Pleiotropic Defects Caused by Loss of the Proteasome-Interacting Factors Rad23 and Rpn10 of *Saccharomyces cerevisiae*

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ABSTRACT

Rad23 is a member of a novel class of proteins that contain unprocessed ubiquitin-like (UbL) domains. We showed recently that a small fraction of Rad23 can form an interaction with the 26S proteasome. Similarly, a small fraction of Rpn10 is a component of the proteasome. Rpn10 can bind multiubiquitin chains *in vitro*, but genetic studies have not clarified its role *in vivo*. We report here that the loss of both Rad23 and Rpn10 results in pleiotropic defects that are not observed in either single mutant. *rad23*Δ *rpn10*Δ displays slow growth, cold sensitivity, and a pronounced G2/M phase delay, implicating overlapping roles for Rad23 and Rpn10. Although *rad23*Δ *rpn10*Δ displays similar sensitivity to DNA damage as a *rad23*Δ single mutant, deletion of *RAD23* in *rpn10*Δ significantly increased sensitivity to canavanine, a phenotype associated with an *rpn10*Δ single mutant. A mutant Rad23 that is unable to bind the proteasome ($^{\Delta UbL}$rad23) does not suppress the canavanine or cold-sensitive defects of *rad23*Δ *rpn10*Δ, demonstrating that Rad23/proteasome interaction is related to these effects. Finally, the accumulation of multiubiquitinated proteins and the stabilization of a specific proteolytic substrate in *rad23*Δ *rpn10*Δ suggest that proteasome function is altered.

THE ubiquitin/proteasome pathway has been implicated in a broad range of activities, primarily those involving protein degradation (Hershko 1991; Hochstrasser 1996; Pickart 1997; Varshavsky 1997). Ubiquitin is attached to proteolytic substrates by ubiquitin-conjugating (E2) enzymes and by substrate recognition (E3) factors that are also known as recognins/ubiquitin-protein ligases. Several lines of evidence indicate that the degradation of a substrate is preceded by the formation of a multiubiquitin (multi-Ub) chain (Chau *et al.* 1989; Pickart 1997), a feature that may increase the affinity of a substrate for the proteasome (Lam *et al.* 1997; Piotrowski *et al.* 1997). Ubiquitinated substrates are degraded by the 26S proteasome, a multicatalytic protease of well-defined composition and activity. The X-ray structure of the yeast 20S catalytic complex has been determined (Groll *et al.* 1997), and the composition of the 19S regulatory particle was described recently (Glickman *et al.* 1998b).

In reconstituted experiments, long, unlinked and substrate-linked multi-Ub chains have an increased affinity for purified proteasomes (Lam *et al.* 1997; Piotrowski *et al.* 1997). The search for a multi-Ub chain-binding protein in the proteasome led to the discovery of S5a in mammals (Deveraux *et al.* 1994), as well as its counterparts in *Saccharomyces cerevisiae* (Rpn10, also called Sun1 and Mcb1, van Nocker *et al.* 1996b) and *Arabidopsis thaliana* (Mbp1, Deveraux *et al.* 1995; van Nocker *et al.* 1996a). Surprisingly, deletion of *RPN10* failed to cause a conspicuous defect, suggesting that the proteasome may contain other subunits that interact with multi-Ub chains (van Nocker *et al.* 1996b; Fu *et al.* 1998). Furthermore, only a small portion of cellular Rpn10 is present in the proteasome at steady-state levels, suggesting that it may form transient interactions. Interestingly, Glickman *et al.* (1998a) reported that Rpn10 stabilizes a discrete subcomplex within the 19S regulatory particle, but the relevance of this activity to its ability to bind multi-Ub chains remains to be determined.

We reported recently that the nucleotide-excision repair protein Rad23 can interact with catalytically active 26S proteasome (Schauber *et al.* 1998a). A striking feature of Rad23 is the presence of an unusual N-terminal ubiquitin-like domain (UbL^{R23}) that mediates the interaction with the proteasome. This interaction may be important for efficient nucleotide-excision repair because removal of UbL^{R23} causes sensitivity to DNA damage (Watkins *et al.* 1993). As with Rpn10, only a small fraction of cellular Rad23 interacts with the proteasome. We therefore examined the possibility that Rpn10 and Rad23 might have overlapping functions. We report here that *rad23*Δ *rpn10*Δ (DLY140) has growth, cold-sensitive, and proteolytic defects that are not observed in either single mutant. DLY140 displays an apparent delay in the G2/M phase of the cell cycle, with cells containing 2N DNA and high levels of the mitotic cyclin Clb2. The canavanine sensitivity that was previously found in *rpn10*Δ was severely exacerbated in *rad23*Δ

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*rpn10*Δ. In addition, we discovered increased levels of multiubiquitinated proteins in *rad23*Δ *rpn10*Δ. Although our findings suggest an overlapping function for Rad23 and Rpn10, their activities are not redundant because the UV sensitivity of *rad23*Δ was not intensified in DLY140. Significantly, we found that the *rad23*Δ single mutant is sensitive to canavanine and is unable to degrade the proteasome substrate Ub-Pro-β-galactosidase (Ub-Pro-βgal). Taken together, these findings for the first time implicate a proteolytic function for Rad23. This conclusion is strengthened by the finding that $^{\Delta UbL}$rad23, a mutant that is unable to interact with the proteasome, failed to complement the canavanine- and cold-sensitive phenotypes of *rad23*Δ *rpn10*Δ.

MATERIALS AND METHODS

Media, yeast strains, and plasmids: Yeast transformations were performed using standard techniques (Guthrie and Fink 1991; Gietz *et al.* 1992) to yield the strains described in Table 2. The *RPN10* gene was amplified by polymerase chain reaction (PCR) with a 5′ *Eco*RI and 3′ *Kpn*I restriction site using the following oligonucleotides: 5′-GCGAATTCATGG TATTGGAAGCTACAGTGTTAGTG-3′ and 5′-GCGGTACC TATTTGTCTTGGTGTTGTTCAGGCTG-3′. The resulting DNA fragment and the plasmid pCS13 (Table 1) were digested with *Eco*RI and *Kpn*I, ligated, and transformed into TOP10F′ to yield pLC97. Similarly, the *RAD23* gene was amplified by PCR with a 5′ *Eco*RI and 3′ *Kpn*I restriction site using the following oligonucleotides: 5′-GCGGTACCTCAGTCGGCAT GATCGCTGAA-3′ and 5′-GCGAATTCATGACGAAGAC CAAAGTAACAGAA-3′. The resulting DNA fragment and plasmid pKM1362 (Madura and Varshavsky 1994) were digested with *Kpn*I and *Eco*RI, ligated, and transformed into TOP10F′ to yield pDL120. pCS19 was constructed by *in vivo* recombination of pCEP10 (Schauber *et al.* 1998a) with *Sma*I-digested pUT11 (generously provided by F. Cross, Rockefeller University, New York) to generate a *TRP1* derivative. DLY140 and DLY152 were constructed by *in vivo* recombination of *Eco*RI-digested pDG28 with MHY960 and DF5, respectively, and selected on synthetic medium lacking uracil.

