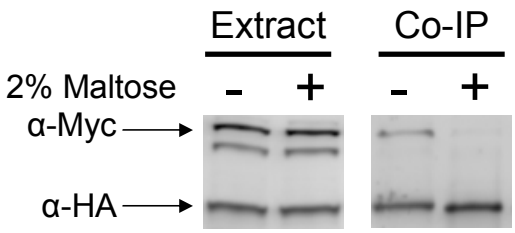
Binding of inducible Mal63/3HA *MAL*-activator to Hsp90 and Hsp70 is regulated by maltose: The results in Figure 5 predict that the *MAL*-activator should be found in association with Hsp70. Bali *et al.* (2003) demonstrated that Mal63/3HA *MAL*-activator co-precipitates with Hsp90 but in this study the His-tagged *HSP82/6His* was over-expressed from the GPD promoter and was carried on a multi-copy plasmid. We decided to explore *MAL*-activator interaction in strains expressing Ssa1 and Hsp82 at normal levels from their native promoters. For this, the genomic copy of *SSA1* and

HSP82 was tagged in strains JN516 and hsc82Δ, respectively. The tag was placed at the C-terminus of the open reading frame by a one-step gene insertion. The *SSA1/Myc* and *HSP82/Myc* gene in each strain is the sole source of the essential chaperone and appears by several measures to be fully functional. Both Myc-tagged strains grow normally and express maltase at rates comparable to the untagged parent (see Materials and Methods). Additionally, glucocorticoid receptor activation is unaffected in the strain containing *SSA1/Myc* as the sole source of this Hsp70 (K. Morano, unpublished results). The C-terminal TPR-binding EEVD domains of Hsp90 and Hsp70 are essential for interaction with Sti1 (BRINKER *et al.* 2002; ODUNUGA *et al.* 2003; SCHEUFLER *et al.* 2000). Our results indicate that, despite the proximity of the Myc epitope to the EEVD domain and its location at the C-terminus, the Myc-tag does not interfere in any measurable way with Sti1 interaction. Moreover, because *HSP82/Myc* and *SSA1/Myc* are carried in the genome and expressed from their native promoter, expression levels should be relatively constant from cell to cell. Thus, we are able to detect differences in the amount of Mal63/3HA activator bound with Myc-tagged chaperone by quantifying the amount of chaperone that co-precipitates with the Mal63/3HA activator and normalizing the intensity of the Myc signal to the HA signal. Control experiments shown in Figure 4 demonstrate that neither Myc-tagged Hsp82/Myc or Ssa1/Myc proteins bind to the HA-sepharose beads in the absence of HA-tagged Mal63 *MAL*-activator.

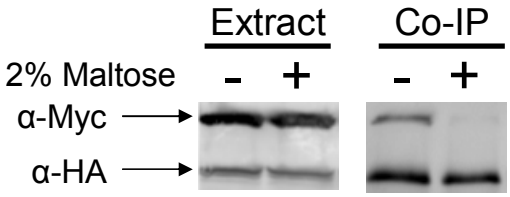
Plasmid-born *MAL63/3HA* was co-expressed in strains containing the genomic Myc-tagged *HSP82/Myc* or *SSA1/Myc* and binding of Mal63 protein to the chaperones determined by co-immunoprecipitation of the chaperone with the triple-HA tagged Mal63p. As can be seen in Figure 6, Mal63/3HA *MAL*-activator binds to Ssa1 and Hsp82

Figure 6: Co-immunoprecipitation of inducible Mal63/3HA *MAL*-activator protein with Ssa1 and Hsp82. Strains CMY1200 (*HSP82/Myc hsc82*Δ) (upper panel) and CMY1300 (*SSA1/Myc ssa2*Δ *ssa3*Δ *ssa4*Δ) (lower panel) were transformed with the plasmid p416GPD-MAL63/3HA harboring the triple HA-tagged inducible *MAL63 MAL*-activator gene. Transformants were grown to mid-log at 30ºC in selective synthetic medium lacking uracil and containing 3% glycerol, 2% lactate, with or without 2% maltose. Nondenaturing protein extracts were prepared as described in Materials and Methods. Co-immunoprecipitation (Co-IP) samples and the total cell extracts from which they were prepared were analyzed by Western blotting using anti-HA and anti-Myc antibodies. Experiments were done at least in triplicate and representative blots are shown.

Co-IP Mal63/3HAp and Hsp82/Myc



Co-IP Mal63/3HAp and Ssa1p/Myc



when cells are grown under uninduced conditions. It should be noted that expression of Mal63/3HA and the chaperones does not appear to be affected by growth in the presence or absence of maltose. In contrast to the results with cells grown in uninduced conditions, association of the chaperones with Mal63/3HA is dramatically reduced by growth in the presence of 2% maltose. This result is consistent with our finding that Ssa1 plays a positive role in *MAL*-activator activation. Moreover, it indicates that growth under induced conditions leads to the release of the activator from its association with both Ssa1 and Hsp82 chaperones.

Noninducible Mal63/3HA mutant *MAL*-activator proteins are defective in their ability to bind to Hsp90: Given these results for inducible Mal63 *MAL*-activator, we asked if mutant *MAL*-activators exhibited differences in their interactions with Hsp70, Hsp90, and other components of the chaperone complex and were these differences reflected in their mutant phenotype. For this, we used a variety of noninducible and constitutive *MAL*-activator mutants characterized by GIBSON *et al.* (1997) and DANZI *et al.* (2003,2000). Table 4 lists the phenotype and the mutant alterations of each compared to inducible Mal63 *MAL*-activator. DANZI *et al.* (2003) isolated the following noninducible *MAL*-activator alleles by charged-cluster to alanine mutagenesis: *mal63-283*, *mal63-331*, *mal63-364*, *mal63-401*, and *mal63-467A9N*. Noninducible *mal63-467S9V* was obtained by random mutagenesis of the C-terminal codons of *MAL63* (DANZI *et al.* 2003).

Triple HA-tagged alleles of each of the noninducible *mal63* mutants were co-expressed in strains carrying genomic Myc-tagged *HSP82/Myc* and *SSA1/Myc* and interaction between the mutant mal63 proteins and the chaperones determined. The

Table 4
***MAL*-activator mutant alleles used in this study**

MAL-activator allele	Mutant alterations compared to Mal63p	Reference
Inducible		
MAL63	None	Kim and Michels (KIM and MICHELS 1988)
Non-inducible		
mal63-283	K283A, D287A	Danzi *et al*. (DANZI *et al.* 2000)
mal63-331	R331A, R335A	Danzi *et al*. (DANZI *et al.* 2000)
mal63-364	E364A, R367A	Danzi *et al*. (DANZI *et al.* 2000)
mal63-391	K391A, E394A	Danzi *et al*. (DANZI *et al.* 2000)
mal63-401	D401A, K405A	Danzi *et al*. (DANZI *et al.* 2000)
mal63-467A9N	D467A, I469N	Danzi *et al*. (DANZI *et al.* 2000)
mal63-467S9V	D467S, I469V	Danzi *et al*. (DANZI *et al.* 2000)
mal63-block3	P362L, R367K, G371D, T373I, K378N	Danzi *et al*. (DANZI *et al.* 2003)
Constitutive		
MAL43-C	31 alterations compared to Mal63p, 27 of which lie in residues 238-461 of	Gibson *et al*. (GIBSON *et al.* 1997)
MAL63/43-C	27 alterations compared to Mal63p mapping to residues 238-461	Gibson *et al*. (GIBSON *et al.* 1997)
MAL63/23-SVI	S392A, V395I, I402V	Danzi *et al*. (DANZI *et al.* 2003)

Table 4 (Continued)
***MAL*-activator mutant alleles used in this study**

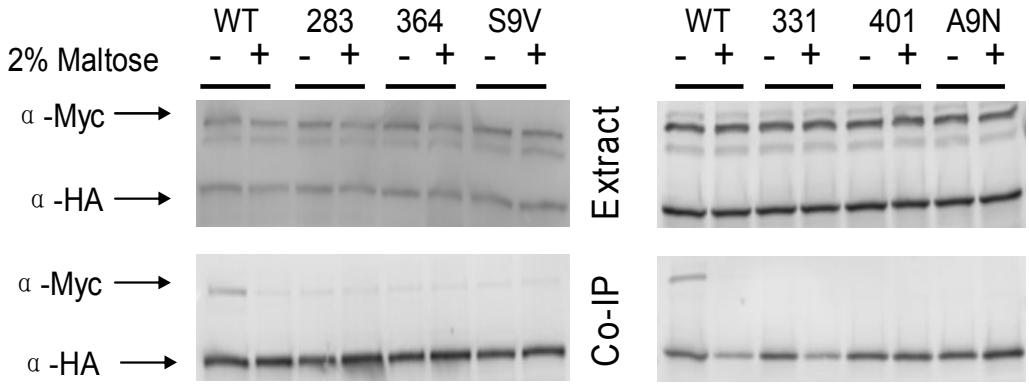
MAL63-block2	L343F, H344Q, K346N, M348T, R349K, M350Δ, N351Δ, L353F, T356A, and T359A	Danzi *et al*. (DANZI *et al.* 2003)
MAL63-NS284-C	C284NS, T247A	Gibson *et al*. (GIBSON *et al.* 1997)

results in Figure 7 demonstrate that the noninducible *MAL*-activator mutant proteins are expressed at levels comparable to inducible Mal63p but exhibit severe defects in their ability to bind Hsp90. The noninducible *MAL*-activator mutant proteins form complexes with Ssa1 chaperone apparently normally. That is, the noninducible mutant proteins bind to Ssa1/Myc chaperone in cells grown in uninduced but not in induced conditions. In contrast, the noninducible mutant activators do not form a complex with Hsp82/Myc even in cells grown in uninduced conditions. It should be noted that the noninducible mutants used in this study map to sites spanning nearly the full length of the regulatory region of Mal63p from residues 283 to 467 and each mutation alters 2-3 residues within a 5-residue window. Thus, it is unlikely that this lack of Hsp82 binding could be explained by a nonspecific phenomenon such as the gross misfolding of the entire region. We conclude, consistent with the findings of BALI *et al*. (2003), that association with Hsp90 is required for *MAL*-activator activation and that, for the mutants studied here, the noninducible phenotype results from an inability to form the intermediate complex and bind Hsp90 chaperone. Additionally, this result indicates that binding of the *MAL*-activator to Hsp70 occurs independent of stable Hsp90 binding and prior to association with Hsp90 in an intermediate complex.

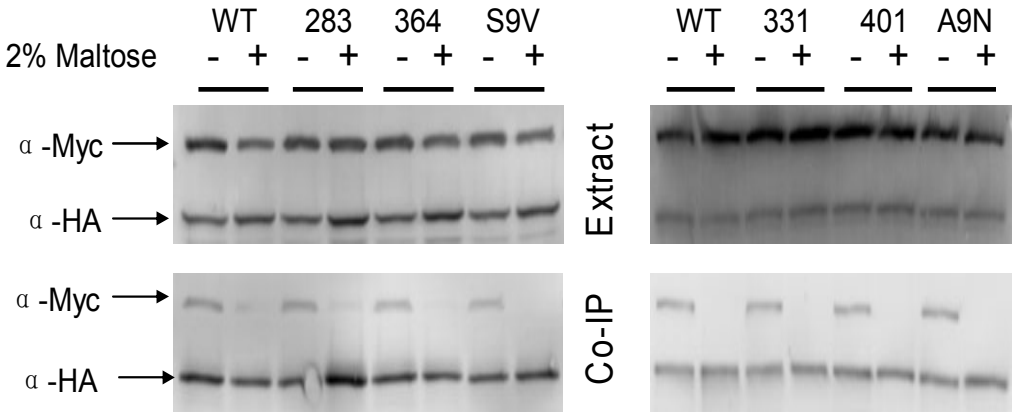
Constitutive *MAL*-activators require Hsp90 but not Hsp70: Strains defective for Hsp90 and Hsp70 were used to determine whether constitutive *MAL*-activator alleles are dependent on Hsp90 and Hsp70, as is seen for inducible *MAL63*. Constitutive *MAL*-activator mutants activate high-levels of *MAL* gene expression in the absence of inducer maltose, even in the presence of glucose (GIBSON *et al.* 1997). *MAL63/43-C* is a hybrid *MAL*-activator containing the first 215 codons of *MAL63* fused to codons 216-470 of

Figure 7: Co-immunoprecipitation of non-inducible *MAL*-activator mutant alleles with Ssa1 and Hsp82. Strains CMY1200 (*HSP82/Myc hsc82*Δ) (upper panel) and CMY1300 (*SSA1/Myc ssa2*Δ *ssa3*Δ *ssa4*Δ) (lower panel) were transformed with plasmids p416GPD-mal63-283, p416GPD-mal63-331, p416GPD-mal63-364, p416GPD-mal63-401, p416GPD-mal63-467S9V, and p416GPD-mal63-467A9N harboring the triple HA-tagged alleles of the non-inducible *MAL*-activator mutant genes (DANZI *et al.* 2003). Transformants were grown as described in Figure 3. Preparation of nondenaturing extracts, co-immunoprecipitation, and Western blot analysis were carried out as described for Figure 6.

Co-IP of noninducible mutant Mal63/3HAp and Hsp82/Myc



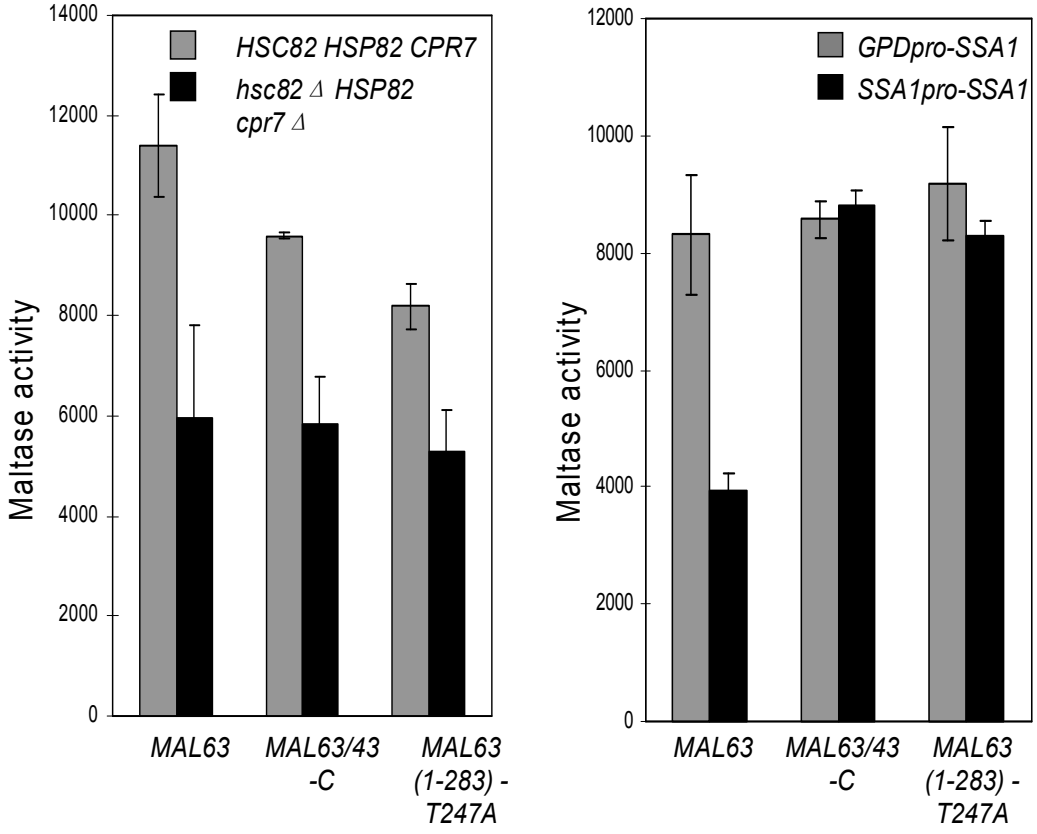
Co-IP of noninducible mutant Mal63/3HAp and Ssa1p/Myc



MAL43-C (GIBSON *et al.* 1997). Mal63p and Mal43-Cp encode 470-residue proteins that differ at 31 residues, 27 of which are in the C-terminal region of the Mal63/43-C fusion protein from residues 238 - 461. Both *MAL43-C* and *MAL63/43-C* exhibit similar, if not identical, phenotypes. Of these 27 alterations, 10 and 8, respectively, are found clustered into two putative negative regulatory domains in residues 343-359 and 419-461 and either cluster of alterations is sufficient to produce the constitutive phenotype when introduced into inducible Mal63p (DANZI *et al.* 2000). *MAL63(1-283)-T247A* was isolated by random mutagenesis of the noninducible nonsense mutation *mal63/NS284* and contains a single base change, T247A, that converts it to a constitutive activator (GIBSON *et al.* 1997).

