Hsp90/Hsp70 Chaperone Machine Regulation of the Saccharomyces *MAL*-Activator As Determined *in Vivo* Using Noninducible and Constitutive Mutant Alleles

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ABSTRACT

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 multichaperone 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 subcomplexes 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.

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 Pratt and Toft 1997, 2003; Wegele *et al.* 2004; Bukau *et al.* 2006; Picard 2006; Caplan *et al.* 2007). 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, 14 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 iden-

tifying 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.* 1996, 2004, 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 on the basis of *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. Finally, 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 enhanced only 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 findings 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, superinducible, 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 homolog), Sti1 (Hop homolog), and Hsp82 (Hsp90 homolog). 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 induc-

tion 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*.

MATERIALS AND METHODS

Yeast strains and plasmids: Table 1 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.

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 2-kb *SacI–KpnI* fragment containing the entire GPD promoter-*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 *NotI* fragment encoding the sequence triple-HA. W303 transformants carrying this plasmid induced maltase expression to normal levels and fermented maltose in 1 day.

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 the GPD promoter. The fragment encoding the mutant 3′ half

TABLE 1

List of strains

Strain	Genotype	Source
W303	*MAT**a** 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
CMY1200	Isogenic to hsc82Δ except *HSP82/Myc*	This study
CMY8001	Isogenic to CMY1200 except *sti1Δ::Hyg^R*	This study
S153	*MAT**a** leu2-3,112 ura3-1 trp1-1 ade2-1 can1-100 GAL SUC hsc82Δ::LEU2 hsp82Δ::LEU2 pGPD-hsp82-T1011*	D. Nathan
cpr7Δ	Isogenic to W303 except *cpr7Δ::HIS3*	A. Duina
hsc82Δ cpr7Δ	Isogenic to W303 except *hsc82Δ::LEU2 cpr7Δ::TRP1*	A. Duina
5CG2	*MATα ura3-52 lys2-801 ade2-101 trp1-63 his3-200 leu2-1 hsc82::URA3 hsp82::GAL1-HSP82::LEU2*	S. Lindquist
JN516	*MAT**a** ura3-52 leu2-3,112 his3-11 trp1Δ1 lys2 SSA1 ssa2::LEU2 ssa3::TRP1 ssa4::LYS2 hem1-Δ100*	E. Craig and L. Zhang
CMY1300	Isogenic to JN516 except *SSA1/Myc*	This study
CMY8002	Isogenic to JN516 except *sti1Δ::Hyg^R*	This study
5B6	*MAT**a** ura3-52 leu2-3,112 his3-11 trp1Δ1 lys2 ssa1::HIS3 ssa2::LEU2 SSA3 ssa4::LYS2 hem1-Δ100 pGAL1-SSA1*	E. Craig and L. Zhang

