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Localization to the Proteasome Is Sufficient for Degradation*

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The majority of unstable proteins in eukaryotic cells are targeted for degradation through the ubiquitin-proteasome pathway. Substrates for degradation are recognized by the E1, E2, and E3 ubiquitin conjugation machinery and tagged with polyubiquitin chains, which are thought to promote the proteolytic process through their binding with the proteasome. We describe a method to bypass the ubiquitination step artificially both *in vivo* and in a purified *in vitro* system. Seven proteasome subunits were tagged with Fpr1, and fusion reporter constructs were created with the Fpr1-rapamycin binding domain of Tor1. Reporter proteins were localized to the proteasome by the addition of rapamycin, a drug that heterodimerizes Fpr1 and Tor1. Degradation of reporter proteins was observed with proteasomes that had either Rpn10 or Pre10 subunits tagged with Fpr1. Our experiments resolved a simple but central problem concerning the design of the ubiquitin-proteasome pathway. We conclude that localization to the proteasome is sufficient for degradation and, therefore, any added functions polyubiquitin chains possess beyond tethering substrates to the proteasome are not strictly necessary for proteolysis.

ATP-dependent protease complexes degrade the majority of unstable cellular proteins, a process that is conserved across all three kingdoms of life. These molecular machines function both generally in protein turnover and specifically in the regulation of processes such as transcription, apoptosis, antigen presentation, and cell cycle progression (1). A high degree of conservation is evident among them; the archaebacterial and eukaryotic 20S proteolytic core particles share both sequence and structural homology (2), whereas eubacteria have functionally related complexes: ClpYQ, ClpXP, and ClpAP (3–5). The 20S core particle is composed of four stacked heptameric rings structured in an α-β-β-α configuration. Access to the proteolytic central chamber is obstructed at both ends of the cylindrical assembly by N-terminal projections of the α-subunits, thus preventing uncontrolled proteolytic degradation (5, 6). In eukaryotes, docking with the 19S regulatory particle (RP)[1] to form the complete 26S proteasome is sufficient to relieve this block, opening a channel into the core (6, 7).

Eukaryotes have evolved an elaborate system that operates in conjunction with the proteasome to facilitate the temporal and specific regulation of intracellular proteolysis. Most proteins are targeted for degradation through ubiquitination, mediated by the E1, E2, E3 ubiquitin (Ub) conjugation machinery. These three consecutively acting enzymes are necessary for target recognition, transfer of a ubiquitin moiety to the substrate, and subsequent elongation of the ubiquitin branched chain (8). Modularity and the large number of E2 Ub-conjugating enzymes and E3 Ub ligases allow for greater specificity and flexibility in recognizing a diverse range of substrates. Once a protein is polyubiquitinated, it is targeted to and degraded by the 26S proteasome.

The polyubiquitin chain is thought to play two possible roles. The first is to target the protein to the proteasome; the second is to initiate the process of degradation. The targeting hypothesis is supported by the identification of several proteasome subunits that either bind or crosslink to ubiquitin chains (9, 10). Hypotheses for how ubiquitin-dependent initiation of degradation might occur include allosteric regulation, channel opening, and assistance in the unfolding of the target (11). However, little data have been reported to support these ideas.

The elucidation of the mechanism for proteolysis of ornithine decarboxylase (ODC) established that polyubiquitination is not necessary for proteasome-mediated degradation (12). ODC is an enzyme whose degradation is mediated by its binding to the cofactor antizyme 1 (AZ1). Once bound, ODC-AZ1 can be recognized by the proteasome, and ODC is degraded in an ubiquitin-independent manner. However, it is unclear whether the means by which AZ1 promotes degradation differs fundamentally from that of polyubiquitin chains. A recent study demonstrated that ODC-AZ1 competes with substrate-linked and free polyubiquitin chains for the occupancy of the same binding site on the proteasome (13). Thus, the mechanism for the degradation of ODC seems to represent a specialized evolutionary adaptation that closely mimics ubiquitination.

The binding of ubiquitin-conjugated substrates (or ODC-AZ1) to the proteasome may itself serve as the activation step in proteolytic degradation. We hypothesize that such an activation step is not necessary, that localization to the proteasome can be sufficient for degradation. In this study, we support this hypothesis by demonstrating proteasome-mediated degradation both *in vivo* and in a purified *in vitro* system, while artificially bypassing the need for ubiquitin-dependence.

