Negative Transcriptional Regulation of Multidrug Resistance Gene Expression by an Hsp70 Protein*

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One of the most common origins of multidrug resistance occurs via the overproduction of ATP-binding cassette (ABC) transporter proteins. These ABC transporters then act as broad specificity drug pumps and efflux a wide range of toxic agents out of the cell. The yeast *Saccharomyces cerevisiae* exhibits multiple or pleiotropic drug resistance (Pdr) often through the overproduction of a plasma membrane-localized ABC transporter protein called Pdr5p. Expression of the *PDR5* gene is controlled by two zinc cluster-containing transcription factors called Pdr1p and Pdr3p. Cells that lack their mitochondrial genome (pO cells) strongly induce *PDR5* transcription in a Pdr3p-dependent fashion. To identify proteins associated with Pdr3p that might act to regulate this factor, a tandem affinity purification (TAP) moiety was fused to Pdr3p, and this recombinant protein was purified from yeast cells. The cytosolic Hsp70 chaperone Ssa1p co-purified with TAP-Pdr3p. Overexpression of Ssa1p repressed expression of *PDR5* but had no effect on expression of other genes involved in the Pdr phenotype. This Ssa1p-mediated repression required the presence of Pdr3p and did not influence Pdr1p-dependent gene expression. Loss of the nucleotide exchange factor Fes1p mimicked Ssa1p-mediated repression of *PDR5*. Co-immunoprecipitation experiments indicated that Ssa1p was associated with Pdr3p but not Pdr1p in yeast cells. Finally, pO cells had less Ssa1p bound to Pdr3p than p+ cells, consistent with Ssa1p-mediated repression of Pdr3p activity serving as a key regulatory step in control of multidrug resistance in yeast.

The same similarities to mammalian cells that have made yeast a powerful model eukaryotic organism restrict the ability to design antifungal drugs without also impacting the health of the animal host. This has led to a limited repertoire of antifungal drugs and makes the development of drug-resistant fungi a serious problem. Even more serious is the acquisition of fungi with a multidrug resistant (Pdr)2 phenotype (reviewed in Ref. 2). Mutations either within genes encoding transcriptional regulators of *PDR* genes or in their regulatory inputs led to overexpression of downstream transporter proteins with associated multidrug resistance. The most extensively studied gene contributing to this pathway in *S. cerevisiae* is *PDR5*, which encodes an ATP-binding cassette (ABC) transporter that exhibits broad spectrum drug efflux activity (3–5). Transcription of *PDR5* and other Pdr pathway genes are controlled in large measure by the Zn(II)2Cys6 zinc finger regulators Pdr1p and Pdr3p (reviewed in Refs. 6 and 7).

Substitution mutant forms of Pdr1p and Pdr3p transcription factors have been identified that lead to high level overexpression of Pdr5p with an associated increase in multidrug resistance (8–10). Genetic experiments indicate that these mutant transcription factors behave as dominant, hyperactive forms of the regulatory proteins. Pdr1p and Pdr3p share partially overlapping function and control expression of their target genes by binding to a sequence element referred to as the Pdr1p/Pdr3p response element (PDRE) (11, 12). The *PDR3* gene itself is also controlled by two PDREs in its promoter and involves an auto-regulatory loop (13). The relative ease of isolation of these hyperactive transcription factors led to the suggestion that both Pdr1p and Pdr3p are subject to negative regulation under normal laboratory conditions, with this negative input eliminated in these mutants (9, 14).

In an effort to find negative regulators of Pdr5p, it was observed that mutants lacking their mitochondrial genome (pO cells) exhibited a striking induction of *PDR5* transcription and drug resistance (15, 16). Interestingly, the elevation in Pdr5p expression and subsequent increased drug resistance was dependent on Pdr3p but not Pdr1p (15). Similarly, overexpression of an Hsp70 protein called Ssz1p (formerly Pdr13p) induced *PDR5* transcription but in a manner dependent only on Pdr1p (17). Even though Pdr1p and Pdr3p share 36% sequence identity and similar domain organizations, these factors respond to different regulatory inputs.

The mitochondrial regulation of Pdr3p function represents the first physiological context in which the Pdr pathway is activated. Analyses of the expression of Pdr3p demonstrate that

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* This work was supported by National Institutes of Health Grant GM49825.