of the *mal63* ORF was amplified using an upstream primer (5'-GGG**GAATTC**CTTCCCTTCGGTGAACAA-3'), which anneals to the *Eco*RI site at the codon 215/216, and a downstream primer (5'-GGG**GTCGAC**CCCGGGATCGATGTGAACAAT AAA-3'), which 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 boldface 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**CTTCCCTTCGGTG AACAA-3'), which contains an *Eco*RI site (in boldface) at codons 215/216 of *MAL63*, and the downstream primer (5'-CCCC**GTCGAC**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 boldface), were used to amplify a 200-bp 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 GGATCCATGCATCATCATCATCATCATGG GGGGTCAAAAGCTGTCGGTATTGAT-3') contains a *Bam*HI site (in boldface) 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'-GGG**CTCGAG**TTAATCAACTTCTTCAACGGT-3') contains an *Xho*I site (in boldface) 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**ATGCATCATCATCATCATCATGGGGGGTCAAAA GCTGTCGGTATTGAT-3') contains a *Bam*HI site (in boldface) 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'-GGG**GCATGC**GTTAGCGATAATCAAGAAGTG-3') contains a *Sph*I site (in boldface) 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**CAAAGGCTCGGTTGTCGACAA-3') contains a *Sac*I site (in boldface) and anneals to a site ~600 bp upstream of the *SSA1* open reading frame. Primer (5'-GGG**GGATCC**ATTATCTG TTATTTACTTGAA-3') contains a *Bam*HI site (in boldface) 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 10 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: 45 bp upstream of the TAA stop codon of *HSP82*, 2 glycine codons, 30 bp of sequence encoding the Myc epitope, a stop codon, and an 18-bp sequence complementary to the 5′ end of the *kan^R* cassette (5′- CCGGTTGAAGAGGTTCCAGCTGACA CCGAAATGGAAGAGGTAGAT GGGGG<u>GAACAAAAACTTAT TTCTGAAGAAGATCTG</u>TAG**CAGCTGAAGCTTCGTACG**-3′). The sequence encoding the Myc tag is underlined; bases homologous to the template are in boldface. The downstream primer contains 45 bp from the 3′ end of *HSP82* followed by 18 bp of sequence from the 3′ end of the *kan^R* cassette (5′-CAT TGTAATGTTTTACCCAGTTATTTC CATGCAGATGCCCTAT TTAC**GCATAGGCCACTAGTGGA**-3′). Bases homologous to the template are in boldface. 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 *hsc82Δ HSP82* and transformants were selected on YPD supplemented with 200 μg/ml geneticin (catalog no. 10131-035; Invitrogen Life Technologies, Carlsbad, CA) (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 units *vs.* 367 ± 57 units, respectively).

SSA1 and *STI1* were Myc tagged by a similar procedure. For *SSA1*, the upstream primer (5′-TCCAGCTCCAGAGGCTGAA GGTCCAACCGTTGAAGAAGTTGATGGGGGG<u>GAACAAAAA CTTATTTCTGAAGAAGATCTG</u>TAA**CAGCTGAAGCTTCGT ACG**-3′) and downstream primer (5′-TTCCTCATTATACCCA GATCATTAAAAGACATTTTCGTTATTAT CAATTGC**GCATA GGCCACTAGTGGA**-3′) were used to amplify the *kan^R* cassette. The PCR product was transformed directly into strain JN516 (from Elizabeth Craig), which 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 units *vs.* 1313 ± 203 units, respectively).

For *STI1*, the upstream primer (5′-CAGACGTTGATCG CTGCTGGTATCATCCGGACTGGCCGCGGGGGG<u>GAACAA AAACTTATTATTTCTGAAGAAGATCTG</u>TAA**CAGCTGAAG CTTCGTACG** -3′) and downstream primer (5′-TTTCCCT CGCCCAAGAAACTTATTATCTTCAAGTTCCGATTTCTCA AA**GCATAGGCCACTAGTGGA**-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 units *vs.* 1240 ± 239 units, respectively).

Construction of *sti1Δ::Hyg^R* strains: 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′-GCCCAAAAGTCTGCTCCCAAA TTCCTCACTGTA GCTACTAAAACAACCTATACGCAAGAAA**ATGGGTAAAAA GCCTGAACTCA**-3′) consists of 62 bp of the *STI1* promoter sequence immediately upstream of the start codon (ATG) of *STI1* followed by 22 bp of the *hyg^R* open reading frame (boldface). The downstream primer (5′-ATGTATGAAAAG CAGTAAAAAAAGAATTCAAGATAATAAAGTTATATTTCGT ATTATTT**TTATTCCTTTGCCCTCGGACG**-3′) consists of 60

bp of sequence homologous to the region immediately downstream of *STI1* and 21 bp 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 (catalog no. 10687-010, Invitrogen Life Technologies). Constructs were confirmed by PCR and Western blotting.

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

Preparation of cell extracts, immunoblot analysis, and coimmunoprecipitation: Strains were grown in the appropriate selective minimal medium to midlogarithmic 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 Vistra ECF kit (Amersham, Piscataway, NJ) 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 (Indianapolis). PGK (phosphoglycerol kinase) was detected by anti-PGK antibody from Molecular Probes (Eugene, OR).

