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Two-hybrid analysis of the *Saccharomyces cerevisiae* 26S proteasome

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Cagney, Gerard, Peter Uetz, and Stanley Fields. Two-hybrid analysis of the *Saccharomyces cerevisiae* 26S proteasome. *Physiol Genomics* 7: 27-34, 2001.—A two-hybrid screen against an activation domain array of *Saccharomyces cerevisiae* proteins was carried out for 31 yeast proteasome proteins. Fifty-five putative interactions were identified: 21 between components of the proteasome complex and 34 between proteasome proteins and other proteins. Many of these latter interactions involved either proteins of the ubiquitin pathway, cell cycle proteins, protein kinases or a translation initiation factor subunit. The role of eleven proteins associated with proteasome function by these screens was analyzed by examining the corresponding deletion strains for temperature sensitivity and canavanine sensitivity and for the stability of a ubiquitin-β-galactosidase fusion protein. These assays additionally implicated three proteins, Bim1, Ump1, and YKL171W, in proteasome function. This study demonstrates the utility of genome-wide two-hybrid assays as an entry point for the further analysis of a large protein complex.

protein degradation; protein interactions; ubiquitin

THE PROTEASOME is a 2-MDa heterocomplex with a central role in protein turnover (27). An extensive variety of substrates are degraded, including misfolded proteins, transcription factors and cell cycle regulatory proteins. The core complex (20S) is composed of two copies each of 14 proteins, which form a hollow cylinder with the proteolytic active sites on the interior (Fig. 1). In the yeast *Saccharomyces cerevisiae* and other eukaryotes, the activity of the core complex becomes ATP dependent in the presence of an ~18-subunit regulatory complex (19S) (5). The 19S regulatory complex may unfold substrates in an ATP-dependent manner to facilitate degradation within the interior of the complex. Additional regulatory complexes, such as the PA28 particle found in mammalian cells, have not been observed in yeast.

Substrates must be modified with several copies of the 76-residue ubiquitin moiety for efficient degradation by the 26S proteasome. Polyubiquitin is recognized by Rpn10, a subunit of the 19S regulatory complex (24). The multistep ubiquitination reaction requires distinct catalytic activities, termed E1, E2 and E3. In the case of *S. cerevisiae*, a single E1 enzyme (Uba1) activates ubiquitin by generating a thioester bond between the enzyme active site and the COOH-terminal glycine of ubiquitin. Eleven E2 enzymes are found in yeast and form similar enzyme-ubiquitin thioester bonds, permitting conjugation of the ubiquitin moiety to the target molecule either by the E2 itself or via another "ligase" (E3 enzyme). Approximately 10 E3 enzymes have been predicted from the yeast genome sequence, although it is believed more await discovery. E3 ligases are key enzymes in the process because target molecules are bound to the E3 ligase before ubiquitin conjugation, and they appear to function at least partially as specificity determinants. Three modes of substrate selectivity by the ubiquitination machinery are known. First, the E2 or E3 enzymes may recognize exposed primary structural features of the target molecules, for example, "N-end rule" degrons (25). Second, many protein targets are recognized following phosphorylation. Third, substrates may be targeted by association with other proteins. These may be single proteins, like Hsc70, or large heterocomplexes, like the anaphase-promoting complex (APC) or the Skp1-cullin-F box complex (SCF).

Despite the proteasome's central role in protein turnover, its location, activity, regulation, and interactions with regulatory complexes and nonproteasomal proteins remain poorly understood. One means to further the functional analysis of the degradative machinery is to identify additional proteins that interact with it, as well as to identify interactions of proteins known to be associated with the proteasome. Recently, an array comprising nearly all *S. cerevisiae* proteins, which is suitable for screening for interactions via the two-hybrid system, was described (23). Here, we use this array to probe the yeast proteome for proteins that interact with proteins of the yeast 26S proteasome.

MATERIALS AND METHODS

Strains and plasmids. S. cerevisiae strains PJ69-4a (10) and PJ69-4α (23) were used as two-hybrid reporter strains expressing Gal4 activation domain-open reading frame (ORF) fusions and Gal4 DNA-binding domain-ORF fusions, respectively. Plasmids pOAD (9) and pOBD2 (23) were used for two-hybrid experiments. pUB23-Arg was a gift from A. Varshavsky (2). For deletion experiments, the parental hap-

