Hsp90 Cochaperone Aha1 Is a Negative Regulator of the Saccharomyces MAL Activator and Acts Early in the Chaperone Activation Pathway*

Received for publication, July 2, 2009, and in revised form, February 13, 2010. Published, JBC Papers in Press, February 22, 2010, DOI 10.1074/jbc.M109.040600

Fulai Ran†,1,2, Nidhi Gadura,‡ and Corinne A. Michels§1

From the †Department of Biological Sciences and Geology, Queensborough Community College–City University of New York, Bayside, New York 11364, and the ‡Graduate School–City University of New York, New York, New York 10016, and the §Department of Biological Sciences and Geology, Queensborough Community College–City University of New York, Bayside, New York 11364

Aha1 is a ubiquitous cochaperone of the Hsp90/Hsp70 chaperone machine. It binds the middle domain of Hsp90 and stimulates ATPase activity, suggesting a function late in the chaperone pathway. Saccharomyces Mal63 MAL activator is a DNA-binding transcription factor and Hsp90 client protein. This study utilizes several MAL activator mutants to investigate Aha1 function in vivo. Deletion of AHA1 enhances induced Mal63-dependent maltase activity levels 2-fold, whereas overproduction of Aha1 represses expression. Maltase expression in strains carrying constitutive and super-inducible mutant activators with alterations near the C terminus (particularly residues 433–463) is unaffected by either aha1Δ or Aha1 overproduction. However, another constitutive activator with alterations outside of this C-terminal region is sensitive to Aha1 regulation. Previously, we showed that in the absence of inducer, Mal63 forms a stable intermediate complex with Hsp70, Hsp90, and Sti1, whereas noninducible mutant activators bind only with Hsp70 in an apparent early complex. Here, we find that triple Myc-tagged Aha1/Myc3 copurifies with all noninducible Mal63 mutant activators tested. Aha1/Myc3 association with inducible Mal63 is observed only in a sti1Δ strain, in which Hsp90 binding and intermediate complex formation are defective. Constitutive and super-inducible mutant activators with C-terminal alterations do not bind Aha1 even in a sti1Δ strain. Mal63 binding to Hsp90 and Hsp70 is enhanced 3-fold by loss of Aha1. These results suggest an interaction between Aha1 and residues near the C terminus of Mal63 thereby regulating Hsp90 association. A novel mechanism for the negative regulation of the MAL activator by Aha1 cochaperone is proposed.

Client protein folding and activation by the Hsp90 chaperone machine utilize a number of associated factors called cochaperones that bind to specific regions of Hsp90 and function to facilitate chaperone activity by regulating Hsp90 ATPase activity. Hsp70–Hsp90 interaction, and client protein binding or release (reviewed in Refs. 1–16). Aha1 cochaperone (for activator of Hsp90 ATPase) is the only cochaperone known to stimulate the ATPase activity of Hsp90 and thus is proposed to positively regulate client protein activation (1, 2). AHA1 was first identified as 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 (17). The 350-residue Aha1 protein exhibits 36% sequence similarity to Hch1 within its N-terminal region (to residue 153). Aha1 cochaperone is a member of a ubiquitous family of eukaryotic proteins, although to date, only Candida albicans has been found to contain an Hch1 homologue (18). Saccharomyces strains lacking HCH1, AHA1, or both are viable, but growth is temperature-sensitive, particularly in the hch1Δ aha1Δ double disruption strain in nonfermentable carbon sources. Aha1 protein binds to Hsp90, and AHA1 expression is up-regulated in heat-stressed and geldanamycin-treated cells, all considered to be characteristics of an Hsp90 cochaperone (18).

In Saccharomyces, deletion of AHA1 causes defects in the activation of GR3 and v-Src kinase (18–20). Similarly, down-regulation of Aha1 expression via small interfering RNA in mammalian cells leads to significant decreases in hormone-dependent GR activation (4). The mechanism of this Aha1 dependence is still a matter of some discussion. In vitro studies demonstrate that Aha1 cochaperone significantly increases eukaryotic Hsp90 ATPase activity (18, 21, 22 and reviewed in Ref. 23). Conflicting reports suggest that the N-terminal domain of Aha1 and the full-length Hch1 activate Hsp90 ATPase activity, but the temperature-sensitive mutation of HSP82 used to isolate these suppressors does not exhibit a defect in ATPase activity at the nonpermissive temperature (4, 5, 18–20, 24). The N-terminal domain of Aha1 binds to the middle region of Hsp90, and this binding competes with the binding of the early cochaperones Hop/Sti1 and p50/Cdc37 and the late cochaperone p23/Sba1, all cochaperones that inhibit Hsp90 ATPase suggesting other possible mechanisms of action (4, 6, 20). Most recently, a variety of in vitro and in vivo methods, including molecular footprinting and cross-linking, and structure-function mutation analysis of Aha1 were used to define sites of interaction between full-length mammalian Aha1 and Hsp90 (25). Koulov et al. (25) demonstrate that full-

* This work was supported, in whole or in part, by National Institutes of Health Grant GM28216 (to C. A. M.).
1 Performed this work in partial fulfillment of the requirements of the Ph.D. degree from the Graduate School of City University of New York.
2 To whom correspondence should be addressed: Biology Dept., Queens College, City University of New York, 65-30 Kissena Blvd., Flushing, NY 11367. Fax: 718-997-3321; E-mail: corinne.michels@qc.cuny.edu.

3 The abbreviations used are: GR, glucocorticoid receptor; HA, hemagglutinin; CFTR, cystic fibrosis transmembrane conductance regulator.
Aha1 Cochaperone, Yeast MAL Activator Negative Regulator

TABLE 1
List of Saccharomyces strains

| Strain     | Genotype                      | Source                  |
|------------|-------------------------------|-------------------------|
| W303       | MATa leu2-3,112 ura3-1 trp1-1 his3-11,15 can1-100 GAL 11C2 | S. Lindquist 28         |
| CMY1200    | Isogenic to W303 except His82/Myc hsc82Δ::LEU2 | 28                      |
| CMY1300    | Isogenic to JN516 except Ssa1/Myc | 28                      |
| CMY8001    | Isogenic to CMY1200 except stlΔ::Htg | 28                      |
| CMY8002    | Isogenic to JN516 except stlΔ::Htg | 28                      |
| CMY8003    | Isogenic to CMY1300 except aha1Δ::Htg | This study             |
| CMY8010    | Isogenic to CMY1200 except aha1Δ::Htg | This study             |
| CMY8015    | Isogenic to W303 except STII/Myc | 28                      |
| CMY8031    | Isogenic to CMY1300 except hch1Δ::Htg | This study             |
| CMY8301    | Isogenic to W303 except AHA1/Myc3 | This study             |
| CMY8305    | Isogenic to CMY8301 except stlΔ::Htg | This study             |

length Aha1 is required for effective stimulation of Hsp90 ATPase, that the N- and C-terminal domains of Aha1 cooperatively bind Hsp90 cross-bridging the Hsp90 dimer, and that the C-terminal domain binds the Hsp90 N-terminal ATPase domain although, as reported earlier, the N-terminal domain of Aha1 binds the middle domain of Hsp90. Finally, Aha1 cochaperone expression is up-regulated in a number of tumor lines, coincident with the activation of several signaling kinases (26, 27). Taken together, these findings are consistent with the proposed role of Aha1 cochaperone as a positive regulator of Hsp90 client proteins, but the mechanism remains unclear.

