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The Hsp40 Molecular Chaperone Ydj1p, Along With the Protein Kinase C Pathway, Affects Cell-Wall Integrity in the Yeast *Saccharomyces cerevisiae*

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ABSTRACT

Molecular chaperones, such as Hsp40, regulate cellular processes by aiding in the folding, localization, and activation of multi-protein machines. To identify new targets of chaperone action, we performed a multi-copy suppressor screen for genes that improved the slow-growth defect of yeast lacking the *YDJ1* chromosomal locus and expressing a defective Hsp40 chimera. Among the genes identified were *MID2*, which regulates cell-wall integrity, and *PKC1*, which encodes protein kinase C and is linked to cell-wall biogenesis. We found that *ydj1*Δ yeast exhibit phenotypes consistent with cell-wall defects and that these phenotypes were improved by Mid2p or Pkc1p overexpression or by overexpression of activated downstream components in the PKC pathway. Yeast containing a thermosensitive allele in the gene encoding Hsp90 also exhibited cell-wall defects, and Mid2p or Pkc1p overexpression improved the growth of these cells at elevated temperatures. To determine the physiological basis for suppression of the *ydj1*Δ growth defect, wild-type and *ydj1*Δ yeast were examined by electron microscopy and we found that Mid2p overexpression thickened the mutant's cell wall. Together, these data provide the first direct link between cytoplasmic chaperone function and cell-wall integrity and suggest that chaperones orchestrate the complex biogenesis of this structure.

MOLECULAR chaperones play vital roles in many cellular processes, such as protein folding, degradation, translocation across membranes, and the rearrangement of multi-protein complexes. While molecular chaperones can work independently, they most commonly function as components of large multi-chaperone assemblies. For example, the Hsp70 and Hsp40 chaperones function together to catalyze a variety of essential processes in the cell (Walsh *et al.* 2004; Hennessy *et al.* 2005; Mayer and Bukau 2005). One Hsp70–Hsp40 pair, which is the focus of this study, is the *Saccharomyces cerevisiae* Ssa1 and Ydj1 proteins.

Hsp70's contain an N-terminal ATPase domain and C-terminal substrate-binding domain (Flaherty *et al.* 1990; Wang *et al.* 1993; Zhu *et al.* 1996), and ATP hydrolysis induces a conformational change that allows the substrate-binding domain to bind peptides with high affinity (McCarty *et al.* 1995; Russell *et al.* 1999). Repeated cycles of ATP hydrolysis lead to successive rounds of peptide binding and release, which is utilized in all Hsp70-dependent processes such as those listed above. However, Hsp70's are intrinsically weak ATPases and thus require cochaperones such as Hsp40's and nucleotide exchange factors (NEFs) for maximal activity. Hsp40's also bind to peptide substrates and can deliver them to Hsp70 (Wickner *et al.* 1991; Langer *et al.* 1992; Szabo *et al.* 1996; Rudiger *et al.* 2001; Han and Christen 2003). All Hsp40's contain an ~70-amino-acid "J domain," which appears to interact with the ATPase domain of an Hsp70, thus stimulating its ATPase activity (Cheetham and Caplan 1998; Gassler *et al.* 1998; Suh *et al.* 1998). The J domain contains four α-helices that form a finger-like projection, and the interaction with Hsp70 is mediated through helix II and an indispensable histidine–proline–aspartic acid (HPD) motif in the loop between helices II and III (Greene *et al.* 1998). Mutations in the HPD loop and in some residues in helix II abrogate the ability of Hsp40's to stimulate Hsp70 ATP hydrolysis (Wall *et al.* 1994; Tsai and Douglas 1996; Genevaux *et al.* 2002; Hennessy *et al.* 2005). In contrast, NEFs work through a variety of mechanisms to catalyze ADP release from Hsp70 (Shomura *et al.* 2005), which is required to free bound peptide substrates.

The Hsp70–Hsp40 chaperones can couple with other chaperone machines in the cell. Specifically, Hsp70 and Hsp40 cooperate with the Hsp90 chaperone complex, which is responsible for folding a specific set of client proteins that have neared their native conformation (Terasawa *et al.* 2005; Zhao and Houry 2005). For example, *in vitro* reconstitution studies indicate that the

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progesterone receptor (PR) is first bound by Hsp40, then delivered to Hsp70, and finally presented to the Hsp90 complex. Hsp90, through its interaction with several additional cochaperones, folds PR and remains associated with the receptor until hormone binds (Smith *et al.* 1992; Hernandez *et al.* 2002a,b; Pratt and Toft 2003).

Another intricate cellular process, which has been best characterized in the model organism *S. cerevisiae*, is cell-wall biogenesis (Levin 2005; Lesage and Bussey 2006), but surprisingly few chaperones have been directly implicated in this process (see below and discussion). The yeast cell wall is composed of two distinct layers. The electron-transparent inner layer is composed primarily of β1,3-glucan chains and small amounts of chitin, which are covalently "glued" together by β1, 6-glucans (Osumi 1998). In contrast, the electron dense outer layer is composed of glycosylphosphatidylinositol (GPI) and Pir glycoproteins (Osumi 1998), which protect the cell wall and aid in cellular recognition. GPI proteins are attached to the β1,3-glucan chains via β1, 6-glucan, whereas Pir glycoproteins are linked directly to β1,3-glucans. Synthesis of β1,3-glucan chains and chitin takes place at the yeast cell wall by known synthases (Douglas *et al.* 1994; Mazur *et al.* 1995; Qadota *et al.* 1996; Santos and Snyder 1997; Valdivia and Schekman 2003), but the location of β1,6-glucan synthesis is unknown, in part because the synthase has not yet been identified (Shahinian and Bussey 2000). β1,6-glucan is entirely localized to the cell wall, suggesting that it is synthesized at this site (Montijn *et al.* 1999). However, β1,6-glucan synthesis clearly requires the secretory pathway because a mutant allele in the gene encoding an ER luminal Hsp70, BiP, reduces the amount of β1,6-glucan at the cell wall when either of the two ER glucosidases are also disabled (Simons *et al.* 1998). Mutations in numerous other genes in the secretory pathway also result in reduced β1,6-glucan at the cell wall (Shahinian and Bussey 2000). Finally, overexpression of several Golgi-resident proteins proposed to aid in β-glucan synthesis suppresses the growth defect in strains mutated for the gene encoding Pkc1p, which is involved in maintaining cell-wall integrity (Roemer *et al.* 1994; Neiman *et al.* 1997).

A third intricate process in which chaperone machines are essential is exemplified by the tumor-causing virus *s*imian *v*irus 40 (SV40) (Brodsky and Pipas 1998; DeCaprio 1999; Sullivan and Pipas 2002). A single viral-encoded protein, large tumor antigen (TAg), interacts with cellular proteins such as the tumor-suppressors p53 and Rb and co-opts their function to trigger SV40 replication (Ali and DeCaprio 2001). These interactions lead to tumor development in rodents and transformation of rodent cell lines in culture. TAg contains a J domain that interacts with Hsp70 and is indispensable for viral replication and viral-induced cellular transformation (Campbell *et al.* 1997; Kelley and Georgopoulos 1997; Srinivasan *et al.* 1997). The J domain of TAg binds to and stimulates Hsp70 ATPase activity, leading to the release of E2F transcription factors from Rb and triggering cell cycle progression (Sullivan *et al.* 2000). Previous observations indicate that additional unknown factors—perhaps other chaperones—are also probably involved in this process (C. S. Sullivan and J. M. Pipas, unpublished data).

To better define why chaperone activity is required for SV40 function and ultimately to identify these other factors, loss-of-function mutations in the TAg J domain were uncovered in a yeast screen using a chimeric T-Ydj1 protein. This chimera contains the TAg J domain fused to the C terminus of a yeast Hsp40, Ydj1p, and its expression rescues the temperature-sensitive growth defect of *ydj1-151* mutant yeast (Fewell *et al.* 2002). Of the 14 mutant alleles of *T-TDJ1* that failed to rescue the temperature sensitivity of *ydj1-151,* we were intrigued by the K53R mutant because the corresponding lysine maps to the third helix in the J domain, and NMR perturbation experiments suggest that this residue is unaltered by Hsp70 binding (Greene *et al.* 1998; Landry 2003). However, when this mutation was introduced into TAg, the mutant protein was partially defective for stimulating Hsp70 ATPase activity and for releasing Rb from the E2F complex. A recombinant SV40 engineered to express K53R TAg was replication and transformation deficient (Fewell *et al.* 2002). Thus a *T-YDJ1* construct containing the K53R mutation is a promising genetic tool to explore TAg chaperone function and to identify novel chaperone modulators.

To better define TAg chaperone function, we performed a yeast suppressor screen in *ydj1*Δ yeast expressing a *T-YDJ1* construct with the K53R mutation [*T(K53R)-YDJ1*]. As reported here, we identified yeast Hsp70 as a suppressor of the K53R allele and uncovered a previously unknown connection between Ydj1p and yeast cell-wall biogenesis. Specifically, we observed cell-wall defects in *ydj1*Δ and Hsp90 temperature-sensitive mutants and discovered that upregulation of the *p*rotein *k*inase C (PKC) pathway can rescue these defects. These studies are the first to implicate the cytosolic Hsp40 chaperones in maintenance of the yeast cell wall.

