RPN4 is a ligand, substrate, and transcriptional regulator of the 26S proteasome: A negative feedback circuit

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Contributed by Alexander Varshavsky, January 16, 2001

The RPN4 (SON1, UFD5) protein of the yeast *Saccharomyces cerevisiae* is required for normal levels of intracellular proteolysis. RPN4 is a transcriptional activator of genes encoding proteasomal subunits. Here we show that RPN4 is required for normal levels of these subunits. Further, we demonstrate that RPN4 is extremely short-lived ($t_{1/2}$ ≈2 min), that it directly interacts with RPN2, a subunit of the 26S proteasome, and that *rpn4*Δ cells are perturbed in their cell cycle. The degradation signal of RPN4 was mapped to its N-terminal region, outside the transcription–activation domains of RPN4. The ability of RPN4 to augment the synthesis of proteasomal subunits while being metabolically unstable yields a negative feedback circuit in which the same protein up-regulates the proteasome production and is destroyed by the assembled active proteasome.

proteolysis | ubiquitin | N-end rule | UFD pathway | cell cycle

The *Saccharomyces cerevisiae* RPN4 gene (its earlier names are *SON1* and *UFD5*) (1) was originally identified through mutant *rpn4* alleles that suppressed the growth defect of *sec63–101* cells, which bore a temperature-sensitive (ts) variant of SEC63, an essential component of the protein translocation channel in the endoplasmic reticulum membrane (2). More recent studies have shown that mutations in *RPN4* inhibit the degradation of normally short-lived proteins that are targeted by the N-end rule pathway, by the *u*biquitin/*f*usion/*d*egradation (UFD) pathway, and apparently also by other pathways of the ubiquitin (Ub)–proteasome system (3, 4). These findings suggested that the ability of *rpn4* mutations to suppress the conditional lethality of *sec63–101* may stem from stabilization of the mutant but partially active SEC63–101 against degradation at nonpermissive temperature.

Regulated proteolysis by the Ub/proteasome system plays essential roles in the cell cycle, differentiation, stress responses, and many other processes (5–7). Ub is a 76-residue protein whose covalent conjugation to other proteins marks these proteins for degradation by the 26S proteasome, an ATP-dependent multisubunit protease. Ub conjugation involves the formation of a thioester between the C terminus of Ub and a specific cysteine of the Ub-activating (E1) enzyme. The Ub moiety of E1~Ub thioester is transesterified to a cysteine in one of several Ub-conjugating (E2) enzymes. The Ub moiety of E2~Ub thioester is conjugated via the isopeptide bond to the ε-amino group of either a substrate's Lys residue or a Lys residue of another Ub moiety, the latter reaction resulting in a substrate-linked multi-Ub chain (7, 8). Most E2 enzymes function in complexes with proteins called E3 (9–11). The functions of E3s include the initial recognition of degradation signals (degrons) in substrate proteins, with different E3s recognizing different classes of degrons (12–14). The E2–E3 complexes, referred to as Ub ligases (this term is also used to denote E3s alone), mediate the formation of substrate-linked multi-Ub chains (15, 16). Ubiquitylated substrates are processively degraded by the 26S proteasome, which consists of the 20S core proteasome and two 19S particles (17–19). *In vivo*, the 20S proteasome exists in complexes with either the 19S particle or the 11S particle (of a distinct protein composition). The latter particle stimulates the peptidase but not the protease activity of the 20S proteasome (18). One 19S and one 11S particle can be bound to each end of the same 20S proteasome, a configuration of likely physiological significance (20, 21). The 19S particle mediates the binding and ATP-dependent unfolding of a substrate protein before its transfer to the interior of the 20S core (17). The biogenesis of the 20S proteasome has been analyzed in some detail (ref. 22 and refs. therein). Most, if not all, of the genes encoding the stoichiometrically present subunits of the *S. cerevisiae* 26S proteasome have been identified (1, 23–26), but regulation of these genes remains to be understood.

Consistent with the effects of *rpn4* mutations on Ub/proteasome-dependent proteolysis, RPN4 was reported to cofractionate with a partially purified 26S proteasome (27). However, RPN4 was not detected among proteasomal subunits in other analyses of purified 26S proteasomes (23, 25). Recent work identified a specific sequence motif in the promoters of yeast proteasomal genes and demonstrated that RPN4 binds to this motif and functions as a transcriptional activator of the motif-containing promoters (28).

In the present study, we showed that RPN4 is required for normal levels of proteasomal subunits in the cell. Further, we found that RPN4 is an extremely short-lived protein, that it directly interacts with at least one specific subunit of the 26S proteasome, and that cells lacking RPN4 are perturbed in their progression through post-G_1 phases of the cell cycle. We also characterized the degron of RPN4, locating it outside the putative transcription-activating domains. Our findings indicate that the ability of RPN4 to augment the synthesis of proteasomal subunits while being metabolically unstable yields a negative feedback circuit in which the intracellular proteolysis is up-regulated by a protein that is destroyed by the assembled active proteasome.