Nondenaturing total cell extracts were prepared as follows. An aliquot of the culture containing ∼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° for at least 20 min. Cells were resuspended in 1 ml of a nondenaturing extraction buffer containing 50 mM sodium molybdate, 20 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 1 mM EDTA plus a cocktail of protease inhibitors [Roche, complete, mini, EDTA-free protease inhibitor tablets (catalog no. 1836170), yeast protease inhibitor cocktail (catalog no. P8215, Sigma, St. Louis), 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 were then combined with 30–40 μl of anti-HA agarose slurry (Roche anti-HA affinity matrix, catalog no. 11 815 01 6001) and incubated with gentle mixing at 4° overnight. The beads were washed three times with 300 μl of cold extraction buffer, 20 μl of cold extraction buffer, and 40 μl of 2× sample buffer were added to the beads, and the suspension incubated at 75° in a heating block for 15 min 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 1 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.

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
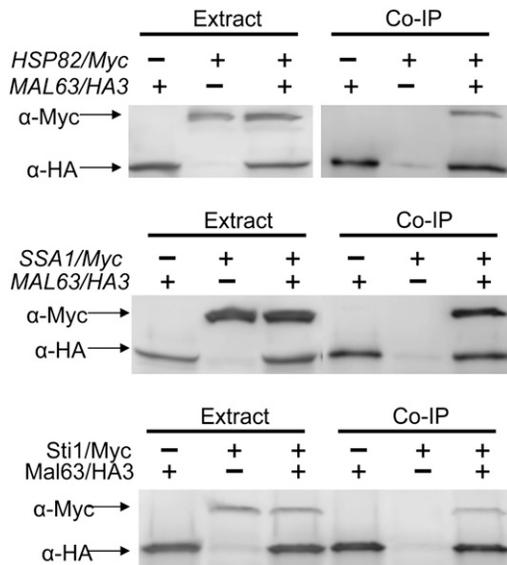
FIGURE 1.—Myc-tagged Hsp82, Ssa1, and Sti1 do not immunoprecipitate in the absence of HA-tagged Mal63. Strains carrying the genomic Myc-tagged alleles and the parental strains lacking the Myc-tagged *HSP82* (strains hsc82Δ and CMY1200), *SSA1* (strains JN516 and CMY1300), and *STI1* (strains W303 and 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 midlog at 30° 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.

FIGURE 2.—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 30° in selective synthetic medium lacking uracil and tryptophan and containing 3% glycerol and 2% lactate (v/v) plus the indicated concentration of maltose. Maltase activity was assayed as described in MATERIALS AND METHODS and is expressed as nmoles PNPG (*p*-nitrophenol-α-glucopyranoside) produced per milligram of protein per minute (top). 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 MATERIALS AND METHODS on total cell extracts prepared from transformants grown at the indicated maltose concentration (bottom). 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.