To test dependence on Hsp90, plasmid constructs carrying *MAL63* or the two constitutive mutant genes expressed from the high-level GPD promoter were introduced into the strains W303 (*HSC82 HSP82*) and hsc82Δ cpr7Δ (*hsc82Δ HSP82 cpr7Δ*) and the affect on maltase expression determined (Figure 8, left panel). All three transformed strains expressed very high levels of maltase approximately 5- to 8-fold higher than the induced levels of maltase expression in strains carrying the inducible *MAL63* expressed from its native promoter. This is likely due to the high level of *MAL*-activator expression from the GPD promoter. Nonetheless, maltase expression is decreased approximately 30 to 50% in the double deletion strain *hsc82*Δ *cpr7*Δ carrying either *MAL63* or the constitutive mutant *MAL*-activator alleles. Comparable results were obtained using the *MAL63* or *MAL43-C* genes expressed from their native promoters in this Hsp90 defective strain (data not shown). Thus, *MAL* gene activation by both the inducible and constitutive *MAL*-activators is similarly dependent on Hsp90 chaperone activity.

Figure 8: Maltase expression in strains carrying constitutive *MAL*-activators is dependent on Hsp90 but not Hsp70. Strains W303 (*HSC82 HSP82*) and hsc82Δ cpr7Δ (*hsc82Δ HSP82 cpr7Δ*) (left panel) were transformed with plasmids p416GPD-MAL63/3HA, carrying the triple HA-tagged inducible *MAL63* or p416GPD-MAL63/43-C and p416GPD-MAL63(1-283)-T247A, carrying the triple HA-tagged constitutive *MAL*-activator mutant genes *MAL63/43-C* and *MAL63(1-283)-T247A*, respectively (Danzi *et al.* 2000; Gibson *et al.* 1997). The strains expressing *SSA1* from the GPD and native *SSA1* promoters described above in Figure 2 were transformed with plasmids p416GPD-MAL63/3HA, p416GPD-MAL63/43-C, and p416GPD-MAL63(1-283)-T247A (right panel). Transformants were grown at 30ºC in selective synthetic medium lacking the appropriate nutrients for plasmid selection and containing 3% glycerol and 2% lactate (vol/vol) with 1% maltose added to transformants carrying *MAL63/3HA*. Maltase activity was assayed on at least three independent transformants as described in Figure 5.

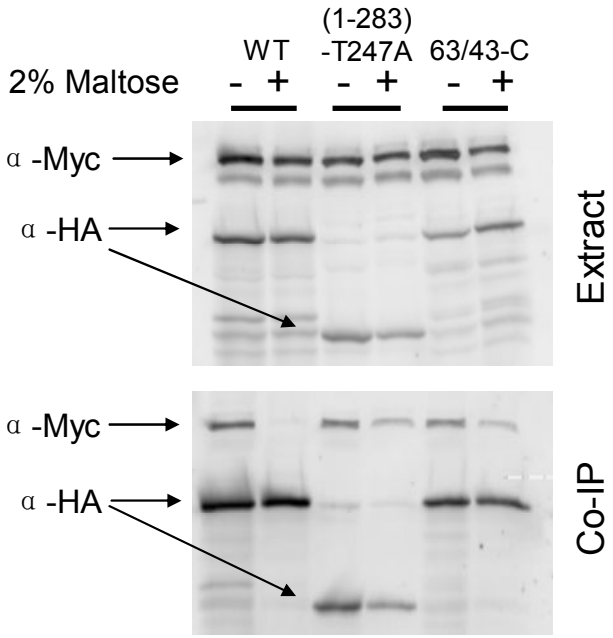

14000
12000
10000
8000
6000
4000
2000
0
Maltase activity
HSC82 HSP82 CPR7
hsc82 Δ HSP82
cpr7 Δ
MAL63
MAL63/43 -C
MAL63 (1-283) - T247A
12000
10000
8000
6000
4000
2000
0
Maltase activity
GPDpro-SSA1
SSA1pro-SSA1
MAL63
MAL63/43 -C
MAL63 (1-283) - T247A

Dependency of the constitutive *MAL*-activators on Hsp70 was tested using the strain series expressing different levels of *SSA1/His* described above. Much to our surprise, maltase expression in the strains carrying the constitutive *MAL*-activator alleles is not affected by reduced Ssa1 expression while inducible maltase expression is approximately 50% reduced in the strain with low levels of Ssa1. A more pronounced effect on maltase expression is observed in Figure 5 where Mal63p is expressed at a lower level from its native promoter suggesting that the apparent independence from Hsp70 of the constitutive alleles may be buffered somewhat by high *MAL*-activator expression but similar results were obtained with the native *MAL43-C* carried on a CEN plasmid (data not shown). Nonetheless, these results suggest that the constitutive *MAL*-activators are significantly less dependent than inducible Mal63p on Hsp70 for activation/maturation. Based on the findings shown in Figure 8 we investigated constitutive *MAL*-activator binding to Hsp70 and Hsp90.

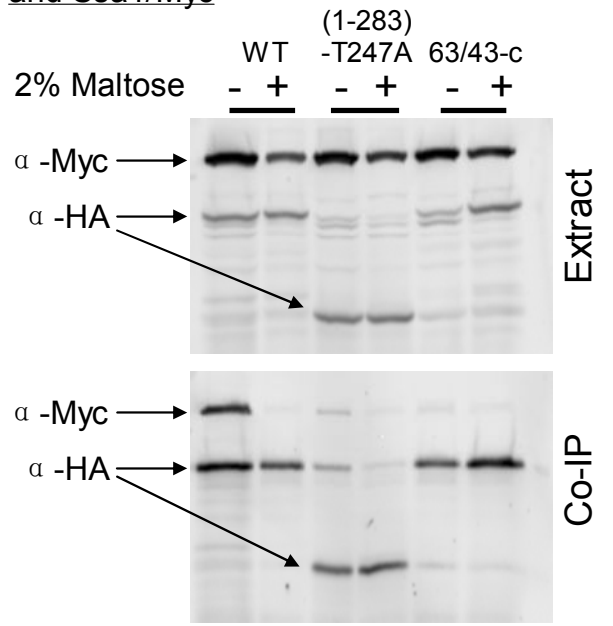
Altered binding of constitutive *MAL*-activator mutant proteins to Hsp70: The constitutive *MAL*-activators encoded by *MAL43/63-C* and *MAL63(1-283)-T247A* were tested for their ability to associate with Hsp70 and Hsp90. The results are shown in Figure 9. Consistent with their dependency on Hsp90, both of the constitutive *MAL*-activator proteins bind Hsp82/Myc. The finding that the truncated Mal63(1-283)-T247A protein binds well to Hsp90 indicates that the full length protein is involved in this interaction not just the C-terminal regulatory region. Some residual binding of Hsp82/Myc with the constitutive *MAL*-activator proteins is sometimes observed even in cells grown in maltose, although this is usually quite modest (see also Figure 11). The results for Hsp70 binding are quite different but again consistent with the apparent lack of

Figure 9: Co-immunoprecipitation of constitutive *MAL*-activator mutant proteins with Ssa1 and Hsp82. Strains CMY1200 (*HSP82/Myc hsc82*Δ) (upper panel) and CMY1300 (*SSA1/Myc ssa2*Δ *ssa3*Δ *ssa4*Δ) (lower panel) were transformed with the plasmids p416GPD-MAL63/43-C and p416GPD-MAL63(1-283)-T247A harboring the triple HA-tagged constitutive *MAL*-activator mutant genes *MAL63/43-C* and *MAL63(1-283)-T247A*. Transformants were grown as described in Figure 6. Preparation of nondenaturing extracts, co-immunoprecipitation, and Western blot analysis were carried out as described for Figure 6.

Co-IP of constitutive Mal63/HA3 mutants and Hsp82/Myc



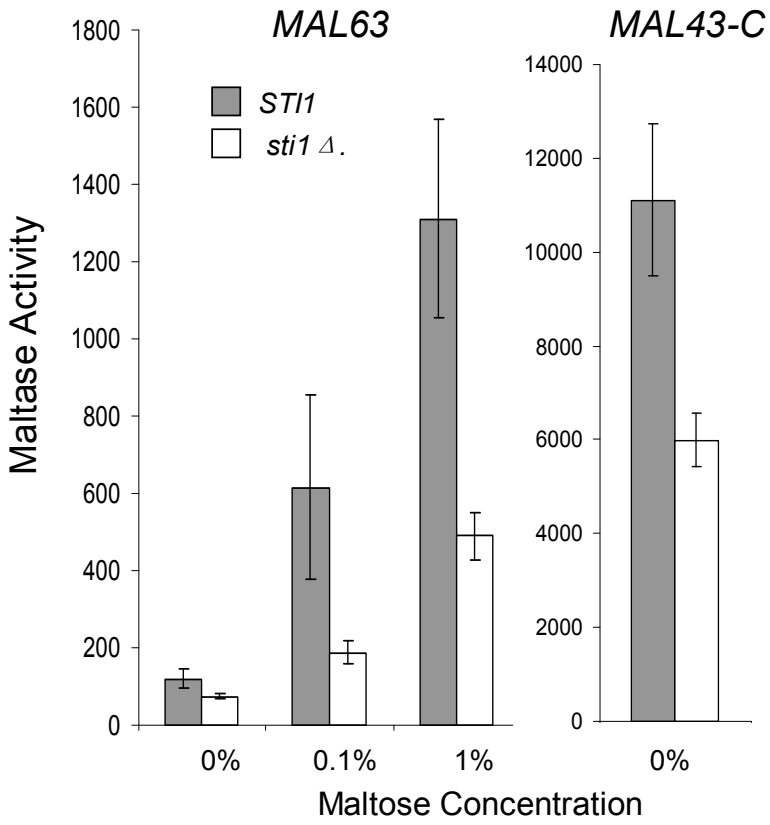
Co-IP of constitutive Mal63/HA3 mutants and Ssa1/Myc



dependency on Hsp70 reported in Figure 8. Binding of the constitutive *MAL*-activator proteins to Ssa1/Myc is very weak or nonexistent, even in extracts prepared from cells grown in uninduced conditions, and addition of maltose to the growth medium further reduces association of the constitutive activators to Ssa1/Myc Hsp70 (see also Figure 11). Therefore, in the absence of inducer, constitutive activators form a stable complex containing Hsp82 but not Ssa1.

Constitutive *MAL*-activator is dependent on *STI1*: The finding that constitutive *MAL*-activators exhibit very modest levels of binding to Ssa1 (Figure 9) and are independent of Ssa1 levels (Figure 8) suggests the possibility that constitutive mutant proteins might bypass Hsp70 and, instead, bind to Hsp90 without the participation of Hsp70 or the formation of complexes containing cochaperones such as Sti1. We tested this possibility by investigating the effect of the loss of *STI1* on *MAL*-activator chaperone binding. *STI1* encodes the *Saccharomyces* Hop/p60 homologue (CHANG *et al.* 1997). Sti1 functions as a scaffold protein that binds to Hsp70 and Hsp90 promoting the formation of the so-called intermediate complex and stimulating transfer of the client protein from Hsp70 to Hsp90 (reviewed in (PRATT and TOFT 2003); (WEGELE *et al.* 2004)). Null mutations of *STI1* are viable but loss of Sti1 reduces glucocorticoid receptor activity in *Saccharomyces* and increases its binding to Hsp40 (Ydj1) (CHANG *et al.* 1997). *STI1* was deleted in the strain carrying *HSP82/Myc* and maltase expression assayed in transformants carrying plasmid-borne inducible *MAL63* or constitutive *MAL43-C*. Loss of Sti1 causes an approximate 50% decrease in maltase activity in transformants carrying either allele suggesting that the inducible and the constitutive activators both require Sti1(Figure 10). *HSP82/Myc* and *SSA1/Myc* strains containing *sti1Δ* were transformed

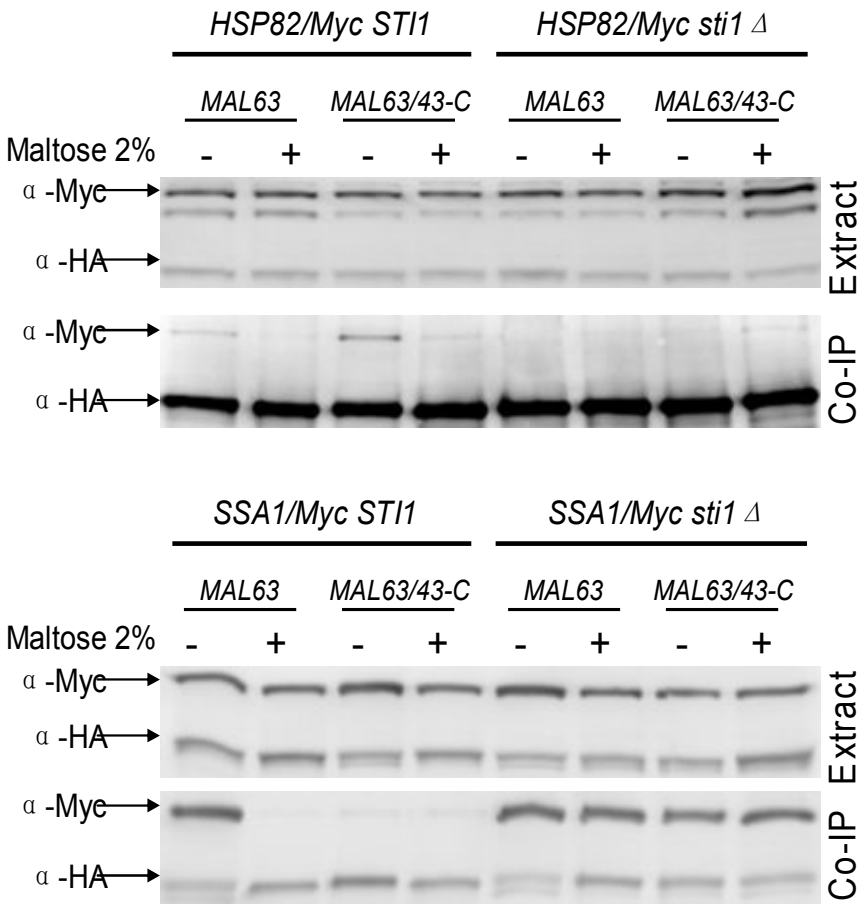
Figure 10: Maltase expression is dependent on *STI1*. Strains CMY1200 (*STI1 HSP82/Myc hsc82*Δ) and CMY8001 (*sti1Δ HSP82/Myc hsc82*Δ) were transformed with plasmid YCp50-MAL63 or YCp50-MAL43-C carrying the inducible *MAL63* and constitutive *MAL43-C MAL*-activator alleles, respectively. Transformants were grown at 30ºC in selective synthetic medium lacking uracil and containing 3% glycerol and 2% lactate (vol/vol) plus the indicated concentration of maltose. Maltase activity was assayed in three independent transformants as described in Figure 5.