MATERIALS AND METHODS

Yeast strains and methods: *S. cerevisiae* yeast strains used in this study are listed in Table 1. Unless otherwise indicated, all yeast cultures were grown in yeast extract–peptone–dextrose (YPD) or selective synthetic complete medium (SC) with 2% glucose at room temperature or at 26° (Adams *et al.* 1997). Cell-wall phenotypes were tested on YP or SC–ura medium with the addition of 0.4 m NaCl, 1 m sorbitol, or 20 μg/ml calcofluor white (CW), and 2% glucose. Yeast transformation was performed by the lithium acetate procedure (Ito *et al.* 1983). For all serial dilutions, overnight cultures were diluted back to early log phase (0.3–0.4 OD) and allowed to grow 2–5 hr. Cell densities were normalized to the lowest OD and cells were serially diluted 10-fold. Unless specifically indicated, all

TABLE 1

Yeast strains used in this study

Yeast strain	Genotype	Source
W303	*MATα ade2-1 leu2-3,112 his3-11,15 trp1-1ura3-1 can1-100*	This lab
ACY95b	*MATα ade2-1 leu2-3,112 his3-11,15 trp1-1 ura3-1 can1-100 ydj1-2::HIS3 pAV4*	Caplan *et al.* (1992)
JN516	*MATα leu2-3,112 his3-11,15 ura3-52 trp1-Δ1 lys2 SSA1 ssa2::LEU2 ssa3::TRP1 ssa4::LYS2*	Becker *et al.* (1996)
JB67	*MATα leu2-3,112 his3-11,15 ura3-52 trp1-Δ1 lys2 ssa1-45::URA3 ssa2::LEU2 ssa3::TRP1 ssa4::LYS2*	Becker *et al.* (1996)
p82a	*MAT***a** *ade2-1 leu2-3,112 his3-11,15 trp1-1 ura3-1 can1-100 hsc82::LEU2 hsp82::LEU2 pTGPD-HSP82*	Nathan and Lindquist (1995)
G313N	*MAT***a** *ade2-1 leu2-3,112 his3-11,15 trp1-1 ura3-1 can1-100 hsc82::LEU2 hsp82::LEU2 pTGPD-HSP82-G313N*	Fliss *et al.* (2000)
G170D	*MAT***a** *ade2-1 leu2-3,112 his3-11,15 trp1-1 ura3-1 can1-100 hsc82::LEU2 hsp82::LEU2 pTGPD-HSP82-G170D*	Nathan and Lindquist (1995)
STI1/SSE1	*MAT***a** *GAL2 his2-11,15 leu2-3,112 lys1 lys2 trp1Δ1 ura3-52*	Nicolet and Craig (1989)
sti1Δsse1Δ	*MAT***a** *GAL2 his2-11,15 leu2-3,112 lys1 lys2 trp1Δ1 ura3-52 sti1::HIS3 sse1::KANR*	Liu *et al.* (1999)

growth assays were performed at 26°, 30°, 35°, and 37° for 3–7 days.

Molecular techniques: All plasmids used in this study are listed in Table 2. *T-YDJ1* and *T(K53R)-YDJ1* constructs were derived from the pOW4 *T-YDJ1* plasmid, which is derived from plasmid YCplac33 (Gietz and Sugino 1988) and contains the *T-YDJ1* chimeric gene (Fewell *et al.* 2002) expressed from the alcohol dehydrogenase 1 (*ADH*) promoter. This chimera consists of amino acids 1–82 of T antigen, which encompasses most of the J domain, and amino acids 71-409 of Ydj1p, which contains the glycine/phenylalanine-rich and zinc-finger-like regions and thus the putative substrate-binding domain (Johnson and Craig 2001; Kim *et al.* 2001; Fan *et al.* 2005). pOW4 *T(K53R)-YDJ1* was created with the Quikchange site-directed mutagenesis kit (Stratagene, La Jolla, CA) with oligonucleotides 5′-GAGATGAAGAAAAAATGAGGAAAATGAAT ACTCTG-3′ and 5′-CAGAGTATTCATTTTCCTCATTTTTTC TTCATCTC-3′, using pOW4 *T-YDJ1* as a template. The pOW4 *T-YDJ1* and pOW4 *T(K53R)-YDJ1* vectors were digested with *Eco*RI and *Xho*I and the inserts were ligated into p*TEF*414 (Mumberg *et al.* 1995) to create *TEF*414 *T-YDJ1* and *TEF*414 *T(K53R)-YDJ1*. *TEF*414 *T(H42R)-YDJ1* was created with the Quikchange mutagenesis kit using *TEF*414 *T-YDJ1* as a template and oligonucleotides 5′-AAATGCAAGGAGTTTCGTCC TGATAAAGGAGGAG-3′ and 5′-CTCCTCCTTTATCAGGAC GAAACTCCTTGCATTT-3′. The inserts in all constructs were subjected to DNA sequence analysis. An additional mutation, D127G, was detected in all constructs at nucleotide 380 in a nonconserved residue between the glycine/phenylalanine-rich and zinc-finger-like regions of *YDJ1* (Caplan and Douglas 1991); however, the presence of this mutation did not affect the growth of yeast-expressing T-Ydj1p.

The yeast genes *REC102*, *YLR149c*, *CIS1*, and *HSP82* were amplified by PCR from genomic wild-type yeast (W303) DNA with primers created against regions 50–200 bp upstream of the TATA box and 15–250 bp downstream of the stop codon. The PCR products were digested with the appropriate restriction enzymes and ligated into pRS426 (Christianson *et al.* 1992). The sequence of each insert was confirmed by sequence analysis. pRS426 *HSP82* had an A493T mutation at a nonconserved residue in the middle region of Hsp82p. This mutation did not alter Hsp82p function since expression of the corresponding protein restored growth in the *hsp82* G170D and *hsp82* G313N temperature-sensitive strains, and the protein was able to support cell growth as the only copy of Hsp82p in the cell (data not shown). To create a high-copy *BCK1-20* expression vector, an ∼6.5-kb fragment containing *BCK1-20* under the control of the endogenous promoter was removed from *pRS314BCK1-20* (Lee and Levin 1992) using *Xho*I and *Not*I and ligated into pRS426. A high-copy version of $MKK1^{DD}$ was created by removing an ∼1.6-kb fragment containing $MKK1^{DD}$ under the control of the endogenous promoter from *pDLB824* (Harrison *et al.* 2004) using *Eco*RI and *Sac*I and ligating this fragment into pRS426. To obtain the pRS426 *HOG1-HA* expression plasmid, an ∼2.5-kb fragment containing *HOG1-HA* under the control of the endogenous promoter was PCR amplified from *p181HOG1ha3* (Winkler *et al.* 2002) and ligated into pRS426. The *HOG1* PCR primers encompassed DNA ∼125 bp upstream of the TATA box and introduced two stop codons immediately downstream of the 3′ HA tag. All primer sequences used for this study are available upon request.

High-copy suppressor screen: To search for high-copy suppressors of the *T(K53R)-YDJ1* thermosensitive phenotype in *ydj1Δ* yeast, a *ydj1Δ* strain (ACY95b) containing a pRS316-derived *CEN-YDJ1* expression vector (pAV4; Caplan *et al.* 1992), was plated on 5-fluoroorotic acid to select for yeast that had lost pAV4. The surviving *ydj1Δ* cells were then transformed with *TEF*414 *T(K53R)-YDJ1* and grown on SC–trp medium and expression of T(K53R)-Ydj1p was verified by immunoblot analysis using both the anti-TAg J domain antibody 419 (Harlow *et al.* 1981) and an antibody against Ydj1p (Caplan and Douglas 1991) (data not shown). Next, a 2μ-*URA3* yeast genomic library in the YEp24 vector (Carlson and Botstein 1982) was introduced into these cells and transformants were selected on SC–ura–trp at 35° for 4 days. Approximately 80,000 colonies were screened, and 61 colonies appeared to contain plasmids that suppressed the *T(K53R)-YDJ1* growth defect when restreaked onto SC–ura–trp at 35°. The plasmid DNA from these cells was isolated and retransformed into *ydj1Δ* yeast-expressing T(K53R)-Ydj1p. Upon retransformation, 21 plasmids improved growth to varying extents. Of these, 8 unique plasmids were obtained as assessed by DNA sequence and restriction digest analysis.

Biochemical and immunological methods: To prepare cellular proteins for immunoblot analysis, 10 ml of yeast were

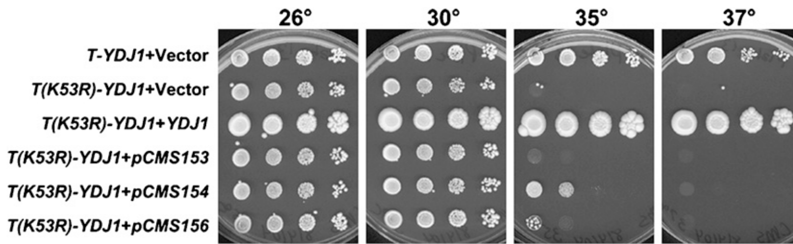

Figure 1.—*pCMS154* in multiple copies moderately rescues the *T(K53R)-YDJ1* growth defect at 35°. Ten-fold serial dilutions of *ydj1*Δ yeast expressing wild-type T-Ydj1p or T(K53R)-Ydj1p and containing a vector control, a Ydj1p expression vector (*pAV4*), or three plasmids recovered in the screen—*pCMS153*, *pCMS154*, and *pCMS156*—were plated on SC–ura–trp and grown for 5 days at the indicated temperatures. The plasmid *pCMS154* contains a yeast genome fragment that includes the genes *REC102*, *CHS5*, and *MID2* and moderately improves the growth of yeast containing the *T(K53R)-YDJ1* expression vector at 35°. The plasmid *pCMS156* contained the genes *STM1* and *YLR149c* and led to papillae colony growth at 35°. Plasmid *pCMS153* did not improve growth of the *ydj1*Δ strain containing the *T(K53R)-YDJ1* expression vector at 35°.

grown to an OD of 0.3–0.9 in the appropriate selective medium at room temperature and the cells were pelleted and resuspended in 0.8 ml of 100 mm Tris, pH 8.0, 20% glycerol, 1 mm dithiothreitol, 1 mm phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml leupeptin, and 0.5 μg/ml pepstatin A. The cells were lysed by agitation with glass beads and crude protein concentrations were determined by measuring the A_{280}. Protein concentrations in each sample were normalized and total protein was resolved by SDS–PAGE, transferred to nitrocellulose, and immunoblotted with the indicated antiserum. Antiserum against ribosomal protein L3 (a kind gift of J. Warner, Albert Einstein College of Medicine) or Sec61p (Stirling *et al.* 1992) was used to establish loading controls. Antisera against Sse1p (Goeckeler *et al.* 2002) and Ydj1p (Caplan and Douglas 1991) were described previously. Antiserum against Hsp82p was provided by A. Caplan, Mount Sinai School of Medicine. Anti-Ssa1p antiserum was prepared in rabbits using a GST-fusion protein that contained amino acids 586–831 of Ssa1p (provided by E. Craig, University of Wisconsin). Antibodies against the HA epitope were obtained from Roche and antibodies against Pkc1p were obtained from Santa Cruz Biotechnology. Bound antibodies were visualized using anti-mouse or anti-rabbit antibodies coupled to horseradish peroxidase and the Supersignal West Pico chemiluminescent substrate kit (Pierce, Rockford, IL). Chemiluminescent signal was detected using a Kodak 440CF Image Station and quantified using Kodak 1D (v. 3.6) software.