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2 The abbreviations used are: Pdr, pleiotropic drug resistance; ABC, ATP-binding cassette; PDRE, Pdr1p/Pdr3p response element; TAP, tandem affinity purification; HA, hemagglutinin; RT, reverse transcription; MRE, metal-response element; MALDI, matrix-assisted laser desorption ionization.
levels of this protein are maintained at an extremely low level in \( \rho^+ \) cells (18). \( PDR3 \) transcription is strongly induced in \( \rho^0 \) mutants, and this autoregulation is required for normal development of multidrug resistance in these cells. Previous data suggest that Pdr3p is regulated post-translationally in \( \rho^0 \) cells to acquire a higher activity state (15, 19). In an effort to find regulators of Pdr3p, we took a biochemical approach in which a tandem affinity purification (TAP)-tagged Pdr3p was used to facilitate purification of this factor and associated proteins. We found that the Hsp70 Ssa1p co-purified with TAP-Pdr3p. Various genetic and biochemical studies revealed that Pdr3p was under negative regulation by this Hsp70 protein. Importantly, Ssa1p-Pdr3p association was reduced in \( \rho^0 \) cells, consistent with this interaction maintaining Pdr3p in a low activity state in the absence of mitochondrial signaling. These studies argue for an important role of the Hsp70-Pdr3p association in the regulation of multidrug resistance.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Media**—Yeast strains used in this study are listed in Table 1. Cells were grown in cultures containing YPD (2% yeast extract, 1% peptone, and 2% glucose) under nonselective or appropriate SC media under selective conditions (20). Drug resistance was measured by the spot test assay on plates with either a single concentration of drug or gradient concentrations (21). Transformation was performed using the LiOAc method of Shevchenko et al. (30). The digested proteins were identified using MALDI time-of-flight mass spectrometry as described by Kinter et al. (31). Samples were analyzed on a Bruker Daltonics, Inc. Biflex III mass spectrometer.

**Real-time PCR**—Total RNA was isolated from each fraction with an RNeasy kit (Qiagen). Synthesis of cDNA was performed using iScript cDNA synthesis kit (Bio-Rad). 1 \( \mu \)g of RNA was used as template in each sample with 1 \( \times \) iScript reaction mix and 1 \( \mu \)l of Moloney murine leukemia virus-derived reverse transcriptase. The reaction mix was incubated for 5 min at 37 \( ^\circ \)C before adding 10 \( \mu \)l of SYBR green PCR master mix (Bio-Rad) and 1 \( \mu \)l of forward and reverse primers to each reaction. Reaction mixtures were incubated for 30 sec at 95 \( ^\circ \)C, followed by 40 cycles of 95 \( ^\circ \)C for 15 sec and 60 \( ^\circ \)C for 1 min.

**TABLE 1**

| Strain           | Genotype                                      | Reference  |
|------------------|-----------------------------------------------|------------|
| SEY6210          | MATa leu2–3, -112 ura3–52 tyr2–801 trp1–1 Δ901 his3–2Δ901 suc2–2Δ9 Mel1 pdr1Δ pdr3Δ | Scott Emr  |
| SEY6210 pdr1Δ    | SEY6210 pdr-1::kanMX4                         | (15)       |
| PBR2             | SEY6210 pdr-1::hisG                           | (15)       |
| PBR4             | SEY6210 pdr-1::hisG pdr3-1::hisG              | (15)       |
| PSY14            | SEY6210 pdr-1::hisG pdr3::TRP1–GAL1–TAP-PDR3  | This study |
| PSY36            | SEY6210 pdr-1::kanMX4                         | This study |
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25 °C, 30 min at 42 °C, and 5 min at 85 °C. For the quantitative PCR reaction, primers were designed using the Primer Select program from DNASTAR. Primer concentrations were optimized for each gene and annealing profiles were analyzed to evaluate nonspecific amplification by primer dimers. Control reactions, including RNA instead of cDNA were performed for each gene and condition. The threshold cycle (Ct) values were determined in the logarithmic phase of amplification for all genes, and the average Ct value for each sample was calculated from three replicates. The Ct value of the gene coding for actin (ACT1) was used for normalization of variable cDNA levels, and induction factors were determined for each gene and condition by three independent experiments. Primers used were ACT1For (5′-TTGGCCGCTAGGATTTGACGTGAC-3′) and ACT1Rev (5′-AGCCTTGTCAATTTGTGAC-3′). The samples were prepared by adding to the cDNA 12.5 μl of the reaction mixture, containing 1× iTaq SYBR Green Supermix with ROX (Bio-Rad) and 200 nM of the both forward and reverse oligonucleotides.