Materials and Methods

Strains, Plasmids, and β-Galactosidase (βgal) Assay. The *S. cerevisiae* strains used were EJY140 (*MATa trp1-Δ63 ura3–52 his3-Δ200 leu2–3, 112 lys2–801 rpn4Δ::LEU2*); JD52 (*MATa trp1-Δ63 ura3–52 his3-Δ200 leu2–3, 112 lys2–801*); AVY302 (*rpn2Δ::URA3* derivative of JD52); Y791 (*MATa cim5–1 ura3–52 his3-Δ200 leu2Δ1*); MHY501 (*MATa trp1–1 ura3–52 his3-Δ200 leu2–3, 112 lys2–801*); MHY1409 (*MATa trp1–1 ura3–52 his3-Δ200 leu2–3, 112 lys2–801 uba1–2*) (3, 29, 37). *RPN1* containing its promoter region was isolated as a suppressor of the toxicity

Abbreviations: ts, temperature sensitive; Ub, ubiquitin; βgal, *E. coli* β -galactosidase; UFD, *ub*/*fusion*/*d*egradation.

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of overexpressed N-end rule pathway (30). *PRE6* (containing its promoter region) and the ORF of *RPN4* were amplified by PCR from total DNA of *S. cerevisiae* YPH500. All constructs were verified by DNA sequencing. *RPN1* and *PRE6* (bearing their promoter regions) were subcloned into the low-copy vector pRS313 (31), yielding p313RPN1 and p313PRE6. The *RPN4* ORF was subcloned into the low-copy vector pRS314CUP1 derived from pRS314 (31), yielding p314CUP1RPN4, in which *RPN4* was expressed from the P_{CUP1} promoter. For immunoblotting and immunoprecipitation, the flag epitope was added to the C termini of RPN1, PRE6, and RPN4. Alternatively, the N terminus of RPN4 was extended with the ha epitope (32). For GST-pulldown assays, the ORFs of *RPN1*, *RPN2*, *RPN9*, *RPN10*, and *RPN12* were fused in frame to the 3′-end of the GST-coding sequence in pGEX-4T-3 (Amersham Pharmacia). The C-terminally flag-tagged derivative of *RPN4* was subcloned into pET-11c (Novagen). *Escherichia coli* BL21 (DE3) was used to express GST fusions, as well as RPN4-flag. The *E. coli lac*Z gene encoding βgal lacking the first eight residues was amplified by PCR, by using pMC1871 (Amersham Pharmacia) as a template. This βgal was expressed as a fusion to the C terminus of $RPN4_{1–151}$ from the P_{CUP1} promoter and pRS315 vector (31). $Ub^{K48R,G76A}$ was expressed from the P_{CUP1} promoter and a high-copy vector (33). Arg-βgal, derived from Ub-Arg-βgal, was expressed from the P_{GAL1} promoter and a high-copy vector (3). The activity of βgal in yeast extracts, from cultures at A_{600} of 0.8–1.0, was determined as described (32).

Immunoblotting, Pulse–Chase, and GST-Pulldown Assays. *S. cerevisiae* transformants were grown to OD_{600} of 0.8–1.0, harvested, and resuspended in lysis buffer (1% Triton X-100/0.15 M NaCl/1 mM EDTA/50 mM Na-Hepes, pH 7.5) containing 1× protease inhibitor mix (Boehringer Mannheim), and lysed by vortexing with glass beads. Equal amounts of extracts were separated by SDS/PAGE, followed by immunoblotting with monoclonal anti-flag antibody (Sigma) or anti-ha antibody (Covance, Berkeley, CA). SDS/PAGE in 6, 8, and 12% gels was used with, respectively, RPN1-flag, RPN4-flag or ha-RPN4, and PRE6-flag or truncated RPN4 proteins. Pulse–chase procedures were as described (30). Briefly, 10-ml cultures (OD_{600} of 0.8–1.2) of wild-type, *rpn2*Δ, *cim5–1*, and *uba1–2 S. cerevisiae* in SD media (30) containing 0.2 mM $CuSO_4$ were labeled for 5 min with 0.15 mCi of [^{35}S]methionine/cysteine (EXPRESS, New England Nuclear). Labeled cells were pelleted, resuspended in 0.8 ml of lysis buffer, and lysed as above. The extracts were centrifuged at 12,000 × *g* for 10 min, and supernatants containing equal amounts of CCl_3COOH-insoluble ^{35}S were used for immunoprecipitation with anti-flag, anti-ha, or anti-βgal antibodies (Promega). For binding assays with GST fusions (GST-pulldown assays), see ref. 30 and the legend to Fig. 1*E*.

Cell Cycle Analysis. Flow cytometric DNA analysis was performed as described previously (34). Briefly, exponentially growing cells (OD_{600} ≈1.0) were fixed in 70% ethanol and treated with RNase A (2 mg/ml) at 37°C for 2 h. Cells were then stained with propidium iodide (50 μg/ml) and analyzed by using Becton Dickinson FACScan. Cells (≈2 × 10^4) were analyzed in each sample. For analyses of synchronous cultures, cells were grown to OD_{600} of ≈1.0 and treated with α factor (5 μg/ml) for 2 h, then washed and resuspended in yeast/peptone/dextrose (YPD medium). Light microscopic determination of the fraction of budded cells was carried out with samples taken after 2-h G_1 arrest and at 20-min intervals after release from arrest, with ≈200 cells analyzed from each sample. Synchronized cultures were also characterized by flow cytometry.