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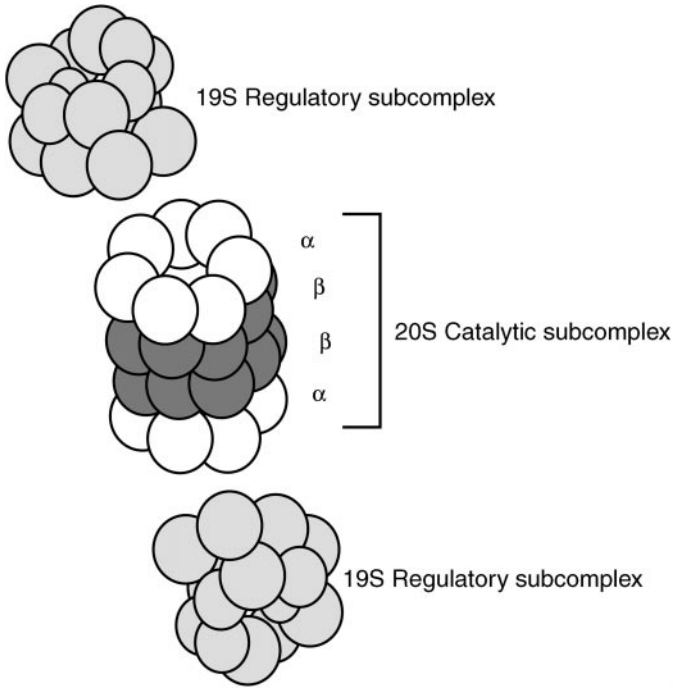

Fig. 1. The 26S proteasome is composed of a core hollow catalytic 20S subcomplex containing two copies each of seven α-subunit and seven β-subunits and a regulatory 19S subcomplex of ~18 subunits positioned at one or both ends of the core subcomplex. The 20S subcomplex may also be found unaccompanied by the 19S subcomplex.

loid *S. cerevisiae* strain was BY4742 [*MATα his3–1 leu2–0 lys2–0 ura3–0*]. Variants with the following genes replaced by a kanamycin-resistance cassette were obtained from Research Genetics (**http://sequence-www.stanford.edu/group/yeast_deletion_project/deletions3.html**): *YDR179C*, *YER144C*, *YNL253W*, *YMR216C*, *YER016W*, *YGR067C*, *YKL171W*, *YPL026C*, *YLR076C*, *YBR173C*, and *YHR200W*. A strain expressing a conditional mutation in the proteasome protein Pre2, MHY792 [MATα *leu2–3,112 ura3–52 lys2–801 trp1–1 gal2* Δ*doa3*::*HIS3 YCplac22-doa3–1*], and the parent strain MHY501 [MATα *his3–200 leu2–3,112 ura3–52 lys2–801 trp1–1 gal2*] (3), were used as controls for the deletion experiments.

Two-hybrid experiments. An array containing most of the ~6,000 *S. cerevisiae* ORFs expressed as fusions to the Gal4 transcriptional activation domain (AD) (23) was used to screen for interacting proteins. A haploid transformant expressing a fusion to the Gal4 DNA-binding domain (BD) was mated to the transformants of the array, and the resulting diploids were pinned onto media selective for the two-hybrid interaction (yeast synthetic media lacking leucine, tryptophan, and histidine and supplemented with 3 mM 3-aminotriazole, except where noted). This array was contained on 16 microtiter plates with 768 colonies per plate (colonies in duplicate). A smaller array comprised all of the colonies from the large array that contained proteins of the 26S proteasome, as well as related proteins. This smaller array consisted of one plate with 96-well spacing (colonies in quadruplicate). The "proteasome" array contained 74 strains expressing the following proteins as Gal4 AD fusions: Ady1, Ame1, Aos1, Bim1, Cdc28, Doa4, Fui1, Gna1, Isa2, Mob2, Nup157, Nup49, Pre1, Pre10, Pre2, Pre3, Pre4, Pre5, Pre6, Pre7, Pre8, Pre9, Prs3, Pup1, Pup2, Pup3, Rad23, Rim13, Rpn1, Rpn10, Rpn11, Rpn12, Rpn2, Rpn3, Rpn4, Rpn5, Rpn6, Rpn7, Rpn8, Rpn9, Rpt1, Rpt2, Rpt3, Rpt4, Rpt5, Rpt6, Rtt101, Scl1, Sha3, Sit4, Skt5, Sky1, Snf11, Ssd1, Ssk22, Sub2, Sui1, Ubi4, Ubp5, Ump1, Xpt1, YBR270C, YDR179C, YFR026C, YGL165C, YGR067C, YGR232W, YIL007C, YKL171W, YLR076C, YLR386W, YNL253W, YOR177C, Yta6. For the smaller array, the presence of DNA capable of expressing the relevant protein was confirmed by PCR. For strains expressing the Gal4 BD-ORF fusions, successful cloning was confirmed by DNA sequencing. For either array, only putative interactions that were observed in at least three of four replicates were scored positive. Proteins appearing as positives in more than 5% of all screens to date (>600) with the larger array were considered false positives and are not reported.