PDR5 forward (5′-GAATATTGGCCAAGTACGCA-3′) and PDR5 reverse (5′-CCACCAACCTGTGACGATC-3′); or SNQ2 forward (5′-TGCTGTGGCTTTGTGACATCC-3′) and SNQ2 reverse (5′-TGAACCGTTTTGGTTGCGTGA-3′). H2O was added to reach the final volume of 15 μl. Each standard sample was prepared in triplicate using serial dilutions starting from 100 ng and ending at 10 ng. The unknown samples were also prepared in triplicate with 50 ng of cDNA and the same reaction mixture as the standard samples. Amplification was carried out in the iCycler apparatus from Bio-Rad, in a two-step process as follows: a denaturation step of 3 min at 95 °C, and 40 cycles of 95 °C for 10 s, with annealing and extension at 60 °C for 45 s each.

The reporter signals were analyzed using the iCycler iQ software (Bio-Rad). These values can be translated into a quantitative result by constructing a standard curve, with the standard sample values. A melting curve was obtained after completion of the cycles to verify the presence of a single amplion. The presented RT-PCR results are mean values of at least three independent experiments.

Co-immunoprecipitation Assay—All immunoprecipitation assays were performed using lysed spheroplasts. In brief, cells growing in log phase were washed with spheroplast solution I (1 M sorbitol, 10 mM MgCl2, 30 mM diethiothreitol, 100 μg/ml phenylmethylsulfonyl fluoride, 50 mM K2HPO4), resuspended in spheroplast solution II (1 M sorbitol, 10 mM MgCl2, 30 mM diethiothreitol, 100 μg/ml phenylmethylsulfonyl fluoride, 50 mM K2HPO4, 25 mM sodium succinate, pH 5.5) containing oxylycine and incubated at 30 °C for 30 min. After chilling on ice for 10 min, cell suspensions were overlaid on a sucrose cushion (20 mM HEPES, 1.2 M sucrose, 0.02% sodium azide). Spheroplasts were pelleted by centrifuging at 5000 rpm for 20 min at 4 °C in a Beckman JA-20 rotor. These spheroplasts were either stored at −80 °C or lysed immediately using Nonidet P-40 lysis buffer (1% Nonidet P-40/Triton X-100, 0.15 M NaCl, 50 mM Tris-HCl, pH 7.2). Glass beads were added to cells suspended in Nonidet P-40 lysis buffer, followed by addition of 2 mM EDTA, 200 μM sodium vanadate, and 50 mM sodium fluoride. Lysis was performed by shaking cell suspensions on a Tomy shaker at 4 °C. Protein extracts were clarified by centrifuging lysates at 14,000 rpm for 5 min in an Eppendorf microcentrifuge. For immunoprecipitation, washed protein A or G beads were treated with either anti-HA, rabbit anti-Ssa1p (from Elizabeth Craig), or anti-Myc antibody for 2 h. These beads were then mixed with cell lysates and incubated for 4 h. Finally, the beads were washed and immunoprecipitated proteins were recovered by adding 3× Laemmli dye (0.125× Tris, pH 6.8, 4% SDS, 20% glycerol, 10% β-mercaptoethanol, 2% bromphenol blue dye).

RESULTS

The Hsp70 Protein Ssa1p Co-purifies with Pdr3p—Genetic experiments strongly suggest that Pdr3p is negatively regulated to maintain this factor in a state of low transcriptional activity. First, missense mutations in various regions of Pdr3p or loss of the mitochondrial genome convert this protein into a hyperactive and constitutive positive regulator of expression (8, 10, 15). Second, rapid depletion of the mitochondrial inner membrane chaperone protein Oxa1p accomplished by use of a temperature-sensitive oxA1 allele (19) triggers rapid induction of PDR5, in a manner believed to be dependent on Pdr3p. To determine if other proteins might associate with Pdr3p to repress the activity of this transcriptional regulator, we constructed a TAP-tagged (27) allele of PDR3. The TAP moiety allows gentle purification measures to be used to help maintain protein-protein interactions and has been successfully utilized to purify rare proteins (28). Expression levels of Pdr3p are very low (32) and to facilitate isolation of the TAP-Pdr3p protein, initial experiments used the GAL promoter to drive this fusion gene. The fusion protein was functional and activated PDR5 expression on galactose-containing medium (data not shown).

Cells containing the TAP-tagged PDR3 were grown, induced with galactose, and lysed. Protein extract was purified using the two-step IgG-Sepharose and calmodulin resin affinity steps as described previously (27) from a 10-liter culture. Polypeptides co-purifying with the TAP-Pdr3p chimera were recovered and subjected to MALDI mass spectrometry to determine their identity. This mass spectrometric analysis indicated that the Hsp70 protein Ssa1p was found in fractions enriched in TAP-Pdr3p and suggested that these proteins might be associated. An important qualification of this analysis comes from the fact that Ssa1p and Ssa2p are 99% identical (33), so it is probable that both or either of these proteins may associate with Pdr3p. SSA1 and SSA2 differ primarily in their regulation and deletion of both these genes is required to uncover phenotypes dependent on these Hsp70 proteins (34, 35). Our analysis will focus on Ssa1p, but it is likely that Ssa2p can also bind and regulate Pdr3p. This point will be considered in the discussion below. To confirm that this association of Ssa1p with Pdr3p has functional relevance, we tested the effect of elevating Ssa1p levels on Pdr3p-dependent drug resistance.