EXPERIMENTAL PROCEDURES

Construction of Parental Strain DY001—All experiments were performed in derivatives of strain DY001 to ensure that the components of the heterodimerization system would minimally interact with endogenous proteins, thus preventing cell cycle arrest and mislocalization of the reporter upon the addition of rapamycin (14). The Fpr1-rapamycin binding domain (nucleotides 5656–6243) of the dominant allele *TOR1*-2 was amplified from strain JHY17–9C (15) and subcloned into the integrating plasmid pRS306 (16). This vector was then digested with HindIII to cut once within *TOR1*-2 and transformed into the strain BY4742 Δ*fpr1*::kan^r (Research Genetics). Integration and subsequent loop-out was selected for on the appropriate plates. The correct strain was verified by PCR and sequencing.

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[1] The abbreviations used are: RP, regulatory particle; Ub, ubiquitin; ODC, ornithine decarboxylase; AZ1, antizyme; HA, hemagglutinin; Tor, (Tor1$^{1883–2078}$); DTL, degradation through localization.

Genomic Tagging of Proteasome Subunits—Tagging of proteasome subunits was performed both by homologous recombination of linear fragments containing 40 bp of flanking homology to the target site and by two-step integration with a non-replicating plasmid.

FPR1 was amplified by PCR from the strain FY4 (17) and subcloned into the plasmid pUG-sp*HIS5* (18) with a C-terminal hemagglutinin (HA)-tag, forming *FPR1*-HA-pUG-sp*HIS5*. Integration primer pairs were designed for tagging each of four proteasome subunits (*PRE10*, *RPN2*, *RPN6*, *RPN11*). For each pair, one primer contained 40 bp of genomic homology to the 3′ end of the proteasome subunit, excluding the stop codon, and 20 bp of homology to the 5′ end of *FPR1* on pUG-sp*HIS5*, excluding ATG. The second primer contained 40 bp of genomic homology ~50 bp downstream of the proteasome subunit gene stop codon and 20 bp of homology to pUG-sp*HIS5* immediately downstream of the sp*HIS5* marker flanked by *lox*P sites. Two confirmatory primers were also designed that flanked the integration site of each proteasome subunit. Strain DY001 was transformed with pSH47, a plasmid with a galactose-inducible *cre* gene and a *URA3* selection marker (19). A 2-kb linear fragment from *FPR1*-HA-pUG-sp*HIS5* was amplified using each integration primer pair to generate linear 2-kb fragments suitable for genomic integration. 15 μg of each fragment was transformed into DY001 carrying pSH47, and selection was performed on SC-URA-HIS. Colonies were then picked and streaked onto SC-URA GAL to induce *cre* and to select for the loopout of the sp*HIS5* marker. Colonies were finally streaked onto a 5-fluoroorotic acid-containing plate to remove pSH47. All tagged subunits were verified by sequencing.

Approximately 400–500 bp of the carboxyl-terminal end (without the stop codon) and 3′ untranslated region of proteasome subunits *RPT2*, *RPT5*, and *RPN10* were amplified from strain FY4. Each pair was subcloned into the integration plasmid pRS306 (16) along with *FPR1* so that the final structure at the cloning site was 5′-proteasome subunit C-term-*FPR1*-proteasome subunit UTR. Each derivative of pRS306 was cut at a unique site within the carboxy terminus of the proteasome subunit and transformed into DY001. Selection for integration was done on SC-URA; loopout of the marker was on 5-fluoroorotic acid-containing plates. All tagged subunits were verified by sequencing. Δ*pdr5* strains were generated by recombination of a *URA3* marker flanked by 40 bp homologous to sequence immediately 5′ and 3′ to genomic *PDR5*.

Preparation of Reporter Plasmids—All versions of the reporters were derivatives of the vector pRS415 (16). *TOR1*(S1972R) is an allele of *TOR1* that has a severely impaired binding affinity to FPR1-rapamycin (14). The sequence corresponding to amino acids 1883–2078 for both Tor1 and Tor1(S1972R) were amplified and inserted into the vector pRS415 along with *HIS3* amplified from strain FY4. HA-tagged versions of the reporters had the hemagglutinin epitope-fused C-terminal to His3.