Growth assays: Yeast cultures were grown for ~15 hr in selective medium, and their densities were normalized to OD_{600} ~1.0 to examine growth properties and OD_{600} ~0.5 to estimate sensitivity to 1 μg/ml canavanine. Tenfold serial dilutions were prepared in sterile water, and 5 μl was plated on YPAD agar medium and incubated at 13°, 25°, and 30° to estimate growth properties. Aliquots were also spotted on synthetic complete (SC) medium and SC supplemented with 1 μg/ml canavanine (but lacking arginine) and incubated at 30°. The growth of the yeast strains was examined after 2–7 days on the basis of incubation temperature. The sensitivity to canavanine was determined after 5 days of growth at 30°.

Microscopy: Yeast cultures were grown for ~15 hr at 30° in liquid culture or plated on YPAD medium for several days at 13°. Cells were collected and fixed in 20% ethanol for 20 min at 4°, sonicated for 5 min, and examined by microscopy. Cells were visualized with an Optiphot-2 light microscope (Nikon, Garden City, NY) using a ×100 objective and photographed with a Nikon FX-35 camera. The fraction of budded and unbudded cells was determined with similar cultures that were not fixed in ethanol.

To examine cells by fluorescence-activated cell sorting (FACS), actively growing cells were fixed for 1 hr in 20% ethanol at 4°, washed in 50 mm Tris, pH 7.5, and treated with 1 mg/ml RNase for 2 hr at 37°. Cells were incubated at 55° for 1 hr in the presence of 0.04 mg/ml proteinase K, washed with PBS, and sonicated for 10 min. Cells were resuspended in 100 μg/ml propidium iodide, stored at 4°, and diluted fivefold prior to sorting.

Ultraviolet sensitivity assays: Yeast cultures were grown overnight in synthetic medium and normalized to an OD_{600} ~ 1.0. Appropriate dilutions were plated on YPAD plates and exposed to 254-nm UV light for 0, 10, 30, and 60 sec at 1.5 J/m^2/sec, followed by incubation at 30° in the dark for 3 days. Colonies were counted and compared to an untreated control.

Protein degradation: Bulk protein degradation assays were performed essentially as described by van Nocker *et al.* (1996b). Yeast cultures were grown to exponential phase at 30° and incubated with ^{35}S-Translabel for 5 min, as described previously (Bachmair *et al.* 1986). The cells were pelleted, washed twice, and resuspended at 25° in chase buffer containing cycloheximide and excess unlabeled methionine and cysteine (Bachmair *et al.* 1986). Aliquots (5 μl) were withdrawn at 0, 10, 20, 30, 40, 50, and 60 min during the incubation in chase medium and were spotted in triplicate on filter disks presoaked in 50% trichloroacetic acid (TCA). Filters were dried, washed in cold 10% TCA for 10 min, and boiled in 10% TCA for 5 min. The filters were dried, transferred to scintillation vials, and quantitated. The radioactivity for each sample was normalized to the value obtained at 0 min.

Yeast strains expressing Ub-Pro-βgal and Clb2-HA were propagated at 30° in raffinose-containing medium. The cells were transferred to medium containing 2% galactose to induce expression of Ub-Pro-βgal and Clb2-HA. Actively growing cells were metabolically labeled for 5 min with ^{35}S-Translabel and chased in medium containing cycloheximide and excess unlabeled methionine and cysteine. Aliquots were removed at 0, 10, 30, and 60 min, combined with buffer A (50 mm

TABLE 1

Plasmids

Plasmid	Description	Reference
pCS13	P_{CUP1}::FLAG-*RAD23 LEU2* 2μ Amp^R	Schauber *et al.* (1998a)
pCS19	P_{GAL1}::*RAD23 TRP1 CEN* Amp^R	This study
pDL120	P_{CUP1}::$^{\Delta UbL}$*rad23 LEU2* 2μ Amp^R	This study
pLC97	P_{CUP1}::FLAG-*RPN10 LEU2* 2μ Amp^R	This study
pPP12	P_{RAD23}::*RAD23 URA3 CEN* Amp^R	A gift from P. Sadhale
pWS945	P_{GAL1}::*CLB2-HA URA3 CEN* Amp^R	Seufert *et al.* (1995)
pUb-P-e^K-βgal	P_{GAL1}::Ub-Pro-βgalactosidase 2μ Amp^R	Bachmair *et al.* (1986)
YCplac22	*TRP1 CEN* Amp^R	Gietz and Sugino (1988)

HEPES, pH 7.5, 5 mm EDTA, 150 mm NaCl, 1% Triton X-100, Bachmair *et al.* 1986), and frozen immediately. The incorporation of ^{35}S into acid (TCA)-insoluble material was determined, and equal cpm were incubated with antibodies and examined as described previously (Schauber *et al.* 1998b). The stability of Ub-Pro-βgal was determined by incubation of 2×10^5 cpm of extract with anti-βgal antibodies (Sigma, St. Louis), while Clb2-HA stability was determined by incubating 1×10^6 cpm of extract with anti-HA antibodies (BabCo, Berkeley, CA). The immunoprecipitates were washed, resolved in an SDS-polyacrylamide, and exposed to X-ray film. Protein levels were quantitated by densitometry.

Immunoblotting: For immunoblotting studies, cell extracts were prepared in lysis buffer (20 mm HEPES, pH 7.5, 100 mm potassium acetate, 5 mm Na · EDTA, 10% glycerol), and equal amounts of extract (1 mg total protein) were incubated with either 20 μl FLAG-agarose (Sigma) or 20 μl protein A-Sepharose (Repligen Co., Cambridge, MA) for a mock control. The immunoprecipitation reactions were adjusted to a final volume of 900 μl using buffer A and were incubated for 6–12 hr at 4°. The immunoprecipitates were washed three times with buffer A, resolved by SDS-polyacrylamide gel electrophoresis, transferred to 0.45 μm nitrocellulose (Bio-Rad, Richmond, CA), and blocked in 5% milk powder. The filter was incubated sequentially with antibodies against Rpt1, Rad23, and the FLAG epitope (Sigma). The immunoblots were developed with enhanced chemiluminescence (Renaissance ECL, New England Nuclear, Boston).

To visualize ubiquitin, 50 μg of total protein was resolved in a 12% SDS polyacrylamide gel and transferred to a 0.2-μm nitrocellulose filter (Bio-Rad). The filter was pretreated as described previously (Swerdlow *et al.* 1986), blocked with 5% milk powder, and incubated with antibodies against ubiquitin (Sigma). The immunoblots were developed with enhanced chemiluminescence (Renaissance ECL, New England Nuclear).

RESULTS

Rad23 and Rpn10 are required for efficient growth: The *RAD23* gene was deleted by homologous recombination from an *rpn10*Δ strain (MHY960, acquired from M. Hochstrasser, University of Chicago) and its wild-type counterpart (DF5), resulting in strains DLY140 and DLY152, respectively (Table 2). Tenfold dilutions of exponential phase DLY140 and the congenic wild-type and single-mutant strains were plated on YPAD medium and incubated at 13°, 25°, 30°, and 37° for 2–7 days (Figure 1A). DLY140 showed extremely poor viability at 13°, and its growth was noticeably impaired at 25° and 30°. DLY140 displayed similar growth rates at 30° and 37°, demonstrating that it is not sensitive to high temperature (data not shown). In contrast, neither single mutant displayed a significant growth defect at any temperature, implicating an overlapping function for these proteasome-interacting factors.