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 2 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 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 2). The turnover rate and expression level of Mal63/HA3p were determined in the strains carrying the GPD- and TEF-expressed *SSA1* and no effect of the reduced level of Hsp70 was 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 (bottom), although growth rates are not particularly affected (data not shown). Decreased expression of Ssa1p severely affects maltase
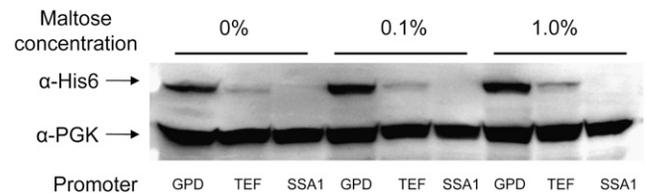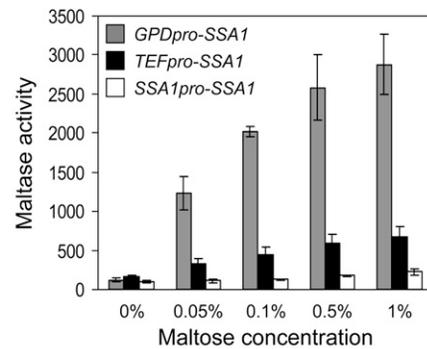
expression at all inducer concentrations and maltase activity correlates with the level of Ssa1p expression (top). While *SSA3* expression may be upregulated in response to the extremely low levels of *SSA1* expression from the construct with its native promoter, it clearly is not sufficient to complement 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.
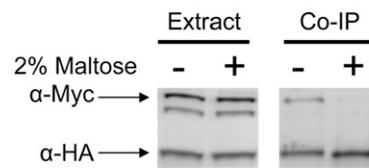
Binding of inducible Mal63/3HA *MAL*-activator to Hsp90 and Hsp70 is regulated by maltose: The results in Figure 2 predict that the *MAL*-activator should be found in association with Hsp70. BALI *et al.* (2003) demonstrated that Mal63/3HA *MAL*-activator coprecipitates with Hsp90 but in this study the His-tagged *HSP82/6His* was overexpressed from the GPD promoter and was carried on a multicopy 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 (SCHEUFLER *et al.* 2000; BRINKER *et al.* 2002; ODUNUGA *et al.* 2003). 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 coprecipitates with the Mal63/3HA activator and normalizing the intensity of the Myc signal to the HA signal. Control experiments shown in Figure 1 demonstrate that neither Myc-tagged Hsp82/Myc nor Ssa1/Myc proteins bind to the HA-sepharose beads in the absence of HA-tagged Mal63 *MAL*-activator.

Plasmid-borne *MAL63/3HA* was coexpressed 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 3, Mal63/3HA *MAL*-activator binds to Ssa1 and Hsp82 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 under 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 if
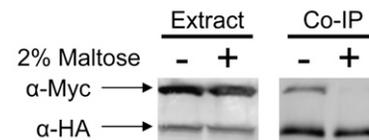

FIGURE 3.—Co-immunoprecipitation of inducible Mal63/3HA *MAL*-activator protein with Ssa1 and Hsp82. Strains CMY1200 (*HSP82/Myc hsc82Δ*) (top) and CMY1300 (*SSA1/Myc ssa2Δ ssa3Δ ssa4Δ*) (bottom) were transformed with the plasmid p416GPD-MAL63/3HA harboring the triple-HA-tagged inducible *MAL63 MAL*-activator gene. Transformants were grown to midlog at 30° 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.

these differences were 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.* (2000, 2003). Table 2 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 coexpressed in strains carrying genomic Myc-tagged *HSP82/Myc* and *SSA1/Myc* and interaction between the mutant mal63 proteins and the chaperones determined. The results in Figure 4 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 under uninduced but not under induced conditions. In contrast, the noninducible mutant activators do not form a complex with Hsp82/Myc even in cells grown under 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

TABLE 2

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

MAL-activator allele	Mutant alterations compared to Mal63p	Reference
Inducible		
MAL63	None	Kim and Michels (1988)
Noninducible		
mal63-283	K283A, D287A	Danzi *et al.* (2000)
mal63-331	R331A, R335A	Danzi *et al.* (2000)
mal63-364	E364A, R367A	Danzi *et al.* (2000)
mal63-401	D401A, K405A	Danzi *et al.* (2000)
mal63-467A9N	D467A, I469N	Danzi *et al.* (2000)
mal63-467S9V	D467S, I469V	Danzi *et al.* (2000)
Constitutive		
MAL43-C	31 alterations compared to Mal63p, 27 of which lie in residues 238–461	Gibson *et al.* (1997)
MAL63/43-C	27 alterations compared to Mal63p mapping to residues 238–461	Gibson *et al.* (1997)
MAL63(1-283)-T247A	C284NS, T247A	Gibson *et al.* (1997)