MAL63
MAL43-C
STI1
sti1 Δ.
Maltase Activity
1800
1600
1400
1200
1000
800
600
400
200
0
14000
12000
10000
8000
6000
4000
2000
0
0%
0.1%
1%
0%
Maltose Concentration

with triple-HA tagged *MAL63* or *MAL63/43-C* and binding of the chaperones to the HA-tagged *MAL*-activator determined. The results, shown in Figure 11, are consistent with our finding that maltase expression in the constitutive mutant is dependent on Sti1. Binding of both inducible Mal63 and constitutive Mal63/43-C *MAL*-activators to Hsp82/Myc protein is severely reduced, almost absent, in the *sti1Δ* strain even when grown in the absence of maltose, the condition in which maximal Hsp82/Myc binding is observed in the *STI1* strain. On the other hand, loss of Sti1 dramatically increases binding of both inducible Mal63 and constitutive Mal63/43-C *MAL*-activators to Ssa1 to comparable levels and binding is observed in cells grown in either uninduced or induced conditions. These results indicate that the constitutive *MAL*-activators bind with Hsp70 during Hsp90 chaperone activation but only transiently. We propose that the alterations present in the constitutive activators weaken binding to Hsp70 and this results in the premature dissociation of the intermediate complex formed by constitutive activators. The weakened Ssa1 binding also appears to result in an apparent loss of dependency on Hsp70.

The results reported in Figures 7 and 9 predict that neither the noninducible nor the constitutive mutant activators would be expected to stably bind Sti1. To investigate this, plasmid-borne inducible *MAL63/3HA*, constitutive *MAL63/43-C/3HA*, and noninducible *mal63-401/3HA* were co-expressed in a strain containing the C-terminal Myc-tagged *STI1/Myc* and binding of the various *MAL*-activators to Sti1/Myc determined by co-immunoprecipitation. Control experiments shown in Figure 4 demonstrate that Sti1/Myc protein does not bind to the HA-sepharose beads in the absence of HA-tagged

Figure 11: Effect of *sti1Δ* on the co-immunoprecipitation of inducible Mal63/3HA *MAL*-activator with Ssa1 and Hsp82. Strains CMY1200 (*STI1 HSP82/Myc hsc82*Δ), CMY8001 (*sti1Δ HSP82/Myc hsc82*Δ), CMY1300 (*STI1 SSA1/Myc ssa2*Δ *ssa3*Δ *ssa4*Δ), and CMY8002 (*sti1Δ SSA1/Myc ssa2*Δ *ssa3*Δ *ssa4*Δ) were transformed with plasmids p416GPD-MAL63/3HA or p416GPD-MAL63/43-C carrying triple HA-tagged *MAL63* and *MAL63/43-C*, respectively. Transformants were grown as described in Figure 3. Preparation of nondenaturing extracts, co-immunoprecipitation, and Western blot analysis were carried out as described for Figure 6.


HSP82/Myc STI1
HSP82/Myc sti1 Δ
MAL63
MAL63/43-C
MAL63
MAL63/43-C
Maltose 2%
- + - + - + - +
α -Myc
α -HA
Extract
α -Myc
α -HA
Co-IP
SSA1/Myc STI1
SSA1/Myc sti1 Δ
MAL63
MAL63/43-C
MAL63
MAL63/43-C
Maltose 2%
- + - + - + - +
α -Myc
α -HA
Extract
α -Myc
α -HA
Co-IP

Mal63 *MAL*-activator. Because *STI1* expression is low, the Myc signal is weak. Nonetheless, as is shown in Figure 9, inducible Mal63 *MAL*-activator binds Sti1/Myc protein in cells grown in uninduced but not induced (maltose) conditions. Along with the results reported in Figure 6, this supports our proposal that inducible Mal63 activator forms a stable intermediate complex in the absence of inducer. Neither the constitutive nor the noninducible mutant *MAL*-activators binds significant levels of Sti1/Myc, even in cells grown in the absence of inducer, suggesting that neither of these mutant activator proteins is found in the so-called intermediate complex.

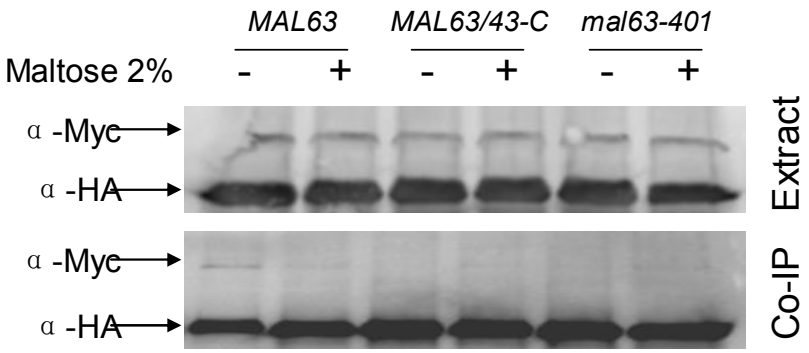
DISCUSSION

This report investigates the roles of Hsp90, Hsp70, and Sti1 in the regulation of the *Saccharomyces* the *MAL*-activator, a DNA-binding transcription activator. Inducible *MAL63* and several noninducible and constitutive *MAL*-activator mutant alleles from our collection are utilized (CHARRON and MICHELS 1987; DANZI *et al.* 2003; DANZI *et al.* 2000; GIBSON *et al.* 1997; NEEDLEMAN 1991). The availability of these *MAL*-activator mutants is unique among Hsp90 client proteins and provides a novel tool to investigate regulation of a specific native client protein *in vivo*. The results demonstrate the formation of distinct stable chaperone complexes with the different classes of *MAL*-activator proteins (inducible, noninducible, and constitutive). Our analysis allows us to propose the *in vivo* pathway of *MAL*-activator regulation by the Hsp70/Hsp90 chaperone machine.

Activation of inducible Mal63 *MAL*-activator by the Hsp70/Hsp90 chaperone machine exhibits significant similarities to the activation process described for other clients but with a few important distinctions. As for other clients, Hsp70 is clearly

required for Mal63 *MAL*-activator activation (Figure 5). However, in the absence of inducer, the stable chaperone complex formed by Mal63 is different from others that have been described in the literature. Under uninduced conditions, inducible Mal63 *MAL*-activator forms a stable complex with Ssa1, Hsp82, and Sti1 (Figures 6 and 12) in what appears to be an "intermediate complex". This contrasts with glucocorticoid receptor which, in the absence of hormone, forms a stable so-called "final complex" with Hsp90, the cochaperone p23, and immunophilin (reviewed in (LAN *et al.* 2004); (PRATT and TOFT 2003); (WEGELE *et al.* 2004); (WEGELE *et al.*2006)). Alternately, the *Saccharomyces* heme-responsive transcription activator Hap1p is bound to Hsp70 in both the absence and presence of heme but is only transiently associated with Hsp90, although Hsp90 binding is enhanced in the presence of heme (LAN *et al.* 2004). Thus, the stable chaperone complexes formed by these three transcription activators are distinct with regard to their component composition. We also found that growth in maltose-induced conditions leads to a dramatic reduction in the relative binding of Mal63p to both chaperones and to Sti1. Similarly, hormone releases glucocorticoid receptor from Hsp90 and allows it to enter the nucleus and bind DNA. In the case of Hap1p, Hsp70 but not Hsp90 is reported to bind to Hap1p-dependent promoters along with Hap1p (XIN *et al.* 2007). Thus, the interactions of *MAL*-activator with the chaperones are similar to those reported for other transcription activator clients but each has unique variations on the common theme. Given that only a small number of transcription activator clients have been studied in detail, our findings suggest that there is a greater variability in the functioning of the components of Hsp90/Hsp70 chaperone machine and that the roles

Figure 12: Co-immunoprecipitation of inducible, constitutive, and noninducible *MAL*-activators with Sti1. Strain CMY8015 (*STI1/Myc*) was transformed with plasmids p416GPD-MAL63/3HA, p416GPD-MAL63/43-C, and p416-mal63-401 carrying the triple HA-tagged alleles of inducible *MAL63*, constitutive *MAL63/43-C*, and noninducible *mal63-401*. Transformants were grown as described in Figure 6. Preparation of nondenaturing extracts, co-immunoprecipitation, and Western blot analysis were carried out as described for Figure 6.


MAL63
MAL63/43-C
mal63-401
Maltose 2%
- + - + - +
α -Myc
α -HA
Extract
α -Myc
α -HA
Co-IP

played by different chaperone and cochaperones with different clients may be more flexible than has been anticipated.

Support for the hypothesis that *MAL*-activator association with Ssa1 and Hsp82 is sequential and in this order comes from studies with the various *MAL*-activator mutants. Noninducible mutants all exhibit normal binding to Ssa1, that is, they bind in uninduced growth conditions and not in induced growth conditions (Figure 7). On the other hand, binding to Hsp82 or Sti1 is not observed under any growth condition (Figures 7 and 12). This suggests that the noninducible mutant proteins are capable of forming the so-called early complex and of being released from Hsp70 in response to inducer but are not able to progress beyond the early complex to form a stable Hsp90-containing intermediate complex. It would appear that sequences required for recognition of the *MAL*-activator by Hsp70 are still functional in the noninducible mutant proteins but sequences required for recognition as an Hsp90 client are altered or unavailable.

The suggestion that inducible Mal63 *MAL*-activator is transferred from the early complex to the intermediate complex comes from the results in Figure 11. These results show that loss of Sti1 leads to Mal63 and Hsp70 binding in the presence of inducer, most likely by inhibiting the formation of the intermediate complex and thereby blocking the normal activation pathway. Additionally, deletion of *STI1* significantly enhances binding of the constitutive *MAL*-activators to Ssa1. This finding, taken together with the dramatic loss of Hsp82 – constitutive *MAL*-activator interaction observed in the *sti1Δ* strain, clearly suggests that the constitutive activator mutants utilize the same chaperone activation pathway as inducible Mal63p. That is, constitutive activators initially bind to Ssa1 and then form an intermediate-like complex with Hsp82, Ssa1, and Sti1, albeit

transiently. Rather than being retained in this intermediate complex, Ssa1 and Sti1 are released thereby allowing the constitutive activators to form a stable complex with Hsp82 alone. Whether this Hsp82 – constitutive activator complex is a final complex similar to that formed by glucocorticoid receptor remains to be determined and will require the analysis of the complex for the presence of other components, such as the cochaperones Sba1 (p23) and the immunophilin Cpr7.

We propose that Ssa1 binding is required to retain the inducible activator in the intermediate complex in the absence of inducer. Moreover, we suggest that the alterations in the constitutive activators weaken Ssa1 binding and facilitate disruption of the intermediate complex formed by constitutive activators without the requirement for inducer. The correlation between decreased Ssa1 binding and constitutivity indicates that, in addition to its positive role in *MAL*-activator regulation, Ssa1 also serves as a repressor of inducible Mal63 *MAL*-activator in the absence of maltose. It is important to note that the constitutive activators bind Hsp82 in cells grown in the absence of inducer, that growth condition in which, by definition, these constitutive activator alleles are active. It is not apparent from our data whether all of the constitutive activator protein is bound to Hsp82 or whether the Hsp82-bound form is capable of DNA-binding, as is seen for Hap1p and Hsp70 (XIN *et al.* 2007).

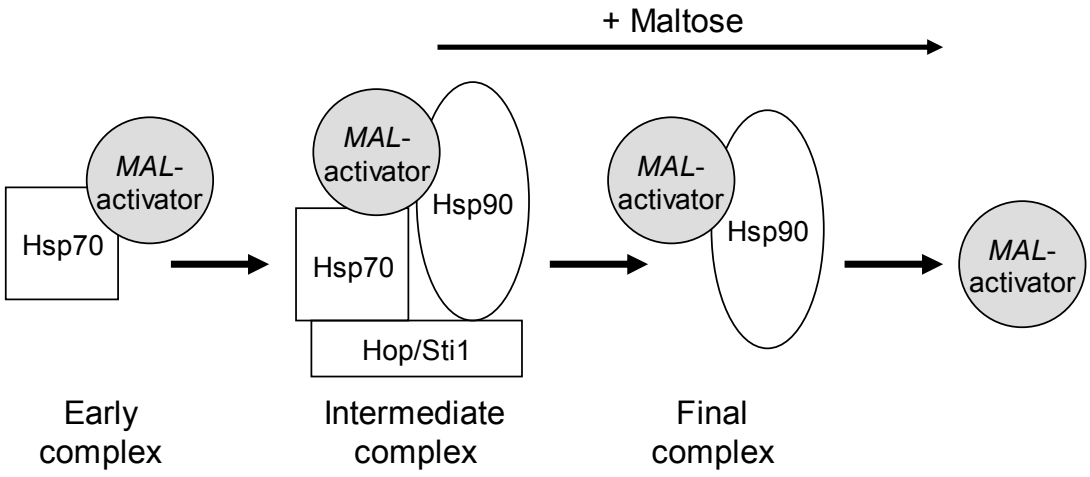
The results above have several interesting implications. Hsp70-bound *MAL*-activator is capable of maltose sensing even when bound in the early complex. This is consistent with the mutation analysis of DANZI *et al*. (2003) which suggests that the maltose-sensing domain of Mal63p is in residues 244-316. All of the noninducible mutants tested here except for *mal63-283* map outside of this region. Additionally, this

finding suggests that Sti1 alone is not sufficient to promote formation of the intermediate complex and raises the possibility that other cochaperones or sequences within the client protein itself are also involved (CHANG *et al.* 1997). Cdc37p is required in the transfer of kinase clients to Hsp90. *CDC37* overexpression suppresses defects in v-Src kinase activation in a *sti1Δ* strain (LEE *et al.* 2002) and the Cdk4 kinase – Cdc37p – Hsp90 complex has been well characterized by X-ray crystallography (VAUGHAN *et al.* 2006). The cochaperones that play this role for other classes of Hsp90 clients remain to be identified, although Aha1p has been suggested as a possible candidate (HAWLE *et al.* 2006).