Increase in Cycloheximide Sensitivity of Cells Overproducing Ssa1p—To test the contribution of Ssa1p to control of Pdr3p-dependent PDR5 regulation, we overproduced Ssa1p using a high copy number plasmid containing SSA1 in an isogenic
series of strains with varying levels of Pdr3p-dependent transactivation. Transformants were grown to mid-log phase, and equal number of cells were spotted on YPD plates containing 0.4 μg/ml 4-nitroquinoline-N-oxide, 75 μM CdSO₄, 0.25 μg/ml cycloheximide or a gradient of this drug and incubated for 2 days at 30 °C (Fig. 1).

Cells lacking their mitochondrial genome (ρ⁰ cells) exhibit increased cycloheximide resistance in a Pdr3p-dependent fashion (15) compared with ρ⁺ cells. Overproduction of Ssa1p reduced cycloheximide in both the ρ⁺ and ρ⁰ strains. When this same analysis was performed in cells lacking Pdr3p, no significant influence of the high copy number SSA1 clone was seen. Note that, in these cells (pdr3Δ), PDR5-dependent transcription and cycloheximide resistance are provided by Pdr1p function (11, 36). Conversely, when cells lacking Pdr1p (pdr1Δ) were used, overproduction of Ssa1p led to a large decrement in cycloheximide resistance, because the only factor driving PDR5 expression and cycloheximide resistance was Pdr3p. Repression of the activity of Pdr3p would be expected to cause a reduction to PDR5-dependent phenotypes, and this is the result we observed. Overproduction of Ssa1p had no significant effect on resistance to 4-nitroquinoline, which can arise due to activation of the SNQ2 ABC transporter-encoding gene (a target of Pdr1p and Pdr3p (25, 37)) or on general growth of the cells when placed on YPD media without drugs. Cadmium resistance was also unaffected by these genotypic changes (data not shown). These data support the view that overproduction of Ssa1p leads to an inhibition of transcriptional activation driven by Pdr3p. This inhibition does not represent a general effect as resistance to another drug is unaffected. To confirm that the decreased cycloheximide resistance is due to diminution of Pdr3p-mediated activation of PDR5 transcription, we assayed PDR5 expression levels.

**Overexpression of Ssa1p Leads to Reduced PDR5 Expression in a Pdr3p-dependent Manner**—To examine the influence of Ssa1p on Pdr3p transcriptional activation, expression of several different genes known to be Pdr3p-responsive was assessed. Previous work has established that the ABC transporter-encoding genes YOR1 and SNQ2, along with PDR5, are regulated by Pdr3p transcriptional control (reviewed in Refs. 7, 38). We used gene fusions between the promoters of these genes and lacZ to evaluate their response to elevated Ssa1p levels. Wild-type cells were transformed with these reporter plasmids and a high copy number plasmid vector containing or lacking the SSA1 gene. Transformants were grown to mid-log phase, and β-galactosidase enzyme activities were determined.

Overproduction of Ssa1p lowered PDR5-dependent β-galactosidase activity from 64 to 34 units/optical density, whereas expression of both YOR1- and SNQ2-lacZ was either unaffected or modestly increased (Fig. 2). These data support the idea that the negative influence of Ssa1p on Pdr3p is linked to repression of PDR5 gene expression with an accompanying decline in cycloheximide resistance. PDR5 expression is responsive to transcriptional activation mediated by both Pdr1p and Pdr3p (11, 36, 39). To assess the relative contributions of these transcription factors to the observed effect of Ssa1p overproduction on PDR5 expression, a series of isogenic ρ⁺ and ρ⁰ strains containing various gene dosages of PDR1 and/or PDR3 was used. These strains were transformed with the PDR5-lacZ fusion gene along with the high copy number plasmid or its SSA1-containing subclone. Transformants...
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The effect of Ssa1p overproduction on expression of the native PDR5 mRNA was also evaluated to eliminate any possible complications that could arise from use of the lacZ gene fusions. Real-time quantitative RT-PCR was employed to measure mRNA levels for the PDR5 and SNQ2 genes. Levels of the actin transcript (ACT1) were also determined to provide an internal control that does not change in response to Pdr pathway activity. Isogenic p+ and p0 cells were transformed with the high copy number clone of SSA1 or the corresponding empty vector plasmid. Transformants were grown to mid-log phase, and total RNA was isolated from each. Transcript levels were then measured by real-time quantitative RT-PCR (Fig. 3).