To confirm that the growth defects of DLY140 were caused by the loss of both Rad23 and Rpn10, we transformed the double mutant with plasmids encoding either FLAG-Rpn10 or single-copy Rad23 expressed from its own promoter. As expected, the unique phenotypes of DLY140 were completely rescued by plasmid-borne copies of either *RPN10* or *RAD23* (Figure 1B), but not by a vector control (Figure 1C). These studies demonstrate the requirement for either Rad23 or Rpn10 for suppressing the defects of DLY140.

The ubiquitin-like domain in Rad23 (UbL^{R23}) is re-

TABLE 2

Yeast strains

Strain	Description	Reference
DF5	*Matα his3-Δ200 leu2-3,112 lys2-801 trp1-1 (am) ura3-52*	Bartel *et al.* (1990)
MHY961	DF5, *rpn10*Δ::*HIS3*	A gift from M. Hochstrasser
DLY140	MHY961, *rad23*Δ::*URA3*	This study
DLY143	DLY140,pLC97	This study
DLY152	DFS, *rad23*Δ::*URA3*	This study
DLY176	DLY140, 5-FOA derivative	This study
DLY180	DLY152, 5-FOA derivative	This study
DLY185	MHY961, pWS945	This study
DLY187	DF5, pWS945	This study
DLY191	DF5, pLC97	This study
DLY192	DF5, pCS13	This study
DLY193	DLY152, pLC97	This study
DLY194	MHY961, pCS13	This study
DLY204	DLY176, pWS945	This study
DLY205	DLY152, pWS945	This study
DLY324	DLY176, pPP12	This study
DLY417	DLY140, pDL120	This study
DLY422	DLY140, YCplac22	This study
LCY600	DLY140, pLC97 and pCS19	This study
LCY602	DF5, pUb-P-e^K-βgal	This study
LCY603	DLY180, pUb-P-e^K-βgal	This study
LCY604	MHY961, pUb-P-e^K-βgal	This study
LCY605	DLY176, pUb-P-e^K-βgal	This study

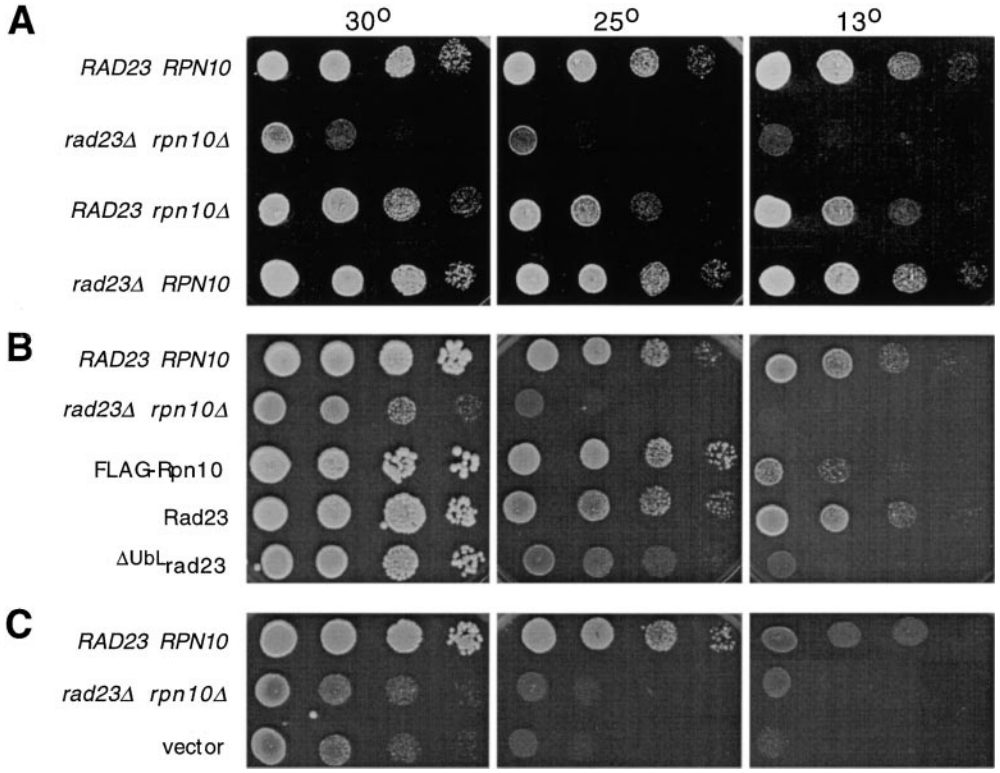
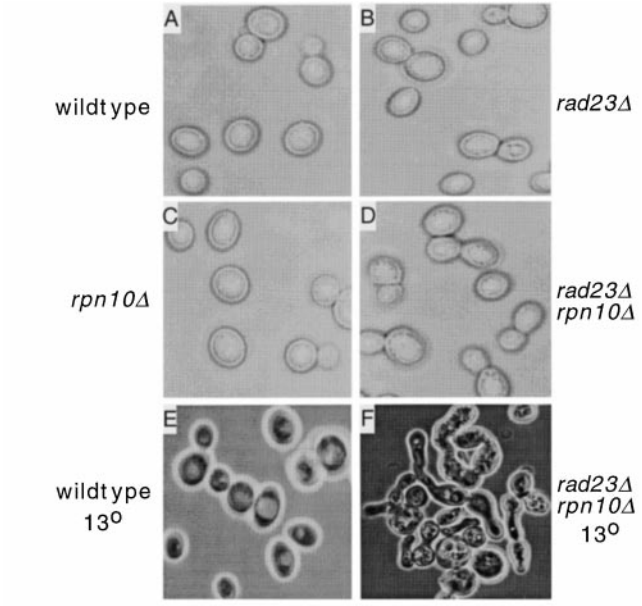


Figure 1.—*rad23*Δ *rpn10*Δ displays slow growth and a cold-sensitive phenotype. (A) Yeast cultures were normalized to an optical density A_{600} ~1, and 10-fold dilutions were spotted on YPAD medium. Agar plates were incubated for 2–7 days at 30°, 25°, and 13°. The growth of *rad23*Δ and *rpn10*Δ was similar to that of the wild-type strain. In contrast, the growth of *rad23*Δ *rpn10*Δ was reduced at 30° and 25° and severely inhibited at 13°. (B) The growth of *rad23*Δ *rpn10*Δ was restored to wild-type levels after transformation with a plasmid encoding FLAG-Rpn10 expressed from P_{CUP1}, or with a *CEN*-based plasmid expressing Rad23 from its own promoter. Significantly, a Rad23 mutant lacking the UbL domain ($^{\Delta UbL}$rad23) did not suppress the cold-sensitive defect of *rad23*Δ *rpn10*Δ and only partially rescued the slow-growth phenotype, demonstrating that UbL^{R23}/proteasome interaction has an important physiological effect. A vector control is shown in C. (The plates in B and C were incubated ~12 hr longer than those in A, accounting for the difference in growth.)

quired for its DNA repair activities (Watkins *et al.* 1993). Our finding that UbL^{R23} can form an interaction with the 26S proteasome (Schauber *et al.* 1998a) implicated a proteolytic function for Rad23 during DNA repair. We investigated whether Rad23/proteasome interaction was also required for the phenotypes associated with DLY140. *rad23*Δ *rpn10*Δ was transformed with a plasmid encoding $^{\Delta UbL}$rad23, a mutant that lacks the UbL domain. Although $^{\Delta UbL}$rad23 partially complemented the slow-growth phenotype of DLY140, it failed to suppress the cold sensitivity of DLY140 (Figure 1B), demonstrating that Rad23/proteasome interaction is important in the absence of Rpn10. These findings present a novel and important genetic link between Rad23 and the ubiquitin/proteasome pathway.