residues 283 to 467 and each mutation alters two to three residues within a five-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 *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 to 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
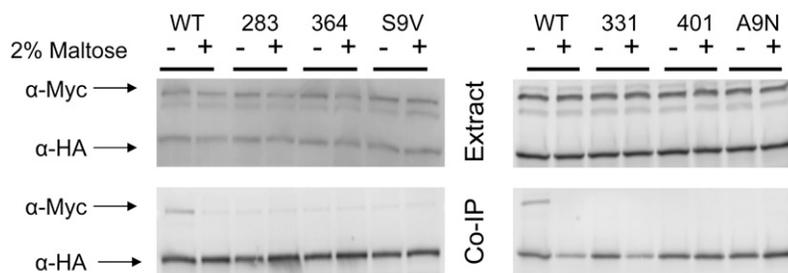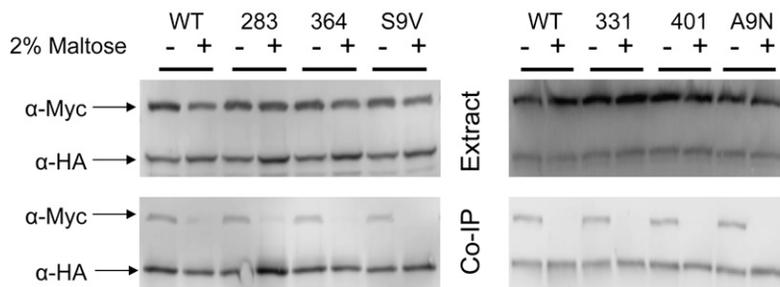

Figure 4.—Co-immunoprecipitation of noninducible *MAL*-activator mutant alleles with Ssa1 and Hsp82. Strains CMY1200 (*HSP82/Myc hsc82Δ*) (top) and CMY1300 (*SSA1/Myc ssa2Δ ssa3Δ ssa4Δ*) (bottom) were transformed with plasmids p416GPD-mal63-283, p416GPD-mal63-331, p416GPD-mal63-364, p416GPD-mal63-401, p416GPD-mal63-467S9V, and p416GPD-mal63-467A9N harboring the triple-HA-tagged alleles of the noninducible *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 3.
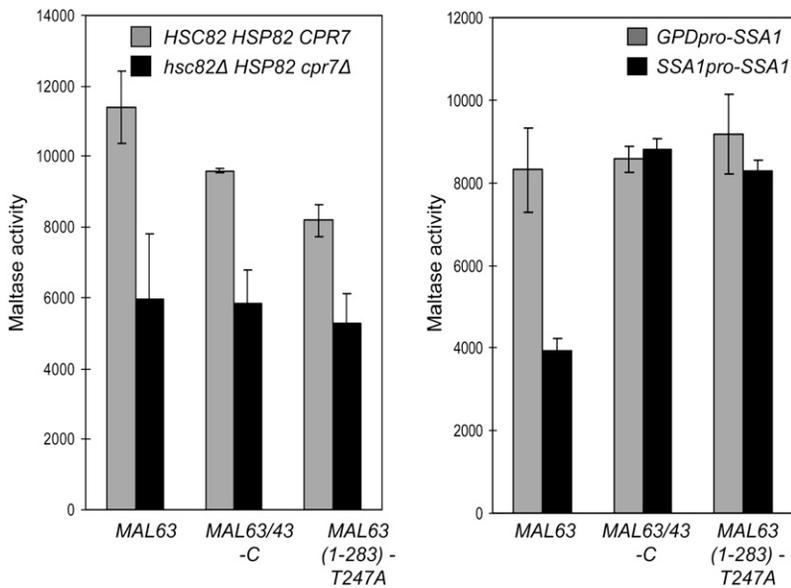
FIGURE 5.—Maltase expression in strains carrying constitutive *MAL*-activators is dependent on Hsp90 but not Hsp70. Strains W303 (*HSC82 HSP82*) and hsc82Δ cpr7Δ (*hsc82Δ HSP82 cpr7Δ*) (left) were transformed with plasmids p416GPD-MAL63/3HA, carrying the triple-HA-tagged inducible *MAL63* or p416GPD-MAL63/43-C 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 (GIBSON *et al.* 1997; DANZI *et al.* 2000). 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). Transformants were grown at 30° in selective synthetic medium lacking the appropriate nutrients for plasmid selection and containing 3% glycerol and 2% lactate (v/v) with 1% maltose added to transformants carrying *MAL63/3HA*. Maltase activity was assayed on at least three independent transformants as described in Figure 2.