Phenotypic analysis of yeast deletion strains. Complete yeast media (YPD), as well as synthetic media lacking arginine and supplemented with 0.6 μg/ml canavanine (Sigma), were used to examine phenotypes. To estimate the stability of ubiquitinated proteins, the deletion strains were transformed with pUB23-Arg (2). The stability of Arg-β-galactosidase in the various deletion strains was measured using a liquid assay for β-galactosidase (1). Briefly, 5 ml of synthetic media lacking uridine and supplemented with galactose were inoculated with each strain and grown overnight at 30°C, at which point the cultures were assayed for β-galactosidase activity.

RESULTS

Screen for interaction of yeast proteasome proteins using protein arrays. Thirty-one ORFs encoding full-length proteasome proteins were cloned into the two-hybrid vector pOBD2 and transformed into yeast strain PJ69-4α as described (23). Strains encoding Gal4 BD-ORF fusion proteins were used to screen for interactions with other *S. cerevisiae* proteins using two arrays. The first array ("genome array") comprised two copies of nearly all ~6,000 proteins as yeast transformants of Gal4 AD-ORF fusions. The second array ("proteasome array") contained four transformants each of proteasome proteins and some proteasome-related proteins, also as Gal4 AD-ORF fusions. The colonies in the proteasome array were at lower density than those of the genome array, and were used to confirm results initially observed with the genome array (Fig. 2).

Fifty-five interactions were found from an equivalent of ~190,000 discrete two-hybrid experiments (Table 1). Forty-two were observed with the genome array, whereas all 55 were observed or confirmed with the proteasome array. Screening with the proteasome array had the advantages that more replicate two-hybrid experiments could be carried out per screen and that spacing of the colonies was only one-quarter the density of the genome array. This smaller array had a slightly lower rate of false negatives. However, the genome array allows much greater coverage of potential interactions. Of the 31 BD fusions screened, four (Pre6, Pup2, Rpn2, Rpt5) were strong self-activators, and seven (Rpn3, Rpn6, Rpn7, Rpn8, Rpn10, Rpt1, Rpt2) produced no reproducible positives. Four other proteins (Pup1, Pup3, Rpn11, Rpn12) also self-activated using standard assay conditions, but two-hybrid

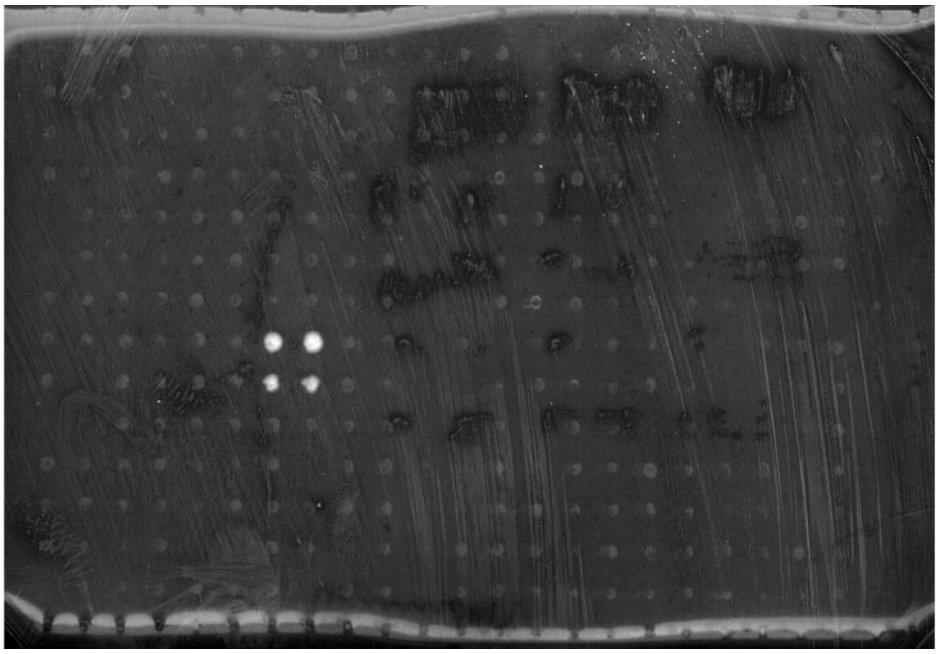

Fig. 2. Two-hybrid interactions of yeast proteasome subunits. A positive interaction between Rpn11 and Rpn8. A yeast strain expressing Rpn11 was mated to the array of transformants carrying proteasome-related proteins, and the diploids were transferred to media selective for two-hybrid interactions (−Leu −Trp −His +15 mM 3-aminotriazole). The four positive replicate colonies are shown after 3 days growth at 30°C.

signals could be obtained by screening under more stringent conditions (Table 1).

