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# The Hsp70-Ydj1 Molecular Chaperone Represses the Activity of the Heme Activator Protein Hap1 in the Absence of Heme

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**In *Saccharomyces cerevisiae*, heme directly mediates the effects of oxygen on transcription through the heme activator protein Hap1. In the absence of heme, Hap1 is bound by at least four cellular proteins, including Hsp90 and Ydj1, forming a higher-order complex, termed HMC, and its activity is repressed. Here we purified the HMC and showed by mass spectrometry that two previously unidentified major components of the HMC are the Ssa-type Hsp70 molecular chaperone and Sro9 proteins. In vivo functional analysis, combined with biochemical analysis, strongly suggests that Ssa proteins are critical for Hap1 repression in the absence of heme. Ssa may repress the activities of both Hap1 DNA-binding and activation domains. The Ssa cochaperones Ydj1 and Sro9 appear to assist Ssa in Hap1 repression, and only Ydj1 residues 1 to 172 containing the J domain are required for Hap1 repression. Our results suggest that Ssa-Ydj1 and Sro9 act together to mediate Hap1 repression in the absence of heme and that molecular chaperones promote heme regulation of Hap1 by a mechanism distinct from the mechanism of steroid signaling.**

Heme is central to oxygen sensing and utilization. Remarkably, heme directly regulates numerous molecular and cellular processes for systems that sense or use oxygen (33). In the yeast *Saccharomyces cerevisiae*, heme directly mediates the effects of oxygen on gene transcription through the heme activator protein Hap1. In response to heme, Hap1 promotes transcription of genes encoding functions required for respiration, for controlling oxidative damage, and for repression of anaerobic genes (62). Hap1 activity is precisely and stringently controlled by the heme concentration. Recent studies in our laboratory show that heme regulation of Hap1 involves a two-tier regulatory mode, with independent control of Hap1 repression in the absence of heme by repression modules (RPMs) and heme binding and activation of Hap1 by heme-responsive motifs (HRMs) (14, 17, 59).

Importantly, previous studies suggested that heme regulation of Hap1 requires the action of certain cellular proteins (11, 58). In the absence of heme, Hap1 is bound by cellular proteins, forming a high-molecular-weight complex (HMC), and binds to DNA with low affinity. Several lines of evidence strongly suggest that HMC formation is directly linked to heme regulation. First, in vitro, as the heme concentration increases the HMC is gradually disrupted, transforming into the dimeric Hap1 complex, with greatly increased DNA-binding affinity (18, 56). Second, Hap1 mutants with deleted or mutated RPMs are derepressed: These mutants gain a high level of activity even in the absence of heme (14). We found that most of the derepressed Hap1 mutants do not form the HMC (14, 60). Third, all repressed Hap1 mutants, like wild-type Hap1, form the HMC (14). Fourth, overexpression of Hap1 titrates certain cellular proteins in the HMC and causes the formation of Hap1 dimeric complexes (18, 56), leading to graded increases in Hap1 DNA binding and transcriptional activities in the absence of heme (18). These results strongly suggest that HMC formation is critical for Hap1 repression and that RPMs mediate Hap1 repression by promoting HMC formation.

To understand how the HMC mediates heme regulation, we purified the HMC (18, 60). We found that at least four proteins are associated with Hap1 in the HMC in the absence of heme (60). By immunodetection, we initially found that two of these Hap1-associated proteins are the yeast Hsp90 (Hsp82/Hsc82) and Ydj1 (60). Hsp90 plays a unique role in the proper functioning of a wide range of signal transducers, such as nuclear hormone receptors and tyrosine kinases (30, 40). Our further analyses showed that Hsp90 is critical for Hap1 activation by heme (60; H. C. Lee, T. Hon, and L. Zhang, unpublished data). Notably, Ydj1 is a cochaperone of yeast Ssa-type Hsp70 proteins (5, 8). Hsp70 is often part of the Hsp90-substrate complexes and cooperates with Hsp90 (30, 36, 38). However, the role of Hsp70 in the regulation of signal transducers remains largely unclear except that Hsp70 is important for the assembly of mature steroid receptor-Hsp90 complexes (31).

In this report, to find out whether the Ssa-type Hsp70 proteins are components of the HMC and to further explore the role of the HMC in heme regulation, we identified two major components of the HMC by two independent mass spectrometric techniques (10). We found that yeast Hsp70 Ssa and Sro9 proteins are two major components of the HMC. Functional analysis showed that Ssa proteins play a major role in Hap1 repression in the absence of heme while Ydj1 and Sro9 play an auxiliary role in Hap1 repression. Our results suggest that the molecular chaperones Ssa-Ydj1 and Sro9 promote heme regulation of Hap1 activity by a novel mechanism.

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## MATERIALS AND METHODS

**Yeast strains and plasmids.** The yeast strains used were JN55Δ*hem1* (*MAT***a** *ura3-52 leu2-3,112 his3-11 trp1*Δ*1 lys2 hem1-*Δ*100*) (wild type), JN516Δ*hem1* (*MAT***a** *ura3-52 leu2-3,112 his3-11 trp1*Δ*1 lys2 ssa2*::*LEU2 ssa3*::*TRP1 ssa4*::*LYS2 hem1-*Δ*100*) (*a2a3a4*), 5B6Δ*hem1* (*MAT***a** *ura3-52 leu2-3,112 his3-11 trp1*Δ*1 lys2 ssa1*::*HIS3 ssa2*::*LEU2 ssa4*::*LYS2 hem1-*Δ*100 pGAL1-SSA1*) (1), MHY200 (*MAT***a** *ura3-52 leu2-3,112 his4-519 ade1-100 hem1-*Δ*100 hap1*::*LEU2*) (16), MHY200Δ*ydj1*, MHY200Δ*sro9*, and JEL1 (*MAT*α *leu2 trp1 ura3-52 nprb1-1122 pep4-3* Δ*His3*::*pGAL10-GAL4*). The *HEM1* gene was deleted from various strains, as described previously (16). The MHY200Δ*ydj1* and MHY200Δ*sro9* strains were generated by using PCR-mediated gene disruption as described previously (52). Briefly, oligonucleotides (sequences available upon request) containing the desired nucleotide sequence of the *YDJ1* or *SRO9* gene and the pRS400 plasmid were used to amplify the kanamycin resistance gene by PCR. The resulting PCR products were transformed into the strain MHY200 and selected on plates containing G418 as previously described (52). Yeast colonies were picked and verified. Yeast cells with the *YDJ1* or *SRO9* gene deleted were confirmed by PCR analysis of the disrupted *YDJ1* or *SRO9* gene and by complementation. The whole Ydj1 or Sro9 coding sequence in the strain was deleted. The UAS1/*CYC1-lacZ* reporter plasmid was described previously (48). The expression plasmids for $His_6$-Hap1 were constructed as previously described (60).

**Preparation of yeast extracts and purification of HMCs containing $His_6$-Hap1.** Yeast cell extracts were prepared according to previously established protocols (56, 60). Briefly, yeast cells bearing expression plasmids were grown to an optical density (OD) of 0.8 to 1.0 in medium containing glucose or 0.3 to 0.5 in medium containing raffinose and then induced with 2% galactose for 5 to 6 h. Cells were harvested and resuspended in three packed cell volumes of buffer (20 mM Tris, 10 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 1 mM dithiothreitol [DTT], 0.3 M NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 μg of pepstatin per ml, 1 μg of leupeptin per ml). Cells were then permeabilized by agitation with four packed cell volumes of glass beads, and extracts were collected as previously described (60). This method consistently yielded extracts with protein concentrations of 5 to 10 mg/ml.