Subcellular fractionation of Mid2p was performed by differential centrifugation as previously described (Kabani *et al.* 2002a) with minor changes. A total of 100 ODs of either wild-type (W303) or *ydj1*Δ yeast-expressing Mid2p-HA were resuspended in 2 ml PLB (20 mm HEPES, pH 7.4, 100 mm NaCl, 20 mm $MgCl_2$, 1 mm PMSF, 1 μg/ml leupeptin, 0.5 μg/ml pepstatin A) and cells were broken by glass-bead lysis. Unbroken cells were removed by centrifugation at 1400 × *g* for 5 min and 1 ml of the supernatant (L) was subjected to a medium-speed spin (16,000 × *g*) for 15 min at 4°. The pellet (P1) was resuspended in 250 μl PLB and half of the supernatant (S1) was saved. The remaining supernatant was subjected to a high-speed spin (150,000 × *g*) for 15 min at 4°. The resulting pellet (P2) was resuspended in 100 μl PLB and the remaining supernatant (S2) was saved. Total protein concentration in L, S1, P1, S2, and P2 was normalized by Coomassie Brilliant blue staining of polyacrylamide gels. The samples were resolved by SDS–PAGE, and immunoblots were performed as described above.

Indirect immunofluorescence and electron microscopy: Indirect immunofluorescence microscopy was performed as described previously (Coughlan *et al.* 2004). Briefly, wild-type (W303) or *ydj1*Δ yeast strains overexpressing Mid2p-HA were grown to midlog phase, fixed in 3.7% formaldehyde, and treated with 20 μg/ml zymolyase for 45 min at 37°. Cells were incubated with primary antibodies [HA 1:250 and Kar2p (1:250; Brodsky and Schekman 1993)] overnight at 4° and in secondary antibodies (Alexa Fluor 488 goat anti-mouse 1:250 and Alexa Fluor 568 goat anti-rabbit 1:250; Molecular Probes, Eugene, OR) for 2 hr at room temperature. To visualize DNA, the cells were incubated in 2 μg/ml 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) for 5 min at room temperature.

Electron microscopy was performed as previously described (Kaiser and Schekman 1990). In brief, the indicated stains containing either the *MID2-HA* expression vector or YEp352 (Hill *et al.* 1986) were fixed in 2.5% glutaraldehyde, processed, sectioned, and affixed to 0.125% formvar-coated grids. Sections counterstained with uranyl acetate and Reynold's lead citrate were examined on JEM-1011 or JEM-1210 (JEOL) transmission electron microscopes. Cell-wall thickness was measured using AMTv542 image capture software (Advanced Microscopy Techniques). A total of 12–16 similarly sized budding cells from each culture were chosen, and the cell-wall thicknesses at 10 points around the mother cell were measured. The average for each cell was calculated and these numbers were averaged for each culture to determine the mean cell-wall thickness.

RESULTS

Identification of suppressors of *T(K53R)-YDJ1* and *ydj1*Δ yeast: We previously reported on a yeast TAg expression system in which the TAg J domain replaced the homologous domain in a yeast Hsp40, Ydj1p. By PCR mutagenesis of the inserted J domain, new mutations in this domain were isolated, some of which conferred strong phenotypes when inserted into full-length TAg and into SV40 (Fewell *et al.* 2002). One mutant allele that conferred profound phenotypes was *T(K53R)-YDJ1*, and to understand better how specific Hsp40 mutations affect yeast cell growth, the *T-YDJ1* chimera with the K53R mutation [*T(K53R)-YDJ1*] was cloned behind a moderate constitutive promoter and introduced into *ydj1*Δ yeast. We found that expression of wild-type *T-YDJ1*, but not *T(K53R)-YDJ1*, restored growth in the *ydj1*Δ strain at 35° (Figure 1). Next, a high-copy screen was performed to uncover suppressors of the temperature-sensitive phenotype, as described in materials and methods. As noted in the Introduction, one goal of this approach was to better define why the TAg chaperone domain is required for viral replication and function. Upon retransformation, eight unique plasmids were obtained that partially

TABLE 2

Plasmids used in this study

Plasmid	Gene	Vector	Reference/source
pCMS39-T-YDJ1	*T-YDJ1*	*pTEF414*	This study
pCMS41-T(K53R)-YDJ1	*T(K53R)-YDJ1*	*pTEF414*	This study
pCMS123-T(H42R)-YDJ1	*T(H42R)-YDJ1*	*pTEF414*	This study
pAV4	*YDJ1*	*pRS316*	Caplan *et al.* (1992)
pCMS125-REC102	*REC102*	*pRS426*	This study
pSR6	*CHS5*	*pRS316*	Santos *et al.* (1997)
pSR23	*CHS5*	*pRS426*	Santos *et al.* (1997)
p1245	*MID2-HA*	*YEp352*	Rajavel *et al.* (1999)
p1300	*MID2-GFP*	*pRS314*	Rajavel *et al.* (1999)
YEp/STM1	*STM1*	*YEp213*	Hata *et al.* (1998)
pCMS119-YLR149c	*YLR149c*	*pRS426*	This study
SYP1pRS316	*SYP1*	*pRS316*	Marcoux *et al.* (2000)
SYP1pRS426	*SYP1*	*pRS416*	Marcoux *et al.* (2000)
YEp351-SSA1	*SSA1*	*YEp351*	E. Craig
pRS426-GPD-(His)$_6$-SSA1	*SSA1*	*pGPD426*	McClellan and Brodsky (2000)
p1657	*SLG-HA*	*YEp352*	Rajavel *et al.* (1999)
pSUS1	*SUS1*	*pRS316*	Rodriguez-Navarro *et al.* (2004)
pFW46	*CYC8*	*pRS316*	Williams and Trumbly (1990)
pRT81	*CYC8*	*YEp24*	Trumbly (1988)
pCMS118-CIS1	*CIS1*	*pRS426*	This study
pSKN1-IV	*SKN1*	*YCp50*	Roemer *et al.* (1993)
pThi4ura3	*THI4*	*pRS416*	Singleton (1997)
pFR22 (YEpU-PKC1)	*PKC1*	*YEp352*	Roelants *et al.* (2004)
pDLB759	*BCK1*	*YEp352*	D. Lew/D. Levin
pRS314 BCK1-20	*BCK1-20*	*pRS314*	Lee and Levin (1992)
pCMS147-BCK1-20	*BCK1-20*	*pRS426*	This study
pDLB823	*MKK1*	*pRS314*	Harrison *et al.* (2004)
pDLB824	*MKK1^{DD}*	*pRS314*	Harrison *et al.* (2004)
pCMS148-MKK1^{DD}	*MKK1^{DD}*	*pRS426*	This study
pDLB758	*MPK1*	*YEp352*	D. Lew/D. Levin
pNC267	*STE7-myc*	2μ, *CYC1*	Zhou *et al.* (1993)
YEp352-Kss1	*KSS1*	*YEp352*	Ma *et al.* (1995)
p181HOG1ha3	*HOG1-HA*	*YEplac181*	Winkler *et al.* (2002)
pCMS155-HOG1-HA	*HOG1-HA*	*pRS426*	This study
p111PBS2	*PBS2*	*YEplac111*	Winkler *et al.* (2002)
p112PBS2	*PBS2*	*YEplac112*	I. Ota
pCMS154-Hsp82	*Hsp82*	*pRS426*	This study

suppressed the *T(K53R)-YDJ1* thermosensitive phenotype at 35° (Table 3). Of note, none of the plasmids rescued the temperature sensitive phenotype to the levels seen when T-Ydj1p was expressed (Figure 1). However, plasmid *pCMS154* moderately rescued the *T(K53R)-YDJ1* phenotype and was the strongest suppressing plasmid uncovered in the screen.