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 non-inducible 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 was determined (Figure 5, left). All three transformed strains expressed very high levels of maltase ∼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 ∼30–50% in the double-deletion strain *hsc82Δ cpr7Δ* 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.
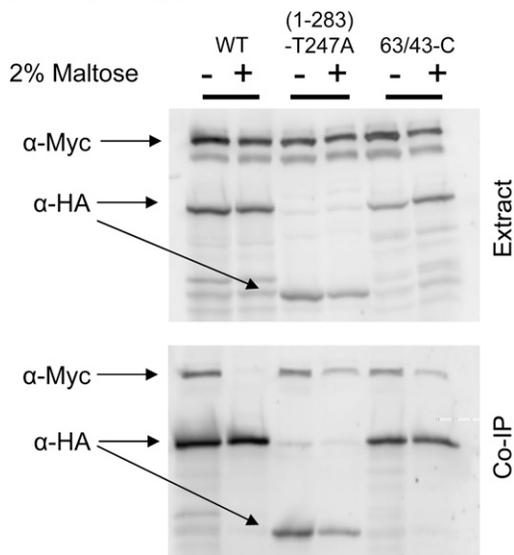
Dependency of the constitutive *MAL*-activators on Hsp70 was tested using the strain series expressing different levels of *SSA1/His* described above. Much to our surprise, maltase expression in the strains carrying the constitutive *MAL*-activator alleles is not affected by reduced Ssa1 expression while inducible maltase expression is ∼50% reduced in the strain with low levels of Ssa1. A more pronounced effect on maltase expression is observed in Figure 2 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. On the basis of the findings shown in Figure 5, 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 6. 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 8).

The results for Hsp70 binding are quite different but again consistent with the apparent lack of dependency on Hsp70 reported in Figure 5. Binding of the constitutive *MAL*-activator proteins to Ssa1/Myc is very weak or nonexistent, even in extracts prepared from cells grown under uninduced conditions, and addition of maltose to the growth medium further reduces association of the constitutive activators to Ssa1/Myc Hsp70 (see also Figure 8). Therefore, in the absence of inducer, constitutive activators form a stable complex containing Hsp82 but not Ssa1.

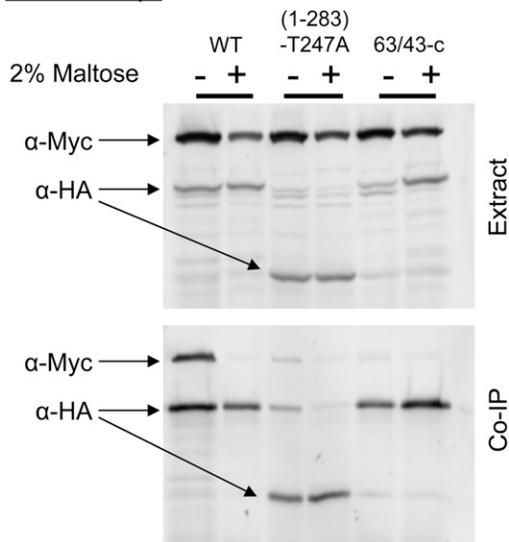## Co-IP of constitutive Mal63/HA3 mutants and Hsp82/Myc

FIGURE 6.—Co-immunoprecipitation of constitutive *MAL*-activator mutant proteins with Ssa1 and Hsp82. Strains CMY1200 (*HSP82/Myc hsc82Δ*) (top) and CMY1300 (*SSA1/Myc ssa2Δ ssa3Δ ssa4Δ*) (bottom) 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 3. Preparation of nondenaturing extracts, co-immunoprecipitation, and Western blot analysis were carried out as described for Figure 3.