Screen and Western Assays—In both assays, rapamycin was added to a final concentration of 1 μM, cycloheximide was added to a final concentration of 30 ng/ml, and PS-341 was added to a final concentration of 200 μM. For liquid cultures, samples were extracted at fixed time intervals (0, 30, 60, 90 min) and whole-cell extracts were made. 25 μg of total protein was used from each sample, resolved by SDS-PAGE, and transferred to polyvinylidene difluoride membranes. Membranes were blocked with 10% powdered nonfat milk in phosphate-buffered saline plus Tween overnight at 4 °C and incubated with anti-HA (3F10) primary antibody (Roche Applied Science) and then HP-conjugated anti-rat secondary antibody. Visualization was done using Amersham Biosciences ECL Western blotting system.

Purification of the Proteasome—26S proteasomes were affinity-purified from strains Pre10-FPR1 and Rpn10 FPR1 using the Rpn11-TEV-ProA tag described previously (20). Cells were grown in YPD, harvested, resuspended in 50 mM Tris-HCl, pH 8, 1 mM EDTA, and lysed by French press. Lysates were centrifuged at 15,000 × *g* for 25 min, filtered, and incubated with IgG resin (ICN) for 1 h at 4 °C. Resin was collected in a column, washed with 100 bed volumes of 50 mM Tris-HCl, pH 8, 50 mM NaCl, 1 mM EDTA buffer and equilibrated with TEV-protease buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol). Elution was performed by incubating the resin in 1.5 bed volumes of TEV-protease buffer containing 50 units of His^6-TEV-protease per ml of resin, at 30 °C for 1 h. One single homogeneous fraction was collected and aliquoted for each prep. The integrity of the 26S complexes and the presence of Pre10-FPR1- and Rpn10-FPR1-tagged subunits was analyzed by Coomassie Blue staining, SDS-PAGE, and immunodetection.

In Vitro Assay—HA-tagged *TOR-HIS3* and TOR_{S1972R}-*HIS3* were cut out of their respective pRS415 vectors, inserted into PROTet.133, and transformed into DH5αPRO cells (BD Biosciences). A 10-ml overnight culture was used to inoculate 1 liter of media, which was subsequently grown for 4 h at 37 °C. The fusion protein production was induced with 100 ng/ml of anhydrotetracycline, and the culture was grown for 4 h at 30 °C. Isolation of purified protein was done according to the manufacturer's instructions, using a bead bed volume of 250 μl. 1.5 ml of the eluate was dialyzed against 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, and concentrated to ~1 mg/ml. The assays were performed in activity buffer (50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM EDTA, 5 mM ATP) containing 4 μg of proteasome and 1.6 μg of Tor-His3 or Tor_{S1972R}-His3, in an initial volume of 100 μl, at 30 °C. Heterodimerization was induced with 1 μM rapamycin, and proteasome activity was inhibited with 100 μM PS-341. Time course fractions were obtained by extraction of equal volumes from the reaction tub in regular intervals. Full details and all primer and plasmid sequence information can be accessed on the World Wide Web at arep.med.harvard.edu/cKD/default.htm.

RESULTS

Chemical inducers of dimerization are a class of reagents that facilitate the regulated association of any two proteins. They have been used in a number of applications such as localization of proteins to subcellular domains, triggering of signal transduction cascades, and control of gene expression (21–23). To test the hypothesis that localization to the proteasome is sufficient for degradation, we modified and utilized such a system in *Saccharomyces cerevisiae*. One part of the heterodimerizing pair of modules is fused to the proteasome, whereas the complementary module is fused to the reporter to be tested for degradation. Addition of the chemical dimerizer in an experimental system brings the two modules together, thus localizing the reporter to the site of the proteasome. If degradation occurs as a result of the drug-induced association, it can be monitored with the appropriate assay (Fig. 1*a*).

In *S. cerevisiae*, the lipophilic macrolide rapamycin has been shown to bind with high affinity to the protein Fpr1, and this complex in turn binds to the ligand-binding domain of Tor1 ($Tor1^{1883–2078}$, or hereafter called Tor) (15). Seven proteasome subunits (Rpn2, Rpt2, Rpt5, Rpn6 Pre10, Rpn10, Rpn11) that range in distance from the 20S proteolytic core were C-terminally fused with Fpr1 (Fig. 1*b*). Strains bearing FPR1-tagged Rpt2, Rpn6, and Rpn11 subunits could not be recovered apparently because of their lethality (data not shown). DY001 strains with Rpn2, Rpt5, Pre10, and Rpn10 FPR1-tagged subunits (henceforth referred to as strain *subunit*-FPR1) were viable and contained correctly integrated fragments, as determined by PCR, and exhibited wild-type expression levels, as determined by Western blotting (data not shown).