To purify the HMC containing $His_6$-Hap1, Ni-nitrilotriacetic acid (NTA) superflow beads (Qiagen) were packed in a column and equilibrated with buffer containing 25 mM Tris-HCl (pH 8), 100 mM NaCl, 6 mM $MgCl_2$, 0.5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1 mM DTT. Then, extracts were loaded onto the column at the rate of approximately 15 ml per h. Columns were subsequently washed with 150 to 200 column volumes of the equilibration buffer containing 20 mM imidazole. The HMC was eluted with buffer containing 250 mM imidazole. The eluate was further concentrated on Centricon 10 (Amicon) columns and analyzed by Bradford assays, sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, and electrophoretic mobility shift assays to determine the protein concentrations and activities.

**Protein mass spectrometry.** Eluates from Ni-NTA columns were first separated on SDS–8 or 9% polyacrylamide gels and then transferred to polyvinylidene difluoride (PVDF) membranes (Boehringer Mannheim). The membranes were stained briefly with Coomassie blue and destained in 50% methanol. The protein bands were excised, and proteins were processed for mass spectrometric fingerprinting as described previously (10). Briefly, tryptic peptide mixtures were partially fractionated on Poros 50 R2 RP microtips, and resulting peptide pools were analyzed by matrix-assisted laser desorption ionization–reflectron time of flight mass spectrometry (MALDI-reTOF MS) using a Reflex III instrument (Brüker Franzen, Bremen, Germany), and in some cases, by electrospray ionization (ESI) MS on an API 300 triple quadrupole instrument (PE-SCIEX, Thornhill, Canada) modified with an injection adaptable fine ionization source (JAFIS) as previously described (12). Selected mass values from the MALDI-reTOF experiments were taken to search a protein nonredundant database (EBI, Hinxton, United Kingdom) using the PeptideSearch algorithm. MS/MS spectra from the ESI triple quadrupole analyses were inspected for the "y" ion series and the resultant information was transferred to the SequenceTag program and used as a search string. Any protein identification thus obtained was verified by comparing the computer-generated fragment ion series of the predicted tryptic peptide with the experimental MS/MS data.

**β-Galactosidase assay.** Yeast cells with the *HEM1* gene deleted were transformed with the UAS1/*CYC1-lacZ* reporter plasmid as described previously (56, 60). Cells were grown in synthetic complete medium containing 2 μg of 5-aminolevulinic acid (ALA) per ml to an OD of approximately 0.5. Cells were then induced with various concentrations of deuteroporphyrin IX (dpIX) for 7 h and harvested for determination of β-galactosidase levels as described previously (56).

**Electrophoretic mobility shift assay and Western blotting.** DNA-binding reactions were carried out in a 20-μl volume with 5% glycerol, 4 mM Tris (pH 8), 40 mM NaCl, 4 mM $MgCl_2$, 10 mM DTT, 3 μg of salmon sperm DNA, 10 μM $ZnOAc_2$, and 300 μg of bovine serum albumin per ml as described previously (56). Approximately 0.01 pmol of labeled oligonucleotides and eluate containing approximately 200 ng of total proteins or 20 μg of crude extracts were used in each reaction. The reaction mixtures were incubated at room temperature for 1 h and then loaded onto 3.5% polyacrylamide gels in Tris-borate-EDTA (diluted 1/3) for gel electrophoresis at 4°C. The intensity of bands representing the HMC and dimeric complex was quantified by using the PhosphorImager system (Molecular Dynamics). Analysis of the protein-DNA complexes formed at the upstream activation sequence (UAS) of *TEF2* and the centromere DNA element I (CDEI) site was carried out as described previously (3, 32, 43).

For Western blotting, proteins were first separated on SDS–7% polyacrylamide gels and then transferred to PVDF or nitrocellulose membranes. Hap1, Ssa, Hsp90, and Ydj1 were detected by using antibodies against Hap1, Ssa, Hsp90, and Ydj1, respectively, and a chemiluminescence Western blotting kit (Boehringer Mannheim) as described previously (1, 60).

**DNA pull-down experiments.** Extracts were prepared from cells expressing Hap1 and various levels of Ssa in the presence of 0.25% galactose plus 1.75% glucose, 1% galactose plus 1% glucose, or 2% galactose. Extracts were incubated with streptavidin-conjugated magnetic beads (Dynal) prebound with the biotinylated wild-type or mutant Hap1-binding site (18) under the same conditions as those for electrophoretic mobility shift assays described above. Nonspecific binding was avoided by preincubating extracts with unbound beads with the Hap1-binding site. The beads were extensively washed and boiled in SDS gel loading buffer to release the bound proteins (41). Proteins were then analyzed by SDS-polyacrylamide gel electrophoresis, followed by Western blotting analysis.

## RESULTS

**Hsp70 and Sro9 are two major proteins associated with Hap1.** To identify the proteins associated with Hap1 in the absence of heme, we purified the HMC by fusing the $His_6$ tag to the N terminus of Hap1. Yeast extracts were prepared from cells expressing high levels of $His_6$-Hap1 and were loaded onto Ni-NTA columns to purify the HMC. Previously, we showed that at least four proteins cofractionate with $His_6$-Hap1 on the Ni-NTA column (Fig. 1A) and on a gel filtration (Superose 6) column (60). When extracts containing untagged Hap1 were loaded onto Ni-NTA columns, these proteins were not found in the eluate (60). In addition, these proteins were selectively cross-linked to Hap1 when partially purified Hap1 complexes were treated with glutaraldehyde (data not shown). These results together strongly suggest that these proteins are directly associated with Hap1 in the absence of heme. The purified proteins also formed the HMC (Fig. 1B), as detected by electrophoretic mobility shift assays. Heme disrupted this complex, permitting Hap1 to bind DNA with high affinity (Fig. 1B). By immunodetection, we found that two proteins associated with Hap1 are yeast Hsp90 and Ydj1, which have molecular masses of approximately 82 and 42 kDa, respectively (60). However, the two major Hap1-associated proteins (bands A and B in Fig. 1A), which have molecular masses of approximately 70 and 60 kDa, remained unidentified. To identify these proteins, we used two independent mass spectrometric techniques, peptide mass fingerprinting using MALDI-reTOF MS and Sequence Tag database searching using limited amino acid sequence data obtained by ESI tandem MS (Table 1) (10). We found that band A represents the products of the yeast Hsp70-coding genes *SSA1* and/or *SSA2*, while band B is the product of the yeast *SRO9* gene (22). Because Ssa1 and Ssa2 share 96% identity (2), we were unable to distinguish between Ssa1 and Ssa2.