Here, we explore the role of Aha1 cochaperone in the activation of a native Saccharomyces client protein, the MAL activator that regulates the maltose-induced expression of the genes for maltose fermentation. This analysis builds on results reported in Ref. 28 that can be summarized as follows. Working with Myc-tagged alleles of the chaperone genes, Ran et al. (28) showed that the in vivo temporal order of Hsp90-Hsp70 chaperone complexes formed during maltose induction of the MAL activator parallels that demonstrated in vitro for induction of the glucocorticoid receptor (see Fig. 10) (reviewed in Refs. 16 and 24) with some variations on this theme. During chaperone-dependent activation, nascent MAL activator progresses sequentially from the so-called early complex, in which it binds with Ssa1, the Saccharomyces Hsp70, and next to the intermediate complex in which it is associated with Ssa1, Hsp82, and Sti1, the Saccharomyces Hsp70, Hsp90, and Hop, respectively. The MAL activator intermediate complex is stable in the absence of inducer maltose, but addition of maltose causes the release of inducible MAL activator from the complex in an active form capable of DNA binding and transcription activation. Ran et al. (28) demonstrated significant differences between the chaperone activation pathway for the MAL activator and that of the glucocorticoid receptor. Primarily, Hsp70 rather than Hsp90 serves as the negative regulator of the inducible MAL activator, and retention of the MAL activator in the intermediate complex in an inactive form, as opposed to the final complex, is essential for inducible regulation.

The temporal order of the MAL activator chaperone pathway proposed by Ran et al. (28) and diagrammed here in Fig. 10 is based on several findings. Deletion of STII causes activation defects for both inducible and constitutive MAL activators and results in the loss of Hsp82 binding and an enhancement of Ssa1 binding even in the presence of inducer maltose, indicating that the temporal order of complex formation is blocked at a point downstream of the early complex. Noninducible activator proteins form complexes with Ssa1 and not with Hsp82 or Sti1 in the absence of inducer, whereas inducible Mal63 and constitutive activators are similarly bound to Ssa1 and not Hsp82, only in sti1Δ. This result suggests that the early complex is formed first for all MAL activators alleles. Additionally, it indicates that constitutive activators must pass from the early complex to the intermediate complex and on to the final complex with Hsp82 to be fully active.

Of particular relevance to the study described here, the work of Ran et al. (28) demonstrates that the different alleles of the MAL activator can be used as markers to distinguish the early, intermediate, and final chaperone complexes. As described above, in the absence of maltose, inducible Mal63 MAL activator is retained in what appears to be an intermediate complex. Thus copurification of a cochaperone with inducible Mal63 MAL activator in the absence of maltose suggests that the cochaperone is a component of the intermediate complex. By contrast, noninducible mutant MAL activators bind to Ssa1 but not Hsp82 or Sti1 and never progress beyond what appears to be an early complex. Copurification of a cochaperone with non-inducible mutant MAL activators in the absence of maltose suggests that this cochaperone is a component of the early complex. Furthermore, constitutive MAL activators are seen to bind only Hsp82, not Ssa1 or Sti1, and, even in the absence of maltose, appear to progress rapidly through the early and intermediate complexes to form a final complex and possibly some unbound activator. Thus, a cochaperone that copurifies with constitutive MAL activators is a candidate component of the final complex.

In this study, we use these different classes of MAL activator mutants as well as a super-inducible mutant, as described previously (29), as markers of the different chaperone complexes to identify the complex or complexes containing the cochaperone Aha1. In contrast to studies with GR and v-Src kinase, the results reported here show that Aha1 cochaperone is a negative regulator of inducible Mal63 MAL activator and that it interacts genetically and physically with the C terminus of certain MAL activators. Alternative models of the mechanism of this novel regulation are presented under the “Discussion.”

EXPERIMENTAL PROCEDURES

Yeast Strains and Plasmids—Table 1 lists the Saccharomyces strains used for this study. Details of their construction are described below or in Ref. 28. All carry naturally occurring defective copies of the MAL1 and MAL3 loci encoding functional maltose permease and maltase genes but nonfunctional
MAL activator alleles and for the expression of the MAL structural genes. Plasmids carrying the triple HA-tagged alleles of MAL63 and the various mutant MAL activators are described in detail in Ref. 28. An untagged version of the plasmid expressing non-inducible mal63–401 was constructed from the plasmid carrying the tagged gene by removal of the Ncol fragment containing the triple HA tag by digestion and religation. The in-frame deletion of the tag fragment was confirmed by sequencing.

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 SalI sites was inserted at the 5’ end of the Kan \( ^{R} \)-containing cassette pFA6–KanMX2 as follows. The following primers were annealed, and the 3’ end was extended by one round of DNA synthesis using TaqDNA polymerase in a thermal cycler in the absence of added template DNA. The upstream primer (5’GGG GTCGAC GGG GGG GAG CAG AAA CTC ATC TCT CTT GAA GAA GAT CTC GGA CAA AAG TTG ATT TCA GAA GAA GAT CTG 3’GAA CAA AAG TTG ATT TCA GAA GAA GAT CTG) consists of the following: a SalI site (in boldface), two glycine codons, followed by the sequence encoding two copies of the Myc epitope tag, and Myc-1 and Myc-2 (underlined). The second copy of the Myc epitope sequence is complementary to and anneals to the Myc-2 sequence (underlined) of downstream primer (5’GAG GTGGC CAG ATC TTC TGG TAA ATT AAT TAA CAG 3’/H11032 TGG TAA ATT AAT TAA CAG 3’/H11032) has a BamHI site (in boldface) and anneals to the first 6 codons of the AHA1/Myc3 open reading frame. The downstream primer (5’GGG ATCGAT TTA ATT TAA CAG ATC TTC TTC TGA AAT CAA CTT TTG TTC 3’/H11032 TTA ATT TAA CAG ATC TTC TTC TGA AAT CAA CTT TTG TTC 3’/H11032) contains Sall site (in boldface), four stop codons (italics), followed by sequences encoding two Myc tags, Myc-2 and Myc-3 (underlined). The PCR product was digested with Sall and cloned into the Sall-digested pFA6–KanMX2 (28) to create plasmid pFA6–Myc3-Stop–kanMX2. The final modified Myc3-Stop–kanR cassette was confirmed by sequencing.

Addition of a Triple Myc Epitope Tag to Genomic AHA1—A PCR-based one-step replacement method was used to insert the triple yc epitope tag at the C terminus of AHA1 in W303 to create strain CMY8301. The amplified product consists of the following sequences in order: 37 bases upstream of the stop codon of AHA1, one copy of the sequence encoding the triple Myc epitope, a stop codon, the Kan \( ^{R} \) gene cassette, and 45 bases downstream of the AHA1 stop codon, and it was synthesized as follows. Plasmid pFA6–Myc3-Stop–kanMX2 was used as a template to amplify the kanamycin (G418) resistance gene (30) with the upstream primer (5’GGG GTGGC CAG ATC TTC TGG TAA ATT AAT TAA CAG GGT TTT GGT GCC GTA TTA GGG GGG GGG GAG CAG AAA CTC 3’) and the downstream primer (5’TGG TAA ATT CCG GGG AGG CCG TCG GCC GCG CGC AAA AGT AGG GCA TAG GCC ACT AGT GGA 3’GGA AGG CCG TCG GCC GCG CGC AAA AGT AGG GCA TAG GCC ACT AGT GGA 3’). The upstream primer contains 38 bases upstream of the TAA stop codon of AHA1 and 21 bases complementary to the 5’ end of the modified triple Myc-Stop–kanR cassette. The downstream primer contains 45 bases from the 3’ end of AHA1 followed by 18 bases from the 3’ end of the Kan \( ^{R} \) cassette. Bases homologous to the template are in boldface. The PCR product was transformed directly into strain W303 and transformants were selected on YPD supplemented with 200 \( \mu \)g/ml geneticin (catalog no. 10131-035, Invitrogen) (28). Epitope tagging of AHA1 was confirmed by PCR and Western blotting analysis.