A Screen for Growth-deficient Phenotypes—A gradient growth assay was used to screen the viable strains containing Fpr1-tagged proteasome subunits for degradation-through-localization (DTL) candidates. The auxotrophic marker *HIS3* encodes a protein involved in histidine biosynthesis and is necessary for growth on histidine-dropout media. All of the Fpr1-tagged strains used in this assay have their chromosomal copy of *HIS3* deleted and therefore require exogenous expression of functional His3 for growth. Two reporter constructs were designed for use in the screen. The reporter Tor-His3 is an amino-terminal fusion of the heterodimerizing module Tor with full-length His3 (Fig. 1*c*). The control reporter Tor_{S1972R}-His3 replaces wild-type Tor with a missense mutant that has a decreased affinity for the rapamycin-Fpr1 complex (14).

Identification of DTL candidates was based on the comparative growth of strains on histidine-dropout solid media, with or without rapamycin. Fpr1-tagged strains that expressed the control reporter Tor_{S1972R}-His3 were not expected to show any difference in growth between the two plates; the fusion protein would not bind rapamycin and, therefore, should not be directed to the proteasome. Tor-His3 binds rapamycin and, therefore, should have an increased association with the proteasome in the presence of the drug. If this association was sufficient for

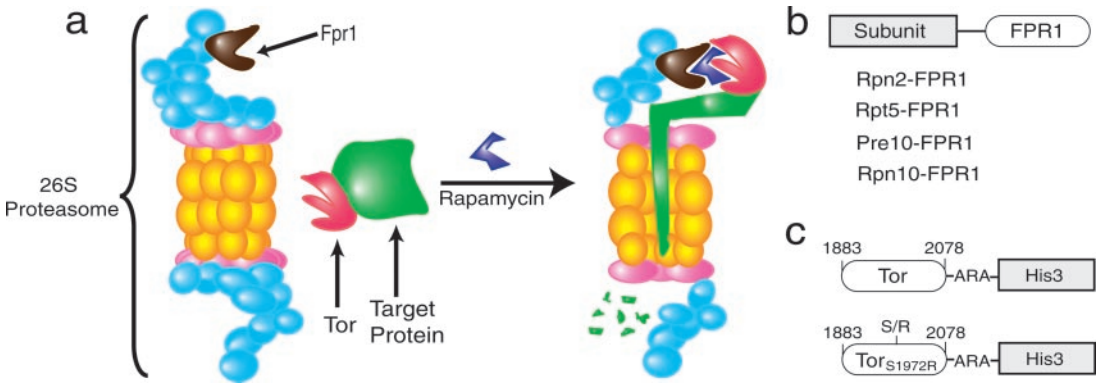

FIG. 1. **Experimental design and fusion constructs for testing whether localization to the proteasome is sufficient for degradation.** *a*, one part of the heterodimerizing pair (Fpr1) is fused to a proteasome subunit. The other part (Tor) is fused to a reporter protein. Heterodimerization of the modules occurs upon addition of the small molecule rapamycin. This brings the reporter protein into close proximity to the proteasome. *b*, the heterodimerizing module *FPR1* was genomically fused immediately downstream of seven proteasome subunits in the strain DY001. Four of the derivative strains were viable. *c*, reporter proteins consist of an N-terminal fusion of the heterodimerizing module Tor to the selective marker His3. The non-heterodimerizing control reporter has an S1972R mutation in Tor that disrupts binding to the Fpr1-rapamycin complex.

degradation, Tor-His3 containing strains would be expected to display a growth-deficient phenotype on the histidine-dropout plates with rapamycin.