**Ssa proteins play a major role in Hap1 repression in the absence of heme.** Ssa proteins (a class of yeast Hsp70 homo-

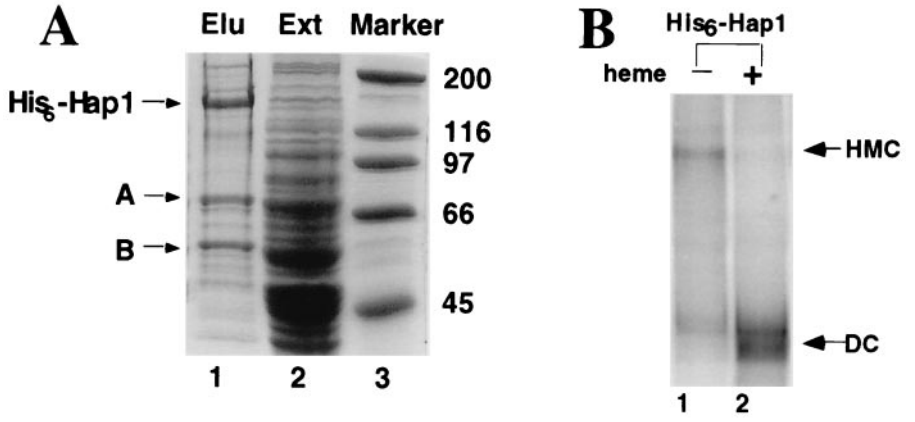


FIG. 1. Analysis of the purified Hap1-associated proteins. (A) Yeast cell extracts containing $His_6$-Hap1 were purified by Ni-NTA columns, and the eluted fractions were analyzed with SDS–10% polyacrylamide gels. The eluted peak fraction (Elu; lane 1) and unpurified whole-cell extracts (Ext; lane 2) are shown. The positions of the protein weight markers (lane 3) are denoted. The two major bands (A and B) are marked and were subjected to mass spectrometric fragment analysis. (B) DNA-Hap1 complexes formed by purified proteins from Ni-NTA columns. The eluate was incubated with radiolabeled DNA in the absence (lane 1) and presence (lane 2) of 2 ng of heme per μl and then analyzed on a 3.5% polyacrylamide gel. The positions of the HMC and dimeric complex (DC) are marked.

logues of *Escherichia coli* DnaK) and Ydj1 (a functional homologue of *E. coli* DnaJ) always function together as molecular chaperones in protein folding in an ATP-dependent manner (8, 20). Ssa proteins are present in both the cytosol and nucleus and play critical roles in nonstress-related processes (44). Ssa proteins possess ATPase activity (61). The Ssa cochaperone Ydj1 stimulates Ssa ATPase activity and may help recruit substrates (1, 6, 7, 28, 29, 47, 61). Previous identification of Ydj1 as a component of the HMC and the identification of Ssa1 or -2 here as the major component of the HMC are consistent with the fact that Ydj1 serves as a cochaperone of Ssa proteins (6–8, 61). Further, Ydj1 can function efficiently in the assembly of a glucocorticoid receptor-Hsp90-Hsp70 complex even when its amount is only one-twentieth that of Hsp70 in the complex (9). Likewise, the Hsp70-Ydj1 chaperone machine in the HMC could be entirely functional, although the amount of Ydj1 was less than that of Hsp70 in the complex (see Fig. 1, lane 1; all bands near the molecular size of Ydj1 are much weaker than band A). The presence of Ydj1 in the complex was determined by Western blotting analysis, as shown previously (60).

To determine the functional importance of Ssa and Ydj1 in heme regulation, we examined the effect of defective Ssa or Ydj1 function on Hap1 activity. In *S. cerevisiae*, the essential *SSA* family includes four genes, *SSA1* to *-4* (2, 53). *SSA1*, which is induced about threefold by heat, and *SSA2* are constitutively

TABLE 1. Summary of results from mass spectrometric fingerprinting

| Band | MS data | | | | |
|---|---|---|---|---|---|
| | Measured (*m/z*) | Calculated ($MH^+$) | ΔDa (ppm) | Residues | Sequence |
| A (Ssa1 or Ssa2) | 1,183.649 | 1,183.647 | 0.002 (1) | 456–466 | (K)FELSGIPPAPR(G) |
| | 1,199.677 | 1,199.674 | 0.003 (3) | 158–169 | (K)DAGTIAGLNVLR(I) |
| | 1,274.693 | 1,274.689 | 0.004 (3) | 234–243 | (R)LVNHFIQEFK(R) |
| | 1,430.760 | 1,430.790 | −0.030 (20) | 234–244 | (R)LVNHFIQEFKR(K) |
| | 1,471.670 | 1,471.706 | −0.036 (24) | 35–47 | (R)TTPSFVAFTDTER(L) |
| | 1,552.700 | 1,552.775 | −0.075 (48) | 346–358 | (K)LVTDYFNGKEPNR(S) |
| | 1,763.796 | 1,763.849 | −0.053 (30) | 55–70 | (K)NQAAMNPSNTVFDAKR(L) |
| | 1,787.919 | 1,787.990 | −0.071 (39) | 170–186 | (R)IINEPTAAAIAYGLDKK(G) |
| | 1,815.908 | 1,815.981 | −0.073 (40) | 323–339 | (K)LDKSQVDEIVLVGGSTR(I) |
| | 1,894.846 | 1,894.929 | −0.083 (43) | 137–153 | (K)VNDAVVTVPAYFNDSQR(Q) |
| | 2,144.119 | 2,144.160 | −0.041 (19) | 92–110 | (K)LIDVDGKPQIQVEFKGETK(N) |
| | 2,167.173 | 2,167.187 | −0.014 (6) | 447–466 | (K)TKDNNLLGKFELSGIPPAPR(G) |
| | 2,183.002 | 2,183.028 | −0.026 (11) | 515–532 | (K)MVAEAEKFKEEDEKESQR(I) |
| B (Sro9) | 1,735.912 | 1,735.938 | −0.026 (14) | 60–75 | (K)QVNLTPAPLPTSSPWK(L) |
| | 1,950.968 | 1,951.065 | −0.097 (49) | 58–75 | (K)SKQVNLTPAPLPTSSPWK(L) |
| | 2,141.019 | 2,140.981 | 0.038 (18) | 170–188 | (K)QQQMKKDGFESAVGEEDSK(D) |
| | 2,269.191 | 2,269.229 | −0.038 (17) | 76–96 | (K)LAPTEIPVSTISIEDLDATRK(K) |
| | 2,307.049 | 2,307.074 | −0.025 (10) | 262–279 | (K)YHNHFHHNQQHPQQPMVK(L) |
| | 2,351.034 | 2,351.151 | −0.117 (49) | 375–395 | (K)EGDNVTGEAKEPSPLDKYFVR(S) |
| | 2,522.109 | 2,522.201 | −0.092 (37) | 260–279 | (R)SKYHNHFHHNQQHPQQPMVK(L) |
| | 2,861.241 | 2,861.332 | −0.091 (32) | 226–250 | (K)FHNSNNAGMPQNQGFPPQFKPYQGR(N) |
| | 2,989.341 | 2,989.427 | −0.086 (28) | 225–250 | (R)KFHNSNNAGMPQNQGFPPQFKPYQGR(N) |

TABLE 2. Effects of *ssa* mutations on Hap1 activity[a]

| Strain | β-Galactosidase activity (Miller units) when grown in the presence of the indicated concentration of dpIX | | | | |
|---|---|---|---|---|---|
| | 0 μg/ml | 0.05 μg/ml | 0.25 μg/ml | 1.0 μg/ml | 4.0 μg/ml |
| Wild type | 0.2 ± 0.1 | 72 ± 4 | 368 ± 55 | 360 ± 9 | 575 ± 69 |
| *a2a3a4* | 2.6 ± 0.4 | 85 ± 10 | 636 ± 88 | 649 ± 99 | 1,087 ± 35 |

[a] Yeast wild-type and *a2a3a4* (with the *SSA2, SSA3*, and *SSA4* genes deleted) cells bearing the Hap1-driven UAS1/*CYC1-lacZ* reporter were grown in the presence of 0, 0.05, 0.25, 1, and 4 μg of the heme analogue dpIX per ml. The *HEM1* gene, encoding ALA synthase, was deleted from these strains to allow the experimental control of heme concentrations. β-Galactosidase activities were detected, and data shown are averages (± standard deviations) of at least three independent transformants.

expressed at a high level (2). *SSA3* and *SSA4* are not constitutively expressed but are greatly induced by heat (2). At least one of the *SSA* gene products must be present in significant quantities to allow viability (1, 53). Note that *SSA3* alone, at the level that it is normally expressed, does not support viability (1).