Constitutive *MAL*-activator is dependent on *STI1*: The finding that constitutive *MAL*-activators exhibit very modest levels of binding to Ssa1 (Figure 6) and are independent of Ssa1 levels (Figure 5) 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 con-
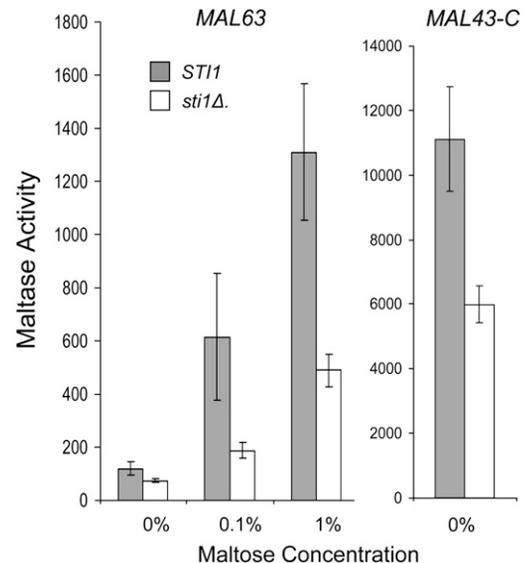

FIGURE 7.—Maltase expression is dependent on *STI1*. Strains CMY1200 (*STI1 HSP82/Myc hsc82Δ*) and CMY8001 (*sti1Δ HSP82/Myc hsc82Δ*) were transformed with plasmid YCp50-MAL63 or YCp50-MAL43-C carrying the inducible *MAL63* and constitutive *MAL43-C MAL*-activator alleles, respectively. Transformants were grown at 30° in selective synthetic medium lacking uracil and containing 3% glycerol and 2% lactate (v/v) plus the indicated concentration of maltose. Maltase activity was assayed in three independent transformants as described in Figure 2.

taining 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 homolog (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 ∼50% decrease in maltase activity in transformants carrying either allele, suggesting that both the inducible and the constitutive activators require Sti1 (Figure 7).

HSP82/Myc and *SSA1/Myc* strains containing *sti1Δ* were transformed 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 8, 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
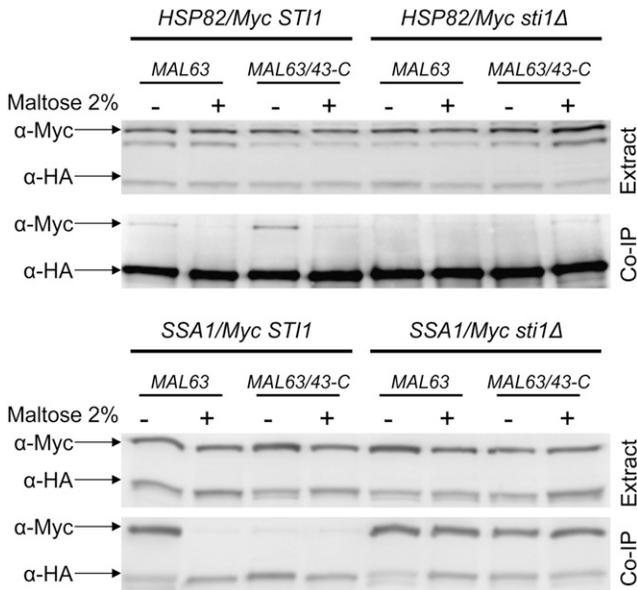
FIGURE 8.—Effect of *sti1Δ* on the co-immunoprecipitation of inducible Mal63/3HA *MAL*-activator with Ssa1 and Hsp82. Strains CMY1200 (*STI1 HSP82/Myc hsc82Δ*), CMY8001 (*sti1Δ HSP82/Myc hsc82Δ*), CMY1300 (*STI1 SSA1/Myc ssa2Δ ssa3Δ ssa4Δ*), and CMY8002 (*sti1Δ SSA1/Myc ssa2Δ ssa3Δ ssa4Δ*) were transformed 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 3.