Construction of High Copy Overexpression Plasmids p423GPD-AHA1/Myc and p423GPD-AHA1C/Myc—Nested PCR was used to amplify the fragment containing the genomic AHA1/Myc3 open reading frame from strain CMY8301. In the first round of PCR, the upstream primer (5’CTT ACT TTC GTT ATT CCT TTC AGT CTT ATT 3’) annealing to the AHA1 promoter region ~30 bp upstream of the open reading frame and the downstream primer (5’CGG ATT CAG TCG TCA CTC AT 3’) annealing to the sequence ~500 bp downstream of the genomic tagged AHA1/Myc3 were used to amplify a product containing the complete AHA1/Myc3 open reading frame with some flanking sequences. This primary PCR product was used as a template in a second round PCR to amplify a smaller fragment containing the AHA1/Myc3 open reading frame with the following primer pair. The upstream primer (5’GGG GGAAC ATG GCC GTG AAT AAC CCA 3’) has a BamHI site (in boldface) and anneals to the first 6 codons of the AHA1/Myc3 open reading frame. The downstream primer (5’GGG ATCGAT TTA ATT TAA CAG ATC TTC TTC TGA AAT CAA CTT TTG TTC 3’) has a ClaI site (in boldface) and anneals to a site downstream of the sequence of the triple Myc tag. The second PCR product was digested with BamHI and ClaI and cloned into the BamHI- and ClaI-digested vector p423GPD placing AHA1/Myc3 under the control of the constitutive GPD promoter. The construct was confirmed by sequencing and Western blotting.

Plasmid p423GPD-AHA1C/Myc3 was constructed by amplifying the C-terminal half of the AHA1 open reading frame from codon 156 using plasmid p423GPD-AHA1/Myc3 as the target. The upstream primer (5’GGG GGATCC ATG GCC GTG AAT AAC CCA 3’) has a BamHI site (in boldface) and an ATG translation start codon and anneals to codons 156–161 of the AHA1 open reading frame (underlined). The downstream primer was the same as the second round downstream primer in the amplification of AHA1/Myc3 (see above). The construct was confirmed by sequencing and Western blotting.

Construction of ha1Δ::Hyg \( ^{R} \), hch1Δ::Hyg \( ^{R} \), and sti1Δ::Hyg \( ^{R} \) Strains—Deletion of STI1 in strains CMY1200 and CMY1300 carrying Myc-tagged HSP82/Myc and SSA1/Myc, respectively, was accomplished 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, shown in Table 2, consists of 62 bp of the STI1 promoter sequence immediately upstream of the start codon of STI1 followed by 22 bp of the Hyg \( ^{R} \) open reading frame (boldface). The downstream primer (Table 2) 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 (30). The PCR product was transformed directly into strains CMY1200 and CMY1300, and transformants were selected on YPD supplemented with 200 mg/liter hygromycin (catalog no. 10687-010 Invitrogen). Constructs were confirmed by PCR and Western blotting analysis.
Western blotting. The other CMY1200 and CMY1300 deletion strains were constructed by a similar procedure with primer sequences listed in Table 2.

**Maltase Activity Assay**—Cultures were grown to mid-log phase in selective minimal media containing the indicated carbon source and lacking appropriate nutrients for plasmid maintenance. Extracts were prepared, and maltase activity was measured in whole cell extracts as described previously (31, 32). Activity is expressed as nanomoles of p-nitrophenol-D-glucopyranoside hydrolyzed per min per mg of protein, measured using the Protein Assay Dye Reagent (Bio-Rad). Assay values are the average of results from three independent transformants assayed in duplicate. Variation is ~20%.

**Preparation of Cell Extracts, Western Blot Analysis, and Coimmunoprecipitation**—Strains were grown in the appropriate selective minimal medium to mid-logarithmic phase (400 nm of 0.2–0.5). Denaturing cell extracts were prepared as described previously (33). Nondenaturing total cell extracts were prepared by glass bead lysis as described previously (28) in an extraction buffer containing 50 mM sodium molybdate, 20 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 1 mM EDTA plus a mixture of protease inhibitors (Roche Applied Science, complete, mini, EDTA-free protease inhibitor tablets (catalog no. 1836170), Sigma yeast protease inhibitor mixture (catalog no. P8215), and a toothpick tip-full of sodium bisulfite), and flash-frozen in liquid nitrogen. Immunoprecipitation was carried out from the nondenaturing total cell extracts as described previously (28) using a slurry of anti-HA-agarose beads (anti-HA affinity matrix, catalog no. 11 815 01 6001, Roche Applied Science).

Western blot analysis was carried out as described previously (28) using standard methods, and the proteins were detected using the Amersham Biosciences Vistra ECF kit in which the secondary antibody is conjugated to a fluorescent dye. The membranes were probed with primary antibodies anti-HA antibody and anti-Myc antibody (Roche Applied Science) and secondary antibody anti-ECL (Roche Applied Science), and protein levels were visualized by using a Molecular Dynamics Storm 860 (GE Healthcare), and the signal was quantified using software provided by the manufacturer.

### RESULTS

**Aha1 Is a Negative Regulator of Inducible Mal63 MAL Activator Induction**—AHA1 and HCH1 were deleted in strain CYM1300 (SSA1/Myc ss2-4Δ AHA1 HCH1) and otherwise isogenic CMY803 (aha1Δ HCH1) and CMY8031 (AHA1 hch1Δ) were transformed with plasmids YCp50-MAL63 or YCp50-MAL43-C carrying the inducible MAL63 or constitutive MAL43-C genes, respectively, in the CEN vector YCp50. Transformants carrying YCp50-MAL63 were grown at 30 °C in selective synthetic medium lacking uracil and containing 3% glycerol and 2% lactate (v/v) plus the indicated concentration of maltose. Transformants carrying YCp50-MAL43-C were grown under the same conditions except the medium lacked maltose. Maltase activity is expressed as nanomoles of p-nitrophenol-α-glucopyranoside produced per mg of protein per min (32, 60). Assays were carried out on at least three independent transformants. The error bars indicate standard deviation from three independent transformants assayed in duplicate.

**FIGURE 1.** Loss of Aha1 cochaperone leads to increased maltase activity in strains carrying inducible MAL63 but not constitutive MAL43-C MAL activators. Strains CMY1300 (SSA1/Myc ss2-4Δ AHA1 HCH1) and otherwise isogenic CMY803 (aha1Δ HCH1) and CMY8031 (AHA1 hch1Δ) were transformed with plasmids YCp50-MAL63 or YCp50-MAL43-C carrying the inducible MAL63 or constitutive MAL43-C genes, respectively, in the CEN vector YCp50. Transformants carrying YCp50-MAL63-MAL63 were grown at 30 °C in selective synthetic medium lacking uracil and containing 3% glycerol and 2% lactate (v/v) plus the indicated concentration of maltose. Transformants carrying YCp50-MAL43-C were grown under the same conditions except the medium lacked maltose. Maltase activity is expressed as nanomoles of p-nitrophenol-α-glucopyranoside produced per mg of protein per min (32, 60). Assays were carried out on at least three independent transformants. The error bars indicate standard deviation from three independent transformants assayed in duplicate.