To determine which of the genes in the eight isolated inserts conferred improved growth, 13 individual genes were chosen for further analysis. These included genes encoding chaperones, transcription factors, and proteins of unknown function, and several that had a common link to cell-wall synthesis or integrity (see below). Each gene was amplified by PCR and cloned into a pRS426 vector or was obtained from colleagues (Table 2). Next, the vectors were transformed into the *ydj1*Δ strain either containing or lacking the *T(K53R)-YDJ1* expression vector and tested for their ability to rescue the temperature-sensitive growth defect. It should be noted that *ydj1*Δ yeast lacking the T(K53R)-Ydj1p expression vector grew poorly at temperatures >26°, and those containing the vector grew poorly >30°, consistent with T(K53R)-Ydj1p exhibiting partial activity (Fewell *et al.* 2002). However, overexpression of 7 genes suppressed the slow-growth phenotype of *T(K53R)-YDJ1* at 35° to varying degrees, and 4 of these (*MID2*, *SLG1/WSC1*, *CYC8*, *SYP1*) also improved the growth of the *ydj1*Δ strain lacking the expression vector variably at 30° (Table 3). Notably, *ydj1*Δ yeast containing *pCMS154* (which harbors *MID2*) or containing a vector engineered specifically for *MID2* overexpression conferred the same degree of rescue (Table 3 and Figure 3A). Overexpression of an eighth gene, *STM1*, did not alter the temperature-sensitive phenotype but led to papillae colony formation [see Figure 1 for data on the abilities of *MID2* (*pCMS154*) and *STM1* (*pCMS156*)-containing

TABLE 3

Summary of the results obtained from the screen

Times recovered	Gene	Plasmid name	Vector	Improves *T(K53R)-YDJ1* growth at 35°?	Improves *ydj1*Δ growth at 30°?
6	*REC102*	*pCMS125-REC102*	*pRS426*	–	–
	CHS5	*pSR6*	*pRS316*	–	–
	CHS5	*pSR23*	*pRS426*	–	–
	MID2-HA	*p1245*	*YEp352*	+++	+++
	MID2-GFP	*p1300*	*pRS314*	ND	+++
5	*STM1*	*Yep/STM1*	*YEp213*	–[a]	–
	YLR149c	*pCMS119-YLR149c*	*pRS426*	–	–
3	*RIM1*	ND	ND	ND	ND
	SYP1	*SYP1pRS316*	*pRS316*	–	–
	SYP1	*SYP1pRS426*	*pRS426*	+	+
	RPS14a	ND	ND	ND	ND
2	*SSA1*	*Yep351-SSA1*	*YEp351*	++	–[b]
	SSA1	*pRS426-GPD-(His)$_6$-SSA1*	*pGPD426*	+	–[b]
	EFB1	ND	ND	ND	ND
	ERP2	ND	ND	ND	ND
2	*SLG1-HA*	*p1657*	*YEp352*	++	+++
2	*SUS1*	*pSUS1*	*pRS316*	–	–
	CYC8	*pFW46*	*pRS316*	–	+
	CYC8	*pRT81*	*YEp24*	++	++
	YBR113w	ND	ND	ND	ND
1	*YDR020c*	ND	ND	ND	ND
	FAL1	ND	ND	ND	ND
	CIS1	*pCMS118-CIS1*	*pRS426*	++	–
	SES1	ND	ND	ND	ND
1	*SKN1*	*pSKN1-IV*	*YCp50*	+	–
	THI4	*pThi4ura3*	*pRS416*	–	–
	ENP2	ND	ND	ND	ND

Eight unique plasmids were isolated in the screen that allowed growth of the *ydj1*Δ yeast-expressing T(K53R)-Ydj1p at 35° upon retransformation, and the number of times each plasmid was identified is indicated. Select individual genes found on each plasmid isolated in the screen were tested for improved growth of *T(K53R)-YDJ1* at 35° and *ydj1*Δ at 30°. Seven genes improved growth at 35° of *ydj1*Δ yeast-expressing T(K53R)-Ydj1p and four genes improved the slow-growth phenotype of *ydj1*Δ yeast at 30°. +++, moderate rescue; ++, weak rescue; +, poor rescue. ND, not determined.

[a] Papillae colonies were detected.

[b] *ydj1*Δ yeast with high-copy *SSA1* expression vectors grew more slowly than empty vector controls.

plasmids isolated from the screen to suppress the temperature-sensitive growth phenotype].

Among the genes tested, only one improved the growth of T(K53R)-Ydj1p-expressing yeast but had a negative effect on *ydj1*Δ yeast lacking the expression vector. The gene is *SSA1*, which encodes a yeast Hsp70 that is known to interact with Ydj1p (Figure 2, A and B) (Cyr *et al.* 1992; Becker *et al.* 1996; McClellan and Brodsky 2000). *SSA1* overexpression in yeast containing YEp351-*SSA1* was verified by immunoblot analysis (Figure 2, C and D). One interpretation of this result is that Ssa1p may directly bind the J domain in T(K53R)-Ydj1p and partially repair the lesion conferred by mutating lysine 53 (since only moderate rescue was observed).

In contrast, four suppressors (*MID2*, *SLG1/WSC1*, *CYC8*, *SYP1*) were identified that suppressed to varying extents the phenotypes associated with both the deletion of the *YDJ1* locus and the phenotype associated with the *T(K53R)-YDJ1* expression vector (Table 3; see Figure 3, A and B for an example of studies with the rescue conferred by *MID2*). To verify the expression of the strongest suppressor, Mid2p, an immunoblot analysis against the HA epitope tag (Figure 3, C and D) was performed and Mid2p resolved as three distinct bands of 40, 47, and 200 kDa. This is consistent with the presence of both immature and highly *O*-glycosylated forms of Mid2p (Lommel *et al.* 2004). To determine if the observed improvement of growth was allele specific, *MID2-HA* was overexpressed in the *ydj1*Δ strain

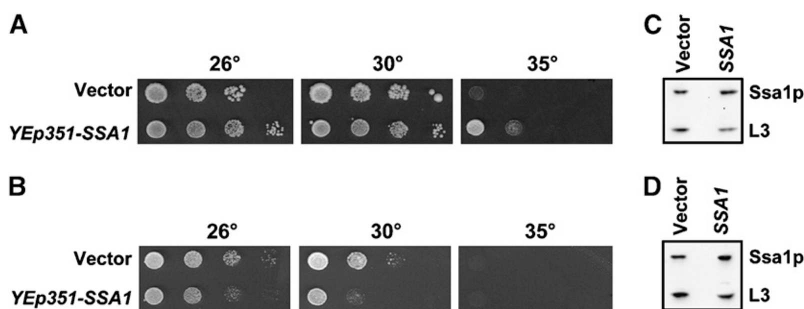

Figure 2.—*SSA1* improves the growth of strains exhibiting the *T(K53R)-YDJ1* thermosensitive phenotype. Cultures of *ydj1*Δ yeast expressing T(K53R)-Ydj1p were transformed with a high-copy *SSA1*-containing vector or an empty vector and were serially diluted onto either SC–ura–trp or SC–ura. Plates were incubated for 4 days. (A and B) High-copy *SSA1* allows some growth of *ydj1*Δ yeast containing the *T(K53R)-YDJ1* expression vector at 35°, but slows the growth of *ydj1*Δ yeast. Immunoblot analysis indicates that Ssa1p is overexpressed (C) 2.1-fold in the *ydj1*Δ strain expressing T(K53R)-Ydj1p and (D) 2.6-fold in the *ydj1*Δ yeast strain.

containing the *T(H42R)-YDJ1* expression vector, which contains an H42R mutation in the conserved HPD motif in the TAg J domain (Fewell *et al.* 2002). We found that *MID2-HA* overexpression also suppressed the slow-growth phenotype of *ydj1*Δ yeast containing the *T(H42R)-YDJ1* expression vector at 30° (data not shown). Taken together, these results suggest that *MID2* suppression is independent of TAg J domain mutant alleles, but instead is the consequence of improved growth of the *ydj1*Δ strain.

***PKC1* and constitutively activated components of the PKC pathway improve the *ydj1*Δ slow-growth phenotype:** Mid2p and Slg1p/Wsc1p are plasma membrane proteins that sense yeast cell-wall stress and activate the PKC pathway (Philip and Levin 2001), which leads to the transcription of genes involved in cell-wall synthesis (Jung and Levin 1999). Since Mid2p and Slg1p/Wsc1p are components of the PKC pathway and several other of our identified suppressors (Table 3)—Syp1p (Marcoux *et al.* 2000), Skn1p (Roemer *et al.* 1994), and Cis1p (Manning *et al.* 1997)—are genetically linked to *PKC1* or *MID2*, the ability of *PKC1* to suppress the *ydj1*Δ growth phenotype was investigated. A 2μ *PKC1* overexpression plasmid was transformed into *ydj1*Δ cells and *ydj1*Δ cells expressing T(K53R)-Ydj1p, and moderate rescue of *T(K53R)-YDJ1* growth at 35° (Figure 4A) and *ydj1*Δ growth at 30° (Figure 4B) was observed. The overexpression of Pkc1p in these strains was verified by immunoblot analysis as shown in Figure 4, C and D.

The PKC-signaling pathway is initiated by the phosphorylation of Pkc1p and terminates with transcription factor activation via the Bck1p, Mkk1p/Mkk2p, and Mpk1 kinases (Levin 2005). To determine if overexpression of downstream members of the PKC pathway also rescued the *ydj1*Δ slow-growth phenotype, plasmids engineered for the expression of constitutively active *BCK1* and *MKK1* alleles were introduced into the mutant strain. *BCK1-20* has an alanine-to-proline mutation at position 1174, immediately upstream of the kinase domain, which is believed to mimic Bck1p phosphorylated by Pkc1p (Lee and Levin 1992). *MKK1^{DD}* contains two serine-to-aspartic acid mutations in the kinase domain, which mimics Mkk1p phosphorylation (Harrison *et al.* 2004). As shown in Figure 4E, overexpression of *BCK1-20* or *MKK1^{DD}* slightly improved the *ydj1*Δ growth defect. In contrast, overexpression of wild-type Mkk1p or Bck1p failed to confer improved growth (data not shown), consistent with the previously reported necessity of using constitutively active forms of these kinases (Lee and Levin 1992; Harrison *et al.* 2004). In any event, these results indicate that activation of the PKC pathway can ameliorate the *ydj1*Δ temperature-sensitive growth phenotype.