Three negative regulatory domains were identified by DANZI *et al.* (2000). Interestingly, these map in close proximity to sites at which alterations cause a noninducible phenotype and inhibit Hsp90 binding (Figure 7). Additionally, DANZI *et al.* (2000) found that very slight changes, even single residue variations, in the C-terminal regulatory domain of Mal63p can convert a constitutive mutant into an inducible allele. These results indicate that multiple, noncontiguous sites in the *MAL*-activator interact with Hsp70 and Hsp90. It appears that alterations in a few critical residues can disrupt or restore binding to the chaperones and impact phenotype in very complex ways. These findings are reminiscent of studies reported for the Src-family kinase Lck which appears to bind to Hsp90 via multiple secondary structure sequences (PRINCE and MATTS 2004). In contrast, the Hsp90-binding site of the glucocorticoid receptor is localized to a relatively defined region of the hormone-binding domain (DALMAN *et al.* 1991; HOWARD *et al.* 1990).

Figure 13 summarizes our current model for the regulation of the *MAL*-activator

Figure 13: Model of *MAL*-activator regulation by the Hsp90/Hsp70 chaperone cycle.


+ Maltose
MAL-
activator
Hsp70
MAL-
activator
Hsp90
Hsp70
Hop/Sti1
MAL-
activator
Hsp90
MAL-
activator
Early
complex
Intermediate
complex
Final
complex

by the Hsp90/Hsp70 chaperone cycle. Binding to Hsp70 is the first step in *MAL*-activator activation but, in uninduced growth conditions, inducible Mal63 *MAL*-activator quickly binds with Hsp90 and Sti1 to form a stable intermediate complex. Binding to Hsp70 in this intermediate complex represses the *MAL*-activator in a conformation that maintains its ability to respond to maltose. Thus, Hsp70 is both required for and is a repressor of *MAL*-activator activation, the role played by Hsp90 in glucocorticoid activation. Addition of maltose causes release of inducible *MAL*-activator in a chaperone-free DNA-binding competent form. While we have no evidence that maltose binds directly to the *MAL*-activator, some of our findings are consistent with this possibility. In summary, while the key players in the chaperone cycle for the *MAL*-activator are the same as those for glucocorticoid receptor and Hap1p, their regulatory function and the components present in the stable complexes formed in the uninduced condition differ substantially. We are currently extending the *in vivo* analysis of the *MAL*-activator chaperone cycle described here to include other *Saccharomyces* cochaperones.

Chapter 2

Aha1 cochaperone is a negative regulator of *Saccharomyces* *MAL*-activator and acts early in its activation by the Hsp90/Hsp70 chaperone machine

INTRODUCTION

Client protein folding and activation by the Hsp90 chaperone machine utilizes a number of associated factors called cochaperones that function to facilitate chaperone activity by regulating Hsp90 ATPase activity, Hsp70 – Hsp90 interaction, and client protein binding (CAPLAN 2003). Hsp90 cochaperones fall into two categories, those containing a tetratricopeptide repeat (TPR) domain (including Hop/Sti1p) and all others (including p23/Sba1p, p50/Cdc37p, and Aha1p). Several of the Hsp90 cochaperones appear to be involved in the Hsp90 chaperone cycle for all types of client proteins. For others, different client proteins appear to require different cochaperone components. For instance, the binding of protein kinase clients to Hsp90 requires Cdc37 cochaperone, which is specific for this class of client protein (MANDAL *et al.* 2007). Moreover, recent studies suggest that particular cochaperones may function differently with different client proteins. Aha1 cochaperone (activator of Hsp90 ATPase) has been reported to be a positive regulator of a number of Hsp90 client proteins including glucocorticoid receptor and v-Src (HARST *et al.* 2005; PANARETOU *et al.* 2002). On the other hand, the ER-regulated secretory pathway of mammalian CFTR (Cystic Fibrosis Transportation conductance Regulator), a putative chloride channel protein mutated in individuals with cystic fibrosis, is negatively regulated by Aha1 cochaperone (WANG *et al.* 2006). Downregulation of Aha1 cochaperone expression rescues the secretory failure of mutant CFTR and enhances localization of mutant CFTR to the plasma membrane. Clarification is needed as to the role played by various Hsp90 cochaperones and how this role differs for different client proteins.

Hsp90 has three functional domains and the various cochaperones bind to the different domains to carry out their function (BUKAU *et al.* 2006; PEARL and PRODROMOU 2000; PEARL and PRODROMOU 2001; PEARL and PRODROMOU 2006; PRATT *et al.* 2004; PRATT *et al.* 2006; WEGELE *et al.* 2004; WEGELE *et al.* 2006). The N-terminal domain of Hsp90 contains the essential ATPase activity. Cochaperone p23/Sba1 binds to the N-terminal domain to client protein- and ATP-bound Hsp90, and appears to inhibit the ATPase activity (HARST *et al.* 2005; MARTINEZ-YAMOUT *et al.* 2006; MAYER *et al.* 2002; SILIGARDI *et al.* 2002). The active form of Hsp90 is a homodimer and dimerization utilizes the C-terminal domain. The C-terminal domain also contains the TPR-binding domain of Hsp90 and is responsible for interactions with Hop/Sti1p, a scaffold protein that facilitates Hsp70 and Hsp90 interaction, and other regulators of Hsp90 function such as the PP5 phosphatase and immunophilins including *Saccharomyces* Cpr6,7 that have peptidyl prolyl isomerase activity (reviewed in (CAPLAN 2003)) . The middle domain appears to be the most complex and responsible for client protein binding and interactions with certain cochaperones including Cdc37p and Aha1p. Activation of GR and v-Src is differentially defective in strains expressing middle domain mutations in Hsp82 suggesting that the middle domain of Hsp90 acts as a "discriminator" of different client proteins (HAWLE *et al.* 2006). Crystallographic analysis of the Cdc37p/Cdc2 kinase/Hsp90 complex demonstrates that the middle domain is the binding site for this kinase client (VAUGHAN *et al.* 2006).

AHA1 is a homologue of *Saccharomyces HCH1*, which was isolated as a high-copy suppressor of the growth defect of a temperature-sensitive mutation in the Hsp90 middle domain, *hsp82-E381K* (NATHAN *et al.* 1999). The 350-residue Aha1 protein

exhibits 36% sequence similarity to Hch1p within its N-terminal region (to residue 153). An Hch1p homologues is found in *Candida albicans* but Aha1p appears to be a member of a ubiquitous family of eukaryotic cochaperones (PANARETOU *et al.* 2002). *Saccharomyces* strains lacking *HCH1*, *AHA1*, or both are viable but growth is temperature sensitive, particularly in nonfermentable carbon sources. Aha1 cochaperone expression is up-regulated in geldanamycin treated and heat stressed cells and binds to Hsp90, both general characteristics of an Hsp90 cochaperone (PANARETOU *et al.* 2002).

In *Saccharomyces*, loss of Aha1 cochaperone causes defects in the activation of the known Hsp90 client proteins GR and v-Src (LOTZ *et al.* 2003; MEYER *et al.* 2003; PANARETOU *et al.* 2002). Similarly, downregulation of Aha1 expression via siRNA in mammalian cells leads to significant decreases in hormone-dependent GR activation (HARST *et al.* 2005). The N-terminal domain of Aha1 cochaperone binds to the middle domain of Hsp90 (MARTINEZ-YAMOUT *et al.* 2006; MEYER *et al.* 2003). Additionally, Hch1p and the N-terminal domain of Aha1p *in vitro* activate Hsp90 ATPase activity (PANARETOU *et al.* 2002). Based on these findings, Aha1 cochaperone is suggested to function as a positive regulator of the Hsp90 chaperone machine.

Chapter 2 explores the role of Aha1 cochaperone in the activation of a native *Saccharomyces* client protein, the activator of the *MAL* genes for maltose fermentation. The analysis builds on results reported in Chapter 1 that show that different *MAL*-activator alleles form different Hsp90 complexes in the absence of inducer and that the Hsp90/Hsp70 cycle for the *MAL*-activator is comparable to that described for GR, at least with regard to the chaperones Hsp70 and Hsp90 and the cochaperone Hop/Sti1p. Chapter 1 reports that noninducible mutant *MAL*-activators form an apparent early complex with

Hsp70, inducible Mal63p forms an intermediate complex with Hsp70, Hsp90, and Sti1, and constitutive *MAL*-activators bind with Hsp90 alone in an apparent final complex. Thus, the different *MAL*-activator alleles can be used to distinguish steps in the Hsp90/Hsp70 chaperone cycle. Chapter 2 shows that Aha1p is a negative regulator of inducible Mal63 *MAL*-activator, interacts genetically with the C-terminus of the *MAL*-activator, and acts early in the Hsp90/Hsp70 chaperone cycle regulating *MAL*-activator activation prior to the formation of the intermediate complex.

RESULTS

Aha1p is a negative regulator of inducible Mal63 *MAL*-activator induction. BALI *et al.* (2003) demonstrated that the *Saccharomyces MAL*-activator encoded by *MAL63* requires Hsp90/Hsp70 chaperone machine for maltose-dependent induction. A variety of cochaperone proteins have been identified that enhance the functioning of Hsp70 and Hsp90, the essential chaperones in this process, and impact client protein maturation (CAPLAN 2003). However, different client protein – Hsp90 complexes are likely to have different arrays of cochaperone components. For example, Cdc37p is required for all kinase clients of Hsp90 but, with the possible exception of the androgen receptor, Cdc37p does not appear to be involved in the activation of other classes of Hsp90 chaperone clients (RAO *et al.* 2001). We investigated the role of several known cochaperones of the Hsp70/Hsp90 chaperone machine to test their effects on maltose induction of the Mal63 *MAL*-activator by creating mutations in the genes encoding several of the known cochaperones. Chapter 2 describes studies on the cochaperone Aha1p and its *Saccharomyces* homologue Hch1p.

AHA1 and *HCH1* were deleted in strain CMY1300 (*SSA1/Myc ssa2-4Δ*) and

maltase expression was determined in transformants carrying inducible *MAL63* and constitutive *MAL43-C MAL*-activator genes (Figure 14). Loss of *HCH1* has no impact on maltase expression. Surprisingly, induced expression of maltase is increased to levels at least two-fold higher in the *aha1Δ* strain suggesting that Aha1p plays a negative role in *MAL*-activator regulation (Figure 14, left panel). No effect of *aha1Δ* is observed in cells grown in the absence of maltose indicating that Aha1p does not play a role in basal expression of maltase which, based on studies by WANG and MICHELS (2004), utilizes transcription regulators other than the *MAL*-activator. We also studied the effect of loss of *AHA1* and *HCH1* on constitutive maltase expression in strains carrying the constitutive *MAL*-activator gene *MAL43-C*. *MAL43-C* encodes a *MAL*-activator that is approximately 93% identical to inducible Mal63p but differs at 31 residues, mostly clustered in two short regions located in the C-terminal regulatory domain of the 470 residue *MAL*-activator (Figure 16; (GIBSON *et al.* 1997)). Mal43-C *MAL*-activator is described in greater detail below. Loss of neither *AHA1* nor *HCH1* alters the high level of constitutive maltase observed (Figure 14, right panel).

To study the impact of the loss of *AHA1* on induction in more detail, we examined the induction of maltase at limiting maltose concentrations (Figure 15). As can be seen, not only is the maximal level of maltase induction approximately 60% higher in the *aha1Δ* strain compared to the parental strain, but also the rate of induction is approximately 2-fold faster. This likely reflects an increased level of maltose permease expression in the *aha1Δ* strain, which would result in increased rates of maltose accumulation at limiting maltose concentrations.

Figure 14: Loss of Aha1 cochaperone leads to increased maltase expression in strains carrying inducible *MAL63* but not constitutive *MAL43-C MAL*-activators. Strains CMY 1300 (*SSA1/Myc ssa2-4Δ AHA1 HCH1*), and otherwise isogenic CMY 8003 (*aha1Δ HCH1*) and CMY 8031 (*AHA1 hch1Δ*) were transformed with plasmids YCp50-MAL63 and YCp50-MAL43-C carrying the inducible *MAL63* and constitutive *MAL43-C* genes, respectively. Transformants carrying YCp50-MAL63 were grown at 30°C in selective synthetic medium lacking uracil and containing 3% glycerol and 2% lactate (vol/vol) plus the indicated concentration of maltose. Transformants carrying YCp50-MAL43-C were grown under the same conditions except the medium lacked maltose. Maltase activity was assayed as described in material and methods and is expressed as nmoles PNPG (p-nitrophenol-α-glucopyranoside) produced per mg protein per minute. Assays were carried out on at least three independent transformants. The error bars indicate standard deviation from three independent transformants assayed in duplicate.