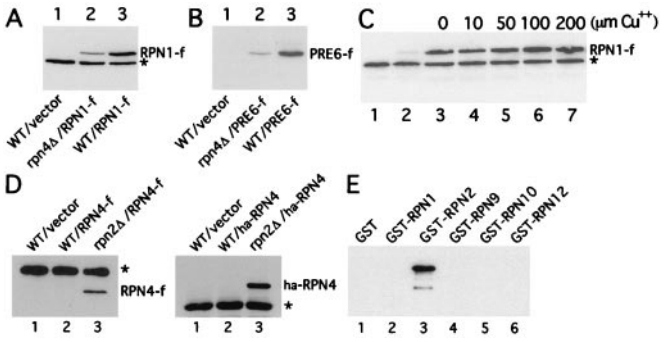

Fig. 1. RPN4 is required for normal expression of proteasomal subunits. (*A* and *B*) Immunoblot analysis of RPN1 (*A*) and PRE6 (*B*) that were C-terminally tagged with flag epitope and expressed from their native promoters and a low-copy plasmid in a *rpn4*Δ *S. cerevisiae* strain (lane 2) and its congenic wild-type counterpart JD52 (lane 3). Lane 1 in *A* and *B*, cells transformed with empty vector. (*C*) Enhanced expression of *RPN4* marginally elevates the level of RPN1. *rpn4*Δ cells were cotransformed with low-copy plasmids expressing, respectively, RPN1-flag and RPN4 from the P_{CUP1} promoter. Increasing concentrations of $CuSO_4$ were used to induce the expression of RPN4 (lanes 3–7). Lanes 1 and 2 in C, *rpn4*Δ cells transformed, respectively, with empty RPN1-flag vector and empty RPN4 vector. (*D*) Immunoblot analyses of C-terminally (RPN4-flag) or N-terminally (ha-RPN4) tagged RPN4 that was expressed from the induced P_{CUP1} promoter and low-copy plasmid either in wild-type (*RPN4*) strain JD52 or in AVY302, a congenic *rpn2*Δ mutant (see legend to Fig. 2 for details). (*E*) RPN4 interacts with RPN2 in GST-pulldown assays. Extracts of *E. coli* expressing RPN4-flag were incubated with glutathione-agarose beads preloaded with the indicated GST fusions or GST alone. The retained proteins were eluted, fractionated, and immunoblotted with anti-FLAG antibody. Approximately equal amounts of different GST fusions were immobilized on glutathione-agarose beads in these assays, as verified by Coomassie staining (data not shown). SDS/PAGE in 6, 8, and 12% gels was used, respectively, in *A* and *C*, in *D* and *E*, and in *B*. The asterisk in *A*, *C*, and *D* indicates a crossreacting band.

Results

RPN4 Is Required for Normal Expression of Proteasome Components. We initially attempted to identify *S. cerevisiae* RPN4-binding proteins by using the yeast two-hybrid assay and observed that RPN4 functioned as a transcriptional activator when fused to the DNA-binding domain of GAL4 (data not shown). This result was consistent with the finding that RPN4 binds to a DNA sequence motif present in the promoters of most genes encoding proteasomal subunits and several other genes of the Ub/proteasome system (28). We then examined the levels of two 26S proteasomal subunits, RPN1 (of the 19S particle) and PRE6 (of the 20S core proteasome) in the presence and absence of RPN4, by using immunoblotting (Fig. 1 *A* and *B*). C-terminally epitope-tagged RPN1-flag and PRE6-flag were expressed from their own promoters on a low-copy vector. The levels of RPN1-flag and PRE6-flag were significantly lower in a *rpn4*Δ strain than in a congenic wild-type strain (Fig. 1 *A* and *B*, lanes 2 and 3). Taken together with the gene expression data (28), these results indicated that RPN4 is a positive transcriptional regulator of genes encoding proteasomal subunits.

RPN4 Is a Short-Lived Protein Degraded by the 26S Proteasome. RPN4 was expressed from the copper-inducible P_{CUP1} promoter on a low-copy vector in the *rpn4*Δ strain that also expressed RPN1-flag from its natural promoter. The expression of *RPN4* from uninduced P_{CUP1} was sufficient to greatly augment the expression of RPN1-flag, but further enhancement of *RPN4* expression elevated the level of RPN1-flag only slightly (Fig. 1*C*, lanes 3–7). In agreement with this result, a strongly increased expression of *RPN4* in the wild-type (*RPN4*) background resulted in at most a slight enhancement of proteasome activity, as

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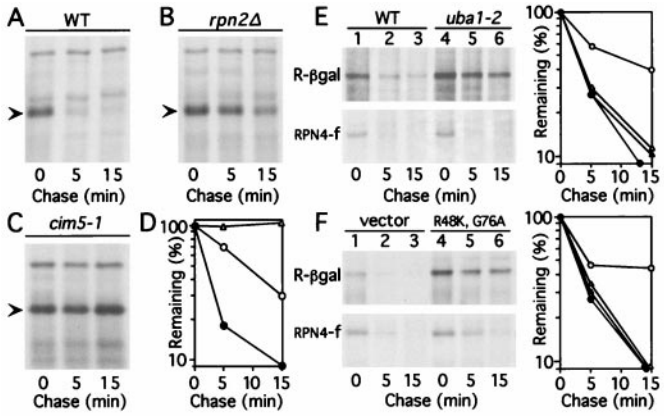