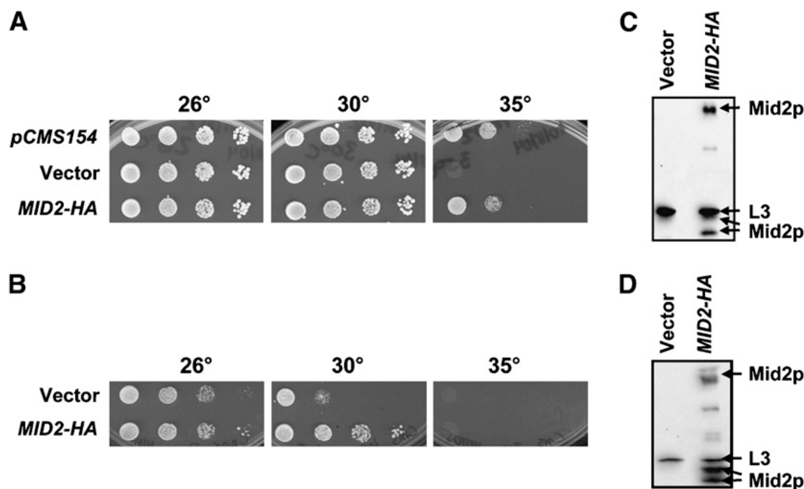

Figure 3.—*MID2* suppresses the thermosensitive growth defect of *ydj1*Δ yeast. (A) *ydj1*Δ yeast expressing T(K53R)-Ydj1p were transformed with the *MID2*-containing multi-copy plasmid isolated in the screen (*pCMS154*), an empty vector, or a multi-copy *MID2-HA*-containing vector, and were serially diluted onto SC–ura–trp. Plates were incubated for 4 days. (B) High-copy *MID2-HA* also suppresses the growth defect in the *ydj1*Δ strain lacking the T(K53R)-Ydj1p expression vector, as assessed in A. Mid2p is expressed in the *T(K53R)-YDJ1*-containing *ydj1*Δ strain (C) as well as the in the *ydj1*Δ strain (D) as indicated by immunoblot analysis.

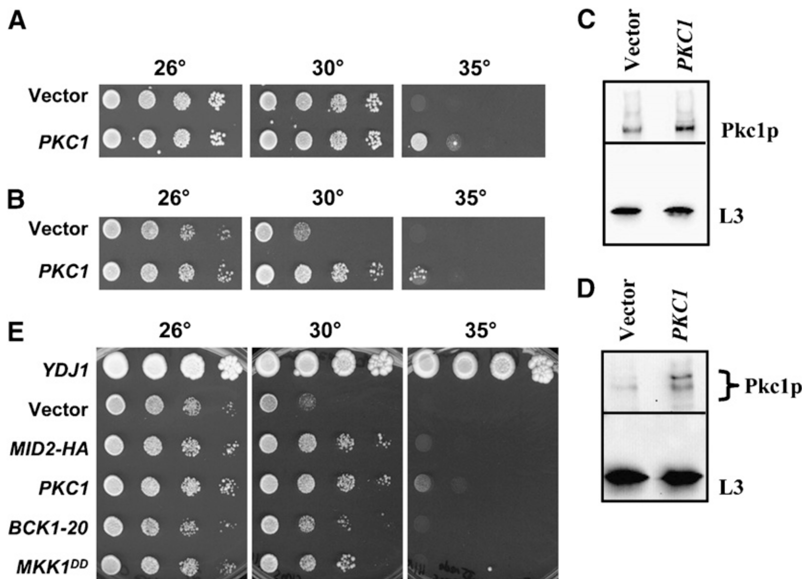

FIGURE 4.—Introduction of a high-copy *PKC1*-containing vector and overexpression of constitutively active components in the PKC pathway improve the slow-growth phenotype of the *ydj1*Δ strain. A multi-copy *PKC1*-containing vector and a vector control were transformed into (A) *ydj1*Δ yeast-expressing T(K53R)-Ydj1p and (B) *ydj1*Δ yeast, and the transformants were serially diluted 10-fold and incubated for 4 days at the indicated temperatures. An immunoblot analysis indicates (C) 1.8-fold overexpression of Pkc1p in the *ydj1*Δ strain expressing T(K53R)-Ydj1p and (D) 3.1-fold overexpression of Pkc1p in the *ydj1*Δ strain. (E) Plasmids containing constitutively active, multi-copy *BCK1-20* and $MKK1^{DD}$ alleles transformed into the *ydj1*Δ strain also improve the *ydj1*Δ temperature-sensitive defect, although to a lesser extent than *MID2* or *PKC1*.

In addition to the PKC pathway, yeast have two other cell-wall integrity pathways, the HOG and SVG pathways (MAGER and SIDERIUS 2002). Each pathway is activated under different conditions, but cross talk between the HOG and SVG pathways is common. The HOG and SVG pathways even contain some common signaling proteins (GUSTIN *et al.* 1998; O'ROURKE and HERSKOWITZ 1998; LEE and ELION 1999). To examine if overexpression of HOG and SVG pathway components affects the growth of *ydj1*Δ yeast, two genes in the HOG pathway (*HOG1* and *PBS2*) and two in the SVG pathway (*KSS1* and *STE7*) were overexpressed in the *ydj1*Δ strain. Hog1p and Kss1p are MAP kinases that initiate gene expression and Pbs2p and Ste7p are the MAPK kinases that phosphorylate Hog1p and Kss1p, respectively (BREWSTER *et al.* 1993; LEE and ELION 1999). Previously, connections between the signaling pathways were examined by overexpressing *STE7* in the *mkk1*Δ*mkk2*Δ strain (YASHAR *et al.* 1995). However, the overexpression of these proteins had no effect on the *ydj1*Δ slow-growth phenotype (data not shown). These results suggest that rescue of the *ydj1*Δ slow-growth phenotype is limited to the PKC pathway and cannot be remedied by upregulation of alternative pathways that may sense cell-wall integrity.

***MID2* and *PKC1* partially suppress the temperature-sensitive phenotype of *hsp82* mutant strains:** Because Ydj1p functions in multi-protein chaperone complexes with Ssa1p, a cytoplasmic Hsp70, and the yeast Hsp90 homolog, Hsp82p (CYR *et al.* 1992; CAPLAN *et al.* 1995; KIMURA *et al.* 1995; BECKER *et al.* 1996; MCCLELLAN and BRODSKY 2000), each of the seven suppressors was also overexpressed in the temperature-sensitive *SSA1* strain, *ssa1-45*, and Mid2p and Pkc1p were overexpressed in the temperature-sensitive *hsp82* G313N and *hsp82* G170D strains (BOHEN and YAMAMOTO 1993; NATHAN and LINDQUIST 1995; FLISS *et al.* 2000). None of the suppressors, including *MID2* or *PKC1* (data not shown; see Figure 5A for data on *MID2* and *PKC1*), rescued the temperature-sensitive phenotype of *ssa1-45* yeast, even though immunoblots verified that Mid2p and Pkc1p were overexpressed (Figure 5B). In contrast, overexpression plasmids encoding *MID2-HA* and *PKC1* partially suppressed the growth defect of both *hsp82* G313N (Figure 5C) and *hsp82* G170D (data not shown). Since the Hsp90 complex contains several other cochaperones, including Sti1p (Hop), Sba1p (p23), and Sse1p (Hsp110) (CHANG *et al.* 1997; FANG *et al.* 1998; LIU *et al.* 1999), we also examined whether the introduction of *MID2* and *PKC1* overexpression plasmids remedied the *sse1*Δ*sti1*Δ temperature-sensitive phenotype (LIU *et al.* 1999). No effect on cell growth was observed (data not shown). These data suggest that Mid2p or Pkc1p overexpression affects only the growth of the Hsp40 and Hsp90 mutant strains and that Hsp40 and Hsp90 play a role in yeast cell-wall integrity. These combined data also suggest that the mode of Ssa1p-mediated suppression of the *ydj1*Δ strain when T(K53R)-Ydj1p is expressed (see above) is distinct.

Because overexpression of Mid2p and Pkc1p improved the growth of *hsp82* mutant strains, we investigated whether overexpression of Hsp82p could rescue the *ydj1*Δ slow-growth phenotype and if, like Ssa1p, Hsp82p also suppressed the *T(K53R)-YDJ1* growth defect. To this end, the *HSP82* locus was PCR amplified from the yeast genome and inserted in the high-copy pRS426 vector, and the plasmid was transformed into both the *ydj1*Δ strain and the *ydj1*Δ strain expressing T(K53R)-Ydj1p. Despite two- to threefold overexpression, *HSP82* was unable to rescue either the *T(K53R)-YDJ1* or *ydj1*Δ temperature-sensitive phenotypes (data not shown).