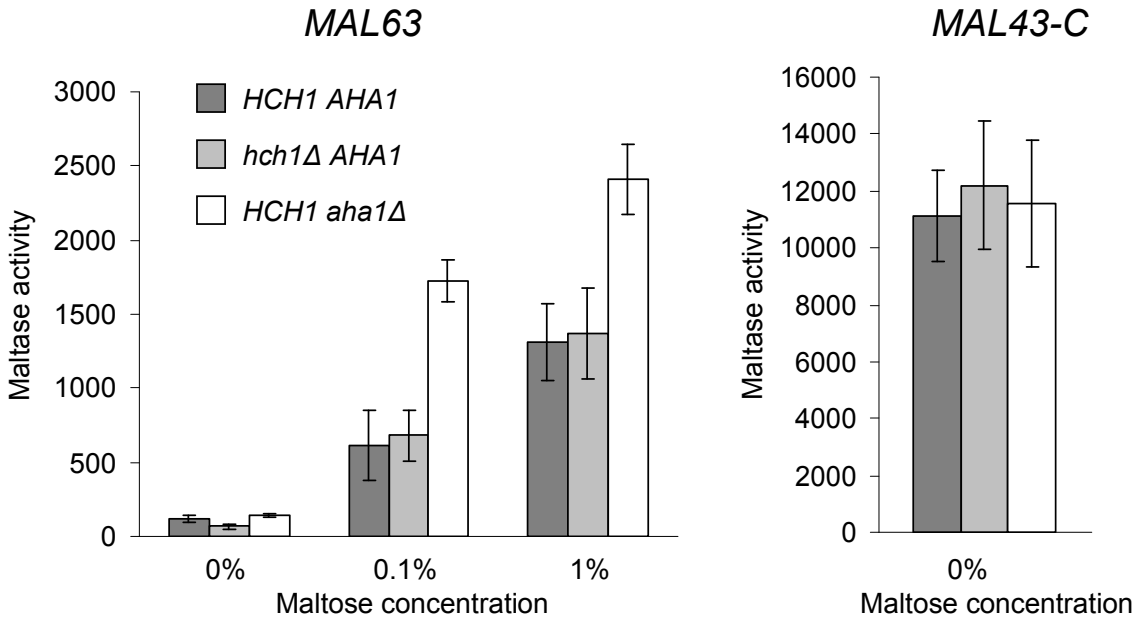
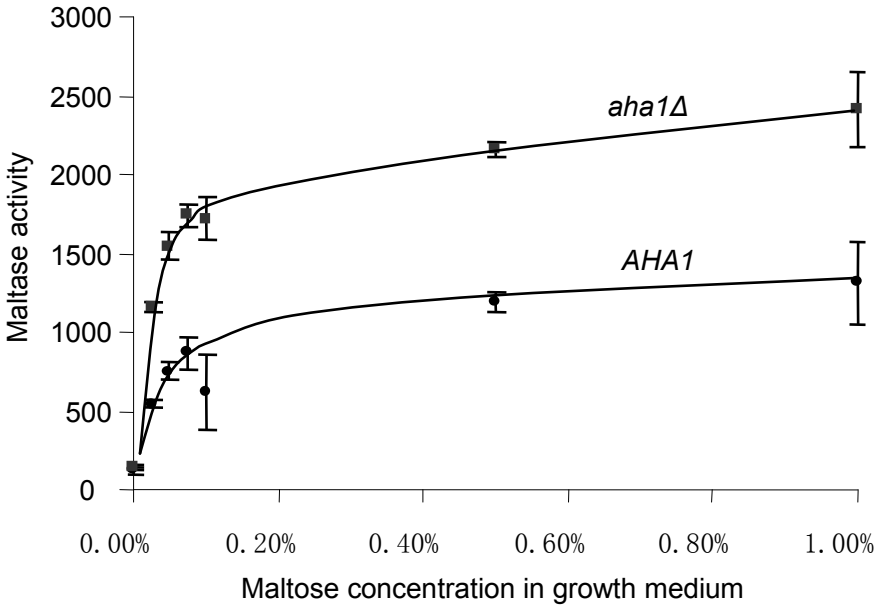

MAL63
HCH1 AHA1
hch1Δ AHA1
HCH1 aha1Δ
Maltase activity
3000
2500
2000
1500
1000
500
0
0%
0.1%
1%
Maltose concentration
MAL43-C
Maltase activity
16000
14000
12000
10000
8000
6000
4000
2000
0
0%
Maltose concentration

Figure 15: Comparison of maltose-dependent maltase induction in *AHA1* and *aha1Δ*. Strains CMY 1300 (*SSA1/Myc ssa2-4Δ AHA1*) and otherwise isogenic CMY 8003 (*aha1Δ*) were transformed with plasmid YCp50-MAL63 carrying inducible *MAL63*. Transformants were grown at 30°C in selective synthetic medium lacking uracil and containing 3% glycerol and 2% lactate (vol/vol) plus the following concentration of maltose: 0%, 0.025%, 0.05%, 0.075%, 0.1%, 0.5%, and 1%. Maltase activity was assayed as described for Figure 14.


3000
2500
2000
1500
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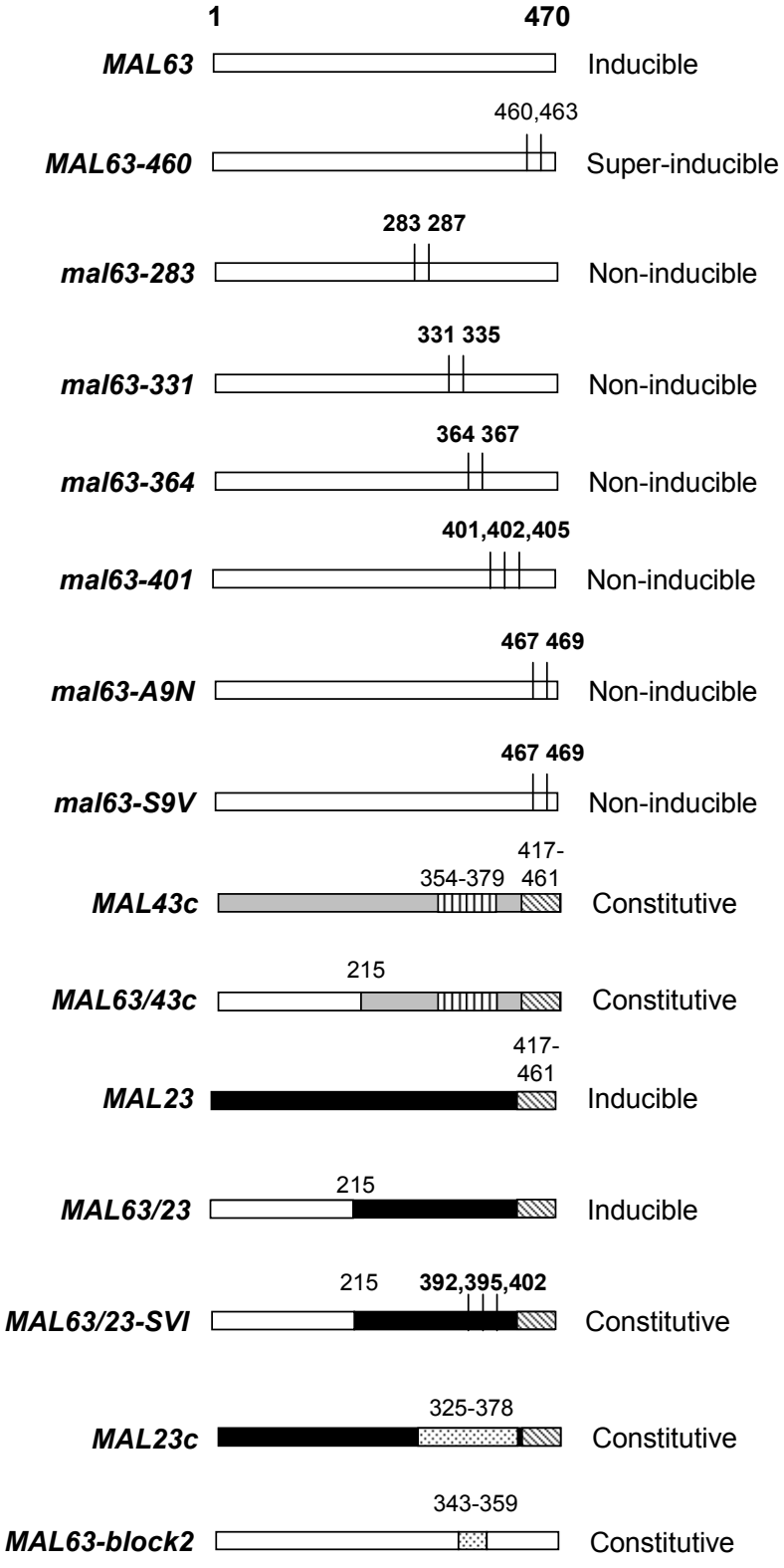
500
0
Maltase activity
aha1Δ
AHA1
0. 00%
0. 20%
0. 40%
0. 60%
0. 80%
1. 00%
Maltose concentration in growth medium

Identification of the *MAL*-activator region that genetically interacts with Aha1p. Previous work from the Michels laboratory described an array of *MAL*-activator mutant alleles with a noninducible, constitutive, and super-inducible phenotypes (DANZI *et al.* 2003; DANZI *et al.* 2000; GIBSON *et al.* 1997). A number of these mutant alleles were used to define the genetic interaction between the cochaperone Aha1p and the *MAL*-activator. Figure 16 lists these alleles and diagrams as simply as possible the derivation of the mutant alleles and the location of significant alterations in each compared to inducible Mal63p. For complete information on these mutant alleles, including the specific alterations and a comparison to the sequence of inducible Mal63p, please refer to DANZI *et al.* (2000, 2003) and GIBSON *et al.* (1997).

To identify sites important for *MAL*-activator regulation, DANZI *et al.* (2003) undertook a mutation analysis of the inducible Mal63 *MAL*-activator C-terminal regulatory domain using clustered-charge to alanine scanning mutagenesis and site-directed random mutagenesis of the C-terminal residues 454-470. Both methods identified noninducible *mal63* alleles containing 2-5 altered residues within a 5-residue window mapping to sites throughout the region from residue 244-469. This study utilizes 6 of these noninducible mutants, diagramed in Figure 16 which indicates the altered residues. Interestingly, alterations near the extreme C-terminus were identified that have a noninducible and, in two cases, super-inducible phenotype. Strains carrying these super-inducible mutations require maltose for induction but induce to levels approximately 2-fold higher than strains expressing Mal63 *MAL*-activator. Super-inducible allele *MAL63-460* is included here to investigate the possible relationship between the super-inducible phenotype and the elevated level of maltase expression observed in the *aha1Δ* strain.

Figure 16: Mutational alterations in the *MAL*-activator mutant alleles utilized in this study. The various mutant alleles are aligned with the inducible activator Mal63p (top). The position of the mutant alteration(s) are diagramed for specific alteration refer to DANZI *et al.* (2000, 2003) and GIBSON *et al.* (1997).


1
470
MAL63
Inducible
460,463
MAL63-460
Super-inducible
283 287
mal63-283
Non-inducible
331 335
mal63-331
Non-inducible
364 367
mal63-364
Non-inducible
401,402,405
mal63-401
Non-inducible
467 469
mal63-A9N
Non-inducible
467 469
mal63-S9V
Non-inducible
417-
354-379 461
MAL43c
Constitutive
215
MAL63/43c
Constitutive
417-
461
MAL23
Inducible
215
MAL63/23
Inducible
215 392,395,402
MAL63/23-SVI
Constitutive
325-378
MAL23c
Constitutive
343-359
MAL63-block2
Constitutive

Mal43p, is a constitutive *MAL*-activator isolated as an X-ray induced maltose-fermenting revertant of a maltose non-fermenting strain (reviewed in (BARNETT and ENTIAN 2005); (CHARRON and MICHELS 1987)). As described above, Mal43-Cp differs from Mal63p at 31 residues, 27 of which are located in the C-terminal regulator domain in residues 238-461. Of these, 10 alterations are clustered in residues 354-379 and 9 others are clustered in residues 417-461. Mal63/43-C *MAL*-activator is a chimera protein containing the first 215 residues of Mal63p and residues 216 to 470 of Mal43-Cp. Since this chimera is constitutive, the alterations in the C-terminal half of the protein are sufficient to cause constitutivity.

Inducible Mal23 *MAL*-activator is approximately 96% identical to Mal63p and differs at 23 residues, 9 of which are clustered at the C-terminus in residues 417-462 and are identical to the sequence of Mal43-Cp in this region. A chimera of the N-terminal 1-215 residues of Mal63p with residues 216-470 of Mal23p contains this C-terminal cluster and is inducible but mutation S392A, V395I, and I402V, which changes these to the sequence found in Mal63p, produces a constitutive allele referred to as *MAL63/23-SVI* (DANZI *et al.* 2000). Thus, except for three very widely scattered single residue differences Mal63/23-SVIp and Mal63p differ by the 9 residues from 417-462, referred to as “block 1”, and these are sufficient to cause constitutivity. A constitutive allele of *MAL23* was isolated by reversion of a nonfermenting *mal23* mutant, referred to as *MAL23-C* (reviewed in (BARNETT and ENTIAN 2005)). Sequencing of *MAL23-C* found that residues 325-378 contained 20 alterations including a two-residue deletion suggesting that *MAL23-C* resulted from a gene conversion from an unknown genomic site. DANZI *et al.* (2000) showed that introduction of the sequence of residues 343-359,

referred to as “block 2”, to replace the comparable region of Mal63p produces a constitutive allele, *MAL63-block 2*, that differs from Mal63p at 10 residues.

To compare the effects of loss of Aha1 cochaperone on the various *MAL*-activator mutant alleles, maltase expression was assayed in strains carrying plasmid-born inducible *MAL63*, non-inducible *mal63-283*, super-inducible *MAL63-460*, and constitutive *MAL63/43-C* and *MAL63/23-SVI MAL*-activator genes (Figure 17). Loss of Aha1p increases the level of maltase expression only in strains expressing inducible Mal63 and constitutive Mal63-block2 activators. Both of these alleles are identical in sequence at the C-terminus, residues 380 to 470. Maltase expression in strains expressing the constitutive Mal63/43-C and Mal63/23-SVI activators and the super-inducible Mal63-460 activator are unaffected. These mutant alleles each contain alterations in residue 417 to 461 compared to Mal63p. While maltase expression in the strain carrying the noninducible mutant activator mal63-283p is very low, it also does not appear to be affected by loss of Aha1p. This suggests that the noninducible phenotype of this mutation is unrelated to Aha1p regulation.

To further explore this hypothesis, Aha1 cochaperone was overexpressed using a high copy plasmid carrying a triple-Myc tagged allele of *AHA1/3Myc* expressed from the high-level GPD promoter. This was introduced into in a strain containing a single genomic copy of *AHA1/3Myc* in which the triple Myc-tag was placed by a PCR-based insertion method at the C-terminus of *AHA1* so as not to alter expression from the native promoter. Western blotting analysis (Figure 18 inset) indicates that Aha1/3Myc protein expression is elevated approximately 10 times over genome expression by the plasmid-borne copy. The impact of *AHA1/3Myc* overexpression on maltase expression was

Figure 17: Deletion of *AHA1* relieves maltase expression activated by selected *MAL*-activator mutant alleles. Strains CMY1200 (*hsc82Δ HSP82/Myc AHA1*) and otherwise isogenic CMY8010 (*aha1Δ*) were transformed with plasmids pUN30-MAL63, pUN30-mal63-283, pUN30-MAL63-460, pUN30-MAL63/43-C, pUN30-MAL63/23-SVI, and pUN30-MAL63-block 2 carrying inducible *MAL63*, noninducible *mal63-283*, super-inducible *MAL63-460*, and constitutive *MAL63/43-C*, *MAL63/23-SVI*, and *MAL63-block 2*, respectively (see Figure 16). Transformants carrying *MAL63*, noninducible *mal63-283*, and super-inducible *MAL63-460* were grown to mid-log in 30ºC in selective synthetic medium lacking tryptophan and containing 3% glycerol and 2% lactate (vol/vol) plus 1% maltose. Transformants carrying the constitutive activator genes were grown under the same conditions but in medium lacking maltose. Maltase activity was assayed as described for Figure 14.