**TABLE 2**

| Gene deletion | Primer sequences |
|---------------|-----------------|
| aha1Δ::HygR   | Upstream: 5' TCTATGGTCTTACTTTTGTTATTCTTTTGTTATTAATCATGTTTA  |
|               | Downstream: 5' TATGCCAATCTATTTAGTATATTCTTTTTCTTTGTTACGGAGC |
| hch1Δ::HygR   | Upstream: 5' CTAACACACTTACAAAGATGGTAGGCAAAGTTACCA  |
|               | Downstream: 5' AAATTAAGGGGGCGGTGGTCTACTAATGACAGCCC |
| mtl1Δ::HygR   | Upstream: 5' GCCCAAAAGTCTGCCAATACCTACGTAGCTACTAAAA  |
|               | Downstream: 5' GCACCTATAGCAAGAAGATGGTGAAAGAAGCCTGAGC |

**Interacts with Aha1**

**TABLE 2**

**One-step gene deletion primers**

| Gene deletion | Primer sequences |
|---------------|-----------------|
| aha1Δ::HygR   | Upstream: 5' TCTATGGTCTTACTTTTGTTATTCTTTTGTTATTAATCATGTTTA  |
|               | Downstream: 5' TATGCCAATCTATTTAGTATATTCTTTTTCTTTGTTACGGAGC |
| hch1Δ::HygR   | Upstream: 5' CTAACACACTTACAAAGATGGTAGGCAAAGTTACCA  |
|               | Downstream: 5' AAATTAAGGGGGCGGTGGTCTACTAATGACAGCCC |
| mtl1Δ::HygR   | Upstream: 5' GCCCAAAAGTCTGCCAATACCTACGTAGCTACTAAAA  |
|               | Downstream: 5' GCACCTATAGCAAGAAGATGGTGAAAGAAGCCTGAGC |

**FIGURE 1.** Loss of Aha1 cochaperone leads to increased maltase activity in strains carrying inducible MAL63 but not constitutive MAL43-C MAL activators. Strains CMY1300 (SSA1/Myc ss2-4Δ AHA1 HCH1) and otherwise isogenic CMY803 (aha1Δ HCH1) and CMY8031 (AHA1 hch1Δ) were transformed with plasmids YCp50-MAL63 or YCp50-MAL43-C carrying the inducible MAL63 or constitutive MAL43-C genes, respectively, in the CEN vector YCp50. Transformants carrying YCp50-MAL63 were grown at 30 °C in selective synthetic medium lacking uracil and containing 3% glycerol and 2% lactate (v/v) plus the indicated concentration of maltose. Transformants carrying YCp50-MAL43-C were grown under the same conditions except the medium lacked maltose. Maltase activity is expressed as nanomoles of p-nitrophenol-α-glucopyranoside produced per mg of protein per min (32, 60). Assays were carried out on at least three independent transformants. The error bars indicate standard deviation from three independent transformants assayed in duplicate.

**TABLE 2**

**One-step gene deletion primers**

| Gene deletion | Primer sequences |
|---------------|-----------------|
| aha1Δ::HygR   | Upstream: 5' TCTATGGTCTTACTTTTGTTATTCTTTTGTTATTAATCATGTTTA  |
|               | Downstream: 5' TATGCCAATCTATTTAGTATATTCTTTTTCTTTGTTACGGAGC |
| hch1Δ::HygR   | Upstream: 5' CTAACACACTTACAAAGATGGTAGGCAAAGTTACCA  |
|               | Downstream: 5' AAATTAAGGGGGCGGTGGTCTACTAATGACAGCCC |
| mtl1Δ::HygR   | Upstream: 5' GCCCAAAAGTCTGCCAATACCTACGTAGCTACTAAAA  |
|               | Downstream: 5' GCACCTATAGCAAGAAGATGGTGAAAGAAGCCTGAGC |
Aha1 Cochaperone, Yeast MAL Activator Negative Regulator

FIGURE 2. Comparison of maltose-dependent maltase induction in AHA1 and aha1Δ. Strains CMY1300 (SSA1/Myc ssa2-4 AHA1) and otherwise isogenic CMY8003 (aha1Δ) were transformed with plasmid YCp50-MAL63 carrying inducible MAL63 in YCp50. Transformants were grown at 30 °C in selective synthetic medium lacking uracil and containing 3% glycerol and 2% lactate (v/v) plus the following concentrations of maltose: 0, 0.025, 0.05, 0.075, 0.1, 0.5, and 1%. Maltase activity was assayed as described for Fig. 1.

ducible, constitutive, or super-inducible phenotypes (29, 35, 36). Several of these mutant alleles are used here to define the genetic interaction between the cochaperone Aha1 and the MAL activator. Fig. 3 lists these mutant alleles and diagrams the location of the alterations in each in relation to the inducible Mal63. For complete information on these mutant alleles, including the specific alterations, see Refs. 29, 35, 36. A brief overview is presented below.

Six of the ∼30 noninducible mal63 alleles isolated in Ref. 29 were used in this study. Each contains 2–3 altered residues within a 5-residue window and maps to sites confined within the MAL activator regulatory domain from residues 244 to 469. The altered residues are indicated in Fig. 3. Interestingly, noninducible and, in two cases, super-inducible mutant alleles were identified that contain alterations near the extreme C terminus of Mal63. Strains carrying the super-inducible alleles require maltose for induction but induce to levels ~2-fold higher than strains expressing Mal63 MAL activator, which we found reminiscent of the aha1Δ phenotype (29). Super-inducible allele MAL63–460 is included in this study (Fig. 3).

The constitutive MAL activator allele MAL43-C was isolated as an x-ray-induced maltose-fermenting revertant of a maltose nonfermenting strain (reviewed in Refs. 37, 38). Mal43-C activator differs from Mal63 at 31 residues, 27 of which are contained in the constitutive Mal63/43-C MAL activator chimera made by fusing the promoter region and the first 215 residues of Mal63 to residues 216 to 470 of Mal43-C. Of these 27 alterations, 10 are clustered in residues 344–379 (Fig. 3, shown as a region of vertical lines) and 9 others are clustered in residues 417–461 (cross-hatched lines). In addition, Mal63/43-C MAL activator has another eight largely conservative alterations scattered outside of these clusters at residues 238, 250, 279, 307, 319, 326, 388, and 404 (not shown in Fig. 3).

Constitutive Mal63/23-SVI MAL activator is a chimera of the N-terminal 1–215 residues of Mal63 and the C-terminal residues 216–470 of Mal23 and also contains alterations S392A, V395I, and 1402V (36). Thus, except for three very widely scattered single residue differences (not shown in Fig. 3), Mal63/23-SVI and Mal63 MAL activators differ only by nine residues from 417 to 461, which are the same nine alterations found in this region of Mal43-C MAL activator. Replacement of residues 343–359 of Mal63 (Fig. 3, shown as a stippled region) with 10 clustered alterations found in the constitutive Mal23-C MAL activator (referred to as block 2) produces a constitutive allele, Mal63-block2 MAL activator. This allele is identical to Mal63 outside of block 2, which contains eight altered residues and a two-residue deletion compared with Mal63 (36).

To compare the effects of loss of Aha1 cochaperone on the various MAL activator mutant alleles, maltase expression was assayed in strains carrying plasmid-borne inducible MAL63, noninducible mal63–283, super-inducible MAL63–460, and constitutive MAL63/43-C, MAL63-block2, and MAL63/23-SVI MAL activator genes (Fig. 4). Loss of Aha1 increases the level of maltase activity only in strains expressing inducible Mal63 and constitutive Mal63-block2 activators. Both of these alleles are identical in sequence except in residues 343–359 (Fig. 3). Maltase activity in strains expressing the constitutive Mal63/43-C and Mal63/23-SVI activators and the super-inducible Mal63–460 activator are unaffected by aha1Δ. Compared with Mal63, these three mutant activators each contain multiple yet different alterations mapping to residues 417–463, with 2–8 clustered changes in residues 433–463 (Fig. 3). Thus, insensitivity to Aha1 regulation correlates with mutational changes in residues 417–463 of Mal63 suggesting that this region of the MAL activator could be the target of Aha1 cochaperone action. Maltase activity in the strain carrying the noninducible mutant activator mal63-283 is very low and does not appear to be affected by loss of Aha1.