Fig. 2. RPN4 is a short-lived protein degraded by a proteasome-dependent pathway. (*A–C*) Pulse–chase analysis of C-terminally tagged RPN4 (RPN4-flag) that was expressed from the induced P_{CUP1} promoter and low-copy plasmid either in wild-type (*RPN4*) strain JD52 (*A*), in a congenic *rpn2*Δ mutant (*B*), or in a *cim5–1* mutant (*C*). Cells were labeled at 28°C and chased at 28°C in *A* and *B*, and at 37°C in *C*. The kinetics of RPN4 degradation in JD52 were similar at 28 and 37°C (data not shown). Arrowheads indicate the band of RPN4-flag. (*D*) Quantitation of the patterns in *A–C*, by using PhosphorImager (Molecular Dynamics) ●, wild-type cells. ○, *rpn2*Δ cells. △, *cim5–1* cells. (*E*) Pulse–chase analyses of Arg-βgal (R-βgal), derived from Ub-Arg-βgal (3, 14), and of RPN4-flag (expressed as in *A*) in *uba1–2* (37) and wild-type cells. Quantitation: ● and ○, Arg-βgal in wild-type and *uba1–2* cells, respectively. ▲ and △, RPN4-flag in the same strains. (*F*) The same test proteins in wild-type cells and cells overexpressing $Ub^{K48R, G76A}$, with quantitation on the right; same designations. The *rpn2*Δ locus, in the strain AVY302 (see *Materials and Methods*), was a disruption allele. It was produced through the integration of *URA3* at the *Hin*dIII site of *RPN2* (codon 146) and was identical to the *rpn2*::*URA3* allele described by Yokota *et al.* (36). The phenotypes of AVY302 and the previously described strain (36) were similar as well (data not shown). Two other studies reported that *rpn2*Δ cells were inviable (4, 56). The disruption of *RPN2* in these works was carried out with either *TRP1* or *ADE2*, by using the *RPN2 Bgl*II site at codon 38. It remains to be determined whether different integration sites account for different phenotypes described for *rpn2*Δ strains.

assessed by measuring the steady-state levels of test substrates that are targeted by either the N-end rule pathway or the UFD pathway (data not shown), both pathways being Ub/proteasome-dependent (3, 35). By contrast, expression of *RPN4* in *rpn4*Δ cells greatly increased the activity of these proteolytic pathways (data not shown).

Given these results, we expressed either a C-terminally tagged RPN4-flag or an N-terminally tagged ha-RPN4 from the P_{CUP1} promoter and determined the levels of RPN4 protein at different concentrations of the promoter-inducing $CuSO_4$. Neither of these tagged derivatives of RPN4 could be detected by immunoblotting even on the induction of P_{CUP1} (Fig. 1*D*), suggesting that RPN4 was a short-lived protein, a property that could account for the relative insensitivity of RPN4-dependent promoters to the level of transcriptional activity of an RPN4-expressing gene. Pulse–chase assays were then carried out with RPN4-flag in wild-type (*RPN4*) cells, revealing that the *in vivo* half-life of RPN4-flag was ≈2 min (Fig. 2 *A* and *D*). The half-life of N-terminally tagged ha-RPN4 was indistinguishable from that of C-terminally tagged RPN4-flag (data not shown), ruling out the epitope tags as the cause of RPN4 metabolic instability. *S. cerevisiae* cells lacking RPN2, a protein of the 19S component of the 26S proteasome, are partially defective in the degradation of natural substrates of the proteasome (36) (see also the legend to Fig. 2). Both RPN4-flag and ha-RPN4 could be detected by immunoblotting in *rpn2*Δ cells, in contrast to wild-type (*RPN2*) cells (Fig. 1*D*). Consistent with these results, pulse–chase assays indicated a significant decrease in degradation of RPN4-flag in *rpn2*Δ cells, in comparison to wild-type cells (Fig. 2 *B* and *D*). In addition, RPN4-flag was found to be long-lived in the *cim5–1* mutant (Fig. 2 *C* and *D*), which bears a ts mutation in RPT1, an essential ATPase of the 19S particle (29). We conclude that the *in vivo* degradation of RPN4 is mediated by the 26S proteasome.