The untagged DY001 parental and the four viable Fpr1-tagged strains were individually transformed with the experimental and control reporter constructs. All strains grew equally well on control plates containing histidine (data not shown). Each transformant was then spotted as a 10-fold dilution series (10^3-10^5) on two sets of experimental plates: histidine-dropout media either containing or lacking rapamycin (Fig. 2). Strain Rpn10-FPR1 displayed the most striking rapamycin-dependent phenotype. Strain Pre10-FPR1 had a milder growth-deficient phenotype that was still significant and reproducible. Strains Rpn2-FPR1 and Rpt5-FPR1 did not show comparative growth phenotypes consistent with degradation of the reporter constructs. Possible uses for these latter two strains will be addressed under "Discussion."

Western Assays Confirm DTL—The aforementioned growth assay was a convenient tool for isolating strains to test the main hypothesis. However, on its own, the growth assay did not prove that localization was sufficient for degradation because His3 function could have been compromised in a rapamycin-dependent but degradation-independent manner. To address this point, degradation of the reporters was measured directly. Strains Pre10-FPR1 and Rpn10-FPR1 were singly transformed with plasmids that expressed HA epitope-tagged versions of Tor-His3 and Tor_{S1972R}-His3. The transformants were grown in liquid culture to early log phase, whereupon cycloheximide was added to halt protein translation. Each culture was then split into two parts, and rapamycin was added to one part. Samples were collected at various times, and whole-cell protein extracts were made and used for Western analysis (Fig. 3, *a*–*e*). Consistent with our hypothesis, both strains, Pre10-FPR1 and Rpn10-FPR1, displayed an increase in the rate of degradation of the reporter Tor-His3 when rapamycin was added to the cultures, with half-lives of ~20–30 min (Fig. 3*f*). No difference in the rate of degradation was seen with Fpr1-tagged strains expressing the mutant reporter, Tor_{S1972R}-His3. To demonstrate that degradation of the reporter was mediated by the proteasome and not processed through other pathways, the experiments were repeated with the addition of the proteasome inhibitor PS-341 (Fig. 3, *g* and *h*; Ref. 24). As expected, addition of PS-341 halted degradation of Tor-His3, even in the presence of rapamycin. These experiments confirm that the growth-deficient phenotype seen on histidine-dropout media was due to the degradation of the Tor-His3 reporter, and that the 26S proteasome is necessary for this to occur.

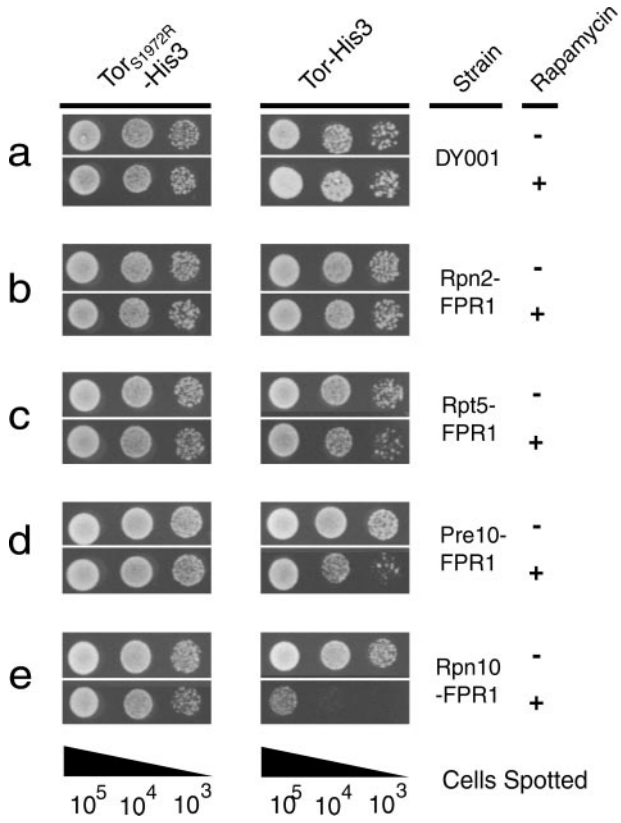

FIG. 2. **Screen for Fpr1-tagged proteasome strains that exhibit growth-deficient phenotypes.** *a*, the DY001 control strain grows equally well when expressing either Tor-His3 or Tor_{S1972R}-His3, in the presence or absence of rapamycin. *b* and *c*, strains Rpn2-FPR1 and Rpt5-FPR1 show little or no difference in growth under all conditions. *d* and *e*, strains Pre10-FPR1 and Rpn10-FPR1 show decreased growth when expressing Tor-His3 and spotting on media containing rapamycin.