To further explore the importance of this C-terminal region of Mal63, we investigated the impact of Aha1 cochaperone overproduction on this same series of MAL activator alleles. AHA1 overexpression was achieved using a high copy plasmid carrying a triple Myc-tagged allele of Aha1/Myc3 (tagged at the C terminus) expressed from the high level GPD promoter. To demonstrate the extent of overproduction of Aha1/Myc3, this plasmid was introduced into a strain containing a single genomic copy of Aha1/Myc3 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 (Fig. 4, inset) indicates that Aha1/Myc3 protein expression is elevated ~10 times over genome expression by the plasmid-borne copy.

The Aha1/Myc3 overexpression plasmid was introduced into a strain containing genomic AHA1 and carrying plasmid-borne copies of the indicated MAL activator genes. As shown in Fig. 4, overexpression of Aha1/Myc3 cochaperone represses maltase expression in strains carrying MAL63 and MAL63-block2 (by ~40%) but does not significantly affect the activity of the other MAL activator alleles tested. (The significance of the impact of aha1Δ and Aha1/Myc3 overproduction was determined by one-way analysis of variance analysis.) Thus, only MAL63 and MAL63-block2 activity are sensitive to Aha1 regulation, *i.e.* their transcription activator activity is enhanced by loss of Aha1 cochaperone (aha1Δ) and repressed by Aha1 cochaperone overproduction. The other alleles tested are insensitive to Aha1 regulation. What distinguishes Mal63 and
Mal63-block2 from Mal63/43-C, Mal63–460, and Mal63/23-SVI? We propose it is the sequence of the C-terminal region, approximately residues 417–463. Mal63 and Mal63-block2 are identical in this region and different from the other alleles, all of which carry multiple altered residues in this region. Taken together, these results suggest a genetic and possibly physical interaction between the Aha1 cochaperone and residues in the region near the C terminus of the Mal63 MAL activator, specifically 417–463.

**Aha1 Cochaperone Binds to Noninducible Mutant MAL Activators in an Apparent Early Complex**—Previously, Ran et al. (28) described an in vivo analysis of the Hsp90/Hsp70 chaperone activation pathway of the MAL activator utilizing a number of MAL activator mutant alleles to tease apart the steps in the pathway and identify regulatory mechanisms. For this, Ran et al. (28) constructed a series of strains (listed in Table 1) in which the genomic Hsp90 (HSP82), Hsp70 (SSA1), and Hop (STI1) genes were epitope-tagged at their C terminus with a single copy of the sequence encoding the Myc epitope. Triple HA epitope-tagged alleles of the various MAL activator mutants were expressed in these strains, and the components contained in the complexes formed between the MAL activator proteins and the chaperones were characterized by coimmunoprecipitation. In summary, Ran et al. (28) demonstrated that, in the absence of inducer maltose, inducible Mal63 MAL activator protein forms a stable intermediate complex with Hsp82/Myc, Ssa1/Myc, and Sti1/Myc; noninducible MAL activator mutant proteins form a stable early complex with Ssa1/Myc without Hsp82/Myc and Sti1/Myc; and constitutive MAL activator proteins form a stable final complex with Hsp82/Myc and without Ssa1/Myc or Sti1/Myc. The converse should also be true, i.e. copurification of a protein with Mal63 MAL activator in the absence of maltose indicates that the protein is a component of the intermediate complex. Similarly, copurification with a noninducible MAL activator in the absence of maltose suggests that the protein is a component of the early complex. We used this approach as a tool to determine which MAL activator-containing chaperone complex contains Aha1 cochaperone.

For our analysis here, we wanted to include the super-inducible mutant Mal63–460 in our studies. Using methods developed previously (28), we investigated the Hsp90 chaperone activation pathway of the super-inducible allele Mal63–460 MAL activator. Plasmid-borne triple HA-tagged inducible Mal63 and super-inducible Mal63–460 were introduced into the series of strains containing the genomic Myc-tagged HSP82/Myc, SSA1/Myc (in a STI1 or sti1Δ background), and STI1/Myc genes (see Table 1) and binding of the HA-tagged MAL activators to Hsp82/Myc, Ssa1/Myc, and Sti1/Myc determined by coimmunoprecipitation from native total cell protein extracts as described previously (28).

---

### Table 1: Mutant allele alterations in the MAL activator alleles utilized in this study.

| Allele     | Effect of aha1Δ on maltase activity | Binds Aha1/3Myc |
|------------|-------------------------------------|-----------------|
| Inducible  | Increased activity                  | Only in sti1Δ   |
| Super-inducible | No change                          | STI1 and sti1Δ |
| Non-inducible | No change                          | STI1 and sti1Δ |
| Non-inducible | No change                          | STI1 and sti1Δ |
| Constitutive | Increased activity                  | ND              |
| Constitutive | No change                          | ND              |
| Constitutive | No change                          | ND              |
| 1           | Inducible                          |                |

![FIGURE 3. Mutant alterations in the MAL activator alleles utilized in this study.](image-url)
**Aha1 Cochaperone, Yeast MAL Activator Negative Regulator**

**FIGURE 4. Impact of deletion versus overexpression of AHA1 on maltase expression activated by selected MAL activator mutant alleles.** Strains CMY1200 (referred to as AHA1), otherwise isogenic CMY8010 (referred to as aha1Δ), and strain CMY1200 carrying the AHA1/Myc overexpression plasmid p423GPD-AHA1/Myc3 were transformed with plasmids pUN30-MAL63, pUN30-mal63-283, pUN30-MAL63–460, pUN30-MAL63/43-C, pUN30-MAL63/23-SVI, and pUN30-MAL63-block2 carrying inducible MAL63, noninducible mal63-283, super-inducible MAL63–460, and constitutive MAL63/43-C, MAL63/23-SVI, and MAL63-block2, respectively (see Fig. 3). Transformants carrying MAL63, noninducible mal63-283, and super-inducible MAL63–460 were grown to mid-log at 30 °C in selective synthetic medium lacking tryptophan and histidine (where appropriate) and containing 3% glycerol and 2% lactate (v/v) plus 1% maltose. Transformants carrying the triple HA-tagged inducible MAL63 and Aha1/3Myc, but it does not impact Aha1–3Myc interaction with six noninducible activators tested or its lack of interaction with super-inducible Mal63–460 (see Figs. 5–7). MAL activator overexpression does appear to attenuate the extent of defects observed in chaperone mutant strains, but the effect is quantitative and not qualitative (see Fig. 5 of Ref. 28 and Figs. 4 and 8 of this study). Thus, we feel that the chaperone interactions with various MAL activators observed here and previously (28) are functionally significant and not the result of its overproduction.

**FIGURE 5. Super-inducible Mal63–460 MAL activator protein coimmunoprecipitates with Ssa1/Myc (Hsp70), Hsp82/Myc (Hsp90), and Sti1/Myc (Hop).** Strains CMY1200 (HSP82/Myc hsc82Δ) (upper panel), CMY1300 (SSA1/Myc ssa2Δ ssa3Δ ssa4Δ STI1) and CMY8002 (SSA1/Myc ssa2Δ ssa3Δ ssa4Δ STI1Δ) (middle panel), and CMY8015 (STI1/Myc) (lower panel) were transformed with plasmids p416GPD-MAL63/3HA or p416-GPD-MAL63–460/3HA harboring the triple HA-tagged inducible MAL63 or super-inducible MAL63–460 MAL activator genes, respectively. Transformants were grown to mid-log at 30 °C in selective synthetic medium lacking uracil and containing 3% glycerol, 2% lactate, with or without 2% maltose. Nondenaturing protein extracts were prepared and incubated with anti-HA-bound agrose beads and bound protein isolated as described previously (28). Coimmunoprecipitation (CoIP) 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.