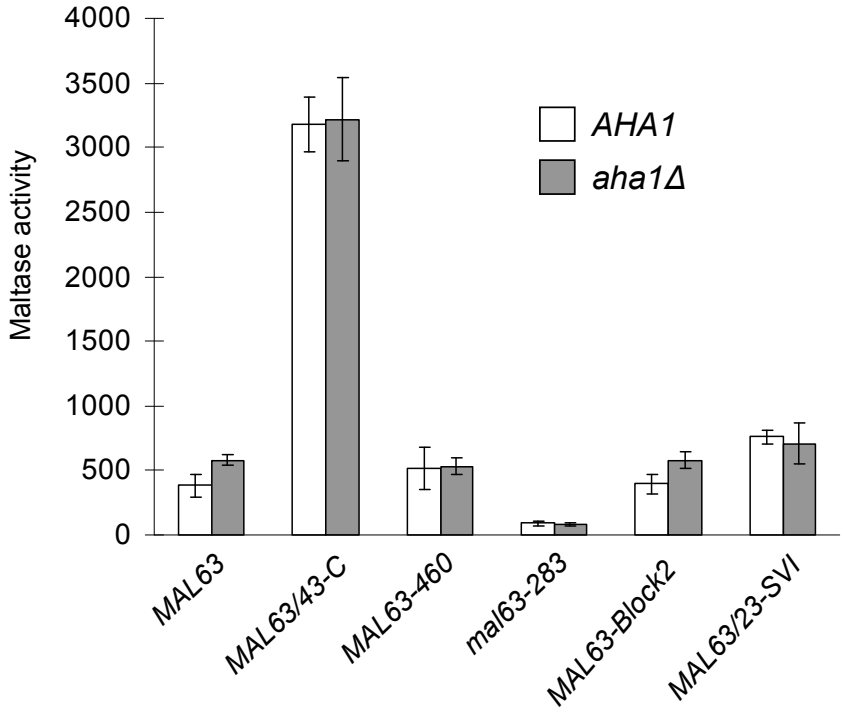
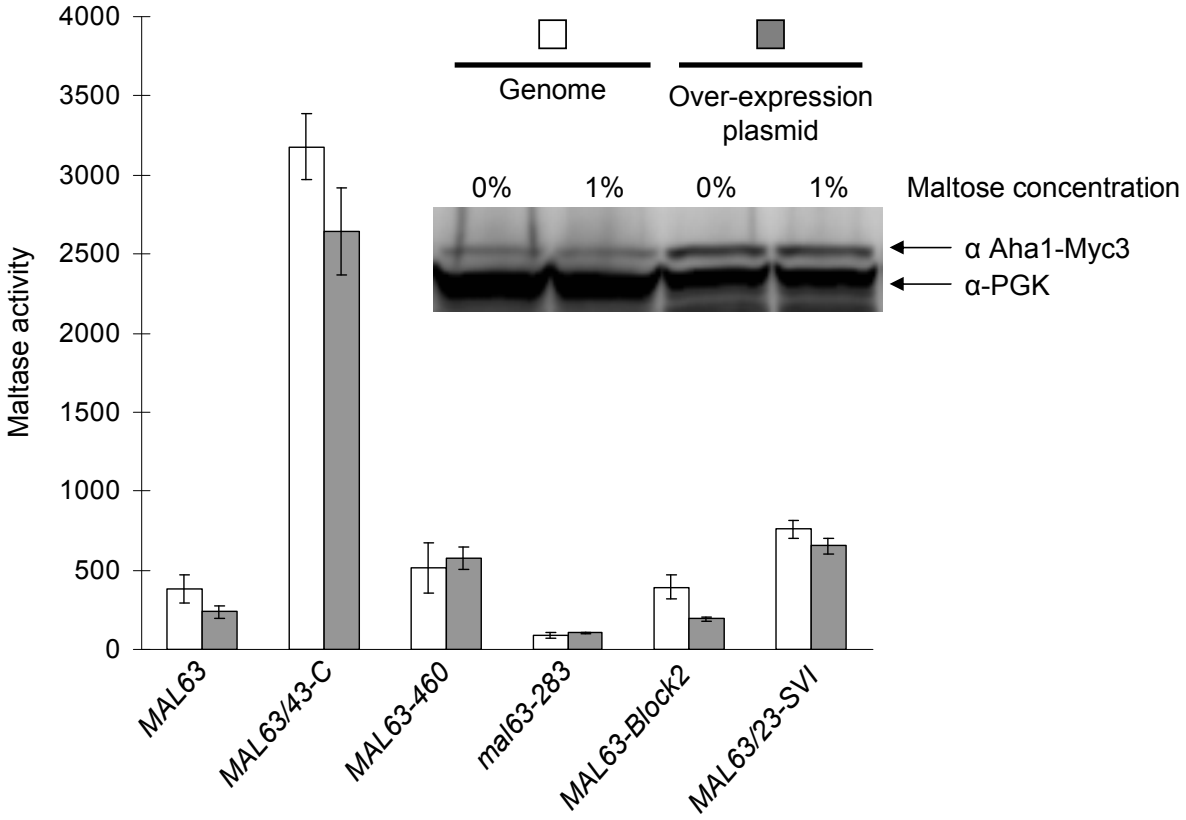

4000
3500
3000
2500
2000
1500
1000
500
0
Maltase activity
AHA1
aha1Δ
MAL63
MAL63/43-C
MAL63-460
mal63-283
MAL63-Block2
MAL63/23-SVI

Figure 18: *AHA1/3Myc* over-expression represses maltase expression activated by selected *MAL*-activator mutant alleles. Strain CMY8301 (isogenic to W303, except *AHA1/Myc3*) was transformed with either the high copy plasmid p423GPD (vector control) or p423GPD-AHA1/Myc3 and with pUN30-MAL63, pUN30-MAL63/43c, pUN30-MAL63-460, pUN30-mal63-283, pUN30-MAL63-block 2, or pUN30-MAL63/23-SVI harboring the triple HA-tagged alleles of *MAL63*, *MAL63/43-C*, *MAL63-460*, *MAL63/23-block 2*, and *mal63-283* (refer to Figure 16). Transformants carrying inducible and super-inducible activator were grown to mid-log in 30ºC in selective synthetic medium lacking tryptophan and histidine and containing 3% glycerol and 2% lactate (vol/vol) plus 1% maltose. Transformants carrying constitutive activators were grown under the same conditions and medium except in the absence of maltose. Maltase activity was assayed as described for Figure 14. Western blot analysis was carried out as described in Material and Methods on total cell extracts prepared from strain CMY8301 transformed with pUN30-MAL63 and either p423GPD or p423GPD-AHA1/Myc3 and grown in the indicated maltose concentration (inset panel). Bolts were probed with anti-Myc antibody and anti-PGK antibody, as a loading control. Analysis was carried out on three independent transformants; a representative blot is shown.

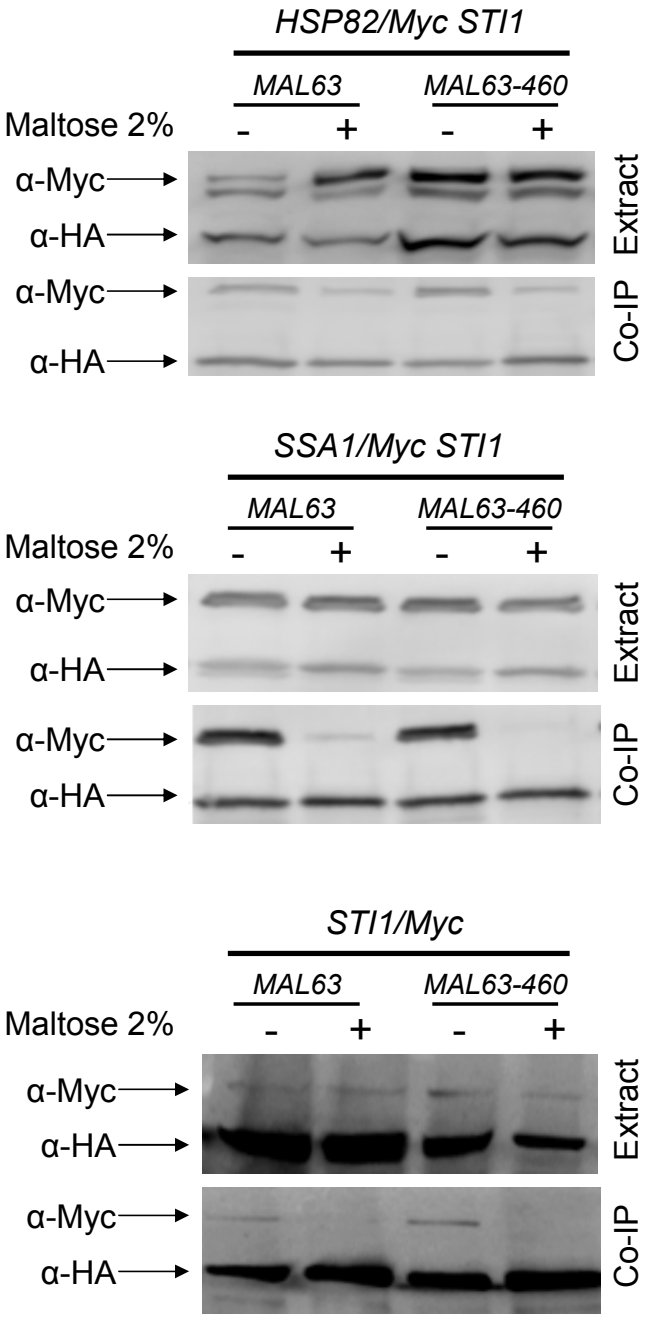

4000
3500
3000
2500
2000
1500
1000
500
0
Maltase activity
Genome
Over-expression plasmid
0%
1%
0%
1%
Maltose concentration
α Aha1-Myc3
α-PGK
MAL63
MAL63/43-C
MAL63-460
mal63-283
MAL63-Block2
MAL63/23-SVI

compared in strains carrying plasmid-borne copies of the indicated *MAL*-activator genes. As shown in Figure 18, overexpression of Aha1/3Myc cochaperone represses maltase expression most dramatically in strains carrying *MAL63* and *MAL63-block 2* (by approximately 40%), although a modest decrease in maltase expression is also observed in strains containing *MAL63/43-C* and *MAL63/23-SVI*.

Taken together, the results reported in Figures 17 and 18 suggest a genetic interaction between Aha1 cochaperone and residues in the region near the C-terminus of the *MAL*-activator. Additionally, the negative regulatory role of Aha1 cochaperone is independent of the negative regulation controlling induction since the three constitutive mutants tested, *MAL63/43-C, MAL63-block 2*, and *MAL63/23-SVI*, respond differently to the loss and overexpression of Aha1 cochaperone. Results reported in Chapter 1 indicate that Ssa1, the *Saccharomyces* Hsp70, is the negative regulator controlling induction by maltose. Thus, Aha1 cochaperone regulates the *MAL*-activator via a different mechanism that is unrelated to the response to maltose.

Defective Aha1p enhances the *MAL*-activator association with Hsp90 and Hsp70. To further explore the mechanism of Aha1p action, we investigated the binding of inducible Mal63 *MAL*-activator to Hsp90 and Hsp70 in strains lacking the cochaperone. Chapter 1 shows that in the absence of maltose inducible Mal63p forms what appears to be an "intermediate complex" containing Ssa1/Myc, Hsp82/Myc, and Sti1/Myc. Figure 19 shows a similar study for the super-inducible allele Mal63-460 *MAL*-activator. As anticipated and similar to inducible Mal63p, Mal63-460p is found bound to Ssa1/Myc, Hsp82/Myc, and Sti1/Myc in the absence of maltose forming what appears to be an "intermediate complex". No binding is observed in extracts prepared from cells grown in

Figure 19: Co-immunoprecipitation of super-inducible allele, Mal63-460 *MAL*-activator protein with Ssa1, Hsp82 and Sti1. Strains CMY1200 (*HSP82/Myc hsc82*Δ) (upper panel) and CMY1300 (*SSA1/Myc ssa2*Δ *ssa3*Δ *ssa4*Δ) (middle panel) and CMY8015(*STI1/Myc*) (lower panel) were transformed with the plasmid p416GPD-MAL63-460/3HA harboring the triple HA-tagged super-inducible *MAL63-460 MAL*-activator gene. Transformants were grown to mid-log at 30ºC in selective synthetic medium lacking uracil and containing 3% glycerol, 2% lactate, with or without 2% maltose. Nondenaturing protein extracts were prepared as described in Materials and Methods. Co-immunoprecipitation (Co-IP) samples and the total cell extracts from which they were prepared were analyzed by Western blotting using anti-HA and anti-Myc antibodies. Experiments were done at least in triplicate and representative blots are shown.


HSP82/Myc STI1
MAL63
MAL63-460
Maltose 2%
- + - +
α-Myc
α-HA
Extract
α-Myc
α-HA
Co-IP
SSA1/Myc STI1
MAL63
MAL63-460
Maltose 2%
- + - +
α-Myc
α-HA
Extract
α-Myc
α-HA
Co-IP
STI1/Myc
MAL63
MAL63-460
Maltose 2%
- + - +
α-Myc
α-HA
Extract
α-Myc
α-HA
Co-IP

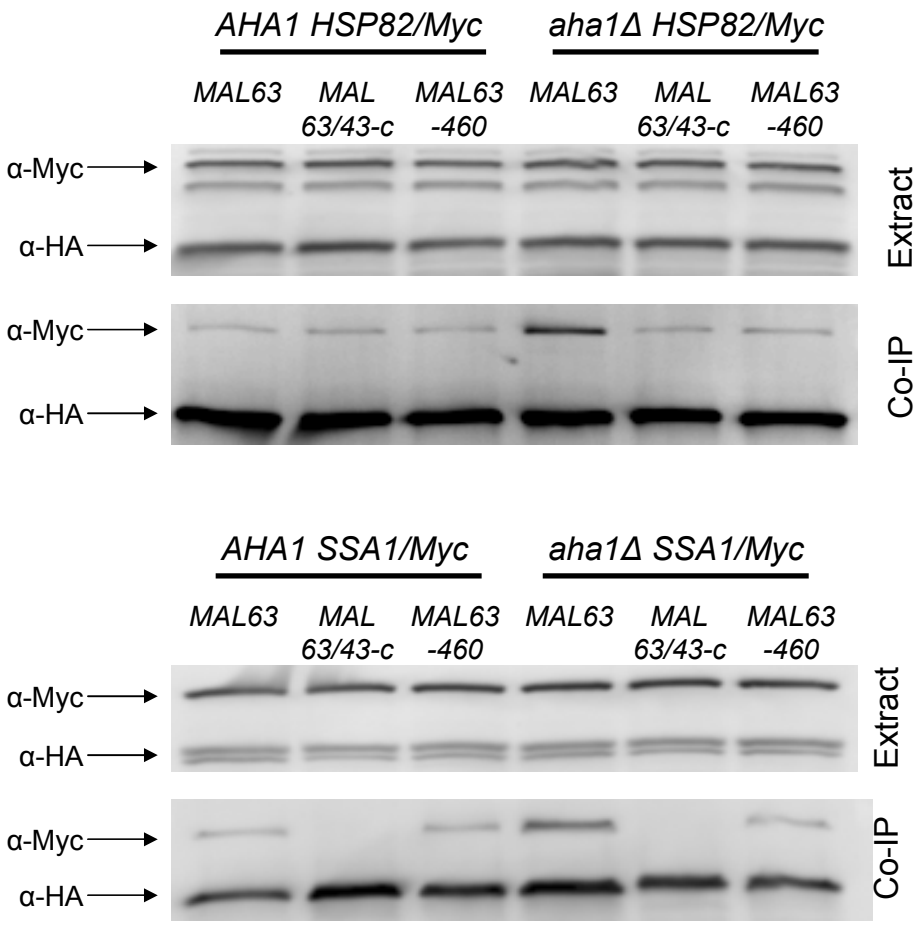
the presence of maltose. In contrast, the constitutive Mal63/43-C *MAL*-activator binds to Hsp82/Myc but binding to Ssa1/Myc and Sti1/Myc is not observed and may proceed to a "final"-like complex even in the absence of maltose.