RPN4 Interacts with RPN2, a Component of the 19S Particle. It was reported that RPN4 cofractionated with the 26S proteasome (27), but RPN4 was not observed in other studies of purified 26S proteasomes (23, 26). Previous work (30) used the GST-pulldown assay to demonstrate that UBR1 and UFD4, the Ub ligase (E3) components of, respectively, the N-end rule and UFD pathways, directly interact with specific subunits of the proteasome's 19S particle. We used the GST-pulldown approach to determine whether RPN4 might interact with specific subunits of the 19S particle. In these experiments, several proteins of the 19S particle were expressed in *E. coli* as fusions to the C terminus of GST. Extracts from *E. coli* expressing RPN4-flag were incubated with glutathione-agarose beads preloaded with GST-RPN1, GST-RPN2, GST-RPN9, GST-RPN10, GST-RPN12, or GST alone. The bound proteins were eluted, fractionated by SDS/PAGE, and immunoblotted with anti-flag antibody. RPN4-flag reproducibly bound to GST-RPN2 but not to any of the other tested subunits of the 19S particle (Fig. 1*E*). It remains to be determined whether RPN2 is the only proteasomal ligand of RPN4, or whether some other proteasomal subunits, among the still untested ones, also interact with RPN4.

Because the steady-state level of RPN4 in wild-type cells was sufficiently low to be undetectable by standard immunoblotting (Fig. 1*D*), RPN4 cannot be a stoichiometric component of the 19S particle. Furthermore, because RPN4 is also a transcriptional regulator (28), it is clear that the population of RPN4 molecules in a cell is dynamically partitioned among several classes of physiologically relevant complexes that include the 26S proteasome and either specific or nonspecific RPN4-binding sites on the chromosomes.

Is Degradation of RPN4 Ubiquitin-Dependent? As demonstrated above (Figs. 1*D* and 2), RPN4 is degraded by the 26S proteasome. Several lines of evidence suggested that ubiquitylation plays at most a minor role in the proteasome-dependent degradation of RPN4. First, the ≈2-min half-life of RPN4 in wild-type cells (Fig. 2*D*) was not significantly changed in the *uba1–2* mutant, which underexpresses the Ub-activating (E1) enzyme and is therefore strongly impaired in ubiquitylation of proteins (37). Specifically, whereas the degradation of Arg-βgal, a substrate of the Ub-dependent N-end rule pathway (14, 38), was decreased in the *uba1–2* mutant, no significant change was observed with RPN4-flag in this mutant (Fig. 2*E*). Second, overexpression of $Ub^{K48R,G76A}$, a Ub mutant that inhibits the formation of Lys^{48}-linked multi-Ub chains, which are essential for a large fraction of the proteasome-dependent proteolysis (33, 39), did not significantly decrease the rate of RPN4 degradation but did decrease the degradation of Arg-βgal (Fig. 2*F*). Third, the kinetics of degradation of RPN4-flag in wild-type cells were indistinguishable from that in mutants lacking one of the following Ub-conjugating (E2) enzymes: UBC1, RAD6 (UBC2), CDC34 (UBC3), UBC4, UBC5, UBC6, UBC7, or UBC8 (data not shown). Analogous pulse–chase assays were also carried out with mutants lacking different pairs of these E2 enzymes. The degradation of RPN4 was slightly decreased in [*ubc4*Δ *ubc5*Δ] cells, which lacked UBC4 and UBC5, two highly similar E2s (data not shown). Because UBC4/UBC5 are a functionally major class of E2 enzymes in *S. cerevisiae* (7), and because [*ubc4*Δ *ubc5*Δ] cells grow slowly and exhibit a number of defects, the observed marginal stabilization of RPN4 in a [*ubc4*Δ *ubc5*Δ] strain could be an indirect result of multiple changes that are caused by the absence of UBC4/UBC5. Fourth, the degradation of RPN4 was not decreased in *S. cerevisiae* mutants that lacked