Purified Proteasomes Are Sufficient for DTL—To demonstrate ubiquitin-independence and that the 26S proteasome is not only necessary but also sufficient for DTL, *in vitro* experiments were performed in which the only components were purified proteasomes and reporter proteins. Whole, functional 26S proteasomes have been previously affinity-purified for use

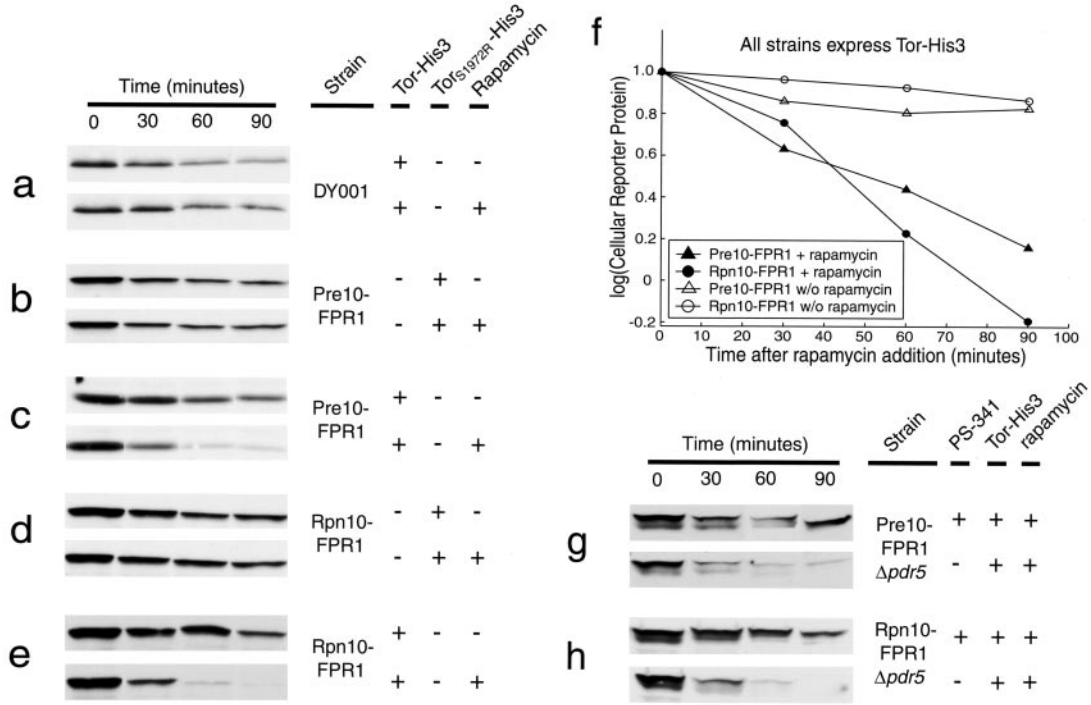

FIG. 3. **Tor-His3 is degraded *in vivo* upon the addition of rapamycin.** *a*, over a 90-min time course, untagged strains grown in culture show no difference in the rate of degradation of the reporter construct in the presence or absence of rapamycin. *b* and *d*, control reporter fusions in strains Pre10-FPR1 and Rpn10-FPR1 show no difference in the rate of degradation in the presence or absence of rapamycin. *c* and *e*, Tor-His3 in both strains Pre10-FPR1 and Rpn10-FPR1 is rapidly degraded in the presence of rapamycin. *f*, quantitation of Tor-His3 degradation in the presence or absence of rapamycin in strains Pre10-FPR1 and Rpn10-FPR1. *g* and *h*, addition of the proteasome inhibitor PS-341 prevents Tor-His3 from degrading in both strains Pre10-FPR1 (*g*) and Rpn10-FPR1 (*h*) even in the presence of rapamycin. *PDR5* encodes a multidrug resistance transporter and was knocked out in both strains Pre10-FPR1 and Rpn10-FPR1 for the purpose of the PS-341 experiments.