Co-immunoprecipitation methods as described in Chapter 1 were used to explore the effect of *AHA1* deletion on the *in vivo* binding of the inducible, constitutive, and super-inducible *MAL*-activators encoded by *MAL63*, *MAL63/43-C*, and *MAL63-460*, respectively, to the chaperones Hsp82/Myc and Ssa1/Myc. The results are shown in Figure 20. All extracts were prepared from cells grown in the absence of maltose.

Hsp82/Myc co-purifies with all three *MAL*-activators tested but loss of Aha1 cochaperone dramatically enhances the level of Hsp82/Myc binding to inducible Mal63p. Quantification of these results, which normalizes to the amount of *MAL*-activator, indicates that there is approximately a 3-fold increase in Hsp82/Myc binding. As reported in Chapter 1, constitutive Mal63/43-Cp binding with Ssa1/Myc is not observed even in the absence of maltose and Figure 20 shows that loss of Aha1 cochaperone does not alter this. Similar to the results for Hsp82/Myc, loss of Aha1p enhances binding of inducible Mal63 *MAL*-activator to Ssa1/Myc and quantification indicates that this enhancement is also approximately 3-fold. As expected, the super-inducible Mal63-460 activator binds Ssa1/Myc but, interestingly, this binding is not enhanced in the *aha1Δ* strain. Also noteworthy, is the finding that the relative binding affinity of super-inducible Mal63-460 activator to Hsp82/Myc and Ssa1/Myc is comparable to that of inducible Mal63p both in the absence and presence of Aha1 cochaperone.

Non-inducible *MAL*-activator mutants bind Aha1 cochaperone. One interpretation of these results reported in Figures 20 is that Aha1 cochaperone negatively

Figure 20: Deletion of *AHA1* enhances the association of Hsp90 and Hsp70with inducible Mal63p but not constitutive Mal63/43-C and super-inducible Mal63-460 *MAL*-activators. Upper panel: Strains CMY1200 (*HSP82/Myc AHA1*) and CMY8010 (*Hsp82/Myc aha1Δ::Hyg*^{R}) were transformed with p416-GPD-MAL63/HA3, p416-GPD-MAL63/43-C/HA3 and p416-GPD-MAL63-460/HA3 carrying the triple HA-tagged inducible *MAL63*, constitutive *MAL63/43-C*, and super-inducible *MAL63-460 MAL*-activator genes, respectively. Lower Panel: Strains CMY1300 (*SSA1/Myc ssa2-4Δ AHA1*) and CMY8003 (*SSA1/Myc ssa2-4Δ aha1Δ*) were transformed with p416-GPD-MAL63/HA3, p416-GPD-MAL63/43-C/HA3 and p416-GPD-MAL63-460/HA3 carrying the triple HA-tagged inducible *MAL63*, constitutive *MAL63/43-C*, and super-inducible *MAL63-460 MAL*-activator genes, respectively. Transformants were grown in selective synthetic medium lacking uracil and containing 3% glycerol, 2% lactate without maltose. Nondenaturing protein extracts were prepared, incubated with anti-HA bound agarose beads, and bound protein isolated as described in Materials and Methods. Co-immunoprecipitation (Co-IP) samples and the total extracts from which they were prepared were analyzed by Western blotting using anti-HA and anti-Myc antibodies. Experiments were done at least in triplicate and representative blots are shown.

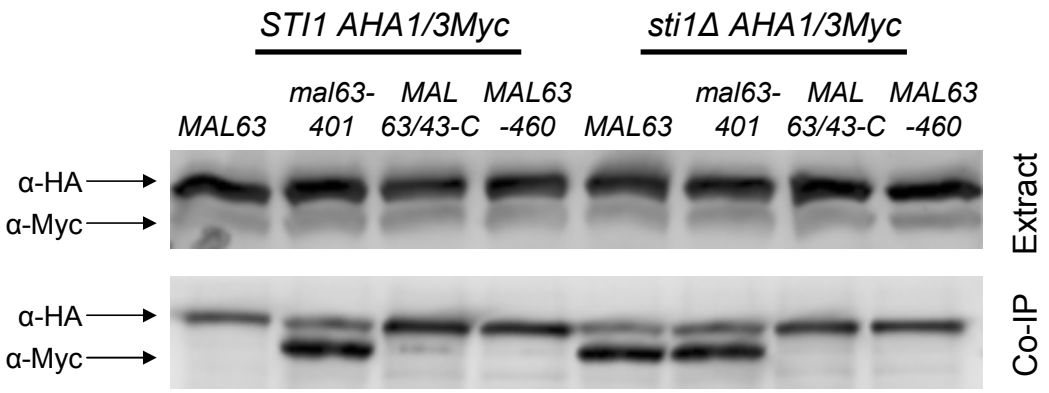

AHA1 HSP82/Myc
aha1Δ HSP82/Myc
MAL63
MAL 63/43-c
MAL63 -460
MAL63
MAL 63/43-c
MAL63 -460
α-Myc
α-HA
Extract
α-Myc
α-HA
Co-IP
AHA1 SSA1/Myc
aha1Δ SSA1/Myc
MAL63
MAL 63/43-c
MAL63 -460
MAL63
MAL 63/43-c
MAL63 -460
α-Myc
α-HA
Extract
α-Myc
α-HA
Co-IP

regulates induction of Mal63 *MAL*-activator by interfering with its delivery to the intermediate complex. Aha1p might deter the formation of intermediate complex by retaining Mal63p in the so-called "early complex" where it is bound to Hsp70 but not Hsp90 or Hop/Sti1. Deletion of *STI1* leads to defects in the formation of the intermediate complex, causes reduced maltase expression in strains carrying either inducible *MAL63* or constitutive *MAL63/43-C*, and dramatically enhances binding of the constitutive Mal63/43-C activator to Ssa1, which is not observed in an *STI1* strain. On the other hand, noninducible mutant *MAL*-activators were shown to bind Ssa1/Myc but not Hsp82/Myc and the results in Chapter 1 suggest that these mutant activators are retained in the early complex and are unable to proceed to the formation of an intermediate complex with Hsp90.

To investigate the role of Aha1 in the chaperone cycle of the *MAL*-activator, we created a strain containing a triple-Myc tagged *AHA1/3Myc* by inserting the tag at the C-terminus of the genomic copy of the gene (see Materials and Methods). The *AHA1* C-terminal modification did not affect maltase expression as compared with the parental strain W303 (data not shown). *STI1* was deleted in this strain using one-step gene disruption techniques. The plasmid-borne triple-HA tagged genes inducible *MAL63*, constitutive *MAL63/43-C*, noninducible *mal63-401*, and super-inducible *MAL63-460* were introduced into both the *STI1* and *sti1Δ* strains and binding of the HA-tagged *MAL*-activators to Aha1/3Myc determined by co-immunoprecipitation.

As shown in Figure 21, only the noninducible mutant mal63-401 activator is found associated with Aha1/Myc3 in the *STI1* strain and this association is not enhanced by loss of Sti1p. In the *STI1* strain, inducible Mal63 activator does not bind to

Figure 21: Effect of *sti1Δ* on *MAL*-activator – Aha1 cochaperone complex formation using inducible Mal63, non-inducible mal63-283, constitutive Mal63/43-C, and super-inducible Mal63-460 *MAL*-activators. Strains CMY8301 (*AHA1/Myc3 STI1*) and CMY8305 (*AHA1/Myc3 sti1Δ*) were transformed with p416-GPD-MAL63/HA3, p416-MAL63-401/HA3, p416-GPD-MAL63/43-C/HA3, and p416-GPD-MAL63-460/HA3, carrying triple-HA tagged inducible *MAL63*, noninducible *mal63-401*, constitutive *MAL63/43-C*, and super-inducible *MAL63-460*, respectively. Cell growth conditions and co-immunoprecipitation sample preparation are as described for Figure 19.


STI1 AHA1/3Myc
sti1Δ AHA1/3Myc
MAL63
mal63-401
MAL63/43-C
MAL63-460
MAL63
mal63-401
MAL63/43-C
MAL63-460
α-HA
α-Myc
α-HA
α-Myc
Extract
Co-IP

Aha1/3Myc but, when *STI1* is deleted thereby promoting the retention of Mal63p in the "early complex" based on results reported in Chapter 1, inducible Mal63 activator is now also found bound to Aha1/3Myc. Neither constitutive Mal63/43-C nor super-inducible Mal63-460 activators are found bound to Aha1/3Myc in either the *STI1* or *sti1Δ* strains. It should be noted that, in the *sti1Δ* strain, binding of the Mal63/43-C activator to Ssa1/Myc is observed. Therefore, this result is consistent with the results reported in Figures 17 and 18 suggesting that Aha1 cochaperone may bind to the C-terminal region of Mal63p but not to Mal63/43-Cp (and possible Mal63-460p) because of the mutant alterations in this activator in residues 417 to 460.

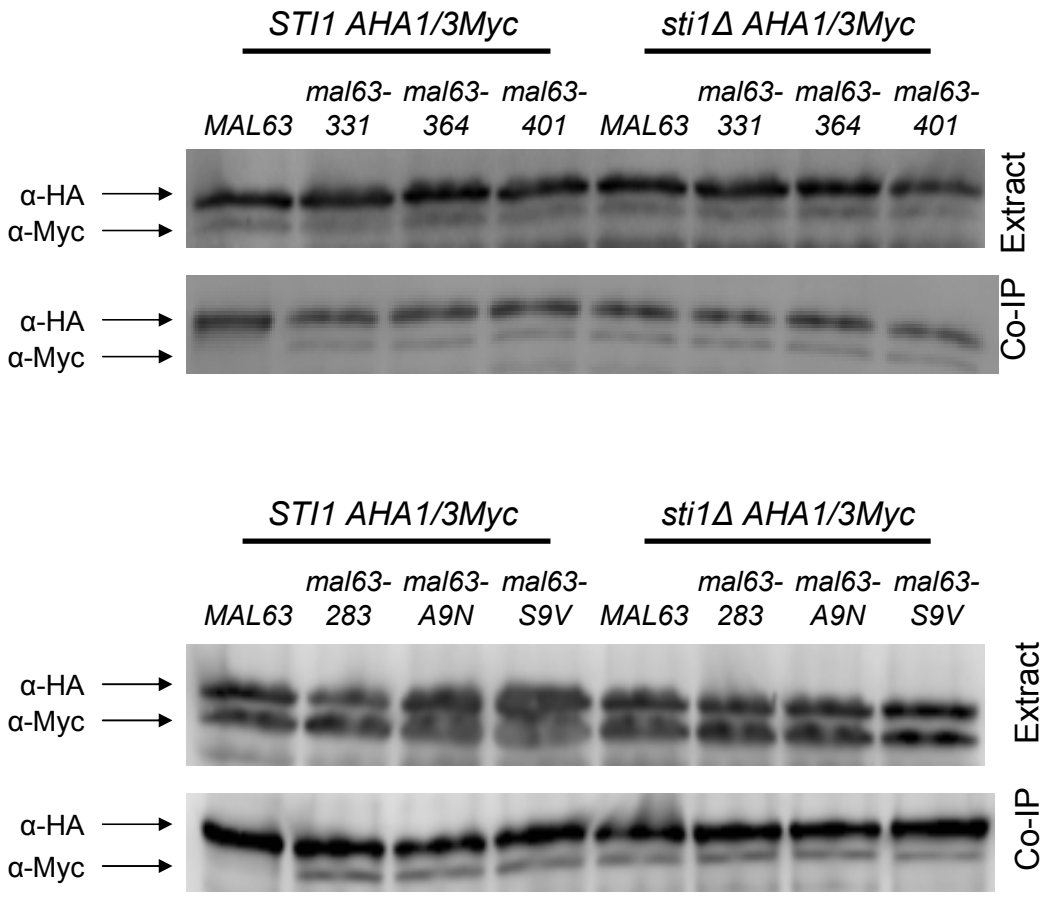
Several other noninducible mutant *MAL*-activators were tested for binding to Aha1/3Myc (Figure 22). These noninducible mutants contain alterations mapping throughout the C-terminal regulatory domain of Mal63p, residues 283 to 405 and residue 469. All of the mutants bind Aha1/3Myc and this binding is not enhanced by loss of Sti1/Hop.

DISCUSSION

The results reported in Chapter 2 make use of our large collection of *MAL*-activator mutant alleles as tools to study the Hsp90/Hsp70 chaperone cycle *in vivo*. This collection is unique among the Hsp90 client proteins and provides novel reagents with which to tease apart the roles of Hsp90, Hsp70, and the various cochaperones that regulate and facilitate the chaperone process. Here we focus on Aha1p cochaperone, a recently identified Hsp90 cochaperone whose function is poorly understood.

Aha1 cochaperone is the only known cochaperone to stimulate the ATPase activity of Hsp90 which is required for client protein activation (CAPLAN 2003). *In vitro*

Figure 22: Effect of *sti1Δ* on *MAL*-activator – Aha1 cochaperone complex formation using several non-inducible *mal63* mutant alleles. Strains CMY8301 (*AHA1/Myc3 STI1*) and CMY8305 (*AHA1/Myc3 sti1Δ*) were transformed with p416-GPD-mal63-331, p416-GPD-mal63-364, and p416-GPD-mal63-401 (upper panel), and with p416-GPD-mal63-283, p416-GPD-mal63-A9N, and p416-GPD-mal63-S9V (lower panel) harboring the triple-HA-tagged alleles noninducible *MAL*-activator mutant genes *mal63-331*, *mal63-364*, *mal63-401*, *mal63-283*, *mal63- A9N*, and *mal63- S9V*, respectively (see Figure 16; (DANZI *et al.* 2003). Cell growth conditions and co-immunoprecipitation sample preparation are as described for Figure 19.


STI1 AHA1/3Myc
sti1Δ AHA1/3Myc
MAL63
mal63-331
mal63-364
mal63-401
MAL63
mal63-331
mal63-364
mal63-401
α-HA
α-Myc
Extract
α-HA
α-Myc
Co-IP
STI1 AHA1/3Myc
sti1Δ AHA1/3Myc
MAL63
mal63-283
mal63-A9N
mal63-S9V
MAL63
mal63-283
mal63-A9N
mal63-S9V
α-HA
α-Myc
Extract
α-HA
α-Myc
Co-IP

studies report that the N-terminal domain of Aha1p and full-length Hch1p activate Hsp90 ATPase activity (HARST *et al.* 2005; HAWLE *et al.* 2006; LOTZ *et al.* 2003; MEYER *et al.* 2003; PANARETOU *et al.* 2002). ATP hydrolysis is reported to be a late step in the Hsp90 chaperone cycle and, in a manner that is poorly understood, provides the energy for the conformational change and ultimate release of the client protein from Hsp90 (ALI *et al.* 2006; DOLLINS *et al.* 2007; FREY *et al.* 2007; MAYER *et al.* 2002; RICHTER and BUCHNER 2006; VAUGHAN *et al.* 2006; YOUNG *et al.* 2001). Additionally, the N-terminal end of Aha1 binds to the middle region of Hsp90 and this binding competes with the binding of the early cochaperones Hop/Sti1p and p50/Cdc37p and the late cochaperone p23/Sba1p, all cochaperones that inhibit Hsp90 ATPase (HARST *et al.* 2005; LOTZ *et al.* 2003; MARTINEZ-YAMOUT *et al.* 2006).