We measured Hap1 activity in the yeast *a2a3a4* strain with the *SSA2*, *SSA3*, and *SSA4* genes deleted (1). To control intracellular heme concentrations in yeast cells, the *HEM1* gene encoding the first enzyme for heme synthesis, ALA synthase (15, 51), was deleted. A low heme concentration, at which wild-type Hap1 remains inactive in wild-type cells, was created by the addition of 2 μg of ALA, a heme precursor (14, 18, 55), per ml. Heme induction was achieved by the addition of various amounts of the heme analogue deuteroporphyrin IX (13, 14, 17, 18, 55–57, 59, 60). Heme induction may also be achieved by growing cells in the presence of 250 μg of ALA per ml (14, 18). As shown in Table 2, in the absence of heme Hap1 activity was notably higher in the *a2a3a4* strain than in the wild-type strain, though still much lower than its activity in the presence of heme, suggesting that defective Ssa function causes partial Hap1 derepression. In the *a2a3a4* strain, the Ssa protein level is about 30% that in the wild-type strain (2). The result suggests that Ssa may help repress Hap1 in the absence of heme.

Western blotting (Fig. 2A) showed that the Hap1 protein level remained the same in the wild-type and *a2a3a4* cells, suggesting that the effect of defective Ssa function on Hap1 activity was not caused by variations in Hap1 protein levels. Further, we examined the Hap1-DNA complexes formed in extracts prepared from these cells (Fig. 2B). Quantification by a phosphorimager showed that the intensity of both the HMC and the Hap1 dimeric complex formed in extracts prepared from *a2a3a4* cells (Fig. 2B, lanes 1 and 2) was about two- to threefold higher than the intensity of those complexes formed in extracts prepared from wild-type cells (Fig. 2B, lanes 3 and 4). To rule out the possibility that the increased DNA-binding activities of Hap1 complexes were caused by nonspecific effects of the *ssa2 ssa3 ssa4* mutations on DNA binding, we examined complexes formed at the UAS of *TEF2*, which is bound by the transcriptional regulator Rap1 (43), and at the CDEI site, which is bound by the transcriptional regulator CP1 (3, 32). Protein complexes formed at these sites are unaffected by glucose and galactose (references 3, 32, 43 and data not shown). Figure 2C shows that the intensity of the DNA-protein complexes formed at these sites was unaffected or somewhat reduced in *a2a3a4* cells (compare lane 1 with lane 2 and lane 4 with lane 5). These complexes were competed out when a large amount of unlabeled DNA sites was included in the DNA-binding reactions (see lanes 3 and 6), suggesting that the detected DNA-binding activities are specific. These results suggest that the increased Hap1 DNA-binding activity is a selective effect of reduced Ssa levels on Hap1 binding at its site, but not on protein binding at the UAS of *TEF2* or the CDEI site, and that increased DNA binding may contribute to partial Hap1 derepression in *a2a3a4* cells.

To further ascertain the effect of low Ssa protein levels on heme regulation of Hap1, we used a strain in which Ssa is expressed from the galactose-inducible, glucose-repressible *GAL1* promoter (1). The *ssa1 ssa2 ssa4* strain with the *SSA1*, *SSA2*, and *SSA4* genes deleted is rescued from lethality by the centromeric plasmid *GAL1-SSA1*. We controlled the Ssa expression level in the *ssa1 ssa2 ssa4 GAL1-SSA1* strain by using media containing various amounts of glucose and galactose. At high Ssa expression levels (2% Gal), Hap1 was repressed in the absence of heme and activated by heme as in the wild-type strain (Table 3). At low Ssa expression levels (in the presence of 1% Gal plus 1% Glc) (14), however, Hap1 gained a high level of activity even in the absence of heme (Table 3). Hap1 exhibited an even higher level of activity in the absence of heme when Ssa expression levels were very low (in the presence of 0.25% Gal plus 1.75% Glc) (14) (Table 3). As a control, we showed that various amounts of galactose and glucose by and large did not affect Hap1 activity in the wild-type strain (Table 3), in which *SSA* genes are intact, and Ssa expression levels were unaffected by glucose or galactose. These results strongly suggest that low levels of Ssa cause Hap1 derepression and that Ssa plays a major role in Hap1 repression in the absence of heme.

To dissect the mechanism by which low levels of Ssa cause Hap1 derepression, we examined Hap1 DNA-binding activity in extracts prepared from *ssa1 ssa2 ssa4 GAL1-SSA1* and wild-type cells. We found that in extracts prepared from *ssa1 ssa2 ssa4 GAL1-SSA1* cells, the intensity of both the HMC and Hap1 dimeric complexes formed in the presence of heme was significantly enhanced when Ssa expression levels were low (0.25% Gal and 1% Gal; Fig. 3A, lanes 1 to 4) compared to when the Ssa expression levels were high (2% Gal; Fig. 3A, lanes 5 and 6). As controls, we show that in extracts prepared from wild-type cells, the HMC and Hap1 dimeric complexes formed in the presence of heme were unaffected by the various amounts of galactose and glucose (Fig. 3B). Further, Fig. 3C shows that the intensity of complexes formed at the UAS of *TEF2* (43) and the CDEI site (3, 32) was unaffected or slightly reduced in extracts prepared from cells induced with 0.25% Gal plus 1.75% Glc (Fig. 3C, lanes 3 and 6) or 1% Gal plus 1% Glc (Fig. 3C, lanes 2 and 5). The data suggest that the increased Hap1 DNA-binding activity in extracts prepared from cells expressing low levels of Ssa was not attributable to nonspecific effects of Ssa on DNA binding.

To ascertain that the differences in the intensity of Hap1-DNA complexes in extracts prepared from *ssa1 ssa2 ssa4 GAL1-SSA1* cells were caused by variations in Ssa protein levels and not in Hap1 protein levels, we examined Hap1 and Ssa protein levels in these extracts. As shown in Fig. 4, Hap1 protein levels (Fig. 4A) were largely unaffected by galactose