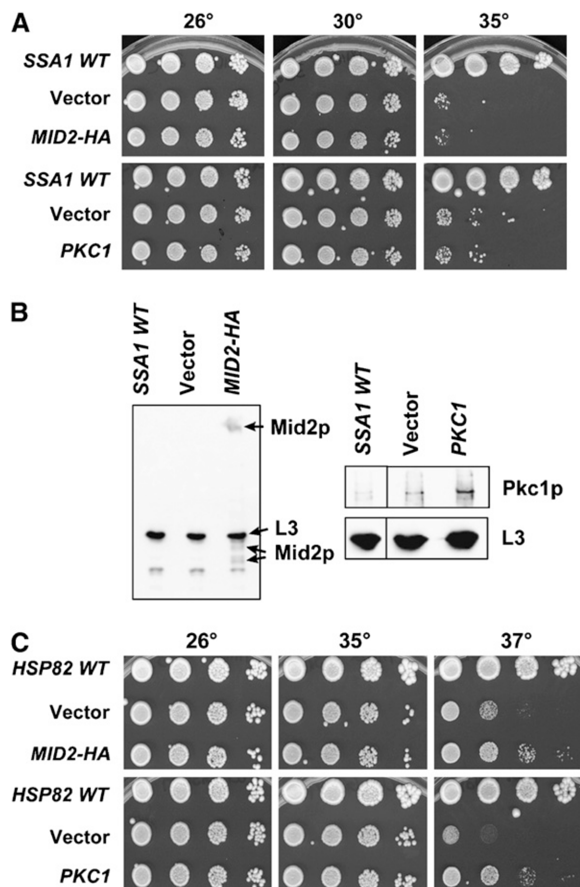

Figure 5.—Multi-copy *MID2* and *PKC1*-containing plasmids improve the temperature-sensitive growth defects of select chaperone mutants. *ssa1-45* or *hsp82* G313N yeast strains were transformed with multi-copy *MID2-HA*- or *PKC1*-containing vectors or with an empty vector. These strains and isogenic strains containing a wild-type version of the mutated genes were serially diluted 10-fold on SC–ura for 3–4 days at the indicated temperatures. (A) *MID2-HA* and *PKC1* do not rescue the temperature-sensitive growth phenotype of *ssa1-45* yeast. (B) Overexpression of Mid2p-HA (left) and Pkc1p (right) in the *ssa1-45* strain were verified by immunoblot analysis. (C) High-copy *MID2-HA* and *PKC1* improve the growth defect of the *hsp82* G313N strain.

Chaperone levels are tightly regulated (Stone and Craig 1990; Hjorth-Sorensen *et al.* 2001) and basal chaperone expression levels might dictate whether a given chaperone can be overexpressed. Thus, endogenous concentrations of Ssa1p and Hsp82p were determined in the presence or absence of Mid2p or Pkc1p overexpression in both the wild-type and *ydj1*Δ strains. Not surprisingly, in *ydj1*Δ cells containing a vector control, Ssa1p is upregulated 1.6- to 2.7-fold and Hsp82p is upregulated 1.4- to 3.2-fold when compared to the wild-type strain (supplemental Figure S1 at http://www.genetics.org/supplemental/, compare lanes 1 and 4). When Mid2p and Pkc1p are overexpressed, we observed a slight additional upregulation of Hsp82p in the *ydj1*Δ strain (supplemental Figure S1, lanes 2 and 3). These results suggest that Hsp82p and Ssa1p and likely other gene products are induced to compensate for the lack of Ydj1p. In addition, this may explain why Ssa1p or Hsp82p overexpression alone does not ameliorate the temperature-sensitive phenotype of *ydj1*Δ yeast (see above).

The *ydj1*Δ and *hsp82* temperature-sensitive strains show phenotypes consistent with cell-wall defects: Since the PKC pathway is involved in yeast cell-wall maintenance (Levin 2005), mutation of pathway members leads to cell-wall defects and several detectable growth phenotypes. For example, the poor growth of *pkc1*Δ yeast is rescued by incubation in osmostabilizing reagents, such as 1 m sorbitol or 0.5 m NaCl (Levin and Bartlett-Heubusch 1992). In addition, *pkc1* mutant yeast are sensitive to the cell-wall dye CW (Schmitz *et al.* 2001), which binds and perturbs the architectural integrity of this structure. Since the introduction of additional copies of *MID2* and *PKC1* affect the growth of *ydj1*Δ and *hsp82* yeast, it is possible that these mutant strains also have cell-wall defects, as hinted at by previous studies (Lussier *et al.* 1997; Yang *et al.* 2006).

To begin to test this hypothesis, *ydj1*Δ cells with or without a *YDJ1* single-copy expression vector (pAV4) were examined on medium supplemented with 0.4 m NaCl. Growth of *ydj1*Δ yeast was partially restored in the presence of salt (Figure 6A). Next, the growth of wild-type and *ydj1*Δ yeast was tested in the presence of sorbitol or CW. As expected for strains having cell-wall defects, the growth of *ydj1*Δ yeast was enhanced on 1 m sorbitol at 30° and was sensitive to CW at 23°. The growth of *hsp82* G313N strains was also restored on high sorbitol at 37° and showed sensitivity to CW at 23° (Figure 6B). Notably, the growth of *ydj1*Δ on sorbitol resembled the growth seen upon overexpression of Mid2p or Pkc1p (Figures 3B and 4B). When Mid2p or Pkc1p was overexpressed on high-sorbitol-containing medium, *ydj1*Δ cells grew at temperatures as high as 37° (supplemental Figure S2 at http://www.genetics.org/supplemental/). Mid2p and Pkc1p overexpression also rescued the CW sensitivity of the *ydj1*Δ strain at 30° (data not shown). These results indicate that *ydj1*Δ and Hsp82 mutant strains have a cell-wall defect and that Mid2p and Pkc1p may repair this defect.

Mid2p is not dramatically mislocalized or aggregated in *ydj1*Δ yeast: Ydj1p is involved in the translocation of nascent polypeptides into the ER (Caplan *et al.* 1992) and is able to retain aggregation-prone proteins in solution (Cyr 1995). We therefore hypothesized that Mid2p might be mislocalized or aggregated in *ydj1*Δ yeast, which would prevent accurate sensing of cell-wall integrity and cause cell-wall defects. But, at least some of the overexpressed Mid2p might escape to the plasma membrane and partially restore cell-wall integrity. To test this hypothesis, the solubility of Mid2p was determined by differential centrifugation in wild-type and *ydj1*Δ yeast strains overexpressing Mid2p-HA. In both wild-type and *ydj1*Δ yeast, the majority of the Mid2p-HA

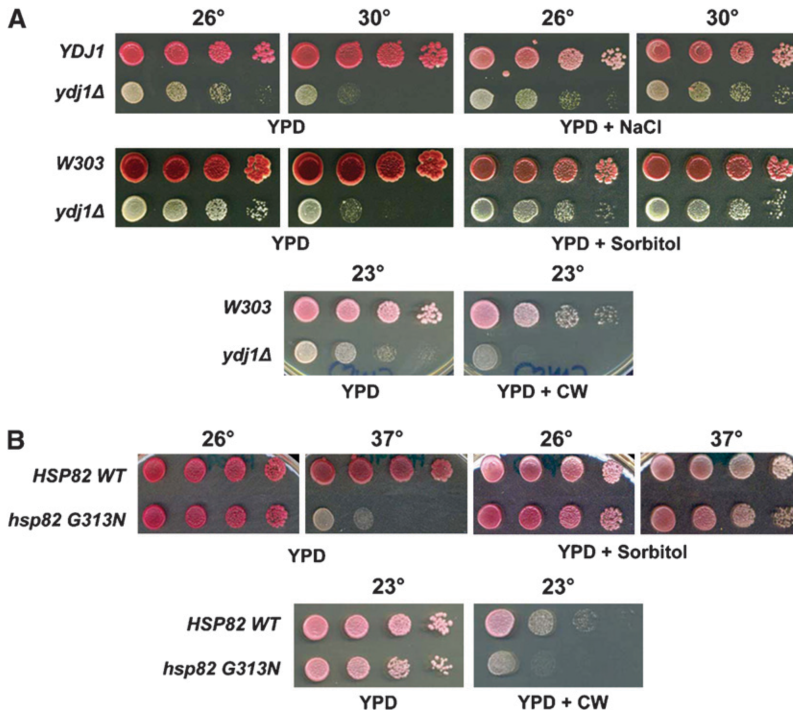

Figure 6.—The *ydj1*Δ and *hsp82* temperature-sensitive mutant strains have phenotypes consistent with defects in cell-wall synthesis. (A) (Top) Tenfold serial dilutions of *ydj1*Δ yeast either expressing Ydj1p ("*YDJ1*") or lacking the expression vector were plated onto YPD either lacking or containing 0.4 M NaCl, 1 M sorbitol, or 20 μg/ml calcofluor white (CW) at the indicated temperatures for 3 days. (Middle and bottom) Wild-type or *ydj1*Δ yeast were incubated on control or sorbitol or CW-containing medium as above. (B) *HSP82* and *hsp82* yeast were grown on either YPD or YPD with either 1 M sorbitol or 20 μg/ml CW at the indicated temperatures for 3 days. All the yeast strains are *ade*⁻ and have a pink coloration. *ydj1*Δ yeast also obtained the pink coloration after longer incubations (not shown).

localized to the first pellet (P1; 76 and 80%, respectively, of the total protein assayed, *i.e.*, the "load") (Figure 7A), consistent with Mid2p membrane residence. Sec61p, a component of the ER membrane translocon (Deshaies *et al.* 1991), fractionated similarly. In wild-type yeast, 14% of the total Mid2p localized to the second pellet (P2), which is nearly identical to the amount of Mid2p found in the P2 from *ydj1*Δ yeast (15%). Intriguingly, only 1.6% of Mid2p fractionated in the second supernatant (S2), which represents clarified cytosol (the cytosolic Sse1p chaperone was used as a marker) in *ydj1*Δ cells, compared to 6.3% in wild-type yeast. The small decrease in the levels of soluble cytosolic Mid2p in fractionated lysates prepared from *ydj1*Δ yeast may be due to an increase in protein aggregation, although only a minor percentage of the total cellular Mid2p is affected.