Interactions of proteins of the catalytic subcomplex. Thirty-three interactions involve known components of the 20S catalytic core, including 13 between members of the complex (Table 1). The core of the yeast proteasome is composed of two inner and two outer seven-member rings, arranged to form a barrel-shaped complex. The outer rings are composed of α-subunits and are likely to form contacts with the 19S regulatory subcomplex (31). No interactions were observed between the 20S and 19S subcomplexes. Electron micrographs show a "wagging" movement of the 19S regulatory subcomplexes relative to the 20S subcomplex (28). It seems likely that multiple protein interactions are involved in 19S-20S docking, and individual interactions may be of insufficient affinity for detection using standard screening approaches.

One interaction was identified between an α-subunit protein (Pre8) and a β-subunit protein (Pup1). In the crystal structure, the α- and β-rings are topologically connected at several points, including Pre8-Pup1 (6). Four interactions among the α-subunit proteins were found, and these are also in accordance with contacts deduced from the crystal structure (6). We found eight interactions among other β-subunit proteins and one with Rpn4, a transcription factor that is associated with the yeast 19S regulatory subcomplex and that binds the proteasome-associated control element (PACE) found upstream of many proteasome genes (13).

Twenty-one pairs describe putative interactions between 20S proteasome components and proteins not known to be part of the proteasome, although in some cases the associations were previously known. The proteasome-associated chaperone Ump1 interacts with the α-subunit protein Scl1 and β-subunit protein Pre2. Pre7 interacts with YIL007C, whose sequence is homologous to the human proteasome regulatory subunit protein p27 (29). Pre3 was identified as a partner of Aos1, a protein that interacts with Uba2 to form a complex capable of activating the ubiquitin-like SUMO-homolog Smt3 protein before transfer to Ubc9 (11). The sumoylation and ubiquitination systems show many similarities, and there is evidence that interplay between the two activities might regulate the function of some proteins (7), although the significance of the interaction observed here is unclear.

Interactions of the proteasome regulatory subcomplex proteins. The 19S regulatory subcomplex confers ubiquitin specificity and ATP dependence on the proteasome, and the purified subcomplex contains at least 18 proteins (4). Twenty-two interactions involving proteins of the 19S regulatory subcomplex were observed, eight of which were with other 19S components. The proteins comprising the 19S proteasome subcomplex can be functionally dissociated into a base, which contacts the 20S core proteins and contains six ATPase proteins, two non-ATPase proteins, and a lid containing eight other proteins (4). There are conflicting reports on the location of another protein, Rpn10 (17), but it may be at the interface of the base and lid subcomplexes, because dissociation of the two occurs in yeast lacking Rpn10 (4). Of the eight interactions between 19S proteins, only one links the base and lid complexes. This interaction (Rpn9 and Rpt3) may be part of the interface between the base and lid. At least one other report of physical interaction between a base and a lid protein (Rpn9 and Rpn10) has been described (21)

Many proteasome regulatory subcomplex proteins have been identified in screens for other functions, suggesting that many disparate processes are coupled to protein breakdown by the proteasome. Our results