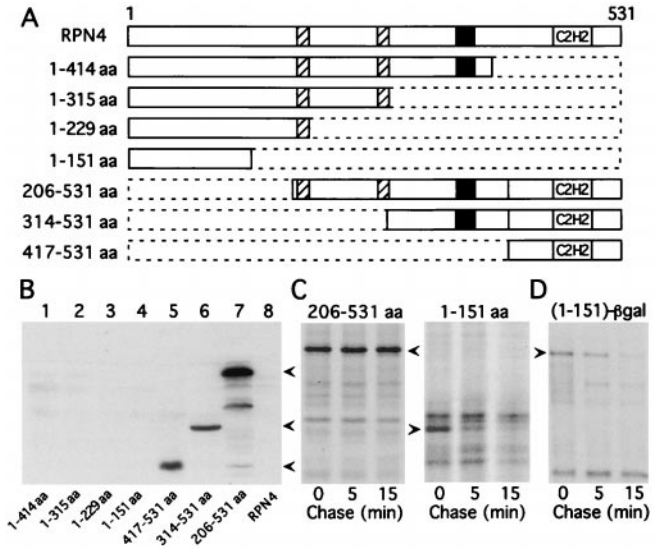

Fig. 3. The N-terminal region of RPN4 contains a portable degradation signal. (*A*) Diagrams of full-length and truncated RPN4. Two putative transcription activation domains are at positions 211–229 and 300–315 (hatched boxes). A putative bipartite nuclear localization signal is at position 381–399 (black box). A putative C_2H_2 finger (residues 477–507) is also indicated. (*B*) N-terminally truncated RPN4 derivatives are long-lived. RPN4 and its truncated derivatives were expressed from the P_{CUP1} promoter and low-copy vector in the JD52 (*RPN4*) strain. Relative steady-state levels of truncated RPN4 proteins (all of them tagged C-terminally with flag epitope) were determined by immunoblotting, by using SDS/12% PAGE and anti-flag antibody. The bands of proteins with expected sizes are indicated by arrowheads. (*C*) $RPN4_{1–151}$-flag is short-lived. Pulse–chase assays, by using SDS/12% PAGE, were carried out in wild-type (*RPN4*) cells with $RPN4_{206–531}$-flag and $RPN4_{1–151}$-flag (indicated by arrowheads) essentially as described in Fig. 2, except that they were performed at 30°C instead of 28°C. (*D*) The 151-residue N-terminal fragment of RPN4 contains a portable degron. Pulse–chase assay with $RPN4_{1–151}$-βgal fusion (see *Materials and Methods*) expressed from the P_{CUP1} promoter and low-copy vector in the JD52 (*RPN4*) strain, by using SDS/6% PAGE and immunoprecipitation with anti-βgal antibody.

one of the following E3 enzymes: UBR1, UFD4, RSP5, TOM1, HUL4, or HUL5 (data not shown) (3, 14, 40, 41). Thus, it appears, but remains to be established definitively, that the proteasome-dependent degradation of RPN4 (Fig. 2 *A* and *D*) is either largely or completely independent of RPN4 ubiquitylation.

N-Terminal Region of RPN4 Contains a Portable Degron. The degradation signals of transcriptional activators are often located within their activation domains (42–45). We constructed a set of truncated, C-terminally tagged RPN4 derivatives that contained either two, one, or none of the putative transcription-activation domains of the intact RPN4 (Fig. 3*A*). Whereas wild-type RPN4 was undetectable by immunoblotting, because of its rapid *in vivo* degradation (Figs. 1*D* and 2 *A* and *D*), RPN4 derivatives such as $RPN4_{206–531}$, $RPN4_{314–531}$, and $RPN4_{417–531}$, which lacked at least the first 205 residues of RPN4, were readily detectable (Fig. 3*B*, lanes 5–8). By contrast, similarly expressed truncated derivatives that retained this N-terminal region of RPN4 ($RPN4_{1–414}$, $RPN4_{1–315}$, $RPN4_{1–229}$, and $RPN4_{1–151}$) were still largely undetectable by immunoblotting (Fig. 3*B*, lanes 1–4), consistent with the interpretation that the major degron of RPN4 was located within its first 150–200 residues. This region does not contain putative transcription-activation domains (Fig. 3*A*).

Pulse–chase assays with $RPN4_{1–151}$-flag (containing the presumed degron of RPN4) and $RPN4_{206–531}$-flag (lacking this degron) confirmed the inferences from immunoblotting data: $RPN4_{1–151}$-flag was rapidly degraded ($t_{1/2}$ ≈2 min), whereas $RPN4_{206–531}$-flag was long-lived (Fig. 3*C*). The degron of RPN4 is portable, in that ligation of $RPN4_{1–151}$ to the otherwise long-lived 115-kDa βgal moiety resulted in a metabolically unstable $RPN4_{1–151}$-βgal protein ($t_{1/2}$ ≈5 min) (Fig. 3*D*). No sequence similarities between the 151-residue region of RPN4 and the known motifs that act as degrons in other short-lived proteins (13) could be detected, suggesting that this region of RPN4 contains a degradation signal which, to our knowledge, is novel. Interestingly, the C-terminally truncated, degron-containing, metabolically unstable $RPN4_{1–229}$ did not bind to RPN2 of the 19S particle in the GST-pulldown assay (data not shown), in contrast to full-length RPN4 (Fig. 1*E*), suggesting that the *in vivo* degradation of intact RPN4 ($t_{1/2}$ ≈2 min) may be independent of the demonstrated RPN4–RPN2 interaction.

Cells Lacking RPN4 Exhibit Delay in Post-G_1 Phases of the Cell Cycle. Despite the decreased expression of proteasomal components in *rpn4*Δ cells (Fig. 1), they were not only viable but also similar to congenic wild-type cells in their resistance to ≈260-nm UV light and in their ability to grow at high temperature (37°C), on poor nitrogen sources (proline and citrulline), and in the presence of canavanine (a toxic arginine analog, at 1.5 μg/ml), NaCl (at 1 M), $CuSO_4$ (at 0.5 mM), or ethanol (at 3%) (data not shown). At the same time, *rpn4*Δ cells are known to grow more slowly at 30°C on standard media than wild-type cells [(2–4); unpublished data]. To assess the effect of RPN4 absence on cell cycle progression, we carried out flow cytometric analyses of DNA content with unsynchronized exponentially growing cultures of haploid *rpn4*Δ and congenic wild-type cells. The fraction of cells with 2*N* (replicated) DNA content was significantly higher in *rpn4*Δ culture (Fig. 4*A*). Light microscopic examination indicated a significantly higher fraction of budded (post-G_1) cells in exponentially growing *rpn4*Δ cultures, in comparison to wild-type ones (data not shown), suggesting that cells lacking RPN4 are delayed in their progression through one or more of post-G_1 phases of the cell cycle.