To investigate whether the subcellular localization of Mid2p was grossly altered when *YDJ1* was deleted, indirect immunofluorescence microscopy was performed. Mid2p-HA was overexpressed in wild-type and *ydj1*Δ yeast, and its location was determined using an antibody against the HA epitope tag. As shown in Figure 7B, Mid2p clearly localized to the plasma membrane in wild-type yeast, which is in agreement with previously published data (Ketela *et al.* 1999; Rajavel *et al.* 1999). Some proteins also resided in large intracellular bodies, which may be late secretory vesicles. In *ydj1*Δ yeast, Mid2p localization was unchanged. Together, these results suggest that Mid2p is not significantly mislocalized or aggregated in the *ydj1*Δ yeast strain.

In addition to its role in cell-wall integrity, Mid2p has been implicated in actin cytoskeleton rearrangement (Marcoux *et al.* 2000). Therefore, it was formally possible that Mid2p overexpression rescues actin cytoskeletal defects in the *ydj1*Δ strain and thus improves growth. To determine if the actin cytoskeleton was perturbed in the *ydj1*Δ strain, we used fluorescence microscopy to visualize cortical actin patches in wild-type and *ydj1*Δ yeast. In both strains, phalloidin staining showed several punctate "dots" around the yeast cell periphery (data not shown), characteristic of cortical actin staining (Adams and Pringle 1984). This result suggests that the actin cytoskeleton is not grossly affected in the *ydj1*Δ strain and that Mid2p overexpression improves the growth of *ydj1*Δ cells through a different mechanism.

***MID2-HA* overexpression thickens the cell wall of *ydj1*Δ yeast:** Another reason that Mid2p might improve the growth of *ydj1*Δ yeast is that Mid2p overexpression may thicken the yeast cell wall (Marcoux *et al.* 2000), possibly due to an increase in chitin (Ketela *et al.* 1999). Thus, we hypothesized that extra copies of Mid2p would rescue *ydj1*Δ growth and the cell-wall defect by strengthening and/or enlarging this structure. To test this hypothesis, wild-type or *ydj1*Δ cells lacking the *T(K53R)-YDJ1* expression vector but containing the *MID2-HA* overexpression vector or an empty vector control were analyzed by electron microscopy. We first noted that *ydj1*Δ cells were generally larger than wild-type cells and contained an enlarged vacuole (Figure 8A), which is consistent with previously published data (Caplan and Douglas 1991). Next, the distance between the outside of the cell wall and the plasma membrane was measured in similarly sized budding wild-type or *ydj1*Δ cells, and the results were quantified as described in materials and methods. Despite the

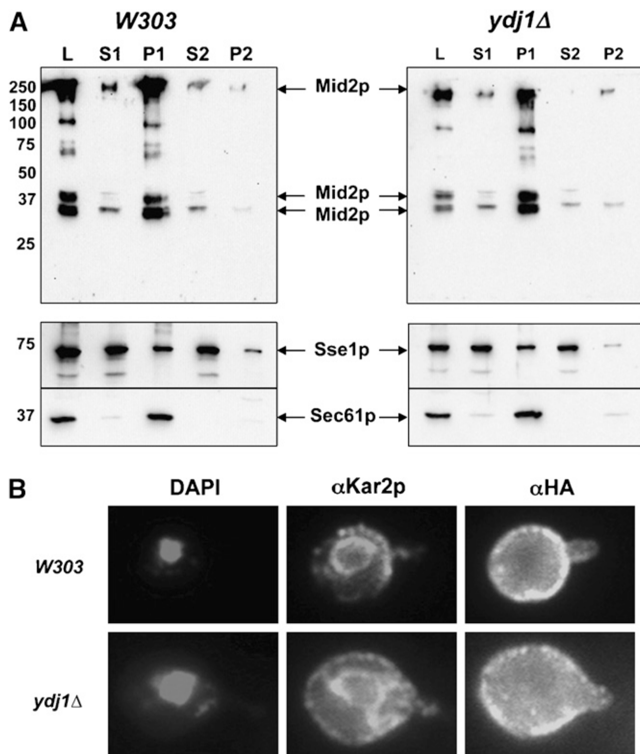

Figure 7.—Only a modest increase in Mid2p aggregation is evident in *ydj1*Δ yeast. (A) Wild-type or *ydj1*Δ yeast overexpressing Mid2p were lysed and the subcellular fractionation of Mid2p was determined by differential centrifugation. Sec61p, a component of the translocon, is an integral membrane protein, whereas Sse1p, a cytoplasmic chaperone, is primarily in soluble fractions. L, load; S1, first medium-speed supernatant; P1, first medium-speed pellet; S2, second high-speed supernatant; P2, second high-speed pellet. (B) Mid2p localization was determined by indirect immunofluorescence using an antibody against the HA epitope in wild-type or *ydj1*Δ yeast overexpressing Mid2p. For comparison, the ER was localized using an antibody against the ER-resident chaperone, Kar2p, and the nucleus was visualized with DAPI staining. The distended ER and enlarged cell volume of *ydj1*Δ yeast is commonly observed.

difference in cell size, the cell-wall thickness of the two strains was similar (Figure 8, A and B). This suggests that the cell-wall defect in *ydj1*Δ yeast arises due to an aberrant cell-wall composition. Strikingly, overexpression of Mid2p in the *ydj1*Δ cells almost doubled the average thickness of the cell wall from 120 to 214 nm. The increased thickness was seen in the inner electron transparent layer of the cell wall, which consists of β1,3-glucan, β1,6-glucan, and chitin (Osumi 1998). Occasionally, the increased cell-wall thickness was localized to a single region of the yeast cell wall (see the region identified by the arrow in Figure 8A), but in most cells the increased thickness was present throughout the cell periphery. Interestingly, overexpression of Mid2p did not uniformly increase the thickness of the cell wall in the wild-type yeast strain, although yeast with a thicker cell wall in limited regions around the cell were observed in some cases. Overall, we conclude that Mid2p overexpression rescues the *ydj1*Δ cell-wall defect by increasing its thickness.

DISCUSSION

As noted in the Introduction, yeast cell-wall biosynthesis is a highly complex process that is subject to multiple regulatory inputs. Because molecular chaperones have well-described roles in regulating processes as diverse as protein transport, enzyme activation, and the assembly of large multi-protein complexes (Fewell *et al.* 2001; Young *et al.* 2003), one might expect many examples in which defects in chaperone function affect cell-wall integrity. Surprisingly, this is not the case, and here we report for the first time that members of the Hsp40 and Hps90 chaperone families are specifically required for cell-wall function. We found that the growth of *ydj1*Δ and *hsp82* temperature-sensitive yeast is improved by conditions that restore the viability of cells with mutations in cell-wall components or regulators of cell-wall biogenesis. We also found that the growth of these strains is hindered by a compound that intercalates into the cell wall. Furthermore, we discovered that the viability of the *ydj1*Δ and *hsp82* temperature-sensitive strains is improved by the overexpression of Mid2p—a cell-wall component that is thought to sense the integrity of this structure and to activate the PKC pathway when cell-wall architecture is compromised—and by the overexpression of Pkc1p and activated components in the PKC pathway, which are known to trigger increased synthesis of cell-wall components. Previous work indicated that the PKC pathway induces the synthesis of cell-wall glycoproteins and proteins involved in cell-wall biosynthesis, including Fks1p and Fks2p (β1,3-glucan synthase) and Chs3p (chitin synthase) (Jung and Levin 1999), and that Mid2p overexpression increases the chitin concentration in the cell wall (Ketela *et al.* 1999). Indeed, we found that Mid2p overexpression is sufficient to thicken the cell wall in yeast lacking *YDJ1*, which provides a rationale for why we identified *MID2* as the strongest suppressor of the *ydj1*Δ slow-growth phenotype.

In addition to identifying *MID2* and the PKC pathway as suppressors of the slow-growth phenotype of *ydj1*Δ yeast, our screen uncovered several other genes with links to cell-wall homeostasis and to the PKC pathway (Table 3). For example, Syp1p was discovered as a *s*uppressor of *y*east *p*rofilin deletion (*pfy1*Δ) (Marcoux *et al.* 2000), and Cis1p was uncovered as a suppressor of a *cik1*Δ yeast strain (Manning *et al.* 1997). Cik1p cooperates with the Kar3p microtubule motor protein to catalyze karyogamy and chromosome segregation (Page and Snyder 1992). Whereas Syp1p helps polarize actin patches, little else is known about Cis1p. Both screens also uncovered Mid2p and Rom2p—another upstream component of the PKC pathway—as suppressors, which suggests potential links between Syp1p and Cis1p and