Table 1. *Interactions of yeast proteasome subunits*

Binding Domain Hybrid			Activation Domain Hybrid				
Protein Name	Chromosomal Locus	Function	Protein Name	Chromosomal Locus	Function	P	G
			19S Base Subcomplex				
RPN1	YHR027C	Proteasome	RAD23	YEL037C	Nucleotide excision repair	•	•
RPN1	YHR027C	Proteasome	SUI1	YNL244C	Subunit of translation initiation factor eIF3	•	•
RPN1	YHR027C	Proteasome	SSK22	YCR073C	MAP kinase kinase kinase involved in the osmoregulation pathway	•	•
RPN1	YHR027C	Proteasome	YKL171W	YKL171W	Serine/threonine protein kinase	•	•
RPT3	YDR394W	Proteasome	AME1	YBR211C	Actin-related protein, regulator of microtubule stability	•	•
RPT3	YDR394W	Proteasome	RPT3	YDR394W	Proteasome	•	•
RPT3	YDR394W	Proteasome	RPT4	YOR259C	Proteasome		•
RPT3	YDR394W	Proteasome	RPT5	YOR117W	Proteasome	•	•
RPT3	YDR394W	Proteasome	SKY1	YMR216C	Serine/threonine protein kinase	•	•
RPT3	YDR394W	Proteasome	RPT6	YGL048C	Proteasome		•
RPT6	YGL048C	Proteasome	RPT3	YDR394W	Proteasome		•
RPT6	YGL048C	Proteasome	SUI1	YNL244C	Subunit of translation initiation factor eIF3	•	•
			19S Lid Subcomplex				
RPN5	YDL147W	Proteasome	DOA4	YDR069C	Ubiquitin-specific protease	•	•
RPN5	YDL147W	Proteasome	SNF11	YDR073W	Component of the Swi-Snf global transcription complex, involved in chromosome remodeling	•	•
RPN5	YDL147W	Proteasome	YDR179C	YDR179C	Unknown	•	•
RPN5	YDL147W	Proteasome	SHA3	YPL026C	Serine/threonine protein kinase	•	•
RPN9	YDR427W	Proteasome	RPT3	YDR394W	Proteasome		•
RPN9	YDR427W	Proteasome	SUI1	YNL244C	Subunit of translation initiation factor eIF3		•
RPN9	YDR427W	Proteasome	YGR067C	YGR067C	Unknown	•	•
RPN9	YDR427W	Proteasome	YLR386W	YLR386W	Unknown	•	•
RPN11*	YFR004W	Proteasome	RPN8	YOR261C	Proteasome		•
RPN12†	YFR052W	Proteasome	RPN8	YOR261C	Proteasome		•
			20S α				
PRE5 (α6)	YMR314W	Proteasome	SUB2	YDL084W	Protein similar to nuclear RNA helicases	•	•
PRE8 (α2)	YML092C	Proteasome	NUP49	YGL172W	Nuclear pore protein	•	•
PRE8	YML092C	Proteasome	PRE9	YGR135W	Proteasome		•
PRE8	YML092C	Proteasome	PUP1	YOR157C	Proteasome	•	•
PRE9 (α3)	YGR135W	Proteasome	SUI1	YNL244C	eIF3 subunit	•	•
PRE10 (α7)	YOR362C	Proteasome	PRE5	YMR314W	Proteasome	•	•
PRE10	YOR362C	Proteasome	PUP2	YGR253C	Proteasome	•	•
PRE10	YOR362C	Proteasome	RIM13	YMR154C	Sporulation protein involved in proteolysis of Rim1	•	•
PRE10	YOR362C	Proteasome	SCL1	YGL011C	Proteasome	•	•
PRE10	YOR362C	Proteasome	SUI1	YNL244C	eIF3 subunit	•	•
PRE10	YOR362C	Proteasome	YLR076C	YLR076C	Unknown	•	•
SCL1 (α1)	YGL011C	Proteasome	UMP1	YBR173C	Proteasome-associated chaperone	•	•
			20S β				
PRE1 (β4)	YER012W	Proteasome	PRE2	YPR103W	Proteasome	•	•
PRE1	YER012W	Proteasome	PRE4	YFR050C	Proteasome	•	•
PRE2 (β5)	YPR103W	Proteasome	PRE1	YER012W	Proteasome		•
PRE2	YPR103W	Proteasome	PRE3	YJL001W	Proteasome	•	•
PRE2	YPR103W	Proteasome	PUP3	YER094C	Proteasome	•	•
PRE2	YPR103W	Proteasome	UMP1	YBR173C	Proteasome-associated chaperone	•	•
PRE3 (β1)	YJL001W	Proteasome	AOS1	YPR180W	Subunit of Smt3-conjugating complex	•	•
PRE3	YJL001W	Proteasome	PUP1	YOR157C	Proteasome	•	•
PRE4 (β7)	YFR050C	Proteasome	BIM1	YER016W	MT-associated protein required for a cell cycle checkpoint	•	•
PRE4	YFR050C	Proteasome	NUP157	YER015C	Nuclear pore protein	•	•
PRE4	YFR050C	Proteasome	RTT101	YJL047C	Protein of the cuillin family, similar to Cdc53	•	•
PRE4	YFR050C	Proteasome	YBR270C	YBR270C	Unknown, probably ATP/GTP-binding protein	•	•
PRE4	YFR050C	Proteasome	YGR067C	YGR067C	Unknown	•	•
PRE4	YFR050C	Proteasome	YNL253W	YNL253W	Unknown	•	•
PRE7 (β6)	YBL041W	Proteasome	Nup157	YER015C	Nuclear pore protein	•	•
PRE7	YBL041W	Proteasome	RPN4	YDL020C	Proteasome-associated transcription factor		•
PRE7	YBL041W	Proteasome	SSK22	YCR073C	MAP kinase kinase kinase involved in the osmoregulation pathway		•
PRE7	YBL041W	Proteasome	YIL007C	YIL007C	Strong similarity to human proteasome modulator p27		•
PUP1† (β2)	YOR157C	Proteasome	PUP3	YER094W	Proteasome	•	•
PUP1†	YOR157C	Proteasome	UBP5	YER144C	Ubiquitin-specific protease	•	•
PUP3* (β3)	YER094W	Proteasome	PUP1	YOR157C	Proteasome		•