To address this question in greater detail, *rpn4*Δ and congenic wild-type cultures were synchronized in G_1 phase with α factor, followed by flow cytometric analysis of cellular DNA content as a function of time after release from G_1 arrest. As shown in Fig. 4*B*, *rpn4*Δ cells exited G_1 phase without delay relative to wild-type cells. However, it took longer for *rpn4*Δ cells to progress through the rest of the cell cycle (Fig. 4*B*). Light microscopy was used to determine the fraction of budded (post-G_1) cells in synchronized cultures at different times after resumption of growth. Consistent with the results of flow cytometric analysis, the budding of *rpn4*Δ and wild-type cells occurred at approximately the same time (peak at ≈80 min) (Fig. 4*C*). However, it took longer, on average, for the budded *rpn4*Δ cells to complete cell division (≈140 min for *rpn4*Δ cells versus ≈120 min for wild-type cells). Note also that a smaller fraction of budded cells exited from M to G_1 in the *rpn4*Δ culture than in the wild-type culture (Fig. 4*C*), consistent with the data for unsynchronized cultures (Fig. 4*A*). Because the ratio of small- to large-budded cells was essentially the same for the synchronized wild-type and *rpn4*Δ cultures at different time points (data not shown), it is likely that the absence of RPN4 affects more than one post-G_1 step, e.g., both the S → G_2 and G_2 → M transitions.

Discussion

RPN4 was originally identified as an extragenic suppressor of *sec63-101*, a ts allele of *SEC63*, which is required for the translocation of proteins into the endoplasmic reticulum and the transport of proteins to the nucleus (2). *rpn4* mutations suppressed the ts growth defect of *sec63-101* but did not reverse its phenotype of mislocalization of proteins that bore a nuclear targeting signal. Moreover, *rpn4* mutants themselves had a

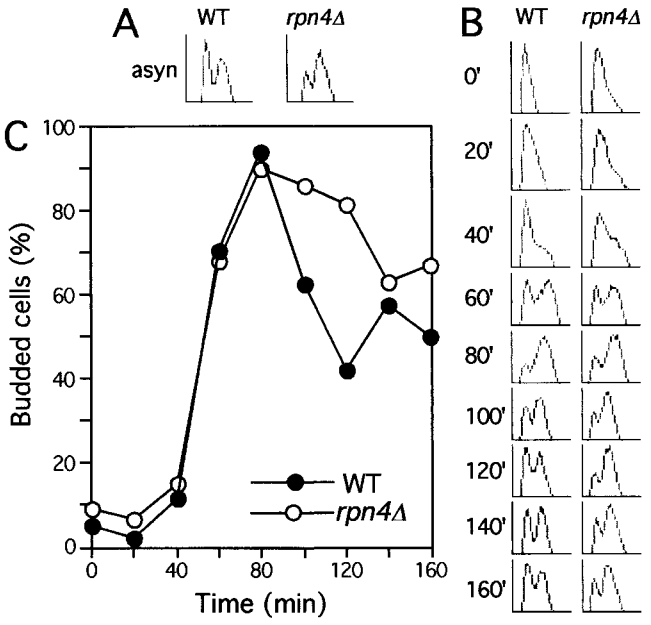

Fig. 4. Cell cycle progression is delayed after G_1 phase in *rpn4*Δ mutant. (*A*) A larger fraction of *rpn4*Δ cells have 2*N* DNA content in unsynchronized cultures. DNA flow cytometry was carried out with wild-type (*RPN4*) and *rpn4*Δ cells. The abscissa ordinate indicate, respectively, the number of cells and relative DNA content. (*B* and *C*) Cell cycle progression of *rpn4*Δ cells is delayed after G_1 phase. Wild-type and congenic *rpn4*Δ cells were synchronized in late G_1 by using α factor. Initial samples were taken after 2 h of G_1 arrest, and subsequent samples were taken at 20-min intervals after release from the arrest. The samples were analyzed by using DNA flow cytometry (*B*) and by determining the fraction of budded cells (*C*). Data shown in *C* are the means of triplicate measurements.

defect in the nuclear protein import (2). Later work has shown that mutations in *RPN4* inhibit the activity of the Ub/proteasome-dependent N-end rule and UFD proteolytic pathways (3). Our findings (Fig. 1 *A* and *B*) and a recent study (28) demonstrated that RPN4 is required for normal expression of subunits of the 26S proteasome and several other components of the Ub system. A parsimonious interpretation is that the observed down-regulation of the Ub/proteasome-dependent proteolysis in *rpn4*Δ cells results primarily from decreased expression of proteasomal subunits and functionally related proteins.

Previous studies did not reach a consensus on whether RPN4 is a component of the 26S proteasome (23, 24, 26, 27). A direct and specific interaction between RPN2 (a stoichiometric subunit of the 19S particle) and RPN4 was demonstrated in the present work (Fig. 1*E*). This result, together with the earlier finding that the E3 enzymes UBR1 and UFD4 interact with specific subunits of the 19S particle (30) and the evidence for interactions between other E2/E3 enzymes and the 26S proteasome (23, 46), indicates that proteasome is at least a transient ligand of many cellular proteins.