in biochemical assays (20). The same procedure was used to isolate proteasomes from strain Rpn10-FPR1. HA epitope-tagged versions of Tor-His3 and Tor$_{S1972R}$-His3 were purified from a bacterial expression system and then mixed with the proteasomes, with or without rapamycin. Samples were extracted at regular intervals, and reporter degradation was monitored by Western analysis (Fig. 4*a*). The *in vitro* results mirrored what was seen with the *in vivo* Westerns blots. All of the experiments using purified Tor$_{S1972R}$-His3 showed no degradation. Degradation of Tor-His3 was observed only in the presence of rapamycin and proteasomes isolated from strain Rpn10-FPR1. The half-life of the reporter was on the order of 15 min (Fig. 4*b*), comparable with what was observed *in vivo*. Once again, proteolysis of Tor-His3 could be halted upon the addition of the proteasome inhibitor PS-341. These results, in combination with the *in vivo* experiments, demonstrate that localization to the proteasome is sufficient for the initiation of degradation.

DISCUSSION

It is not known what role, if any, polyubiquitin chains serve beyond targeting substrates to the proteasome. In this study, we demonstrated that once a substrate is localized to the proteasome, additional signals are not required for degradation to occur. To test our DTL hypothesis, we designed a system using chemical inducers of dimerization to artificially associate reporters with proteasome subunits. We then established a functional screen to identify strain candidates for DTL. If our hypothesis was correct, we expected to isolate Tor-His3-expressing Fpr1-tagged strains that were deficient for growth on rapamycin-containing media. Strains Pre10-FPR1 and Rpn10-FPR1 exhibited such phenotypes. Western blot assays performed on whole-cell extracts from these strains confirmed that inactivation of the reporter proteins was due to their proteolysis. Furthermore, we showed that the proteasome was necessary for this degradation by arresting the process with the addition of the inhibitor PS-341. Finally, in a ubiquitin-free *in vitro* system we demonstrated that purified 26S proteasome alone was sufficient to mediate DTL. Although our results cannot rule out a model in which polyubiquitin chains stimulate or catalyze degradation, they confirm that any additional functions beyond tethering substrates to the proteasome are not strictly necessary for proteolysis to occur.

Seven proteasome subunits were randomly selected for Fpr1-tagging, as it was not known which, if any, would display a phenotype consistent with DTL. The tagged subunit that produced the strongest effect was Rpn10, interestingly one of the few proteasome subunits that has been shown to bind to polyubiquitin chains (9). Relatively little is known about Pre10, an essential protein that forms part of the proteolytic core particle (25) and which showed a mild but reproducible phenotype in our assay. Strains Rpn2-FPR1 and Rpt5-FPR1 showed comparable growth on plates containing or lacking rapamycin.

Western analysis of strains Rpn2-FPR1 and Rpt5-FPR1 established that both fusion proteins migrated at their expected molecular masses and, therefore, were correctly expressed. Rpt5 is another subunit that has been reported to recognize ubiquitin chains as indicated by crosslinking experiments (10). The fact that DTL did not occur for these strains implies that either dimerization of the modules was unsuccessful, or that despite localization to the proteasome, degradation was not initiated. The ability of an Fpr1-tagged subunit to promote DTL is dependent upon a number of factors, including substrate accessibility for the Fpr1-tag, stability of the tag, and proximity of a bound target to any substrate-processing site such as the ATPase assembly. We are currently testing whether dimerization of the modules actually occurs in strains