Interactions observed using the genomic array are indicated in by "•" in column G; those observed using the proteasome array are indicated by "•" in column P. *Two-hybrid assay carried out in presence of 15 mM 3-aminotriazole. †Assay carried out in presence of 50 mM 3-aminotriazole.

support this notion. Several cell cycle-related proteins and protein kinases were found to interact with 19S regulatory subcomplex components. Rad23, a DNA damage repair protein, interacts with Rpt1 and Rpt6 (18). Four protein kinases, Sha3, Sky1, Ssk22, and YKL171W (the last a putative protein kinase based on homology), were found to interact with Rpn5, Rpt3, Rpn1, and Rpn1, respectively.

Phenotypic analysis of deletion strains. Most, if not all, of the 21 interactions for which both partners are proteasome components are likely to be biologically relevant. Of the 34 putative links of proteasome proteins to proteins not known to form part of the mature complex, at least two alternatives are possible. Nonproteasomal proteins may modify components of the proteasome as part of regulatory processes that control proteasome activity. Alternatively, the nonproteasomal proteins may be modified, sequestered, or degraded as a result of the interaction, thus potentially contributing to various catalytic or regulatory processes. We examined the phenotypes of 11 strains with deletions of genes encoding proteins observed to interact with proteasome components. We chose primarily uncharacterized genes and those encoding protein kinases whose deletion resulted in viable strains.

At elevated temperature, or in the presence of the arginine analog canavanine, reduced growth has been associated with some defects in proteasome function. One strain lacking Bim1/YER016W was defective for growth at 38°C (Fig. 3*A*). This strain, ΔYER016W, as well as a strain lacking YKL171W, showed growth defects in the presence of 0.6 μg/ml canavanine relative to the parent strain (Fig. 3*A*). Growth of a strain lacking the proteasome-associated chaperone Ump1/YBR173C was weakly inhibited by elevated temperature and more strongly inhibited by canavanine treatment. A strain deleted for the proteasomal protein Rpn10/YHR200W was both temperature-sensitive and canavanine-sensitive (Fig. 3*A*). A strain derived from a different background, with a defect in the chymotrypsin-like proteasome component Pre2, grew very poorly under all conditions tested (data not shown). Thus strains lacking YBR173C, YER016W and YKL171W had phenotypes similar to, although less severe than, those observed in strains with disrupted proteasome functions. Although these phenotypes are often ob-