A role for Rad23 in cell-cycle progression: To further characterize the growth defect of *rad23*Δ *rpn10*Δ, we examined late exponential phase cells by microscopy. We found that a high fraction of DLY140 accumulated as large-budded cells in contrast to the wild-type or single-mutant strains (compare Figure 2D to Figure 2, A–C). The slow-growth and cold-sensitive phenotypes observed in DLY140 may be the result of the G2/M phase delay. Since the growth defect of DLY140 is intensified at 13°, we examined yeast cells that were incubated at 13° to determine if the cells arrested with a terminal defect. We observed profound morphological aberrations in *rad23*Δ *rpn10*Δ at 13° in addition to the accumulation of large-budded cells (Figure 2F). In contrast to the congenic wild-type strain (Figure 2E), DLY140 cells contained a large vacuole and numerous small vesicles. We also quantitated the number of cells that contained small and large buds in the four strains. These results (Figure 2G) corroborate the microscopic analysis (Figure 2, A–D). The ability of $^{\Delta UbL}$rad23 to partially complement the slow-growth but not the cold-sensitive phenotype of DLY140 may reflect the complex growth defects of the double mutant. For instance, the large-budded phenotype that is observed at 30° may be distinct from the other morphological defects that are apparent only at low temperatures.

To further characterize the G2/M phase delay, we stained yeast cells with propidium iodide and examined them by FACS. We found that ~70% of DLY140 and ~40% of wild-type and single-mutant cells contained 2N DNA (Figure 3A), consistent with the microscopic analysis (Figure 2, A–D). In agreement with the FACS analysis, we found a significant increase in the fraction of *rad23*Δ *rpn10*Δ cells that contained large buds, and a decrease in the proportion of unbudded cells (Figure 2G). We also examined the levels of Clb2-HA in *rad23*Δ *rpn10*Δ, since the degradation of this mitotic cyclin occurs during the transition from mitosis to G1 (Amon *et al.* 1994). We expressed Clb2-HA in each of the four strains and measured stability in asynchronous cultures by pulse-chase methods. We found that the level of Clb2-



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Strain	○	○°	○○
wildtype	45	15	40
rpn10Δ	37	21	42
rad23Δ	40	26	34
rad23Δ rpn10Δ	22	19	59

Figure 2.—*rad23*Δ *rpn10*Δ displays distinct morphological defects at 30° and 13°. Yeast cells were grown to late exponential phase at 30°, fixed in 20% ethanol, and visualized by microscopy. (A) Wild-type, (B) *rad23*Δ, and (C) *rpn10*Δ cells displayed few large-budded cells, while (D) a high fraction of *rad23*Δ *rpn10*Δ contained large-budded cells. (E) Wild-type cells maintained normal morphology at 13°, but they were noticeably smaller than cells grown at 30°. (F) In contrast, the morphology of *rad23*Δ *rpn10*Δ cells was highly aberrant at 13°, and a large fraction was highly elongated and contained numerous small vesicles. (G) The percentage of unbudded cells and cells containing small and large buds in the strains described in A–D is shown.

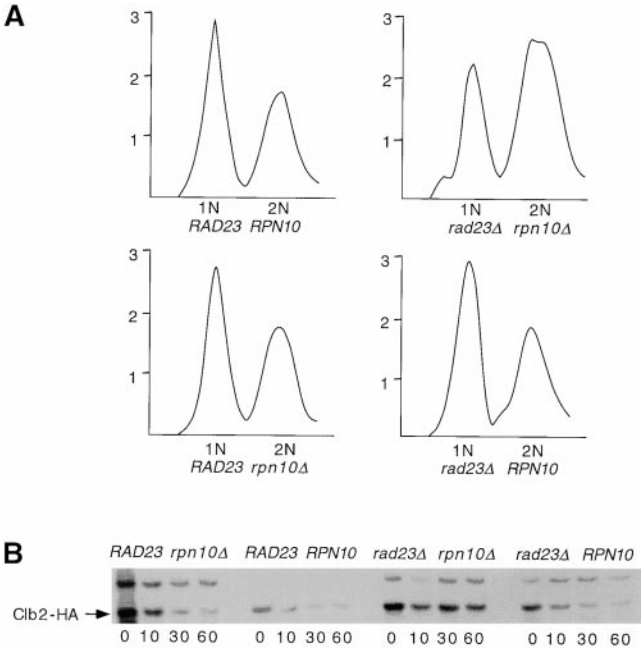

Figure 3.—An increased fraction of *rad23*Δ *rpn10*Δ cells contains 2N DNA and high levels of the mitotic cyclin Clb2-HA, consistent with a delay in G2/M phase. (A) Yeast cells were stained with propidium iodide and examined by FACS. Approximately 40% of wild-type and single-deletion cells contained 2N DNA, while ~70% of *rad23*Δ *rpn10*Δ accumulated with 2N DNA. (B) The stability of Clb2-HA was determined by pulse-chase analysis in asynchronously growing cultures. Clb2-HA accumulated to higher levels and was noticeably more stable in *rad23*Δ *rpn10*Δ compared to wild-type and single-deletion strains, consistent with the delayed progression through the G2/M phase.

HA was ~2.5-fold higher in DLY140 after 30 min in the chase buffer, relative to wild-type and single-deletion strains (Figure 3B). The accumulation of Clb2-HA may contribute to the delay in *rad23*Δ *rpn10*Δ, although our data do not exclude the possibility that the stabilization of Clb2-HA is simply a consequence of the G2/M-phase delay in DLY140. Nonetheless, the accumulation of Clb2-HA in the double mutant provides a useful biochemical marker that reflects the delay in G2/M, which was predicted by the microscopic and FACS analyses. Further studies will be required to precisely define the point of delay during the cell cycle.

Rad23 and Rpn10 form independent interactions with the 26S proteasome: Plasmids encoding FLAG-Rad23 and FLAG-Rpn10 were transformed into *rpn10*Δ and *rad23*Δ cells, respectively. We prepared cell extracts from actively growing cells and incubated equal amounts of protein with FLAG-agarose beads. The beads were washed, and the bound proteins were separated in an SDS-polyacrylamide gel, transferred to nitrocellulose, and incubated with antibodies against Rpt1, a subunit in the 19S regulatory particle of the proteasome (Glickman *et al.* 1998b). We found that Rpt1 could be coprecipitated with both FLAG-Rad23 and FLAG-Rpn10, even in the absence of the other protein (Figure 4A), demonstrating that Rad23 and Rpn10 form independent interactions with the proteasome.