Taken together, these findings have been interpreted to indicate that Aha1p acts late in the Hsp90 chaperone cycle to stimulate ATPase and client protein release. Thus, Aha1p is proposed to function as a positive regulator of Hsp90 client protein activation and this has proven to be the case for several known Hsp90 clients. Activation of the Hsp90 clients proteins v-Src kinase and GR is dependent on Aha1 cochaperone, both when tested in the heterologous host *Saccharomyces* and mammalian cells (HARST *et al.* 2005; HAWLE *et al.* 2006; LOTZ *et al.* 2003). Moreover, Aha1 cochaperone expression is up-regulated in a number of tumor lines, coincident with the activation of several signaling kinases (HOLMES *et al.* 2008).

In contrast to these results, recent findings regarding mammalian cystic fibrosis transmembrane conductance regulator (CFTR) suggest that Aha1 cochaperone negatively regulates mutant ΔF508 CFTR (WANG *et al.* 2006). CFTR is a 12 transmembrane domain

integral membrane protein that folds in the ER and is delivered to the plasma membrane where is functions as a chloride transporter. The ΔF508 allele is the most commonly occurring mutation in human populations. During normal expression of CFTR, about 80% of the protein is degraded in the ER but for ΔF508 CFTR this increases to 100%. (WANG *et al.* 2006) used proteomic approaches to identify CFTR and ΔF508 CFTR binding proteins. They identified a large number of cytoplasmic and ER luminal chaperones and cochaperones, including Aha1 cochaperone, and found that these were differentially bound by the two CFTR alleles. Analysis of other cochaperones, p23 and FKBP8, indicated a positive role in ΔF508 CFTR expression. In contrast, decreased Aha1p expression enhanced plasma membrane localization of ΔF508 CFTR and over-expression of Aha1p increased ΔF508 CFTR degradation. Their data suggests that loss of Aha1 cochaperone facilitates progress of ΔF508 CFTR through the Hsp90 pathway enhancing plasma membrane expression and directing the mutant protein away from the degradation pathway. Hsp70, presumably in the early complex, appears to be the decision point at which a client protein is directed either to the Hsp90 folding pathway or to the degradation pathway via interaction with the Hsp70-binding ubiquitin ligase CHIP (CONNELL *et al.* 2001; CYR *et al.* 2002; JIANG *et al.* 2001; MURATA *et al.* 2003; PRATT *et al.* 2006). (WANG *et al.* 2006) are focusing their efforts on the site of action of Aha1 cochaperone in an effort to explain the mechanism of the Aha1 cochaperone effect on ΔF508 CFTR maturation. The work described here on the *Saccharomyces MAL*-activator may help address this question.

Aha1p is a negative regulator of *MAL*-activator activation by the Hsp90/Hsp70 chaperone machine and interacts genetically with its C-terminal

domain of the *MAL*-activator: The results reported in Figure 14 and 15 indicate that Aha1p cochaperone, but not its homologue Hch1p, is a negative regulator of *MAL* gene induction by Mal63 *MAL*-activator. Deletion of *AHA1* in a strain expressing only the *SSA1* Hsp70 gene caused over a 2-fold increase in maltase expression (Figure 14). Hch1p is approximately half the length of Aha1p and the homology between the proteins is restricted to the N-terminal half of Aha1p (PANARETOU *et al.* 2002). This raises the possibility that the impact of Aha1 cochaperone on *MAL*-activator regulation may be distinct from its ability to stimulate Hsp90 ATPase activity, which limited to its N-terminal domain and Hch1p (LOTZ *et al.* 2003; PANARETOU *et al.* 2002). Additionally, it should be noted that maltase expression is induced at significantly lower concentrations of maltose (Figure 15) suggesting that maltose permease induction is also affected by *aha1Δ* (WANG *et al.* 2002).

DANZI *et al.* (2000, 2003) created a large number of mutant *MAL*-activator alleles by altering residues in the C-terminal region from residues 250 to 470. Alteration of residues D460A and D463A (*MAL63-460*) or S462A alone (*MAL63-462*), produced super-inducible alleles in which the induced level of maltase expression is comparable to the expression level observed here for *aha1Δ*. Moreover, Aha1p over-expression only weakly repressed Mal63-460 dependent maltase expression compared to its impact on Mal63-dependent expression (Figure 18). This result suggested that the negative regulatory effect of Aha1 cochaperone acted via this region of the protein, the region that DANZI *et al.* (2003) proposed has a role in folding and maintenance of the *MAL*-activator. Chapter 1 shows that Hsp70 is the negative regulator of Mal63p maltose induction and that defects in Hsp70 binding cause constitutivity. Loss of Aha1 cochaperone does not

lead to constitutivity. In fact, two of the three constitutive *MAL*-activator alleles tested, *MAL63/43-C* and *MAL63/23-SVI*, were unaffected by *aha1Δ* and these two were also largely insensitive to repression by Aha1p over-expression. Both of these mutants contain several alterations in the C-terminus compared to Mal63p: N433H, D437Q, G445S, T449D, K454R, Q457R, N461D. The constitutive allele *MAL63-block2*, whose sequence is identical to Mal63p in the C-terminus, is sensitive to repression by Aha1 cochaperone.

Taken together, these results suggest that Aha1 cochaperone regulation of *MAL*-activator activation acts via an interaction with the C-terminal region of the *MAL*-activator and suggests a possible direct physical interaction.

Aha1p negatively controls *MAL*-activator – Hsp90 intermediate complex formation: Deletion of *AHA1* increases Hsp82/Myc and Ssa1/Myc binding to inducible Mal63 *MAL*-activator approximately 3-fold (Figures 20). The co-immunoprecipitation analysis was carried out using extracts prepared from cells grown in the absence of maltose. As demonstrated in Chapter 1, inducible Mal63p is found in an apparent intermediate complex in the absence of inducer maltose and is bound to Hsp82/Myc, Ssa1/Myc, and Sti1/Myc. The results reported here suggest that loss of Aha1 cochaperone leads to an increase in the percentage of Mal63 *MAL*-activator that is able to form the intermediate complex. Formation of the intermediate complex is the first step in the Hsp90 chaperone cycle and is likely to function as the gatekeeper regulating activation of a client protein. Thus, the increased level of Mal63p binding to Hsp82/Myc and Ssa1/Myc in the absence of Aha1p is consistent with the observed negative regulation of inducible Mal63p by Aha1 cochaperone.

Super-inducible Mal63-460 *MAL*-activator also is present in an apparent intermediate complex in the absence of maltose, that is, it is bound to Hsp82/Myc, Ssa1/Myc, and Sti1/Myc (Figures 19). Binding of super-inducible Mal63-460 *MAL*-activator to Hsp82/Myc, Ssa1/Myc, and Sti1/Myc is unaffected by loss of Aha1 cochaperone, which is not unexpected based on the results in Figure 17. One might have expected an increased level of binding to these proteins even in the presence of Aha1p but this is not the case. The amount of chaperone-bound Mal63-460 activator in both the absence and presence of Aha1 cochaperone is comparable to that observed for the inducible Mal63 activator in cells expressing Aha1 cochaperone. Since super-inducible Mal63-460 activator requires maltose for induction, it is likely that this mutant activator protein remains bound in the intermediate complex in the absence of inducer, like inducible Mal63p. Thus, this finding suggests that, distinct from the lack of Aha1p regulation, the chaperone complex may be more efficient at the activation of super-inducible Mal63-460 protein.

Similarly, the binding pattern of constitutive Mal63/43-C *MAL*-activator is not altered in the *aha1Δ* strain. Constitutive Mal63/43-C *MAL*-activator does not bind Ssa1/Myc or Sti1/Myc and binds Hsp82/Myc alone in what appears to be a final complex (Chapter 1). Whether significant amounts of this constitutive activator are active and unbound to any chaperone has not been shown and, given the extremely high level of maltase expression observed in strains expressing *MAL63/43-C*, it is probable that this is the case.

These results suggest that Aha1 cochaperone inhibits the rate at which inducible Mal63 *MAL*-activator forms an intermediate complex with Hsp82/Myc, Ssa1/Myc, and Sti1/Myc and that this is a rate limiting step in inducible Mal63 *MAL*-activator activation.

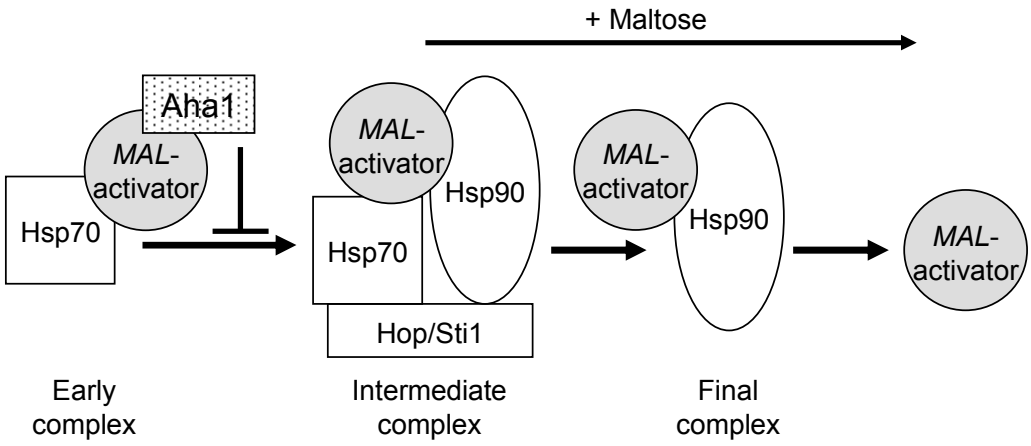
Aha1p binds initially to the *MAL*-activator in an apparent early complex: The results reported here suggest that Aha1 cochaperone interacts with the *MAL*-activator in the early complex while it is bound to Ssa1/Myc but not associated with Hsp82/Myc or Sti1/Myc. The findings in Chapter 1 indicate that noninducible mutant *MAL*-activators remain bound to Ssa1/Myc and are unable to progress to the intermediate complex. Thus, association with noninducible mutant *MAL*-activators can be used as a marker for the early complex. As seen in Figure 21, Aha1/3Myc protein binds to noninducible mutant mal63-401 activator in the absence of maltose and deletion of *STI1* does not enhance this binding. Six noninducible *MAL*-activator alleles were tested that carry alterations in sites spanning the regulatory domain of the *MAL*-activator from residues 283-401. Based on results reported in Chapter 1, all of these mutant proteins bind Ssa1/Myc in an apparent early complex and Figure 22 shows that all also bind Aha1/3Myc.

Inducible Mal63p forms a stable intermediate complex in the absence of maltose but, in an *sti1Δ* strain, intermediate complex formation is defective and Mal63p is found bound to Ssa1/Myc in an apparent early complex (Chapter 1). The presence of Mal63p in an early complex in the *sti1Δ* strain also allows for the co-purification of Aha1/Myc and inducible Mal63p (Figure 21). Constitutive Mal63/43-C activator does not form a stable intermediate complex and rapidly progresses to a final complex bound to Hsp82/Myc and not Ssa1/Myc or Sti1/Myc (Chapter 1). Deletion of *STI1* enhances binding of Mal63/43-C activator to Ssa1/Myc (Chapter 1), indicating that it is in an early complex, but

Aha1/3Myc is not observed (Figure 21). The results reported in Figure 20 indicate that super-inducible Mal63-460 activator forms an intermediate complex bound to Hsp82/Myc, Ssa1/Myc, and Sti1/Myc. Experiments are currently underway to determine if deletion of STI1 promotes early complex formation for Mal63-460, as is found for Mal63p and Mal63/43-C, but this is expected. Nonetheless, binding of Mal63/43-Cp to Aha1/3Myc is not observed even in the *sti1Δ* strain. In summary, reduced binding to Hsp82/Myc, as caused by *sti1Δ* or for noninducible mal63 mutant activators, impedes the Hsp90 chaperone cycle and promotes Ssa1/Myc binding in an apparent early complex. In this early complex, and only in the early complex, is Aha1/3Myc binding to *MAL*-activator alleles observed. Nonetheless, even in the *sti1Δ* strain in which the constitutive Mal63/43-C and super-inducible Mal63-460 activators are presumed to be in an early complex, Aha1/3Myc binding is not observed. This is consistent with the genetic analysis discussed above that suggests that constitutive Mal63/43-C and super-inducible Mal63-460 are insensitive to the negative regulation of Aha1 cochaperone and that the alterations in the C-terminal residues of these activators lead to defects in Aha1p binding and resistance to its negative regulatory effects.

Model of Aha1 cochaperone regulation of inducible Mal63*MAL*-activator: Figure 23 presents our model of the mechanism by which Aha1 cochaperone negatively regulates the *MAL*-activator. We propose that Aha1 cochaperone binds to the C-terminus of Mal63 *MAL*-activator while bound to Hsp70 in the "early complex" and fine-tunes the transfer of the *MAL*-activator from the "early complex" to the "intermediate complex". Binding of Aha1p to the C-termini of the *MAL*-activator inhibits the transfer and thus Aha1 cochaperone acts as a "governor" decreasing the rate at which the *MAL*-activator

Figure 23: Model of Aha1 cochaperone regulation of the *MAL*-activator


+ Maltose
Aha1
MAL-
activator
Hsp70
MAL-
activator
Hsp90
Hsp70
Hop/Sti1
MAL-
activator
Hsp90
MAL-
activator
Early
complex
Intermediate
complex
Final
complex

enters the Hsp90 pathway. The negative regulation by Aha1p is distinct from the negative regulation of *MAL*-activator induction by Hsp70 and is distinct from maltose induction.

In summary, our results with the *MAL*-activator and those of others working with other clients, such as glucocorticoid receptor and v-Src kinase, suggest that Aha1 cochaperone may function as either a positive or a negative regulator of client protein activation depending upon the particular client protein (LOTZ *et al.* 2003; PANARETOU *et al.* 2002). CHIP (C-terminal Hsp70 Interacting Protein) that had long been recognized as an E3 ubiquitin ligase responsible for the degradation of misfolded Hsp70/Hsp90 client proteins has recently been found to exhibit chaperone activity with certain clients under certain conditions (ROSSER *et al.* 2007; TRIPATHI *et al.* 2007). Clearly, one should be open to an unexpected plasticity in the function of the cochaperones. Our findings highlight the importance of investigating the roles of the various components of the Hsp70/Hsp90 molecular chaperone machine with a variety of different client proteins.

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