Overproduction of Ssa1p lowered the relative PDR5 transcript levels to ~50% in p+ cells and to 36% in p0 cells compared with these same strains with wild-type SSA1 gene dosage. These reductions agree well with those reported above for assays using the PDR5-lacZ reporter gene. Importantly, levels of SNQ2 transcription were not seen to change significantly in response to variation in Ssa1p expression level. This analysis indicates that the wild-type PDR5 gene is negatively regulated upon Ssa1p overproduction as expected from the decrease in cycloheximide resistance seen in Fig. 1.

PDR5 Expression Is Reduced in fes1Δ Cells—A complicating feature of the analysis of Hsp70 phenotypes in many eukaryotic cells is the existence of multiple, closely related species of these proteins. S. cerevisiae is no exception to this and contains three different Hsp70 proteins sharing striking sequence identity with Ssa1p. Ssa2p is 96% identical to Ssa1p, whereas Ssa3p and Ssa4p share ~80% identity with Ssa1p (40). Furthermore, Pdr3p repression would represent only one of the vast array of functions influenced by these Ssa proteins, including protein translation (41), protein insertion into the endoplasmic reticulum (42), prion production (43), and even mRNA turnover (44). Loss of either SSA1 or SSA2 individually has little effect on the cell as the remaining gene appears to compensate for the absence of its homologue (35). Cells that lack both SSA1 and SSA2 are temperature-sensitive for growth in most backgrounds, because elevated expression of both SSA3 and SSA4 cannot provide sufficient Hsp70 function in the absence of the other genes (35). We constructed an ssa1Δ ssa2Δ strain but found (data not shown) that this mutant grew poorly even in the

were grown, and β-galactosidase enzyme activities were measured as described above.

In p+ cells grown on glucose-containing medium, Pdr1p and Pdr3p contribute roughly equally to expression of PDR5 (11, 36). Both wild-type and pdr1Δ cells were found to exhibit ~50% less PDR5-dependent β-galactosidase activity when Ssa1p was overproduced (Fig. 2). Importantly, neither a pdr3Δ nor a pdr1Δ pdr3Δ double mutant strain was found to be significantly influenced by elevating the level of Ssa1p. This same qualitative response was observed when the isogenic p+ cells were assayed for PDR5 expression. The magnitude of PDR5-lacZ enzyme activities was much higher, because Pdr3p is activated in the p0 genetic background. Only p0 cells, in which Pdr3p was still expressed, showed a decrease in β-galactosidase enzyme activity. We interpret these data to argue that elevation of Ssa1p levels led to a decrease in Pdr3p-dependent transactivation of the PDR5 promoter, whether Pdr3p is in the low activity state (p+ cells) or the high activity state (p0 cells). Pdr1p function was not detectably influenced by changes in Ssa1p levels.

FIGURE 2. Control of Pdr3p-dependent gene expression by elevated Ssa1p levels. A, low copy number reporter genes were transformed into wild-type cells along with a high copy number plasmid carrying SSA1 (2 μm SSA1) or the empty (YEp351) plasmid (Vector). Transformants were grown to mid-log phase and plasmid-dependent β-galactosidase levels assessed as described earlier (23). B, isogenic p+ and p0 cells were transformed with a low copy number plasmid carrying the PDR5-lacZ fusion gene along with vector and high copy SSA1-containing plasmids described above. Transformants were grown and assayed for PDR5-dependent lacZ expression as above.

PDR5-lacZ

wt pdr1Δ pdr3Δ pdr1Δ pdr3Δ

wt pdr1Δ pdr3Δ pdr1Δ pdr3Δ

Vector 2μm SSA1

β-galactosidase Activity (OD600)

0 10 20 30 40 50 60 70

PDR5-lacZ

YOR1-lacZ SNQ2-lacZ

PDR5-lacZ

2μm SSA1

Vector

β-galactosidase Activity (OD600)

0 20 40 60 80 100 1200
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FIGURE 3. Overproduction of Ssa1p leads to a decrease in native PDR5 mRNA levels. Real-time quantitative RT-PCR analysis of PDR5, SNQ2, and ACT1 (actin) in p* (top panel) and p0 (bottom panel) cells. These cells were also transformed with vector or high copy SSA1 plasmids as above. Data are presented as a ratio of either PDR5 or SNQ2 normalized to ACT1 transcript levels (relative transcription level). The difference in scales is due to the large induction of PDR5 expression in p0 cells.

absence of drugs and was defective in expression of several different reporter genes, including TRP5-lacZ (not subject to Pdr control) (11). These findings indicate that loss of both SSA1 and SSA2 causes a broad range of physiological issues as mentioned above and makes the use of this strain untenable for analysis of the role of Ssa1p and Ssa2p in selective control of Pdr3p function. Because it was important to demonstrate that the observed influence of Ssa1p on Pdr3p was not merely a consequence of overproduction, we used a mutant strain that lacks a key regulator of Ssa1p to evaluate changes in normal chromosomal levels of Hsp70 activity on PDR5 expression.