To examine these interactions further, we expressed FLAG-Rpn10 and galactose-inducible Rad23 in DLY140. Actively growing cells were transferred from raffinose- to galactose-containing medium to induce expression of Rad23. Aliquots were withdrawn at the times indicated, and protein extracts were prepared. Equal amounts of protein were applied to FLAG-agarose, and Rpn10-interacting proteins were examined by SDS-PAGE and immunoblotting. We found that the interaction between Rpn10 and the proteasome was not affected by increasing levels of Rad23 (Figure 4B), because the level of Rpt1 that coprecipitated was essentially unchanged. Significantly, Rad23 was also detected in

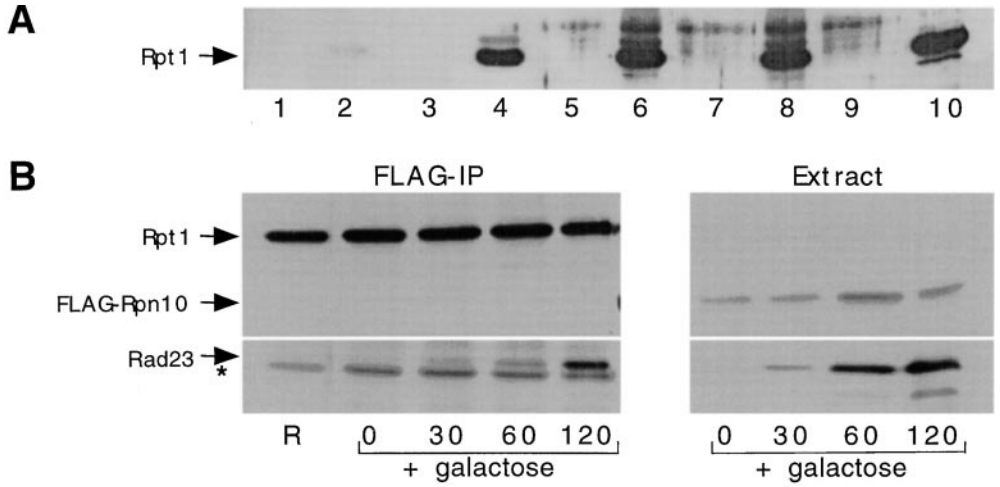

Figure 4.—Rad23 and Rpn10 form independent interactions with the proteasome. (A) Yeast extracts were prepared from *rad23*Δ and *rpn10*Δ strains expressing FLAG-Rpn10 and FLAG-Rad23, respectively. Equal amounts of protein were incubated with FLAG-agarose (lanes 2, 4, 6, 8, and 10) or protein A-Sepharose as a mock control (lanes 1, 3, 5, 7, and 9), and the bound proteins were examined by immunoblotting with antibodies against Rpt1. Lanes 1 and 2 represent a wild-type strain that did not express a FLAG-tagged protein. The amount of Rpt1 that coprecipitated with FLAG-Rad23 from wild-type (lane 4) and *rpn10*Δ strains (lane 8) was essentially the same. Similarly, FLAG-Rpn10 coprecipitated an equivalent amount of Rpt1 from wild-type (lane 6) and *rad23*Δ (lane 10) cells. (B) Rad23 and Rpn10 do not compete for binding to the proteasome. Yeast cells expressing galactose-inducible Rad23 (P_{GAL1}::*RAD23*) and FLAG-Rpn10 were grown in raffinose-containing medium. After the addition of 2% galactose, aliquots were withdrawn at intervals (0, 30, 60, and 120 min) and equal amounts of extract were incubated with FLAG-agarose (FLAG-IP). The immunoprecipitated proteins were analyzed sequentially in an immunoblot with antibodies against Rpt1 and Rad23. Despite the rapid increase of Rad23 levels, after the addition of galactose (Extract), there was no appreciable decrease in the amount of Rpt1 that coprecipitated with FLAG-Rpn10 (FLAG-IP). The abundance of FLAG-Rpn10 was relatively constant (Extract). Significantly, Rad23 was also detected in the FLAG-Rpn10 immunoprecipitates, demonstrating that both Rad23 and Rpn10 can bind the same proteasome. The asterisk represents a cross-reaction against the immunoglobulin heavy chain. FLAG-Rpn10 was expressed at similar levels in raffinose and galactose medium (data not shown).

the FLAG-Rpn10 immunoprecipitate, demonstrating that Rpn10 and Rad23 can bind the same proteasome. The immunoprecipitation reactions were performed without the addition of exogenous ATP, which are conditions that promote the dissociation of the 19S and 20S particles of the proteasome. We speculate, therefore, that Rpn10 and Rad23 occupy different binding sites on the same 19S particle.

The UV sensitivity of *rad23*Δ is unaffected in *rad23*Δ *rpn10*Δ: Rad23 enables Rad14 and RNA polymerase II transcription factor IIH (TFIIH) to assemble into the nucleotide-excision repair complex *in vitro* (Guzder *et al.* 1995a,b). Rad23 can also be purified in a complex with Rad4 in yeast and XP-C in humans, both of which are essential components of the nucleotide-excision repair complex. Mutations in XP-C cause xeroderma pigmentosum, which is characterized by a predisposition to skin cancer, ataxia, and neurological impairment (Cleaver and Kraemer 1994; Friedberg *et al.* 1995). The biochemical activity of Rad23/Rad4 is unknown (Masutani *et al.* 1994; Guzder *et al.* 1995a) even though the dimer can preferentially bind damaged DNA (Guzder *et al.* 1998). We reported previously that Rad4 can be copurified through several chromatographic steps with GST-Rad23 and subunits of the 26S proteasome (Schauber *et al.* 1998a). On the basis of this finding, we proposed that Rad23 might play a role in delivering proteolytic substrates to the proteasome. It is not known, however, if Rad4 constitutes one of these substrates.

We investigated whether the DNA repair defect of *rad23*Δ was further exacerbated in *rad23*Δ *rpn10*Δ because of the overlapping roles of Rad23 and Rpn10. We measured the survival of DLY140 after different doses of UV light and found that its sensitivity was similar to that of *rad23*Δ (Figure 5). This finding is significant because it demonstrates that the functions of Rad23 and Rpn10 are not redundant, but overlapping. Similarly, Biggins *et al.* (1996) reported that Rad23 and Dsk2 (another UbL-containing protein) have overlapping functions. Significantly, the UV sensitivity of *rad23*Δ was not further aggravated in *rad23*Δ *rpn10*Δ, and, similar to *rpn10*Δ, the *dsk2*Δ single mutant does not display any conspicuous growth defects. We have recently discovered that the UbL domain in Dsk2 (UbL^{DSK}) can interact with the proteasome (data not shown), suggesting that other UbL-containing proteins might also have proteolytic functions that intersect with Rad23 and Rpn10.

Degradation of most short-lived proteins is unaffected in *rad23*Δ *rpn10*Δ: We determined whether the phenotypes of *rad23*Δ *rpn10*Δ were caused by an underlying defect in the ubiquitin/proteasome system. To test this idea, the degradation for short-lived proteins was measured in the wild-type and *rad23*Δ *rpn10*Δ strains, as described previously (van Nocker *et al.* 1996b). Proteins were labeled with ^{35}S-Translabel for 5 min, followed by incubation at 25° in chase buffer containing excess unlabeled methionine and cysteine. Aliquots were removed at intervals during the chase, and the amount of ^{35}S-labeled protein that was degraded was determined (see materials and methods). Interestingly, DLY140 and wild-type cells showed similar levels of degradation

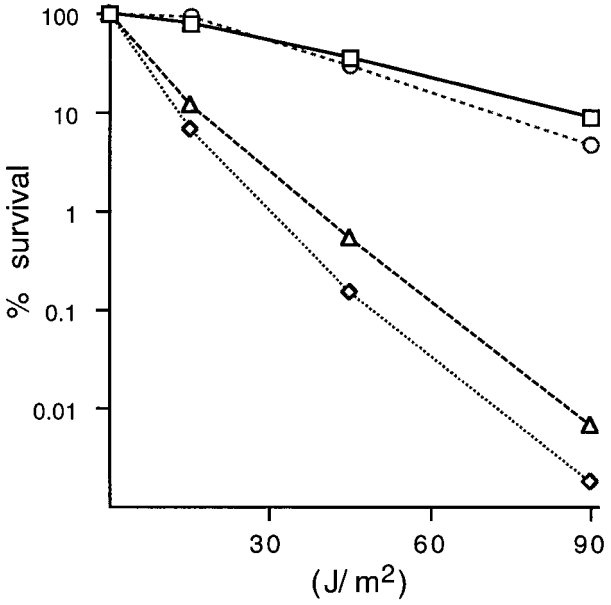

Figure 5.—Rpn10 is not required for the DNA repair function of Rad23. Yeast cells were exposed to 254 nm UV light at a fluence of 1.5 J/m^2/sec, and survival was determined. Wild-type (○) and *rpn10*Δ (□) cells displayed similar sensitivity. In contrast, *rad23*Δ *rpn10*Δ (△) and *rad23*Δ (◇) were sensitive to UV light.