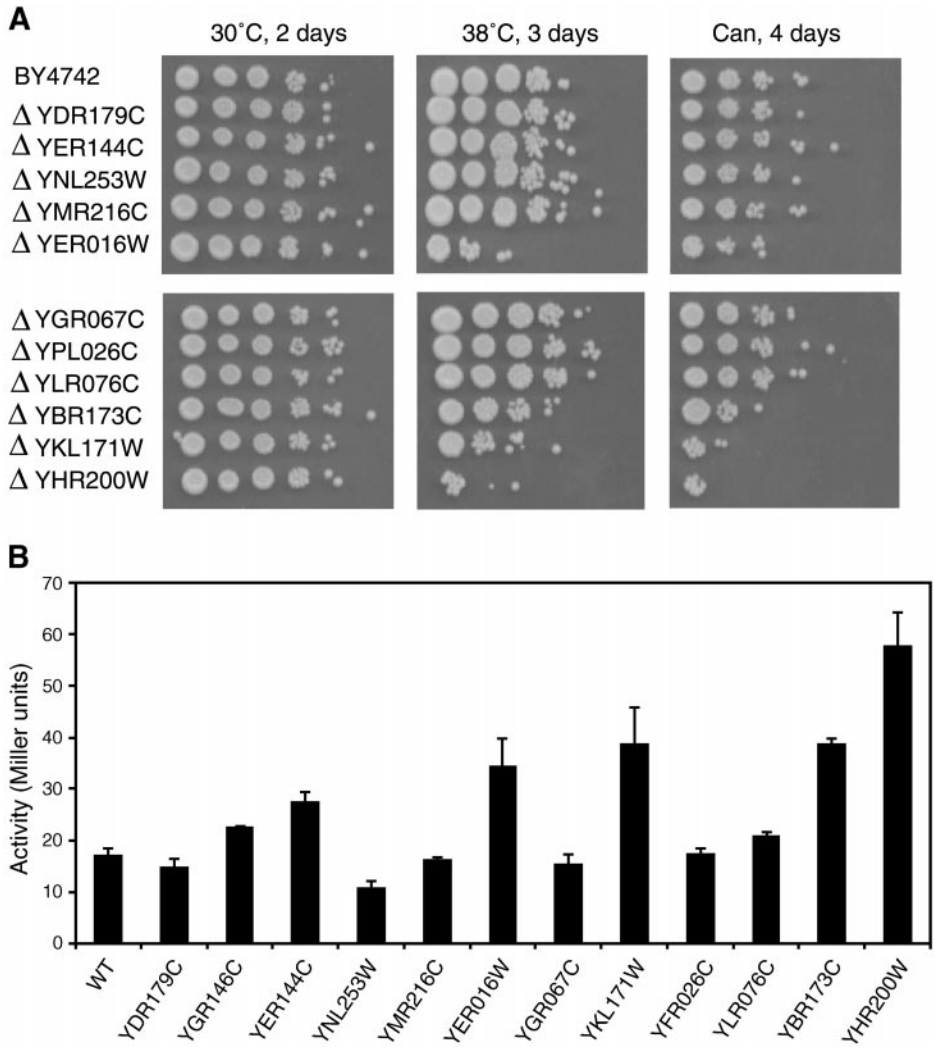

Fig. 3. Strains deleted for proteasome-interacting proteins show proteasome-related defects. *A*: yeast strains with selected individual genes replaced by a kanamycin-resistance cassette (30) were grown on complete medium at 30°C and at 38°C for 3 days and on plates containing 0.6 μg/ml canavanine at 30°C for 4 days. *B*: the strains were transformed with a plasmid expressing a ubiquitinated β-galactosidase and grown overnight before measuring β-galactosidase activity. WT, wild type.

served in proteasome mutants, defects in other essential cell functions could also account for these traits.

To investigate the efficiency of proteasome function in the deletion strains more directly, we measured the stability of a ubiquitin-β-galactosidase fusion protein with a half-life of ~2 min. Defects in proteasome activity are linked to extended half-life of ubiquitinated proteins. Three strains, ΔYER016W, ΔYKL171W, and ΔYBR173C, showed moderately elevated levels of β-galactosidase activity relative to parental strains (Fig. 3*B*). A strain deleted for YHR200W also showed elevated levels of β-galactosidase (Fig. 3*B*). Taken together, these data suggest that Bim1 (YER016W), a microtubule-binding protein involved in cell cycle control, YKL171W, a serine-threonine kinase, and Ump1 (YBR173C), a proteasome-specific chaperone, influence the efficiency of proteasome function in yeast.

DISCUSSION

In a comprehensive set of two-hybrid screens of the yeast 26S proteasome, we found 55 interactions between pairs of proteasome components or between proteasome components and nonproteasomal proteins. Over a third of the interactions were in the former class, strongly suggesting that the interactions were for the most part highly specific. The proteasome is involved in cellular protein turnover, but this biochemical definition masks a variety of specific molecular functions that overlap with those of cell cycle control proteins, transcriptional regulators, signaling molecules, metabolic proteins, nuclear proteins, as well as proteins involved in proteolysis. Defects in known proteasome components have been shown to generate phenotypes typical of all these functions as well as others (27). Interacting proteins identified in two-hybrid screens of proteasome proteins might consist of proteasome proteins, regulators and co-factors, or substrates.

Cases in which proteins of the proteasome interact with other such proteins should reflect the higher order architecture of the proteasome. For both 19S and 20S subcomplexes, all interactions identified were between proteins of the same subcomplex. For the 20S catalytic core, the observed two-hybrid interactions can be compared with interactions predicted by the crystal structure. Of the 14 interacting pairs for the 20S core, 3 involved proteins that are not direct neighbors in the crystal structure. For nonneighboring proteins, first, the contacts may be via processes distinct from the final structural and catalytic functions of the assembled proteasome structure. Such processes might include substrate recruitment or recognition, mature complex formation, or modification of catalytic activity. The proteasome assembly pathway involves several steps, with immature variants of β-subunit proteins contributing to the formation of proteasome precursors (19). For instance, the Ump1 maturation factor, found here to interact with Scl1 and Pre2, is associated with proteasome precursor complexes but not with the mature 20S proteasome (16). Second, alternative forms of proteins may be found in the proteasome substituting for each other. Thus apparently nonneighboring proteins might interact transiently in response to dynamic transformations of the overall complex. Third, pairs of proteins that do not interact directly may score positive in a two-hybrid assay, because the interaction is assisted by one or more bridging proteins.