Fes1p was demonstrated to serve as a nucleotide exchange factor for Ssa1p (45). Additionally, work from several laboratories identified the Sse1p/Sse2p proteins as a second Ssa1p nucleotide exchange factor (46–48). Our attempts to use sse1Δ mutants to assess the effect of loss of this important Ssa1p regulator were compromised by the poor growth characteristics of these sse1Δ mutants (data not shown). For this reason, we focused our attention on Fes1p. Fes1p binding to Ssa1p triggers ADP release and is believed to inhibit Ssa1p ATPase activity. Interestingly, fes1Δ strains have been reported to exhibit cycloheximide sensitivity and temperature-sensitive growth (45). We constructed a fes1Δ mutation in our standard wild-type genetic background to determine the consequences of loss of normal Hsp70 activity regulation to the observed influence of Ssa1p on PDR5 expression. Isogenic wild-type and fes1Δ strains were tested for their relative drug resistance in response to normal SSA1 gene dosage but with or without the Fes1p nucleotide exchange factor, respectively. Cells were grown to mid-log phase and assayed for drug resistance as described above (Fig. 4).

Loss of FES1 led to hypersensitivity to cycloheximide as described before (45). To determine if the fes1Δ-dependent cycloheximide hypersensitivity correlates with decreased PDR5 expression, the isogenic pair of FES1/fes1Δ cells was transformed with PDR5- and SNQ2-lacZ reporter plasmids. Transformants were assayed for β-galactosidase activity as before (Fig. 4).

The presence of the fes1Δ allele led to a modest but reproducible decrease in PDR5-lacZ expression that was nearly equivalent to that caused by overproduction of Ssa1p. Importantly, loss of Fes1p had no significant influence on SNQ2 expression, demonstrating that fes1Δ mutant strains were not globally compromised in gene expression. These lacZ data were confirmed by quantitative RT-PCR experiments (Fig. 4). The negative effect on PDR5 transcription upon loss of Fes1p is reminiscent of a depression in prion propagation in the absence of this nucleotide exchange factor (49). We conclude from these data that regulation of normal Hsp70 activity is required to properly control PDR5 expression, consistent with the model that Ssa1p acts to inhibit Pdr3p transcriptional activation. These data also support the view that wild-type levels of Hsp70 proteins act to modulate transcription and drug resistance.

Ssa1p Interacts Preferentially with Pdr3p in Vivo—The biochemical data described above, suggesting that Ssa1p directly binds to Pdr3p, were derived from cells overproducing this transcriptional regulator. Additionally, the genetic epistasis experiments indicate that, although Pdr3p is required for the influence of Ssa1p on PDR5 gene expression, its close relative, Pdr1p, is not. Pdr1p and Pdr3p share 36% sequence identity across their lengths (36). To evaluate the interaction of Pdr1p and Pdr3p with Ssa1p under normal expression levels of all proteins, co-immunoprecipitation analysis was performed. Epitope-tagged versions of Pdr1p and Pdr3p were used that have been previously demonstrated to faithfully reproduce the regulator behaviors of the native proteins (19, 32). Both epitope-tagged alleles were carried on low copy number plasmids and were under control of their wild-type promoter sequences.

The plasmids described above were introduced into a pdr1Δ pdr3Δ mutant strain. The empty low copy number vector was included as a control. Transformants were grown to mid-log phase, and protein extracts were prepared. An aliquot of these extracts was withdrawn in each case to serve as an input control for the levels of each factor of interest. Equal amounts of protein extracts were then incubated with protein A-agarose beads.
expression. This autoregulation is required for the response to the mitochondrial signal. Activated Pdr3p then engages two PDREs that Pdr3p is post-translationally activated by an unknown size that Pdr3p is post-translationally activated by an unknown JOURNAL OF BIOLOGICAL CHEMISTRY 26828 membrane, proteins of interest were detected by Western blotting transfer of the resolved polypeptides to a nitrocellulose mem-

were recovered and electrophoresed through SDS-PAGE. After the association of Pdr3p and Ssa1p occurs under conditions of nor-

ations (15), no Ssa1p-Pdr1p interaction was seen. This reflects the specificity of the Pdr3p-Ssa1p complex seen in these cells. Although equal levels of HA-Pdr3p were recovered by anti-HA immunoprecipitation, less Ssa1p was found in the precipitate indicating that these two proteins associate (Fig. 5).