It should be noted that the MAL activators in these coimmunoprecipitation studies and in those of Ref. 28 are expressed from the high level constitutive GPD promoter. Initial concerns that the observed chaperone interactions might be an artifact of this overexpression were not supported by the evidence reported previously (28). First, the chaperone-MAL activator interactions observed in the absence of inducer are not seen in maltose-grown cells, which would be unexpected if the interaction were a function of overexpression. In addition, chaperone interactions with the MAL activator are consistent within a phenotypic class of activators, such as noninducible or constitutive, which differ in the type of chaperones and cochaperones that bind. Moreover, deletion of STI1 enhances interaction between Hsp70 and inducible Mal63, Hsp70, and constitutive Mal63/43-C and inducible Mal63 and Aha1/3Myc, but it does not impact Aha1–3Myc interaction with six noninducible activators tested or its lack of interaction with super-inducible Mal63–460 (see Figs. 5–7). MAL activator overexpression does appear to attenuate the extent of defects observed in chaperone mutant strains, but the effect is quantitative and not qualitative (see Fig. 5 of Ref. 28 and Figs. 4 and 8 of this study). Thus, we feel that the chaperone interactions with various MAL activators observed here and previously (28) are functionally significant and not the result of its overproduction.

Similar to inducible Mal63, the results in Fig. 5 indicate that in the absence of maltose super-inducible Mal63–460 activator binds to Ssa1/Myc, Hsp82/Myc, and Sti1/Myc forming what appears to be an intermediate complex. Binding is not observed...
Aha1 cochaperone to a MAL activator containing Hsp90/Hsp70 chaperone complex. For this, we created a strain containing a triple Myc-tagged AHA1/Myc3 by inserting the tag at the C terminus of the genomic copy of the gene by a PCR-based one-step gene insertion method creating strain CMY8301 (see “Experimental Procedures” and Table 1). The AHA1 C-terminal modification does not affect the level of maltase activity as compared with the parental strain W303 (data not shown). STI1 was deleted in strain CMY8301 to create CMY8305 using one-step gene disruption techniques (see “Experimental Procedures” and Table 1). The plasmid-borne triple HA-tagged 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/Myc3 was determined by coimmunoprecipitation from native total cell protein extracts as described previously (28). It should be noted that overexpression of MAL63 does not qualitatively alter the impact of AHA1 deletion or overexpression on maltase expression (data not shown) but only the magnitude of the effect.

or is significantly reduced in extracts prepared from cells grown in the presence of maltose. Additionally, deletion of STI1, encoding the yeast Hop homologue, leads to deficits in the formation of the intermediate complex and dramatically enhances binding of super-inducible Mal63–460 and inducible Mal63 to Ssa1/Myc in cells grown in the presence of inducer maltose to levels comparable with those observed in the absence of maltose in the STI1 and sti1Δ strains. Therefore, super-inducible Mal63–460 MAL activator, like inducible Mal63 (28), progresses sequentially through the Hsp90/Hsp70 chaperone pathway from the early to the intermediate complex and, in the absence of maltose, is retained in the intermediate complex.

Using the MAL activators encoded by inducible MAL63, super-inducible MAL63–460, noninducible mal63-401, and constitutive MAL63/43-C as markers, we set out to localize
The results in Fig. 6 demonstrate that only the noninducible mutant Mal63–401 activator is found associated with Aha1/Myc3 in the STII strain, and this association is not enhanced by loss of Sti1/Hop. In the STII strain, inducible Mal63 activator does not bind to Aha1/Myc3 but, when STII is deleted, thereby promoting the retention of Mal63 in the early complex (28), inducible Mal63 activator is now also found bound to Aha1/Myc3. Aha1/Myc3 binding to constitutive Mal63/43-C or super-inducible Mal63–460 activators is not observed in either the STII or sti1Δ strains. This result is notable, because, in the sti1Δ strain, both Mal63/43-C and Mal63–460 activators bind to Ssa1/Myc indicating that they are retained in an early-like complex in strains lacking Sti1/Hop (Fig. 5) (28).

Aha1 cochaperone binding to noninducible Mal63–401 is not unique. Other noninducible mutant MAL activators, each reported to form only early complexes (28), were tested for binding to Aha1/Myc3. The noninducible mutants tested contain alterations mapping throughout the C-terminal regulatory domain of Mal63, including the region from residues 283 to 405 and mal63-A9N and mal63-S9V, each of which contains alterations in residues 467 and 469 (Fig. 3). All of the mutants bind Aha1/Myc3, and this binding is not enhanced by loss of Sti1/Hop (Fig. 7).

The C-terminal half of Saccharomyces Aha1 protein is the region that exhibits the greatest sequence homology among eukaryotic Aha1 proteins (25). To determine whether this region of Aha1 is sufficient to bind the MAL activator, we constructed AHA1C/Myc3 in which the sequence encoding residues 156–350 is expressed from the high level constitutive GPD promoter. Plasmids expressing full-length Aha1 (AHA1/Myc3) and the C-terminal domain (AHA1C/Myc3) were transformed into strain W303 along with noninducible mal63-401/3HA, and binding of the C-terminal fragment was determined by communoprecipitation from native total cell protein extracts as described previously (28). The results shown in Fig. 8 (upper left panel) demonstrate that both the C-terminal fragment and the full-length Aha1 copurify with the noninducible mutant Mal63–401/3HA MAL activator and that comparable amounts of each are bound to the activator. As control, AHA1C/Myc3 and untagged mal63-401/NT were coexpressed in W303, and the same culture, extract preparation, and immunoprecipitation procedures were used (Fig. 8, lower left panel). Unfortunately, the level of Aha1C/Myc3 protein expressed in this strain is very limited. Strains that overexpress full-length Aha1 grow slowly, but those overexpressing the C-terminal domain grow even more slowly, particularly when coexpressed with untagged Mal63–401/NT. This slow growth rate could suggest that overproduction of Aha1 and Aha1C are cytotoxic thereby resulting in plasmid instability. Nonetheless, we feel that the result is sufficient to conclude that the C-terminal fragment of Aha1 does not independently bind to the beads.

Additionally, the C-terminal fragment is capable of repressing Mal63 MAL activator. AHA1/Myc3 and AHA1C/Myc3 were expressed at high levels in the aha1Δ strain CMY8010. As is shown in Fig. 8 (right panel), the full-length and the C-terminal fragments of Aha1 are both able to repress maltase activity to similar levels. Taken together, the results in Fig. 8 demonstrate that the C-terminal domain of Aha1 cochaperone is sufficient to bind to the MAL activator, and it represses MAL activator function.

**Deletion of AHA1 Enhances MAL Activator Association with Hsp82/Myc and Ssa1/Myc—**To explore the mechanism of Aha1 cochaperone regulation, we investigated 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 cochaperones Hsp82/Myc and Ssa1/Myc. The results are shown in Fig. 9.

Approximately equal levels of each of the MAL activators tested in Fig. 9 are found in the total extracts prepared from the AHA1 and aha1Δ strains indicating that deletion of AHA1 does not appear to affect their levels of accumulation. Most importantly, loss of Aha1 cochaperone enhances the level of Hsp82/Myc binding to inducible Mal63 but does not significantly alter binding to constitutive Mal63/43-C or super-inducible Mal63–460 activators. Quantification of these results, which was normalized to the amount of MAL activator protein, indicates that there is approximately a 3-fold increase in Hsp82/Myc binding. Similarly, loss of Aha1p enhances binding of inducible Mal63 MAL activator to Ssa1/Myc, and quantification indicates that this enhancement is also ~3-fold. Super-inducible Mal63–460 activator binds Ssa1/Myc, but this binding is not enhanced in the aha1Δ strain. As reported previously (28), constitutive Mal63/43-C binding with Ssa1/Myc is not observed even in the
absence of maltose, and Fig. 9 shows that loss of Aha1 cochaperone does not alter this.