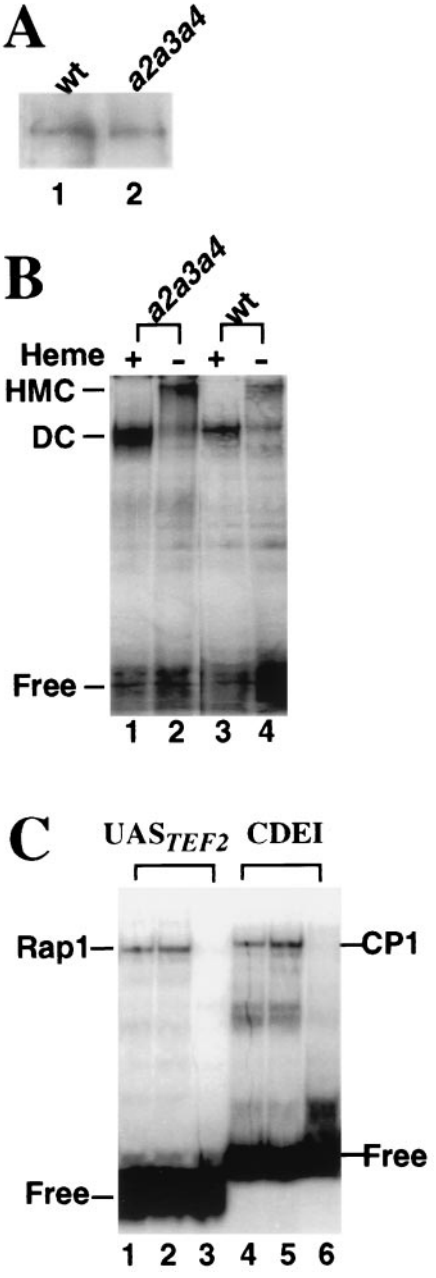


FIG. 2. (A) Western blot showing Hap1 protein levels in wild-type and mutant *ssa* cells. Cell extracts (50 μg) prepared from wild-type (wt; lane 1) and *a2a3a4* (lane 2) cells expressing Hap1 from a 2-μm plasmid (35) were analyzed on an SDS-polyacrylamide gel, transferred to a PVDF membrane, and probed with an antibody against Hap1. (B) Effect of Ssa proteins on Hap1 DNA binding. Yeast cell extracts were prepared from wild-type (lanes 3 and 4) and *a2a3a4* (lanes 1 and 2) cells. Electrophoretic mobility shift assays were carried out. Extracts (20 μg) containing Hap1 prepared from yeast cells were incubated with radiolabeled DNA in the presence (lanes 1 and 3) or absence (lanes 2 and 4) of 2 ng of heme per μl. The reaction mixtures were analyzed on 3.5% polyacrylamide gels. The positions of the HMC and dimeric complex (DC) are marked. (C) Protein binding at *TEF2* UAS and CDEI sites in wild-type and mutant *ssa* cells. Extracts (20 μg) prepared from wild-type (lanes 2, 3, 5, and 6) or mutant *a2a3a4* (lanes 1 and 4) cells were incubated with radiolabeled synthetic DNA containing *TEF2* UAS (43) (lanes 1 to 3) or the CDEI site (3, 32) (lanes 4 to 6). In lanes 3 and 6, 1 μg of cold synthetic *TEF2* UAS (lane 3) or CDEI site (lane 6) was included in the DNA-binding reactions, respectively. The reaction mixtures were analyzed on a 4% polyacrylamide gel.

and glucose whereas Ssa protein levels (Fig. 4C) were significantly reduced in extracts prepared from cells grown in medium containing 0.25% Gal plus 1.75% Glc (lane 1) or 1% Gal plus 1% Glc (lane 2). These results strongly suggest that reduced Ssa levels caused a significant increase in Hap1 DNA-binding activity, whether or not heme was present. The increased DNA-binding activity may in part account for Hap1 derepression in the absence of heme in cells expressing low levels of Ssa.

To better understand the molecular basis for the increased Hap1 DNA-binding and transcriptional activities in cells expressing low levels of Ssa, we examined the composition of the Hap1-DNA complexes by DNA pull-down assays. DNA pull-down experiments were carried out in the absence of heme, when the HMC is formed (56, 60) (Fig. 3). Extracts prepared from cells expressing low or high levels of Ssa were incubated with a biotinylated wild-type or mutant (for control) Hap1-binding site (18) (see Materials and Methods). Nonspecific binding to the beads was minimized by preincubating extracts with beads that were not bound with DNA. The bound Hap1, Ssa, Hsp90, and Ydj1 proteins were analyzed by Western blotting (Fig. 4B, D, F, and H). When the mutated Hap1-binding site (18) was used in the pull-down experiments, no bound protein was detected and thus is not shown. For comparison, proteins in original extracts were also detected in parallel (Fig. 4A, C, E, and G). As expected, similar levels of Hap1 were bound to DNA in extracts prepared from cells expressing various levels of Ssa, while less Ssa was bound to Hap1 in extracts prepared from cells expressing low levels of Ssa (compare Fig. 4B and D, particularly lanes 2 and 3). Intriguingly, when low levels of Ssa were expressed and bound to Hap1 (Fig. 4C and D, lanes 1 and 2), a significantly higher level of Hsp90 (Fig. 4F, lanes 1 and 2) and a slightly higher level of Ydj1 (Fig. 4H, lanes 1 and 2) were bound to Hap1. The increased levels of Hsp90 and Ydj1 bound to Hap1 in extracts prepared from cells expressing low levels of Ssa were not caused by increased protein expression levels (Fig. 4E and G). The data suggest that high levels of Ssa and low levels of Hsp90 correlate with low Hap1 DNA-binding and transcriptional activities, whereas high levels of Hsp90 and low levels of Ssa correlate with high Hap1 DNA-binding and transcriptional activities. The increased binding of Hsp90 to Hap1 in the presence of low levels of Ssa is consistent with the previous finding that Hsp90 is critical for Hap1 activation (60; Lee et al., unpublished data) and provides an explanation for increased Hap1 DNA-binding and transcriptional activities in the absence of heme. The increased binding of Ydj1 to Hap1 in the presence of low levels of Ssa may reflect an increased need for Ydj1 in maintaining Hap1 folding and stability when Ssa is limiting.

**The N-terminal residues of Ydj1 are important for Hap1 repression in the absence of heme.** Ydj1 is a cochaperone of Ssa and is a component of the HMC. We therefore examined the effects of defective Ydj1 function on Hap1 activity. Although Δ*ydj1* cells grow very poorly and assays are difficult to perform, we managed to measure Hap1 activity in these cells. We found that the deletion of *YDJ1* caused partial Hap1 derepression in heme-deficient cells (Hap1 activity was 20 ± 3 [mean ± standard deviation], compared to 2 ± 0.4 in wild-type cells). Because we initially did not detect an effect of various Ydj1 mutants (21) on Hap1 repression, we reasoned that Hap1

TABLE 3. Effect of low levels of Ssa on Hap1 activity[a]

| Concentration (%) of Gal plus Glc | β-Galactosidase activity (Miller units) when grown in the presence of the indicated concentration of dpIX | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | *GAL1-SSA1* | | | | | Wild type | | | | |
| | 0 μg/ml | 0.05 μg/ml | 0.25 μg/ml | 1.0 μg/ml | 4.0 μg/ml | 0 μg/ml | 0.05 μg/ml | 0.25 μg/ml | 1.0 μg/ml | 4.0 μg/ml |
| 2 | 1.4 ± 0.1 | 116 ± 13 | 445 ± 12 | 776 ± 27 | 1,215 ± 148 | 1.4 ± 0.1 | 73 ± 11 | 687 ± 36 | 703 ± 58 | 826 ± 40 |
| 1 + 1 | 73 ± 8 | 219 ± 14 | 644 ± 89 | 837 ± 57 | 1,499 ± 53 | 1.1 ± 0.1 | 72 ± 15 | 558 ± 19 | 736 ± 27 | 1,080 ± 138 |
| 0.25 + 1.75 | 205 ± 35 | 284 ± 34 | 513 ± 43 | 915 ± 216 | 1,314 ± 296 | 1.0 ± 0.1 | 59 ± 6 | 577 ± 11 | 740 ± 8 | 729 ± 11 |

[a] Yeast wild-type cells or the 5B6 cells (*GAL1-SSA1*) (Δ*ssa1* Δ*ssa2* Δ*ssa4 pGAL1-SSA1*) were grown in medium containing 2% galactose for a high Ssa1 expression level, 1% galactose plus 1% glucose for a low (5 to 10% of high) Ssa expression level, or 0.25% galactose plus 1.75% glucose for a very low (<2% of high) Ssa1 expression level in the presence of the indicated amounts of dpIX. The *HEM1* gene was deleted from all these strains to allow the experimental control of heme concentration. β-Galactosidase activities were detected, and data shown are averages (± standard deviations) of at least three independent transformants.

activity gained from partial derepression in Δ*ydj1* cells may be too low to reveal Ydj1 mutants' effects. Thus, to examine Ydj1 mutants' effects, we decided to use a hyperactive Hap1 variant, Hap1-18 (25), with a Gly-to-Arg mutation in the C6 zinc cluster which does not affect heme regulation but drastically increases Hap1 activity in heme-sufficient cells. As shown in Table 4, in the absence of Ydj1 (in cells bearing the empty vector), Hap1-18 activity in heme-deficient cells (low heme level) was higher than that of wild-type Hap1, although the activity was still about 10% of its activity in heme-sufficient cells (high heme level). This higher activity led to the revelation that one point mutation, F47L, and two deletions, N104 and N134, caused partial Hap1 derepression in heme-deficient cells (Table 4). Further, the absence of Ydj1 (Fig. 4I, lane 2) or Ydj1 mutants, such as F47L (Fig. 3D, lane 3), did not affect Hap1 protein levels, suggesting that the increase in Hap1 activity caused by the lack of Ydj1 or Ydj1 mutants in the absence of heme was not attributable to greatly increased Hap1 protein levels. The absence of Ydj1 or Ydj1 mutants also did not significantly affect Hap1 DNA binding or HMC formation in the absence or presence of heme (data not shown).