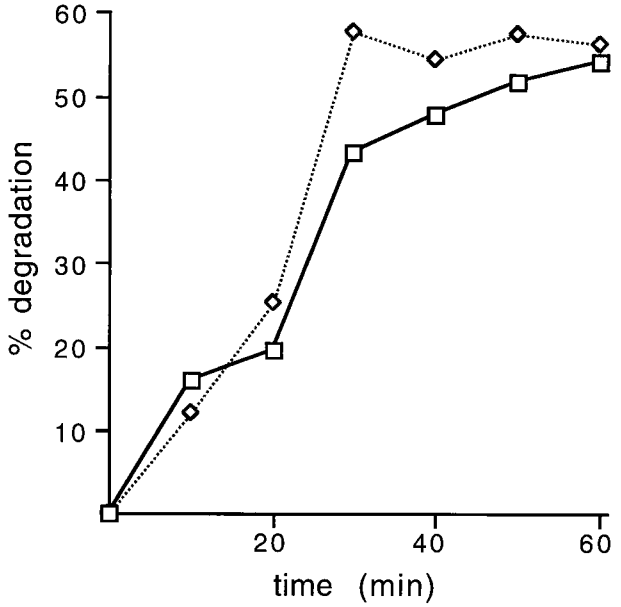

Figure 6.—The degradation of bulk short-lived proteins is not affected in *rad23*Δ *rpn10*Δ. Yeast cells were grown to exponential phase at 30°, labeled with ^{35}S-Translabel for 5 min, washed, and incubated at 25° in medium containing excess cold methionine and cysteine. Aliquots (5 μl) were withdrawn at 10-min intervals and treated as described in materials and methods. The turnover of generally short-lived proteins was equivalent in the wild type (□) and *rad23*Δ *rpn10*Δ (◇). The values reflect measurements that were obtained in triplicate, and they are representative of two independent experiments.

of bulk short-lived proteins (Figure 6). Furthermore, the rates of degradation were not affected significantly in the presence of canavanine (data not shown) despite the sensitivity of *rad23*Δ *rpn10*Δ to this amino acid analog (see below).

The proteolytic defects of *rpn10*Δ are intensified by loss of Rad23: The growth of many mutants in the ubiquitin/proteasome pathway is inhibited by canavanine (Seufert and Jentsch 1990; van Nocker *et al.* 1996b; Ramos *et al.* 1998), an amino acid analogue whose incorporation into proteins causes misfolding. Loss of Rpn10 was previously shown to cause moderate sensitivity to canavanine (van Nocker *et al.* 1996b). We show here that the loss of Rad23 also causes the cells to become sensitive to canavanine. However, loss of both Rad23 and Rpn10 resulted in extreme sensitivity to canavanine, and plating assays showed >100-fold reduced survival (Figure 7A). Significantly, the canavanine sensitivity of DLY140 was suppressed by either FLAG-Rpn10 or Rad23, but not by $^{\Delta UbL}$rad23 (Figure 7B). Similarly, the cold sensitivity was not suppressed by $^{\Delta UbL}$rad23 (Figure 1B), demonstrating the importance of Rad23/proteasome interaction. These findings suggest that the proteolytic defect in *rad23*Δ *rpn10*Δ may occur at the level of the proteasome.

To investigate the potential proteolytic role of Rad23, we examined the levels of multiubiquitinated proteins in wild-type, *rad23*Δ, *rpn10*Δ, and *rad23*Δ *rpn10*Δ strains. We resolved 50 μg of total protein from each strain by SDS-PAGE and incubated an immunoblot with antiubiquitin antibodies. We detected higher levels of multiubiquitinated proteins in DLY140, relative to the wild-type and single-deletion strains, consistent with the idea that Rad23 and Rpn10 provide overlapping proteolytic functions (Figure 8A).

The loss of Rpn10 caused stabilization of Ub-Pro-βgal (van Nocker *et al.* 1996b), a well-characterized substrate of the ubiquitin/proteasome pathway (Johnson *et al.* 1992, 1995). Ub-Pro-βgal was also stablilized in the *rad23*Δ single mutant, providing compelling evidence of a proteolytic role for Rad23. Curiously, however, despite the stabilization of Ub-Pro-βgal, its levels were significantly lower in *rad23*Δ than in the other strains. As expected, Ub-Pro-βgal was completely stabilized in DLY140 and accumulated to high levels (Figure 8B). We also detected a distinct 90-kD degradation product in *rpn10*Δ and the wild-type strain. The release of this proteolytic fragment, which is indicative of the degradation of Ub-Pro-βgal (Bachmair *et al.* 1986), was detected in neither *rad23*Δ nor *rad23*Δ *rpn10*Δ.

DISCUSSION

The N-terminal, ubiquitin-like domain in Rad23 is required for efficient nucleotide-excision repair, and a regulatory function has been proposed for this motif (Watkins *et al.* 1993). We discovered that UbL^{R23} forms a stable interaction with catalytically active proteasomes