condition under 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 under 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 4 and 6 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 coexpressed 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 1 demonstrate that Sti1/Myc protein does not bind to the HA-sepharose beads in the absence of HA-tagged Mal63 *MAL*-activator. Because *STI1* expression is
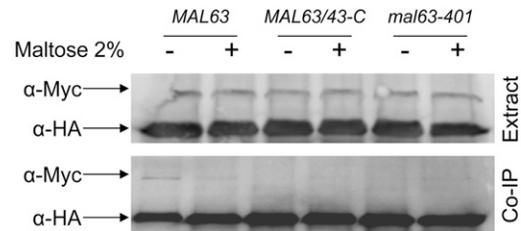

FIGURE 9.—Co-immunoprecipitation of inducible, constitutive, and noninducible *MAL*-activators with Sti1. Strain CMY8015 (*STI1/Myc*) was transformed with plasmids p416GPD-MAL63/3HA, p416GPD-MAL63/43-C, and p416-mal63-401 carrying the triple-HA-tagged alleles of inducible *MAL63*, constitutive *MAL63/43-C*, and noninducible *mal63-401*. Transformants were grown as described in Figure 3. Preparation of nondenaturing extracts, co-immunoprecipitation, and Western blot analysis were carried out as described for Figure 3.

low, the Myc signal is weak. Nonetheless, as is shown in Figure 9, inducible Mal63 *MAL*-activator binds Sti1/Myc protein in cells grown under uninduced but not induced (maltose) conditions. Along with the results reported in Figure 3, 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 *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; NEEDLEMAN 1991; GIBSON *et al.* 1997; DANZI *et al.* 2000, 2003). 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 2). 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 3 and 9) 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 PRATT and TOFT 2003; LAN *et al.* 2004; WEGELE *et al.* 2004, 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 under maltose-induced conditions leads to a dramatic reduction in the relative binding of Mal63p to both chaperones and 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 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 under uninduced growth conditions and not under induced growth conditions (Figure 4). On the other hand, binding to Hsp82 or Sti1 is not observed under any growth condition (Figures 4 and 9). 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 8. These results show that loss of Sti1 leads to Mal63 and Hsp70 binding in the presence of inducer, most likely by in-

hibiting 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 the 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
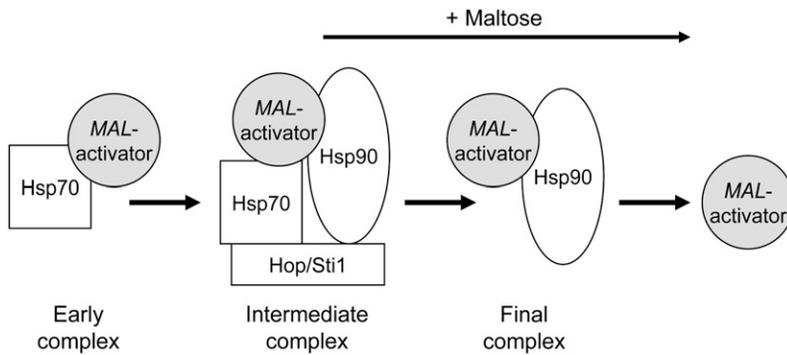
FIGURE 10.—Model of *MAL*-activator regulation by the Hsp90/Hsp70 chaperone cycle.

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 4). 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 affect phenotypes 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 (HOWARD *et al.* 1990; DALMAN *et al.* 1991).

Figure 10 summarizes our current model for the regulation of the *MAL*-activator by the Hsp90/Hsp70 chaperone cycle. Binding to Hsp70 is the first step in *MAL*-activator activation but, under 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 under 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.

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