Together, these results show that Ydj1 helps repress Hap1 in the absence of heme. However, Ydj1 appears to play an auxiliary role while Ssa plays a major role in Hap1 repression in the absence of heme, because the extent of Hap1 derepression caused by low levels of Ssa (Table 3) was much greater than that caused by the absence of Ydj1 (see above). In addition, our results (Table 4) show that Ydj1 residues 1 to 172 containing the J domain, and not the substrate-binding region (28), are necessary and sufficient for repressing Hap1. Although here we did not observe a Hap1 activation defect in cells lacking Ydj1 or expressing Ydj1 mutants, Johnson and Craig observed an activation defect in cells expressing Ydj1 but lacking the C terminus of Ydj1 (19). This difference may be attributable to strain differences. Perhaps Ydj1 exerts multiple, but auxiliary, effects on Hap1. Thus, depending on the strain background, the absence of Ydj1 or Ydj1 mutants may reveal one Ydj1 function, while the other functions may be compensated for by other factors involved in heme regulation of Hap1. When Ssa is limiting, Ydj1 may play a greater role in maintaining Hap1 function, as suggested by the somewhat increased binding of Ydj1 to Hap1 (Fig. 4H).

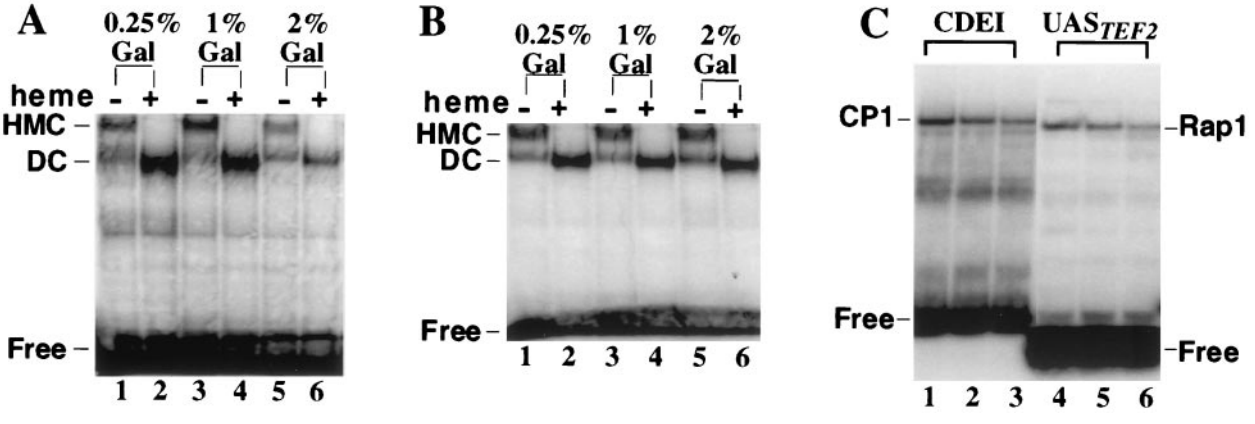


FIG. 3. (A) Effect of low levels of Ssa on Hap1 DNA binding. Yeast 5B6 cells (Δ*ssa1*Δ*ssa2* Δ*ssa4 pGAL1-SSA1*) were grown in medium containing 2% galactose (lanes 5 and 6) for a high Ssa expression level, 1% galactose plus 1% glucose (1% Gal; lanes 3 and 4) for a low (5 to 10% of high) Ssa expression level, or 0.25% galactose plus 1.75% glucose (0.25% Gal; lanes 1 and 2) for a very low (<2% of high) Ssa1 expression level. Cell extracts were prepared from these cells, and DNA-binding reactions were carried out in the presence (lanes 2, 4, and 6) or absence (lanes 1, 3, and 5) of 2 ng of heme per μl. (B) Hap1 DNA binding in wild-type cells was unaffected by various amounts of galactose and glucose. Yeast wild-type JN55 cells were grown in medium containing 2% galactose (lanes 5 and 6), 1% galactose plus 1% glucose (lanes 3 and 4), or 0.25% galactose plus 1.75% glucose (lanes 1 and 2). Cell extracts were prepared from these cells, and DNA-binding reactions were carried out in the presence (lanes 2, 4, and 6) or absence (lanes 1, 3, and 5) of 2 ng of heme per μl. (C) Protein binding at *TEF2* UAS and CDEI sites in cells expressing various levels of Ssa. Extracts (20 μg) prepared from 5B6 cells grown in medium containing 2% galactose (lanes 1 and 4), 1% galactose plus 1% glucose (lanes 2 and 5), or 0.25% galactose plus 1.75% glucose (lanes 3 and 6) were incubated with radiolabeled synthetic DNA containing the CDEI site (3, 32) (lanes 1 to 3) or *TEF2* UAS (43) (lanes 4 to 6). The reaction mixtures were analyzed on a 4% polyacrylamide gel.