An operationally useful definition of a bona fide subunit of the 26S proteasome should stipulate that a large (predetermined) fraction of the proteasome particles in a cell is associated with this subunit *in vivo*. By contrast, and similarly to terminology in the ribosome field, specific protein ligands of the proteasome that interact with it transiently and/or are bound to small, dynamically determined subsets of the 26S proteasome, can be called proteasome-interacting proteins. In this terminology, RPN4, UBR1, and UFD4 are proteasome-interacting proteins, as distinguished from the 26S proteasomal subunits such as PRE6, RPT6, or RPN2.

RPN4 is not essential for cell viability under normal conditions. As described above, *rpn4*Δ cells are not hypersensitive to a variety of physical and chemical stresses, suggesting that a strongly decreased concentration of the 26S proteasome in *rpn4*Δ cells is sufficient to maintain cell viability and growth even under conditions of stress. At the same time, given the diminished activity of at least the N-end rule and UFD pathways in *rpn4*Δ cells (3), one would expect an impairment of some physiological functions that require these pathways. The post-G_1 abnormality in the cell cycle progression of *rpn4*Δ cells described in the present work (Fig. 4) is unlikely to be caused by inhibition of the N-end rule pathway, because *ubr1*Δ cells, which lack this pathway, lose chromosomes at a greatly increased frequency but are similar to congenic wild-type cells in the kinetics of cell cycle progression (47). [The chromosome-loss phenotype of *ubr1*Δ cells is caused largely by metabolic stabilization of the ESP1-produced fragment of SCC1, a prosubstrate of the N-end rule pathway and a component of the chromosome-bound cohesin complex (47).] Because RPN4 functions as a transcriptional activator of many nonproteasomal genes as well (48), the cell cycle defect of *rpn4*Δ cells may stem from down-regulation of these other genes. For example, the expression of *CDC48*, which is essential for the cell cycle progression and encodes an AAA-type ATPase, is decreased in *rpn4*Δ cells (28). However, we found that overexpression of *CDC48* from a heterologous (P_{MET25}) promoter did not rescue the cell cycle abnormality of *rpn4*Δ cells (data not shown).

One of our main results is the striking metabolic instability of RPN4 ($t_{1/2} \approx 2$ min). The degradation of RPN4 is proteasome-dependent (Fig. 2 *A–D*). At the same time, several lines of evidence (Fig. 2 *E* and *F*; see also above) suggest that degradation of RPN4 is largely independent of ubiquitylation. Proteins whose *in vivo* degradation is proteasome-dependent but Ub-independent include ornithine decarboxylase (49) and the cyclin-dependent kinase inhibitor $p21^{Cip1}$ (50, 51).

The mapping of RPN4 degron localized it to the first 150 residues of the 531-residue RPN4 (Fig. 3), outside of its putative transcription activation domains. This location of a degron is an exception to the previously established pattern in which the activation domains and degrons tend to overlap in a transcriptional regulator (42–45). As to the mechanics of RPN4 degron, the properties of RPN4 suggest two possibilities. The N-terminal degradation signal of RPN4 may function as a canonical bipartite degron of the Ub/proteasome system (12, 13, 52). The first determinant of such a degron is bound by a degron-specific E2-E3 Ub ligase, whereas the second determinant is an internal Lys residue of a substrate protein. Alternatively, RPN4 might be targeted for degradation directly through its demonstrated interaction with RPN2, a subunit of the 19S particle (Fig. 1*E*). This model is made unlikely (but not definitively precluded) by the fact that the degron-containing, short-lived $RPN4_{1–229}$ fragment was unable to bind to RPN2, in contrast to full-length RPN4 (see above).

Metabolic instability of RPN4 identifies it as a member of a growing class of components of the Ub/proteasome system that are also substrates of this system and are destroyed by it, either constitutively or conditionally. One example is UMP1, a chaperone that is required for efficient assembly of the 20S proteasome and becomes its first substrate on the completion of assembly (22, 53). Other examples are *S. cerevisiae* F-box proteins CDC4, GRR1, and MET30, which are short-lived substrate-recognition subunits of, respectively, SCF^{Cdc4}, SCF^{Grr1}, and SCF^{Met30} Ub ligases (54, 55).

The unique ability of RPN4 to augment the transcription of genes encoding proteasomal subunits while being metabolically unstable yields a negative feedback circuit in which the same protein up-regulates the proteasome production and is destroyed by the assembled active proteasome.

We are grateful to Xiaofeng Qin and Wenge Lu for advice and assistance with flow cytometric analysis, to Christopher Byrd (California Institute of Technology), Jürgen Dohmen (University of Cologne, Cologne, Germany), Mark Hochstrasser (Yale University, New Haven, CT), Jon Huibregtse (Rutgers University, Piscataway, NJ), Erica Johnson (Thomas Jefferson University, Philadelphia, PA), Yoshiko Kikuchi (University of Tokyo, Tokyo, Japan), Robert Swanson (University of Chicago, Chicago, IL), Glenn Turner (California Institute of Technology), and Fred Winston (Harvard Medical School, Boston, MA) for strains and constructs, and to Keiji Tanaka (Institute of Medical Science, Tokyo) for anti-RPN4 antibody. This work was supported by grants to A.V. from the National Institutes of Health (NIH) (DK39520 and GM31530). Y.X. was supported in part by a postdoctoral fellowship from the NIH.

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