**DISCUSSION**

The results reported here and in Ref. 28 make use of our large collection of MAL activator mutant alleles as tools to study the Hsp90/Hsp70 chaperone pathway in vivo. This collection is unique among the Hsp90 client proteins and provides novel reagents with which to dissect the roles of Hsp90, Hsp70, and the various cochaperones that regulate and facilitate the chaperone process. Here, we focus on the Aha1 cochaperone, the most recently identified Hsp90 cochaperone whose role in client protein regulation by Hsp90 chaperone remains to be fully clarified. Published studies of Aha1 cochaperone indicate that Aha1 binds Hsp90 and stimulates its ATPase activity suggesting that this cochaperone acts late in the Hsp90 chaperone pathway to accelerate client protein activation and release via the energy from ATP hydrolysis (2, 4, 5, 7, 14, 15, 18–22, 25, 39–43). Thus, Aha1 should function as a positive regulator of Hsp90, and this has proven to be the case with regard to the client proteins GR and v-Src kinase (1, 18–20, 44).

In contrast, published studies of mammalian cystic fibrosis transmembrane conductance regulator (CFTR) suggest that Aha1 cochaperone plays a negative regulatory role in CFTR expression, particularly impacting mutant ΔF508 CFTR expression and cell surface localization (45). CFTR is a 12-transmembrane domain integral membrane protein that folds in the endoplasmic reticulum and is delivered to the plasma membrane where it functions as a chloride transporter. The ΔF508 allele is the most commonly occurring mutation in human populations. During normal expression of wild-type CFTR, about 80% of the protein is degraded in the ER, but for ΔF508 CFTR, this increases to 100%. Using proteomic approaches, Wang et al. (45) identified cytoplasmic and ER luminal chaperones and cochaperones that differentially bind the two CFTR proteins, including Aha1 cochaperone. They showed that decreased Aha1 expression enhances plasma membrane localization of ΔF508 CFTR, and overexpression of Aha1 increases ΔF508 CFTR degradation suggesting that loss of Aha1 cochaperone facilitates the progress of ΔF508 CFTR through the Hsp90 pathway thereby enhancing plasma membrane expression. This hypothesis is supported by Sun et al. (46), who demonstrate that overproduction of a fusion peptide derived from ΔF508 CFTR disrupts the association of the mutant transporter with Aha1 and rescues plasma membrane delivery of the mutant transporter. Most recently, Koulov et al. (25) demonstrated that overproduction of mutant Aha1 defective in its ability to stimulate Hsp90 ATPase is significantly less effective compared with wild-type Aha1 in its ability to impede CFTR production and cell surface delivery. Koulov et al. (25) propose that the decreased stimulation of Hsp90 ATPase by this defective Aha1 lengthens the time that the CFTR client is held in the chaperone complex, and this increased “dwell time” provides more opportunity for the client to fold properly.

Together, these published studies suggest that Aha1 cochaperone functions as either a positive or a negative regulator, depending on the specific client protein and specific characteristics of its folding requirements. Specifically, delayed release from Hsp90 resulting from low ATP turnover may benefit the activation of some clients but cause defects in the activation of others. This leads one to ask the following question. How do the chaperoning requirements of these clients differ such that the decreased Hsp90 ATPase and the proposed increased dwell time caused by loss of Aha1 enhance activation of some clients and impede activation of others? The work described here uses another Hsp90 client, the Saccharomyces MAL activator, and in vivo approaches to explore the mechanism of Aha1 cochaperone function for another client protein in which Aha1 is acting as a negative regulator. Our results suggest an alternative hypothesis for Aha1 negative regulation.

**Aha1 Cochaperone Is a Negative Regulator of Mal63 MAL Activator and Interacts with Sequences near the C Terminus—** The results reported here indicate that Aha1 cochaperone is a negative regulator of maltose-induced MAL gene expression by Mal63 MAL activator. Deletion of AHA1 causes approximately a 2-fold increase in the level of Mal63-dependent maltase activity (Figs. 1 and 2). Not only is the induced level of maltase higher but inducer sensitivity is increased at limiting maltose concentrations, possibly because of enhanced basal level maltose permease expression. Additionally, overexpression of Aha1 cochaperone decreases the induced level of maltase activity (Fig. 4).

Interestingly, we found that MAL activators whose sequences differed from Mal63 at just a few sites in the region of residues 433–463 were insensitive to negative regulation by Aha1. Alteration of residues D460A and D463A in MAL63–460 produces a super-inducible allele in which the induced level of maltase activity is comparable with that observed in an aha1Δ strain expressing Mal63 MAL activator in Fig. 4. Moreover, maltase levels in the strain carrying MAL63–460 are not enhanced in the aha1Δ strain, and Mal63–460-dependent maltase expression is largely insensitive to repression by Aha1 overexpression. Similarly, maltase activity in strains carrying two of the three constitutive MAL activator alleles tested, MAL63/23–C and MAL63/23–SVI, is not affected by aha1Δ, i.e. loss of Aha1 cochaperone does not cause an increase in maltase activity, and Aha1 overproduction does not significantly repress activation of these two constitutive alleles. These constitutive alleles both contain several alterations in the C terminus compared with Mal63: N433H, D437Q, G445S, T449D, K454R, Q457R, and N461D. Conversely, the constitutive allele MAL63/23–block2, whose sequence is identical to inducible Mal63 in the C-terminal region, is sensitive to repression by Aha1 cochaperone overexpression, and activation of maltase expression by MAL63/23–block2 is enhanced by deletion of AHA1. These results provide genetic evidence that negative regulation by Aha1 cochaperone acts via sequences close to the C terminus of Mal63, approximately residues 433–463, the region that Danzi et al. (29) suggest has a role in folding, activation, and maintenance of the MAL activator.

It is important to note that the negative regulation of Mal63 MAL activator by Aha1 cochaperone reported here is distinct from the negative regulation by Hsp70 described previously (28). Hsp70 is the negative regulator of maltose-stimulated Mal63 induction, and defects in binding between Hsp70 and Mal63 MAL activator cause constitutivity. Super-inducible
Mal63–460, which is insensitive to Aha1 negative regulation (Fig. 4) and does not bind Aha1 (Figs. 6 and 9), is nonetheless still inducible. Moreover, loss of Aha1 cochaperone does not lead to constitutivity in strains expressing inducible Mal63, nor does it affect basal uninduced maltase expression or expression in strains carrying noninducible mutant activators. Finally, although some constitutive MAL activators (MAL63/43-C and MAL63/23-SVI) are insensitive to repression by Aha1 cochaperone, others (MAL63/23-block2) remain sensitive. Thus, Aha1 cochaperone is not involved in regulating induction but regulates the MAL activator via a different mechanism that is unrelated to the response to maltose.

**Aha1 Cochaperone Associates with Mal63 MAL Activator and Noninducible Mal63 Mutant Activators in an Apparent Early Complex**—The results reported here demonstrate that Aha1 cochaperone interacts with the MAL activator in what appears to be an early complex although it is bound to Ssa1/Myc and not to Hsp82/Myc or Sti1/Myc. As reported in Ref. 28, noninducible mutant MAL activators remain bound to Ssa1/Myc in the absence of maltose and are not observed bound with Hsp82/Myc or Sti1/Myc. Addition of inducer maltose releases the noninducible activators from all chaperone binding. Ran et al. (28) proposed that these mutant activators 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. Six noninducible *mal63* MAL activator alleles were tested. These carry alterations in sites spanning the regulatory domain of the MAL activator from residues 283–401 (Fig. 3). All of these noninducible mutant proteins bind Ssa1/Myc and are never observed bound to Hsp82/Myc or Sti1/Myc (28). All of these noninducible activators also form a complex with Aha1/Myc3 (Fig. 7). Inducible Mal63 forms a stable intermediate complex in the absence of maltose but, in a *stiΔ* strain, intermediate complex formation is defective, and Mal63 is found bound to Ssa1/Myc in an apparent early complex (28). Copurification of Aha1/Myc3 and inducible Mal63 is observed in the *stiΔ* strain but not the *STI1* strain (Fig. 6) indicating that defects in Hsp90 association, due in this case to the loss of Sti1/Hop, allows for the binding of Aha1 cochaperone to Mal63.