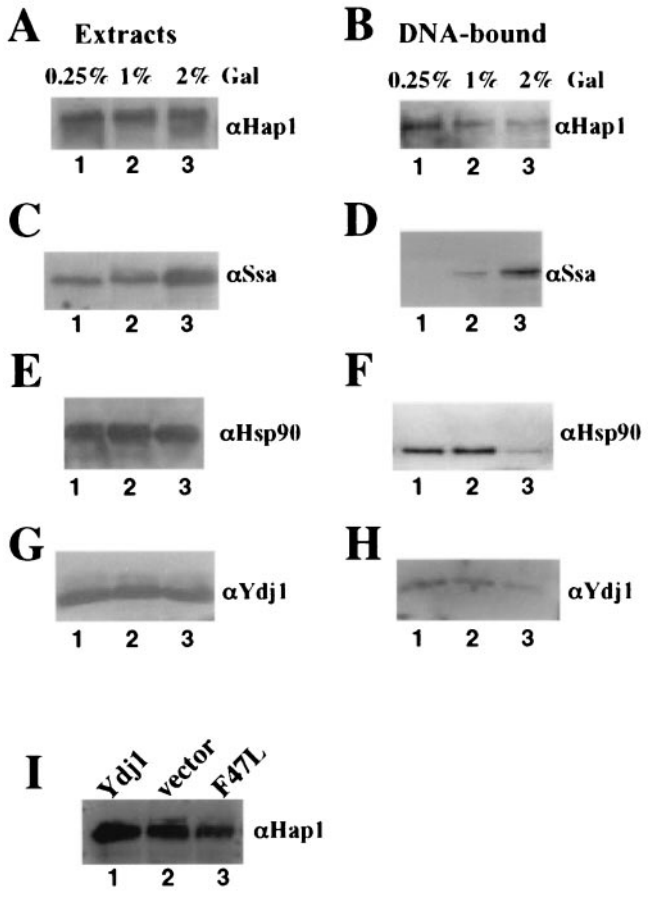


FIG. 4. (A to H) Effects of low levels of Ssa on the composition of the DNA-bound Hap1 complexes. Yeast 5B6 cells (Δ*ssa1* Δ*ssa2* Δ*ssa4* *pGAL1-SSA1*) were grown in medium containing 2% galactose (lane 3) (A to H), 1% galactose plus 1% glucose (lane 2) (A to H), or 0.25% galactose plus 1.75% glucose (lane 1) (A to H). Extracts were incubated with streptavidin-conjugated magnetic beads (Dynal) prebound with the biotinylated synthetic wild-type or mutant Hap1-binding site (18) in the absence of heme. The beads were extensively washed and boiled in SDS gel loading buffer to release the bound proteins (41). Bound proteins (B, D, F, and H) and proteins in the original extracts (A, C, E, and G) were subsequently electrophoresed on SDS-polyacrylamide gels, transferred to PVDF membranes, and probed with antibodies against Hap1 (A and B), Ssa (C and D), Hsp90 (E and F), and Ydj1 (G and H). No bound protein was detected when a mutated Hap1-binding site was used in the pull-down experiments. These experiments were repeated twice. (I) Western blot showing Hap1 protein levels in Δ*ydj1* cells. Yeast MHY200Δ*ydj1* cells expressing Hap1 and bearing the expression plasmid for wild-type Ydj1 (lane 1), the empty vector (lane 2), or the mutant F47L (lane 3) were prepared and subjected to Western blotting analysis with an anti-Hap1 antibody. The same result was obtained when Hap1-18 was expressed in the cells.

**Sro9 is functionally important for Hap1 repression in the absence of heme.** To determine whether Sro9 is functionally relevant to heme regulation of Hap1, we examined the effect of deletion of *SR09* on Hap1 activity (Table 5). We found that the absence of Sro9 caused partial Hap1 derepression in the absence of heme and at lower heme concentrations. Hap1 protein levels and DNA-binding activities were unaffected by the deletion of *SRO9*, as detected by Western blotting and electrophoretic mobility shift assays (data not shown). The results show that the association of Sro9 with Hap1 is not an artifact but is functionally important. Evidently, Ssa plays a major role while Sro9 plays an indispensable, but auxiliary, role in Hap1 repression, because the extent of derepression caused by low levels of Ssa (Table 3) was much greater than that caused by the absence of Sro9 (Table 5).

## DISCUSSION

In this report, we have shown that reduced levels of Ssa (Hsp70) caused Hap1 derepression in the absence of heme and increased Hap1 DNA-binding activities. In addition, deletion of *YDJ1* or *SRO9* causes partial Hap1 derepression. Our data suggest that Ssa plays a major role while Ydj1 and Sro9 play an auxiliary role in Hap1 repression. These and previous results suggest that the molecular mechanism governing the actions of molecular chaperones in heme regulation is analogous but distinct from the mechanism by which molecular chaperones promote steroid signaling (4, 36). Heme regulation of Hap1, like steroid signaling, involves the actions of molecular chaperones in repression and activation. The activation of both Hap1 and steroid receptors requires the action of Hsp90, while the repression of Hap1 and steroid receptors involves Ssa and Hsp90, respectively. Steroid receptors bind to DNA constitutively when stripped of Hsp90 (23, 42). Similarly, Hap1 DNA-binding and transcriptional activities increase when Hap1 is overexpressed, causing the formation of Hap1 dimeric complexes in the absence of heme (18, 56), or when Ssa is underexpressed (Fig. 2 and 3 and Tables 2 and 3), altering the composition of the HMC (Fig. 4). However, our data also suggest that Hsp90 and Hsp70 molecular chaperones promote heme regulation of Hap1 in ways distinct from those in promoting steroid signaling.

**Ssa (Hsp70) plays a major role in Hap1 repression in the absence of heme.** Our results suggest that Ssa plays a major role in Hap1 repression in the absence of heme. Even a slight reduction of the Ssa protein level caused partial Hap1 derepression in *a2a3a4* cells (Table 2). Strikingly, low Ssa levels allowed Hap1 to gain a high level of activity even in the absence of heme but had virtually no effect on Hap1 activity at higher heme concentrations (Table 3). Such an in vivo effect of reduced levels of Hsp70 on a transcriptional activator contrasts with the effect of reduced levels of Hsp90 on the activities of steroid receptors (37) and Hap1 (60; Lee et al., unpublished data). Reduced levels of Hsp90 cause reduced Hap1 activity at heme concentrations that permit Hap1 activation but have no impact on Hap1 repression in the absence of heme (60; Lee et al., unpublished data). Thus, it appears that Hsp90 and Hsp70 play distinct roles in heme regulation of Hap1. In contrast, both Hsp90 and Hsp70 promote the formation of a mature steroid receptor complex capable of high-affinity ligand binding (31).

How may Ssa (Hsp70) repress Hap1? Our data suggest that Ssa may repress Hap1 by inhibiting Hap1 DNA-binding and transcription-activating activities in the absence of heme. As shown in Fig. 2B and 3A, reduced levels of Ssa caused increased Hap1 DNA-binding activities. The increased DNA-binding activities may in part account for the high Hap1 transcriptional activity gained in the absence of heme. In addition, Ssa may repress Hap1 transcription-activating activity by interfering with the Hap1 activation domain. This idea is consistent

TABLE 4. Effects of Ydj1 mutants on Hap1-18 activity[a]

| Heme level | β-Galactosidase activity (Miller units) in presence of Ydj1 or Ydj1 mutant | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Ydj1 | Vector | F47L | N104 | N134 | N172 | N206 | N274 | N363 | G315E |
| Low | 2.2 ± 0.6 | 124 ± 6 | 39 ± 4 | 24 ± 1.5 | 18 ± 1 | 2.6 ± 0.4 | 2.4 ± 0.3 | 3.5 ± 0.6 | 5.3 ± 0.5 | 5.3 ± 1.2 |
| High | 1,240 ± 257 | 1,531 ± 281 | 1,491 ± 237 | 1,351 ± 122 | 1,054 ± 24 | 1,060 ± 34 | 885 ± 150 | 1,254 ± 157 | 1,066 ± 136 | 990 ± 56 |

[a] Yeast Δ*ydj1* Δ*hem1* cells expressing Hap1-18 and bearing the expression plasmid for wild-type Ydj1, the indicated mutants, or the empty vector and a Hap1-driven reporter were grown in the presence of a low (2.5 μg/ml) or high (250 μg/ml) level of heme precursor ALA. β-Galactosidase activities were detected, and data shown are averages (± standard deviations) of at least three independent transformants.

with the previous hypothesis suggesting that the Hap1 DNA-binding domain and activation domain are both repressed in the HMC (18, 57).

The role of Ssa in heme regulation of Hap1 may be similar to the role of Hsc70 in the regulation of the heme-regulated inhibitor (HRI) (46, 50). In the case of HRI, Hsc70 plays dual roles, a positive one in HRI folding, maintenance, and transformation and a negative one in attenuating the kinase activity of activated HRI (46, 50). The role of Hsc70 in HRI repression is independent of its role in the assembly of the Hsp90-HRI complex (49). Existing data are also consistent with the idea that Ssa may repress Hap1 independently of Hsp90. Perhaps Hsp70 plays an Hsp90-independent role in the repression of a distinctive class of substrates including Hap1 and HRI.