FIGURE 5. Ssa1p associates specifically with Pdr3p. Low copy number plasmids expressing either HA-Pdr3p or Myc-Pdr1p were introduced into pdr1Δ pdr3Δ cells along with an empty pRS315 vector control (Vector). Transfor-
mants were grown to mid-log phase and whole cell protein extracts prepared. Aliquots of these extracts were reserved to serve as input controls (Input) and the remainder of each extract was immunoprecipitated using rabbit polyclonal anti-Ssa1p antiserum. Immunoprecipitates were washed and then electrophoresed, along with input controls through SDS-PAGE. After electrophoresis, the proteins were transferred to nitrocellulose and analyzed by Western blotting with the indicated antibodies.

The Binding of Ssa1p to Pdr3p Is Reduced in a mitochondrial signal that induces Pdr3p activity and supports the view that Ssa1p acts as a negative regulator of Pdr3p function. To examine the regulated association of Ssa1p and Pdr3p, we wanted to avoid the complication that is produced by the positive autoregulation of PDR3. Because Pdr3p levels are induced by >10-fold in ρ0 cells, this would create a problem in terms of assuring equal levels of Pdr3p in immunoprecipitates to be assayed for levels of associated Ssa1p. The autoregulatory circuit was eliminated by replacing the PDREs in the PDR3 promoter with binding sites for the copper-inducible Ace1p transcription factor (50). These binding sites will be referred to as metal-response elements (MREs). This construct has been previously characterized and confers copper-regulated Pdr3p production on cells (18). This plasmid will be referred to as MRE-HA-Pdr3p and was constructed in the low copy number vector pRS315 backbone, which served as a control for this analysis.

Isogenic pdr1Δ pdr3Δ double mutant ρ0 and ρ0 cells were transformed with the vector or MRE-HA-Pdr3p plasmids. Transformants were grown to mid-log phase in the presence of 75 μM copper to induce the Ace1p-responsive fusion gene. Protein extracts were prepared for co-immunoprecipitation as described above, but the immunoprecipitating antibody in this case was mouse anti-HA coupled to protein G-agarose beads. Immunoprecipitates were recovered and analyzed, along with aliquots of the initial protein extract (input control), by Western blotting (Fig. 6).

Although equal levels of HA-Pdr3p were recovered by anti-HA immunoprecipitation, less Ssa1p was found in the immunoprecipitates from ρ0 cells compared with those from an isogenic ρ+ strain. Levels of both Ssa1p and HA-Pdr3p were identical in the input control samples. These data indicate that Ssa1p-Pdr3p association is reduced in response to a mitochondrial signal that induces Pdr3p activity and supports the view that Ssa1p is a negative regulator of Pdr3p function.
The role of Hsp70 proteins in maturation of steroid hormone receptors is a well known example of the function of these chaperones in transcription factor regulation (reviewed in Ref. 51). More recently, it has been appreciated that Hsp70 proteins can influence the activity of many transcription regulatory proteins, including heat shock transcription factor (reviewed in Ref. 52), GATA-1 (53), and S. cerevisiae Hap1p (54). The experiments reported here add the zinc cluster-containing Pdr3p transcription factor to the list of Hsp70 client proteins and implicate Ssa1p/2p as functional regulators of multidrug resistance in S. cerevisiae.

The role of Ssa1p in regulation of Hap1p has been extensively analyzed (54–57). Ssa1p is found associated with Hap1p in a constitutive fashion that does not respond to changes in Hap1p transcriptional activation (58). This is in marked contrast to the interaction of Pdr3p with Ssa1p. Pdr3p:Ssa1p association decreased in pdr1Δ pdr3Δ cells in which Pdr3p activity was strongly stimulated (Fig. 5). This finding suggests the simple model that Ssa1p binding to Pdr3p keeps this protein in a low activity state. Because Pdr3p is autoregulated (13), maintenance of this factor in a repressed status is important to ensure that levels of this protein are kept low in the absence of retrograde signaling. Previous work has demonstrated that this autoregulatory loop involving Pdr3p is critical for activation of PDR3 expression and ultimately multidrug resistance in pdr1Δ cells (reviewed in Ref. 59).

The finding that association with Ssa1p acts to inhibit Pdr3p activity has important implications for the understanding of the molecular basis of the many hyperactive dominant forms of Pdr3p that have been described (8–10). Single amino acid substitution mutations have been found that convert Pdr3p to a strong activator of downstream gene expression and drug resistance. These mutations are scattered across the C-terminal region of Pdr3p and have eluded a simple explanation to explain their increased transcriptional activation. We hypothesize that these gain-of-function mutant forms of Pdr3p may be due to decreased Ssa1p binding as a result of changes in the conformation of the central regulatory domain of this transcription factor. We are currently testing this hypothesis.