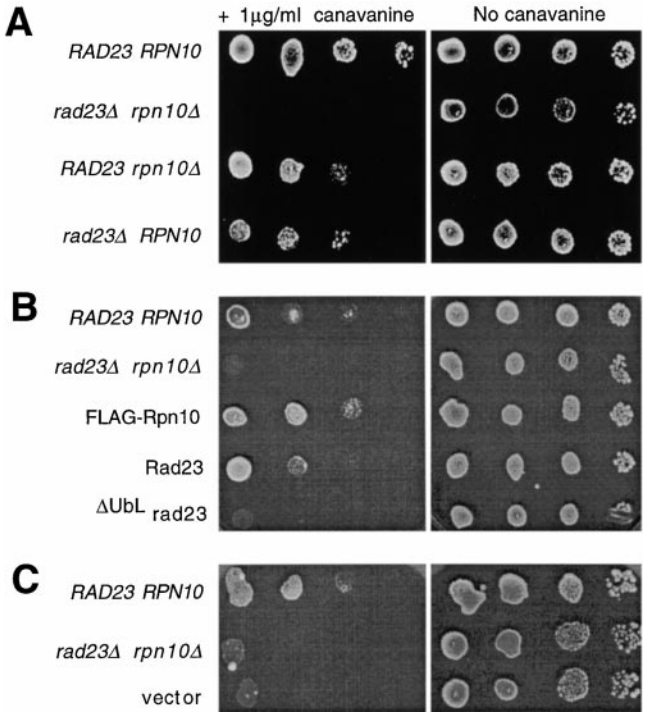

Figure 7.—The canavanine sensitivity of *rpn10*Δ is exacerbated by the loss of *RAD23*. (A) Late exponential phase cultures were normalized to an optical density of A_{600} ~0.5. Tenfold dilutions were plated on synthetic medium lacking arginine and supplemented with 1 μg/ml canavanine, and they were incubated at 30° for 5 days. *rad23*Δ *rpn10*Δ displayed extremely poor growth on medium containing canavanine. In contrast, *rad23*Δ and *rpn10*Δ displayed moderate sensitivity to canavanine. The growth of these strains on medium lacking canavanine was similar to that observed in Figure 1A, although the longer incubation of these plates at 30° permitted *rad23*Δ *rpn10*Δ to form a patch of growth. (B) The sensitivity of *rad23*Δ *rpn10*Δ to canavanine was alleviated by expression of FLAG-Rpn10 or Rad23, but not by $^{\Delta UbL}$rad23, confirming the requirement for Rad23/proteasome interaction. A vector control is shown in C.

(Schauber *et al.* 1998a), and recent studies indicate that this interaction occurs with the 19S regulatory particle (data not shown). These findings have led to the hypothesis that Rad23 might perform a role at the proteasome that is relevant to its DNA repair functions.

Rpn10 is a component of the 19S regulatory particle of the 26S proteasome (van Nocker *et al.* 1996b; Glickman *et al.* 1998b). Rpn10 and its mammalian counterpart (S5a) were isolated by their ability to interact with multiubiquitin chains, suggesting that Rpn10/S5a could play a central role in proteasome-mediated degradation. Surprisingly, Rpn10 is not required for cellular viability since a deletion of *RPN10* caused only subtle defects, including stabilization of an engineered substrate, moderate sensitivity to an amino acid analog, and instability of the 19S particle under high-salt conditions (Glickman *et al.* 1998a). Similar to Rad23, only ~5% of Rpn10 is associated with the proteasome at steady-state levels, suggesting that a similar mechanism might regulate their interactions with the proteasome.

We report the discovery of a novel genetic interaction between the proteasome-interacting factors Rad23 and Rpn10. The simultaneous deletion of both genes caused pleiotropic defects, including slow growth, cold sensitivity, G2/M phase delay, increased sensitivity to the amino acid analog canavanine, and the accumulation of multiubiquitinated proteins. The suppression of the sensitivity of *rad23*Δ *rpn10*Δ to canavanine and cold temperature required UbL^{R23}, indicating a need for Rad23/proteasome interaction. The sensitivity of *rad23*Δ *rpn10*Δ to canavanine and the accumulation of multiubiquitinated proteins in this mutant indicate that the defects are caused, at least in part, by impairment of the function of the ubiquitin/proteasome pathway.

The elimination of canavanyl proteins requires the activities of the ubiquitin/proteasome pathway (Seufert and Jentsch 1990). Nonetheless, some mutants of the ubiquitin pathway are sensitive to canavanine but do not display an obvious biochemical defect in proteolysis. We note that although *rad23*Δ *rpn10*Δ is highly sensitive to growth on canavanine-containing medium, its ability to degrade bulk short-lived proteins is similar to that of the wild-type counterpart. It is therefore possible that the sensitivity of *rad23*Δ *rpn10*Δ to canavanine is not related to impaired proteolysis. However, the significant accumulation of multiubiquitinated proteins in *rad23*Δ *rpn10*Δ is consistent with a proteolytic defect. We suggest, therefore, that the cellular sensitivity to canavanyl proteins may be a much more acute sensor of a proteolytic defect than the measurement of bulk protein turnover.

The sensitivity to canavanine was less severe in *rpn10*Δ (van Nocker *et al.* 1996b) than in *rad23*Δ (Figure 7A). Significantly, the double mutant was ~100-fold more sensitive to canavanine than either single mutant. Taken together, the defects of *rad23*Δ *rpn10*Δ, which include canavanine sensitivity, accumulation of multiubiquitinated proteins, and stabilization of Ub-Pro-βgal are consistent with altered proteolytic function. We note that the stabilization of Ub-Pro-βgal in *rad23*Δ and the sensitivity of this mutant to canavanine have revealed an Rpn10-independent proteolytic function for Rad23. The accumulation of Clb2-HA in *rad23*Δ *rpn10*Δ provides a plausible explanation for the delay in the G2/M phase of the cell cycle (Figure 3B). However, our results do not resolve whether Clb2 stabilization is caused directly by the loss of both Rad23 and Rpn10, or by an indirect effect resulting from the delayed progression through G2/M phase.

To determine if the functional overlap between Rad23 and Rpn10 was of a specific nature, we examined the effect of deleting *RAD23* from a strain lacking another proteasome subunit, Son1/Ufd5. Fujimuro *et al.* (1998) showed that Son1 can be isolated in association with the proteasome. A proteolytic function for Son1