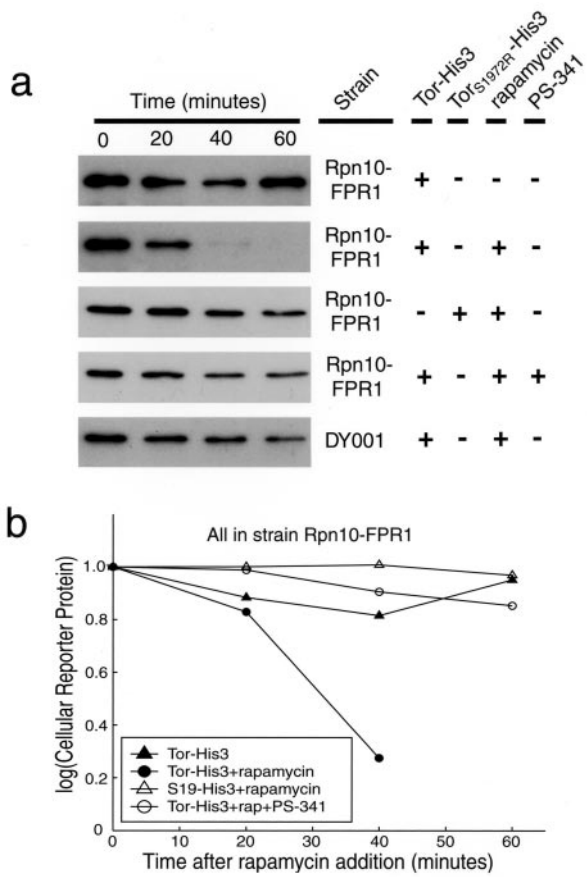

FIG. 4. **Tor-His3 is degraded *in vitro* upon the addition of rapamycin.** *a*, over a 60-min time course, purified Tor-His3 is rapidly degraded when mixed with Rpn10-FPR1-tagged purified proteasome complexes in the presence of rapamycin. There is no appreciable degradation of the reporter with the mutant construct or with untagged proteasome, tagged proteasome in the absence of rapamycin, and tagged proteasome in the presence of rapamycin and a proteasome inhibitor. *b*, quantitation of Tor-His3 degradation in the presence or absence of rapamycin in experiments containing purified Rpn10-FPR1 proteasomes (for the purpose of the figure, Tor$_{S1972R}$-His3 is labeled as S19-His3).

Rpn2-FPR1 and Rpt5-FPR1. If so, these strains could be used to recruit factors to the proteasome without their proteolysis.

In addition to being used as a screen, the gradient growth assay also served as an important functional control. There are reports of observations of ubiquitin-independent degradation of denatured substrates *in vitro* (26, 27). There is also evidence that the proteasome will degrade functional but highly unstable substrates in a ubiquitin-independent manner (28–30). In our experiments, the substrates were neither denatured nor unstable. The reporters were fully functional, as demonstrated by the growth assay and also by the ability of Tor to bind to Fpr1-rapamycin. Furthermore, we know that binding of rapamycin itself did not destabilize the substrates; a growth-deficient phenotype was not observed in the untagged DY001 parental strain expressing free Fpr1 in addition to the reporters (data not shown).

Unlike denatured or labile polypeptides, stable proteins need to be unfolded to enter the narrow channel into the 20S proteolytic core (5, 31). This process is thought to be facilitated by the six ATPase subunits, which exhibit both chaperone-like and nonspecific unfoldase activity and form part of the base for the 19S regulatory particle (31–33). The simplest model consistent with these data is that substrates held at the proteasome are driven toward an unfolded state through their association with the ATPase subunits, and that degradation follows once this is achieved (34, 35). The results of our study support this model as opposed to more elaborate models that postulate additional steps for proteolysis to occur.

We observed a range of rates in DTL that was dependent upon the FPR1-tagged strain used. That degradation was observed at all suggests that precise positioning of the substrate with respect to the proteasome is not strictly necessary for proteolysis. However, the positional bias observed in our study suggests that there is an optimal topological arrangement for presenting substrates to the ATPases for unfolding. This is the likely conformation assumed by proteins bound to the proteasome through polyubiquitin chains. The rapamycin-induced half-life of the reporter protein in the strain Rpn10-FPR1 was ~20 min, which is rapid and comparable with that of polyubiquitinated substrates.

Our results suggest a novel approach to protein therapeutics. The current paradigm in screening small molecule libraries is to find inhibitors of enzymatic processes that are causal in disease. However, the fraction of small molecules that both specifically bind to and inhibit the function of a protein target is extremely small. The continuing development of PROTACS (36, 37) seeks to potentially exploit the superset of "binders but not inhibitors" by using phosphopeptide-small molecule chimeras to artificially target proteins for ubiquitination. We propose to bypass the ubiquitination step and generate small molecules that will promote the degradation of disease-causing proteins through heterodimerization with the proteasome.

In addition, we are investigating the possibility of expanding the system used in this study into a generalized method for the facile construction of conditional protein knockdowns. Fusing the Tor module to endogenous proteins of choice in an Rpn10-FPR1 strain background should permit rapamycin-dependent control of degradation. One advantage such a system would have over technologies such as RNA interference (38) and promoter shutoff assays (39) is that it would directly target proteins for degradation, whereas the two referenced technologies affect transcript levels and are, therefore, dependent upon the natural half-lives of targets for functional knockdowns.

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