Pdr1p shares 36% sequence identity with Pdr3p across the length of the factors and is capable of heterodimerization (32). Analogous hyperactive mutant forms of Pdr1p have also been isolated that convert this protein to a strong activator of target gene transcription (9). Interestingly, not only do we find that Ssa1p fails to associate with Pdr1p, previous work has demonstrated that Pdr1p is positively regulated by another Hsp70 protein called Ssz1p (17). Although both Pdr1p and Pdr3p are regulated by Hsp70 proteins, these chaperone proteins have opposite effects on the transcriptional regulators of multidrug resistance. Ssz1p seems most likely to exert its positive effect on Pdr1p via an indirect mechanism, because this Hsp70 protein is a component of a set of interacting proteins called the ribosome-associated complex (60), which is required for normal protein synthesis. Additionally, localization data indicated that the majority of Ssz1p was found in the cytoplasm (61), consistent with the important role of this protein in translation. However, some experiments have argued that only extra-ribosomal Ssz1p is responsible for control of drug resistance (62) and suggest the possibility that Ssz1p might more directly interact with Pdr1p, which is found in the nucleus (61, 63). Further investigation is required to evaluate the basis of the positive regulation of Pdr1p by Ssz1p.

Association of overproduced proteins with Hsp70 family members can occur due to the role of these chaperone proteins in translation or protein folding. We believe that Ssa1p–Pdr3p association reflects an authentic regulatory interaction for these two proteins for several reasons. First, binding can be demonstrated when Pdr3p and Ssa1p are expressed at normal chromosomal levels. Second, high level expression of Ssa1p exerts a negative effect on Pdr3p- but not Pdr1p-responsive gene expression. Third, loss of the Fes1p nucleotide exchange factor, which blocks the normal catalytic cycle of cytosolic Hsp70 members (like Ssa1p), leads to a decrease in drug resistance and a drop in PDR5 expression. Finally, overproduction of Ssa1p lowered cycloheximide resistance but had no significant effect on other resistance phenotypes such as 4-nitroquinoline-N-oxide or cadmium (data not shown). Cells overproducing Ssa1p exhibited no change in growth properties in the absence of drug challenge, consistent with high levels of this chaperone being well tolerated by the cell.

The finding that Ssa1p negatively regulates Pdr3p is unexpected when considering the levels of expression of these two proteins. Estimates using TAP-tagged proteins indicate that Ssa1p levels are roughly 1000 times that of Pdr3p (64). This does not account for the levels of Ssa2p that are similar to those of Pdr3p.
Ssa1p Represses Pdr3p Function

of Ssa1p. Because we believe that either Ssa1p or Ssa2p can negatively regulate Pdr3p, the combined levels of these two chaperone proteins are several orders of magnitude greater than the level of Pdr3p. This vast difference in abundance reflects the wide range of activities carried out by the Ssa1p/2p Hsp70 proteins and is illustrative of the fact that control of Pdr3p activity is but one of these many functions.

The integration of Ssa1p/2p chaperones with Pdr3p is also likely to have important consequences to our understanding of the mitochondrial control of multidrug resistance called retrograde regulation (reviewed in Ref. 65). In S. cerevisiae and the pathogenic yeast Candida glabrata (66), loss of mitochondrial DNA (ρ0) cells triggers induction of a variety of genes, including ABC transporters that in turn strongly elevate resistance to a wide variety of antifungal agents. Surprisingly, this induction of multidrug resistance is not seen in the many petite mutants that maintain their organellar genome of the mitochondrial ATPase (67), whereas this is not typically the mitochondrial membrane is linked to loss of the Fo component (68). Perhaps a high degree of misfolding associated with the aberrant membrane morphology acts as a competitor for Ssa1p/2p binding to Pdr3p and ultimately induces PDR5 expression. Mutations in the yeast heat shock factor gene have been described that cause temperature-sensitive defects in mitochondrial protein folding likely due to failure to induce SSA1 (69). This simple model may explain the ρ0-mediated induction of PDR5 in S. cerevisiae and possibly in other yeasts.

Acknowledgments—We thank Elizabeth Craig, Karl Kuchler, and Jeff Brodsky for providing reagents and strains; Rob Piper, Jeff Brodsky, and William Walter for helpful discussions and advice; and Yalan Li and the University of Iowa Molecular Analysis Facility for carrying out the mass spectrometric analysis.

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