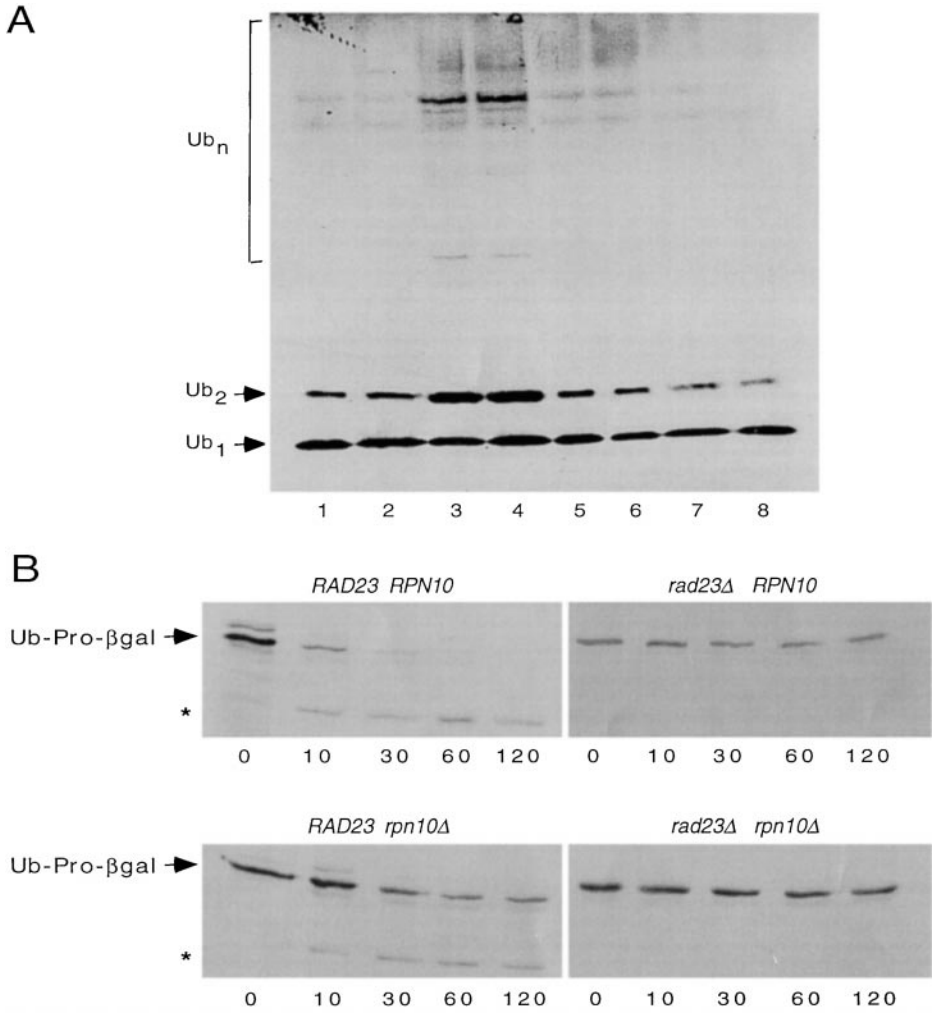

Figure 8.—Accumulation of multiubiquitinated proteins and stabilization of Ub-Pro-βgal in *rad23*Δ *rpn10*Δ. (A) The levels of multiubiquitinated proteins were examined in extracts prepared from wild-type (lanes 1 and 2), *rad23*Δ *rpn10*Δ (lanes 3 and 4), *rpn10*Δ (lanes 5 and 6), and *rad23*Δ (lanes 7 and 8). Multiubiquitinated proteins were present at higher levels in *rad23*Δ *rpn10*Δ than in wild-type or single-mutant strains. The levels of multiubiquitinated proteins increased slightly in *rad23*Δ *rpn10*Δ in the presence of 3 μg/ml canavanine (even-numbered lanes). (B) The stability of Ub-Pro-βgal was measured by pulse-chase analysis in wild type, *rad23*Δ, *rpn10*Δ, and *rad23*Δ *rpn10*Δ. Actively growing yeast cells were incubated with ^{35}S-Translabel, and β-gal was immunoprecipitated from equal cpm of extract. Ub-Pro-βgal was noticeably stabilized in the double deletion relative to *rpn10*Δ and wild-type strains although there was also significant stabilization in *rad23*Δ. The asterisk represents a 90-kD degradation product. Significantly, the 90-kD fragment was detected neither in *rad23*Δ *rpn10*Δ nor *rad23*Δ.

was also revealed in studies by Johnson *et al.* (1995), who found that it is a component of the ubiquitin fusion degradation (UFD)-targeting pathway. Unlike *rad23*Δ *rpn10*Δ, we found that *rad23*Δ *son1*Δ failed to display any synthetic defects. Similarly, deletion of *RAD23* from *ufd2*Δ (*rad23*Δ *ufd2*Δ) had no adverse effects. Ufd2 was recently reported to encode a novel factor that can bind and extend short multi-Ub chains (Koegl *et al.* 1999). Ufd2 is also required for the UFD-targeting system (Johnson *et al.* 1995). These findings suggest that the genetic link between Rad23 and Rpn10 is specific, and is not a general consequence of mutating two unrelated proteolytic factors.

Several proteasome mutants, including *cim3-1, cim5-1* (Ghislain *et al.* 1993), and *nin1-1* (Kominami *et al.* 1995), display a G2/M phase cell-cycle defect. Significantly, *RPN10* was previously isolated as a high-copy suppressor of both *nin1-1* and *nin1*Δ, implicating Rpn10 in progression through mitosis (Kominami *et al.* 1997). Biggins *et al.* (1996) showed that loss of both Rad23 and Dsk2 caused cells to arrest growth at 37° with 2N DNA content because of a failure to duplicate the spindle pole body. The previous findings and our studies described here collectively suggest that Rad23 may also play an important role in cell-cycle progression, although its activity is expected to be redundant with other factors, including Rpn10 and Dsk2.

On the basis of our findings, we propose that Rad23 could augment either of the two known activities of Rpn10: the recognition of multiubiquitinated substrates by the proteasome (van Nocker *et al.* 1996b) or the stabilization of the 19S regulatory particle of the proteasome (Glickman *et al.* 1998a). Alternatively, the possibility that Rad23 might mediate a novel mechanism for translocating substrates to the proteasome is suggested by the copurification of the DNA repair protein Rad4 with Rad23 and the proteasome (Schauber *et al.* 1998a). It is not known, however, if Rad4 constitutes one of the Rad23-specific substrates. An underlying assumption of this idea is that proteolytic substrates could be targeted to the proteasome by either Rad23 or Rpn10. The translocation of these substrates or their recognition by the proteasome would fail in strains lacking both Rad23 and Rpn10, leading to the pleiotropic defects observed in *rad23*Δ *rpn10*Δ. The stabilization of Ub-Pro-βgal (Figure 8B) in *rad23*Δ *rpn10*Δ is consistent

with a requirement for both Rad23 and Rpn10 in the degradation of specific proteasomal substrates. However, because the degradation of bulk short-lived proteins is unaffected in *rad23*Δ *rpn10*Δ (Figure 6), we believe that only a subset of cellular substrates is targeted for degradation by Rad23 and Rpn10.

Recent studies have shown that several proteins contain significant sequence and structural similarity to ubiquitin (Hochstrasser 1998). Some of these UbL sequences are processed and post-translationally ligated to other cellular proteins in a reaction that is identical to ubiquitin conjugation (Johnson and Blobel 1997; Johnson *et al.* 1997). However, the significance of these modifications is not known. In contrast, the UbLs present in a distinct set of proteins that includes Rad23 and Dsk2 are not processed and ligated to other proteins (Watkins *et al.* 1993; Biggins *et al.* 1996). It was shown previously that the *rad23*Δ *dsk2*Δ double mutant has a temperature-sensitive growth defect that is caused by a failure to duplicate the spindle pole body (Biggins *et al.* 1996). This result implicates an overlapping function for Rad23 and Dsk2 that is similar to what we have observed in *rad23*Δ *rpn10*Δ. We discovered that the UbL domain in Dsk2, as well as a UbL present in an uncharacterized open reading frame (YOL111c), can interact with the proteasome (data not shown). On the basis of these findings, we speculate that Rpn10 and UbL-containing proteins might promote the degradation of distinct subsets of proteins through regulated interaction with the proteasome. The degradation of a proteolytic target *in trans* has been described previously with an engineered substrate (Johnson *et al.* 1990). A similar role is performed by antizyme, which facilitates the translocation of ornithine decarboxylase to the proteasome for degradation (Murakami *et al.* 1992a,b).

The requirement for the UbL of Rad23 for suppressing the phenotypes of *rad23*Δ *rpn10*Δ raises several interesting points. It was shown previously that a multi-Ub-chain-binding domain that is present in Rpn10 is dispensible for activity, although it is required for forming a high-affinity interaction with multi-Ub chains *in vitro* (Fu *et al.* 1998). Since the proteolytic role of UbL^{R23} was revealed only in a strain lacking Rpn10, it will be of interest to determine if the multi-Ub chain-binding domain in Rpn10 plays any role in suppressing the phenotypes of *rad23*Δ *rpn10*Δ. Additionally, Rad23, Dsk2, and Rpn10 have been linked functionally using biochemical and genetic strategies. It will therefore be important to determine if Dsk2/Rpn10 also displays a genetic relationship that is analogous to Rad23/Rpn10 and Rad23/Dsk2 (Biggins *et al.* 1996). A detailed study of the biochemical functions of these proteins, and their role during the cell cycle, can provide insights into the significance of proteolysis during the cell cycle in yeast and mammalian cells.

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