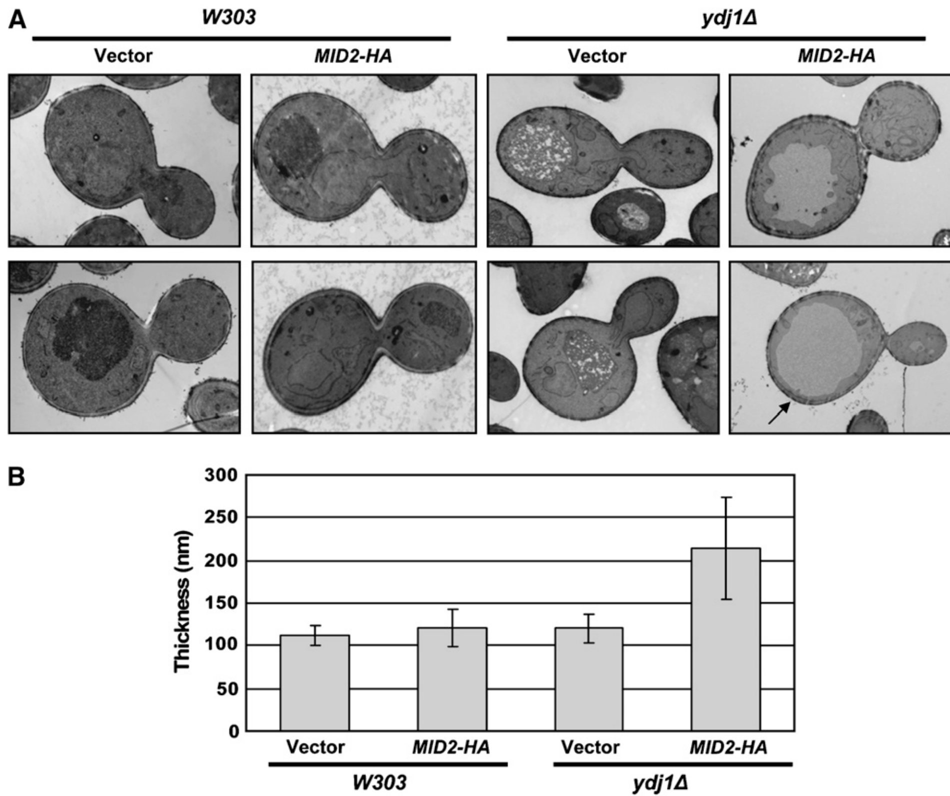

Figure 8.—Overexpression of Mid2p thickens the cell wall of *ydj1*Δ yeast. Wild-type or *ydj1*Δ strains containing a *MID2-HA* expression vector or vector control were analyzed by electron microscopy. (A) Two examples of single budding yeast from each strain are shown. Mid2p overexpression in the *ydj1*Δ strain thickens the entire cell wall (top) or at select locations (bottom, arrow) compared to the vector control. Wild-type yeast are shown at ×∼25,000 magnification and *ydj1*Δ yeast are shown at ×∼15,000 magnification. (B) The average cell-wall thickness for each strain was calculated; cell-wall thickness of *ydj1*Δ cells overexpressing Mid2p *vs.* those containing the vector control, $P < 0.0001$.

the PKC pathway. In addition, Cyc8p/Ssn6p, which is found in a corepressor complex with Tup1p, regulates genes under a wide array of stress conditions, including growth in hypertonic media (Proft *et al.* 2001; Proft and Struhl 2002). Finally, *SKN1* is homologous to *KRE6*, which exhibits synthetic interactions with *PKC1*, *MPK1*, and *MKK1/MKK2*; moreover, Kre6p overexpression can rescue a *pkc1*Δ lysis defect (Roemer *et al.* 1994). In contrast, we were surprised to find that the overexpression of *CHS5*, which is involved in the transport of chitin synthase from the Golgi (Sanchatjate and Schekman 2006), did not rescue the slow-growth phenotype of *ydj1*Δ yeast (Table 3).

Prior to this study, to our knowledge, there were only two other links between yeast cell-wall integrity and chaperone function. First, it was observed that strains containing mutations in *KAR2*, which encodes an ER Hsp70, have decreased amounts of β1,6-glucan in the cell wall when ER-resident glucanases are disabled (Simons *et al.* 1998). Second, some but not all *hsp82* mutant alleles exhibit sensitivity to growth on medium containing high sorbitol, which was interpreted as a connection between Hsp90 function and the HOG signaling pathway even though other *hsp82* mutants were sensitive to CW (Yang *et al.* 2006). On the basis of the growing importance of chaperones in nearly every aspect of cell function and maintenance, we anticipate that additional connections between chaperone function and cell-wall architecture will be uncovered.

The Hsp40 and Hsp90 chaperones commonly associate with Hsp70 to engineer distinct cellular processes, and thus we were surprised that overexpression of Mid2p or components in the PKC pathway failed to rescue the *ssa1-45* thermosensitive phenotype. One interpretation of this result is that Hsp40 and Hsp90, but not Hsp70, play a role in cell-wall biosynthesis, and we note that *in vitro* experiments suggested that Ydj1p and Hsp82p may hold PR in a hormone-binding competent state independent of Ssa1p (Hernandez *et al.* 2002a). However, another interpretation of this result is that allele-specific interactions underlie the ability of Mid2p and Pkc1p to improve the growth of *ydj1*Δ and *hsp82*, but not of *ssa1-45* yeast. It is important to mention that the G170D and G313N mutations are in the Hsp82 ATPase domain and middle region, respectively (Nathan and Lindquist 1995; Fliss *et al.* 2000), and have been shown to affect protein stability and Hsp82p client protein activation (Bohen 1995; Lee *et al.* 2002; Youker *et al.* 2004). In contrast, the *ssa1-45* allele encodes a P417L mutation in the peptide-binding domain of Ssa1p (Becker *et al.* 1996) and has been shown to compromise protein folding, translocation, and ER-associated degradation (Becker *et al.* 1996; Kim *et al.* 1998; Zhang *et al.* 2001). Moreover, the *hsp82* mutations reside in a strain that lacks chromosomal copies of *HSP82* and *HSC82*, whereas the *ssa1-45* mutation resides in a strain that lacks the *SSA2*, *SSA3*, and *SSA4* genes. Unfortunately, no other tight *ssa1* temperature-sensitive strains exist in which we can further explore this

phenomenon, and it is not clear how the necessary strain backgrounds for these studies affect the effects on cell-wall integrity/signaling that we observed.

Germane to our results, the PKC pathway has been previously linked to Hsp90 function. A yeast two-hybrid screen for Hsp90 clients was performed using yeast expressing an E33A mutant form of the protein that inhibits ATP hydrolysis and thus stabilizes client and cochaperone interactions (Millson *et al.* 2005). As anticipated, cochaperones such as Sba1p, Sti1p, Sse1p, and Ydj1p were identified, as well as the following kinases: Hog1p (HOG pathway), Kss1p (SVG pathway), Ste11p (several signaling pathways), and Mpk1p/Slt2p (PKC pathway) (Millson *et al.* 2005). Further, Hsp82 was shown to bind the phosphorylated, stress-activated form of Mpk1p. On the basis of these and other experiments, the authors suggested that Hsp90 folds Mpk1p, which, in turn, is required for proper signaling through the PKC pathway (Millson *et al.* 2005).

One goal of this study was to better understand why the K53R mutation in SV40 Tag compromises J domain function when inserted into a TAg-Ydj1p chimeric protein (Fewell *et al.* 2002). As hoped, we identified several genes that rescued to some degree the thermosensitive phenotype of *ydj1*Δ yeast-expressing T(K53R)-Ydj1p. On the basis of NMR perturbation studies (Greene *et al.* 1998), it was previously suggested that the solvent-exposed region in helix III that is occupied by the conserved K53 residue would not be in contact with Hsp70. Thus, we were surprised to identify the Hsp70-encoding *SSA1* gene in our screen. There are two general explanations for this result. First, it is possible that additional copies of Hsp70 in the cell "fix" the aberrant conformation conferred by the K53R mutation; second, higher levels of Hsp70 might improve the function of a protein or process that lies downstream of the K53R-induced phenotype. At present, we are unable to differentiate between these scenarios.

Another weaker suppressor of the *T(K53R)-YDJ1* phenotype that we identified was *CIS1*. As mentioned above, *CIS1* was identified as a suppressor of a *cik1*Δ phenotype (Manning *et al.* 1997), and uncovering *CIS1* as a weak suppressor of the *T(K53R)-YDJ1* phenotype may be indicative of a link between Ydj1p function and microtubule homeostasis. In fact, Ssa1p and Ydj1p were previously suggested to be important for microtubule formation, and, more specifically, strains either containing an *ssa1* temperature-sensitive allele (*ssa1-134*) or that were deleted for *YDJ1* showed irregular microtubule assembly at the nonpermissive temperature after nocodazole treatment (Oka *et al.* 1998). Moreover, both the *ssa1-134* and *ydj1*Δ alleles exhibit synthetic interactions with *tub4-1*, which is a temperature-sensitive allele in the gene encoding γ-tubulin. Therefore, improved growth of the *T(K53R)-YDJ1*-containing *ydj1*Δ strain by *CIS1* overexpression may be due to the rescue of a residual microtubule defect.

Because suppressors of the *T(K53R)-YDJ1* phenotype could be isolated from the nonbiased screen reported here, we have now begun to examine the effects on T(K53R)-Ydj1p-expressing *ydj1*Δ yeast when known or suggested TAg interactors are simultaneously produced. For example, we are currently investigating whether BAG1 or HspBP1 expression alter the *T(K53R)-YDJ1* thermosensitive phenotype because these mammalian NEFs are known to interact with Hsp70 and to promote ADP dissociation (Raynes and Guerriero 1998; Kabani *et al.* 2002b; Alberti *et al.* 2003). Thus, defects in J domain-Hsp70 function evident in T(K53R)-Ydj1p-expressing *ydj1*Δ yeast might be repaired by producing a factor that enhances the Hsp70 ATPase cycle. We are also testing the effects of expressing Cul7, which, as a member of an SCF ubiquitin ligase complex, helps direct specific proteins for proteasomal degradation (Dias *et al.* 2002; Ali *et al.* 2004). Cul7 binds to the TAg J domain in the extended loop between helices III and IV (Kasper *et al.* 2005), a region that is encoded in the chimeric *T-YDJ1* construct used in our studies. In addition to these directed experiments, we also hope in the future to perform a nonbiased suppressor screen using a mammalian, yeast-expression library to uncover new TAg J domain-binding proteins.

Finally, our study suggests a putative connection between TAg J domain function and the PKC pathway. Previous work demonstrated that TAg blocked the apoptotic response upon EGF withdrawal from mouse embryo cells, and chemical inhibition of the PKC pathway also inhibited apoptosis (A. Slinskey and J. M. Pipas, unpublished data). In addition, the amount of an uncharacterized PKC phosphorylated substrate decreased in mouse intestines upon TAg expression (R. Beerman, M. T. Saenz-Robles and J. M. Pipas, unpublished data). While these results suggest a connection between TAg and PKC, much more work is clearly needed to define how the TAg J domain affects the PKC pathway.

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