Without additional experiments, it is difficult to assess the biological relevance of putative interactions of proteasome proteins with uncharacterized proteins. Interaction of an uncharacterized protein with two or more proteasome components increases the confidence that it is functionally related to the proteasome, as is the case for YGR067C (which interacts with Pre4 and Rpn9). Similarly, YLR386W was found to interact with Rpn9 in this study, as well as with Pre3 in an independent library screen (23). However, it is unclear why a single protein would interact with both the 19S and 20S subcomplexes.

Proteins interacting with proteasome components cluster among a relatively narrow range of functional classes. Several interactions implicate a role in protein degradation for cell cycle proteins. For example, Bim1 is associated with cell cycle checkpoints, and mutations in Ame1 lead to cell cycle arrest. The identification of protein kinases (Sha3, Sky1, Ssk22, and YKL171W) as proteasome-interacting components may reflect the involvement of various signaling pathways. Such pathways might modify a proteasome component to alter its activity; several proteins of the 19S complex are known to be phosphorylated (14). Alternatively, protein kinases and other regulatory enzymes might associate with the proteasome as substrates. The interaction of the nuclear pore complex protein Nup157 with two β-proteins, Pre4 and Pre7, may be indicative of regulated nuclear import. Strikingly, we found that five proteasome components interact with Sui1, a subunit of the eIF3 translational initiation factor. The eIF3 complex, the COP9 signalosome complex, and the regulatory subcomplex of the proteasome share sequence features, and these multiprotein complexes may regulate the catalytic activity of the 20S proteasome (8). If this is the case, then such regulating interactions do not exclusively involve the PCI or MPN domains as both are absent from Sui1 and only one of the five proteasome proteins, Rpn9, contains a PCI domain. Furthermore, it is difficult to account for the binding of a single translation initiation factor to three distinct subcomplexes (the 20S catalytic core, and the base and lid of the 19S subcomplex). Recently, a study using mass spectrometry to identify components of the yeast 26S proteasome found a similarly diverse range of proteasome-interacting proteins (26). Although the mass spectrometry study listed interactions between the proteasome and chaperones, mitotic proteins, the deubiquitinating enzyme Ubp6, and proteins involved in transcription, RNA metabolism, and signal transduction, there was no direct overlap with the nonproteasomal proteins reported here. Presumably, the different approaches used account for this discrepancy.

Following the two-hybrid screen, additional studies were carried out on 11 proteins found to interact with

the proteasome. These proteins were chosen because most were previously uncharacterized or were protein kinases, and viable deletion mutants were conveniently available (30). Mutants deleted for the genes encoding three of these proteins, Bim1, YKL171W, and Ump1, displayed genetic and biochemical phenotypes consistent with involvement in the proteasome pathway, and the strains showed phenotypes similar to those observed in yeast strains with proteasomal defects, including poor growth at elevated temperature or after treatment with canavanine. Although these conditions are believed to result in increased synthesis of misfolded and nonfunctional proteins that overwhelm the protein synthesis and degradation machinery, it is also possible that a defect independent of the proteasome is generating these phenotypes. Additionally, turnover of a proteasome substrate was reduced in the three strains. Because defects in Bim1, YKL171W, and Ump1 appear to affect the activity of the proteasome, it is more likely that these proteins modulate proteasome activity than that they serve as substrates of the complex. Mutations in *BIM1* lead to pleiotropic phenotypes, including microtubule defects, karyogamy defects, cell cycle checkpoint defects, and temperature sensitivity and benomyl sensitivity (20, 22). Ump1 is a proteasome-associated chaperone (16), and disruption of its activity may affect proteasome assembly or the ability of potential substrates to interact with the proteasome.

Our functional analysis of yeast proteasome components demonstrates the power of systematic two-hybrid approaches to rapidly identify protein interactions within a complex, and between the complex and associated proteins. Such an approach can serve as the starting point for further analysis of the significance of newly identified interactions identified. Here we demonstrate the possibility of this approach by investigating 11 largely uncharacterized proteins, 3 of which showed evidence of proteasome-related function.

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