Retention in an apparent early complex is not sufficient to promote Aha1 binding to MAL activators carrying C-terminal alterations. Super-inducible Mal63–460 activator forms an intermediate complex bound to Hsp82/Myc, Ssa1/Myc, and Sti1/Myc, and deletion of *STI1* promotes early complex formation (Fig. 5) but association with Aha1/Myc3 cochaperone is not observed in the *stiΔ* strain, as is found for Mal63. Similarly, constitutive Mal63/43-C activator does not form a stable intermediate complex and rapidly progresses to a final complex bound to Hsp82/Myc but not Ssa1/Myc or Sti1/Myc (28). Deletion of *STI1* enhances binding of Mal63/43-C activator to Ssa1/Myc (28), indicating that it too is in an early complex when entry into the Hsp90 chaperone pathway is blocked or defective. Nonetheless, association of Mal63/43-C activator with Aha1/Myc3 is not observed in the *stiΔ* strain (Figs. 5 and 6). In summary, reduced or defective binding to Hsp82/Myc, as caused by *stiΔ* or as is found in the noninducible *mal63* mutant activators, impedes entry into the Hsp90 chaperone pathway and promotes Ssa1/Myc binding and retention in an apparent early complex. In this early complex, and only in the early complex, is Aha1/Myc3 binding to MAL activator observed. This finding raises the possibility that Aha1 binding to the client and progression of the client to the intermediate complex may be mutually exclusive. Aha1/Myc3 binding is not observed in all MAL activators, and this lack of binding is consistent with the genetic analysis discussed above that finds that constitutive Mal63/43-C and super-inducible Mal63–460, which contain alterations in C-terminal residues compared with Mal63, are insensitive to the negative regulation of Aha1 cochaperone. Taken together, these results suggest that Aha1 cochaperone regulates MAL activator activation by interaction with the C-terminal region of Mal63 MAL activator, possibly by direct physical interaction. Efforts to demonstrate direct interaction between the C-terminal 50 residues of Mal63 MAL activator and Aha1 cochaperone by yeast two-hybrid analysis were not successful (data not shown).

Interestingly, the C-terminal domain of Aha1 from residues 156 to 350 is sufficient to allow this Aha1 fragment to copurify with the noninducible mutant MAL activator Mal63–401 and to inhibit Mal63-dependent activation of maltase synthesis to an extent comparable with that of the full-length Aha1 (Fig. 8). These results suggest that it is this region of the cochaperone that is responsible for MAL activator interaction and the negative regulation of Mal63. It should be noted that strains overexpressing either the full-length Aha1 or the C-terminal domain of Aha1 (residues 156–350) grow extremely slowly (data not shown) implying that activation of other *Saccharomyces* clients may also be defective.

**Mechanism of Aha1 Cochaperone Regulation of Inducible Mal63 MAL Activator**—Similar to the mechanism proposed by Koulou et al. (25) to explain the role of Aha1 in CFTR regulation, one could propose that that loss of Aha1 lengthens the dwell time of Mal63 in the Hsp90-bound complex thereby providing greater opportunity for enhanced chaperone activation. This hypothesis is consistent with our finding that deletion of *AHA1* increases Hsp82/Myc and Ssa1/Myc binding to inducible Mal63 MAL activator ~3-fold (Fig. 9), suggesting that an increased percentage of Mal63 MAL activator is found in the intermediate complex in the absence of Aha1. Nonetheless, this hypothesis does not explain our findings that Aha1/3Myc binds to all noninducible *mal63* mutant activators tested and to inducible Mal63 only in the *stiΔ* strain. Therefore, we propose an alternative hypothesis that we feel conforms better to data reported in this investigation. Our hypothesis focuses on published results showing that Aha1 cochaperone competes with other early and late cochaperones for Hsp90 binding (4, 6). Of particular interest is Sti1/Hop, which Ran et al. (28) demonstrates is a positive regulator of MAL activator regulation.

Formation of the intermediate complex is mediated by cochaperones, including Sti1/Hop and Cdc37, that facilitate Hsp90 binding to distinct subsets of clients (14, 21, 47–51). Cdc37 is utilized almost exclusively as a cochaperone for kinases and facilitates the binding of these kinase clients to Hsp90. Sti1/Hop is a tetra tricopeptide repeat-containing cochaperone that serves as a scaffold protein by binding the C-terminal domains of both Hsp90 and Hsp70 thereby facili-
Aha1 cochaperone acts as a “governor” that decreases the rate at which those MAL activators that bind Aha1 enter the Hsp90 pathway.

In summary, we propose that Aha1 cochaperone may function as either a positive or a negative regulator of chaperone-dependent activation depending upon the client protein (18, 20, 45, 46). The mechanisms by which it carries out these opposing regulatory roles are likely to differ depending on the array of cochaperones utilized in the activation of the client and variations in the Hsp90 chaperone pathway for this client. With regard to the latter, the impact of Aha1 could depend on whether the client is regulated and held in an inactive complex that is stable in the absence of inducer, as are GR and Mal63, or is not regulated and is not retained in any of the Hsp90 pathway complexes, as is the case for the kinases. Moreover, if a client is regulated by retention in a chaperone complex, the specific complex formed in the absence of inducer may be a factor. In its capacity as a negative regulator, Aha1 cochaperone could act in the early complex controlling the sorting of client proteins to alternative fates. Interaction with Hsp70 in the early complex appears to be a decision point at which some client proteins are directed either to the Hsp90 folding pathway via formation of the intermediate complex or to the degradation pathway via interaction with the Hsp70-binding ubiquitin ligase CHIP (C-terminal Hsp70 interacting protein) (12, 53–56). Alternatively, Aha1 could function late in the chaperone pathway by interactions with late cochaperones, like Sba1/p23, or by stimulating the Hsp90 ATPase and decreasing dwell time on the chaperone.

Flexibility in the roles of other components in the chaperone pathway has been described previously. CHIP that had long been recognized as an E3 ubiquitin ligase responsible for the degradation of improperly folded Hsp70/Hsp90 client proteins has recently been found to exhibit chaperone activity with certain clients under certain conditions (57, 58). Cdc37/p50, and essential cochaperone in binding kinase clients to Hsp90 early in their activation, has also been reported to have Hsp90-independent cochaperone functions and to play a role in regulating kinase degradation (14, 49, 59). Plasticity in the function of cochaperones may be more common than anticipated and may differ with different clients. Our findings highlight the importance of investigating the roles of the Hsp70/Hsp90 chaperones and cochaperone components with a variety of different client proteins and using both in vitro and in vivo approaches.

Acknowledgments—We thank Avrom Caplan and Susan Rotenberg for helpful in-depth discussions, Susan Rotenberg for critical reading of the manuscript, and Muhammad Awan for technical assistance. Western blot visualizations were done in the Core Facilities for Imaging, Cell and Molecular Biology of Queens College, City University of New York. We greatly appreciate the assistance of Areti Tsiola with the same.

REFERENCES
1. Caplan, A. J. (2003) Cell Stress Chaperones 8, 105–107
2. Neckers, L., Tsutsumi, S., and Mollapour, M. (2009) Nat. Struct. Mol. Biol. 16, 235–236
3. Bukau, B., Weissman, J., and Horwich, A. (2006) Cell 125, 443–451

FIGURE 10. Model of Aha1 cochaperone MAL activator regulation. The block arrows above each complex indicate the stable complex formed by inducible, super-inducible, noninducible, and constitutive MAL activators in the absence of maltose.