**Ydj1 and Sro9 play an auxiliary role in Hap1 repression in the absence of heme.** Deletion of Ydj1 caused partial Hap1 derepression in the absence of heme (Table 4). Further, Ydj1 mutants containing intact residues 1 to 172 conferred Hap1 repression whereas mutants containing regions smaller than residues 1 to 172 or containing mutations in residues 1 to 172 caused partial Hap1 derepression (Table 4). These results suggest that Ydj1 residues 1 to 172 are necessary and sufficient for Hap1 repression in the absence of heme. Ydj1 residues 1 to 172 contain the J domain, the G/F-rich region, and part of the zinc finger-like domain (5, 8). The N-terminal J-plus-G/F region of Ydj1 permits interaction with Ssa and stimulates Ssa ATPase activity, while the C-terminal residues 179 to 384 bind to unfolded substrates (5, 24, 27, 28). Thus, Ydj1 residues 1 to 172 are able to serve as an Ssa cochaperone and can confer Hap1 repression, indicating that Ydj1 acts by Ssa to promote Hap1 repression. The role of Ydj1 in heme regulation of Hap1 differs from its role in steroid signaling. First, the absence of Ydj1 causes virtually complete glucocorticoid receptor (GR) derepression (21, 26) but only partial Hap1 derepression (see Results). Second, all Ydj1 mutants cause GR derepression, and the whole Ydj1 protein is required for the action of steroid receptors and v-Src (21). In contrast, only Ydj1 residues 1 to 172 appear to be important for Hap1 repression (Table 4). These results suggest that Ydj1 acts on Hap1 and GR by distinct mechanisms.

When Ssa is abundant, Ydj1 very likely acts through Ssa as a cochaperone to repress Hap1. However, when Ssa is limiting, Ydj1 may directly act on Hap1 in maintaining Hap1 function, as suggested by the data shown in Fig. 4H. A direct interaction between Hap1 and Ydj1 is consistent with the previous ideas that Ydj1 recruits substrates, that Ydj1 directly interacts with GR (21), and that Ydj1 acts together with Hsp90 to promote steroid signaling (26). Evidently, the slightly increased association of Ydj1 with Hap1 was not sufficient to repress Hap1 (Table 3 and Fig. 4H), although this increased interaction may be important for Hap1 folding and stability.

Sro9 is a major component of the HMC (Fig. 1 and Table 1). It contains a conserved La motif that permits RNA binding (45) and plays a role in the organization of actin filaments (22). In yeast, La proteins may serve as molecular chaperones for RNA (34). Thus, Sro9 may also serve as a molecular chaperone for Hap1. Although it is not yet clear how Sro9 acts to promote Hap1 repression, our data show that Sro9 is functionally important for heme regulation of Hap1.

**Heme regulation of Hap1: a two-tier regulatory model.** Heme regulation of Hap1 is mediated by two distinct, independent classes of Hap1 elements, RPMs and HRMs. RPMs mediate Hap1 repression in the absence of heme; deletion of an RPM causes Hap1 derepression in the absence of heme. HRMs mediate heme binding and heme activation of Hap1; deletion and mutations of HRMs cause Hap1 to be defective in heme activation. In parallel to these two classes of Hap1 elements, our results suggest that Hsp90 promotes only heme activation of Hap1 (60; Lee et al., unpublished data), whereas Ssa, Ydj1, and Sro9 mediate Hap1 repression in the absence of heme. Evidently, RPMs cooperate with Ssa to repress Hap1, as deletion of RPMs (14) or low levels of Ssa cause Hap1 derepression in the absence of heme (Tables 2 and 3). Ydj1 and Sro9 presumably assist Ssa in Hap1 repression. The separate roles of Hsp90 and Ssa in Hap1 activation and repression are also supported by data from pull-down experiments (Fig. 4). Repressed Hap1 was associated with high levels of Ssa but low levels of Hsp90. In contrast, derepressed (or active) Hap1 was associated with high levels of Hsp90 but low levels of Ssa.

Taken together, we propose a tentative model for how heme

TABLE 5. Effects of deletion of *SR09* on Hap1 activity[a]

| Strains | β-Galactoside activity (Miller units) when grown in the presence of the indicated concentration of dpIX | | | | | | |
|---|---|---|---|---|---|---|---|
| | 0 μg/ml | 0.031 μg/ml | 0.062 μg/ml | 0.125 μg/ml | 0.25 μg/ml | 0.5 μg/ml | 1.0 μg/ml |
| Wild type | 0.6 ± 0.1 | 14 ± 2 | 38 ± 5 | 339 ± 100 | 625 ± 133 | 876 ± 102 | 1,266 ± 94 |
| Δ*sro9* | 8.0 ± 0.4 | 35 ± 3 | 119 ± 8 | 437 ± 64 | 617 ± 130 | 755 ± 104 | 984 ± 40 |

[a] Yeast Δ*sro9* Δ*hem1* Hap1-expressing cells bearing the UAS1/*CYC1-lacZ* reporter were grown in the presence of the indicated concentrations of heme analogue dpIX. β-Galactosidase activities were detected, and data shown are averages (± standard deviations) of at least three independent transformants.

regulation of Hap1 is achieved. In the absence of heme, Hap1 is bound by Ssa-Ydj1, Sro9, and Hsp90. Ssa-Ydj1 and Sro9 likely bind to Hap1 through RPMs, directly or indirectly, while Hsp90 may bind to Hap1 near HRMs (14, 17, 57). Ssa and its cochaperone Ydj1, Sro9, and Hap1 may act together to block the activities of Hap1 DNA-binding and activation domains, thereby keeping Hap1 repressed in the HMC. Ssa and its cohorts appear to repress Hap1 independently of Hsp90 because Hap1 remains repressed even when Hsp90 function is defective (60). When the heme concentration increases, heme binds to Hap1 through HRMs, causing Hap1 conformational changes. Consequently, the interactions of Hap1 with Ssa-Ydj1, Sro9, and Hsp90 are weakened and the inhibition on Hap1 DNA-binding and activation domains is relieved, thereby leading to Hap1 activation. Hsp90 appears to promote Hap1 activation independently of Ssa because low levels of Hsp90 (60; Lee et al., unpublished data), but not low levels of Ssa (Tables 2 and 3), cause defective Hap1 activation. In sum, our data suggest that Hap1 repression and activation are mediated independently by Ssa and its cohorts and by Hsp90, respectively.

This two-tier regulatory model likely allows Hap1 activity to be regulated precisely and stringently according to heme concentrations. The involvement of multiple Hap1 elements and multiple chaperones allows Hap1 to sense small changes in the heme concentration and to respond precisely. This kind of two-tier regulatory model is different from the model of steroid signaling (36, 39, 54). First, the regulation of steroid receptors does not involve two distinct classes of receptor elements. Second, Hsp90 plays a dual role in steroid signaling—in the repression of receptors and in ligand binding and activation of receptors (36, 40). In contrast, our data show that Ssa-Ydj1 and Sro9 act together to repress Hap1, whereas no evidence supporting the role of Hsp90 in Hap1 repression has emerged (60; Lee et al., unpublished data). Third, Ydj1 plays an auxiliary role in Hap1 repression whereas it plays a major role in the repression of steroid receptors (21). In addition, only the N-terminal Ydj1 residues are required for Hap1 repression whereas the whole Ydj1 protein is required for repressing steroid receptors (21). Finally, Sro9 is a new component which has not been previously found in the steroid receptor-Hsp90 complexes. Thus, the HMC may represent a new and distinctive class of multichaperone complexes operating in systems where precise responses to signals with widely varying intensities are needed.

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