Identification of the Preferential Ubiquitination Site and Ubiquitin-dependent Degradation Signal of Rpn4* 

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Lysine selection is a long-standing problem in protein ubiquitination catalyzed by the RING ubiquitin ligases. It is well known that many substrates carry multiple lysines that can be ubiquitinated. However, it has seldom been addressed whether one lysine is preferred for ubiquitin conjugation when all other lysines exist. Here we studied the mechanism underlying ubiquitin-dependent degradation of Rpn4, a transcription activator of the Saccharomyces cerevisiae proteasome genes. We found that the ubiquitin-dependent degradation of Rpn4 can be mediated by six different lysines. Interestingly, we showed through in vivo and in vitro assays that lysine 187 is selected for ubiquitination when all other lysines are available. To the best of our knowledge, this is the first demonstration of a preferential ubiquitination site chosen from a group of lysines susceptible for ubiquitination. We further demonstrated that lysine 187 and a proximal acidic domain constitute a portable degradation signal. The implications of our data are discussed.

The ubiquitin (Ub)2 system is responsible for selectively marking abnormal and regulatory proteins for degradation by the proteasome (1–3). Ubiquitination of a protein substrate is a consecutive process involving multiple enzymes (4). Ub is first activated by the Ub-activating enzyme (E1), forming a thioester between the C-terminal carboxyl group of Ub and a specific cysteine of the E1. The Ub moiety of the E1–Ub thioester is thereafter transferred to one of the Ub-conjugating enzymes (E2). The Ub moiety of the E2–Ub thioester is conjugated, via an isopeptide bond, to the ε-amino group of a lysine residue (Lys) of a substrate or a preceding Ub molecule conjugated to the substrate, the latter reaction resulting in a substrate-linked multi-Ub chain. Most E2s function in complex with one of the E3 enzymes or Ub ligases. A Ub ligase also denotes an E2-E3 complex. Besides this common scenario, multiubiquitination of a subset of substrates also requires a Ub chain elongation factor (E4) (5). Ubiquitination of a specific substrate is mainly regulated through modulation of its degradation signal (degron) and through control of the activity of its cognate E3 (4, 6–8). The isopeptide bond between Ub and a substrate can be hydrolyzed by deubiquitinating enzymes, which provides yet another layer of regulation for substrate ubiquitination (9).

Most E3s are grouped into two families (HECT E3 and RING E3) based on their catalytic modules and features of sequence and structure (4, 7). A HECT E3 can accept Ub moiety from an associated E2–Ub thioester, forming an E3–Ub thioester intermediate and acting as a proximal Ub donor to the substrate that it selects. By contrast, formation of thioester between a RING E3 and Ub has not been detected. The precise mechanism by which a RING E3 catalyzes the transfer of Ub from the E2–Ub thioester to the substrate is still poorly understood. Many known substrates of the RING E3s carry multiple lysines that can be ubiquitinated (10–12). It is unclear whether one lysine is preferred for Ub conjugation when all other lysines susceptible for ubiquitination are also available in the substrate. This question remains one of the central issues in regard to the mechanism of substrate ubiquitination by the RING E3s. It is also uncertain whether or not formation of multiple Ub chains is required for substrate degradation by the proteasome. Failure to address this problem partly results from biased experimental designs that emphasized the essential but not the sufficient lysine for substrate ubiquitination and degradation.

Rpn4 (also named Son1) and Ufd5) is a transcription activator required for normal expression of the Saccharomyces cerevisiae proteasome genes (13, 14). Interestingly, Rpn4 is extremely short-lived and degraded by the proteasome (14). These observations and subsequent reports demonstrated that the proteasome homeostasis is regulated by a negative feedback circuit in which Rpn4 up-regulates the proteasome genes and is destroyed by the proteasome (15, 16). Intriguingly, the proteasomal degradation of Rpn4 can be mediated by two distinct pathways (17). One pathway is Ub-independent, whereas the other involves lysine ubiquitination. Recently, we demonstrated that the Ub-dependent degradation of Rpn4 is mediated by the Rpn10/Rpn2 Ub ligase (18). Ubr2, a RING E3, is a sequence homolog of Ubr1, the E3 component of the N-end rule pathway (18–20). The Ubr2-mediated ubiquitin-dependent degradation of Rpn4 has been shown to play an important role in controlling the steady-state levels of Rpn4 and the proteasome (18).

In the current work, we investigated the molecular mechanism underlying the Ub-dependent degradation of Rpn4. We found that the Ub-dependent degradation of Rpn4 can be mediated by six different lysines. Remarkably, among these six lysines, Lys-187 is the preferential ubiquitination site. We further demonstrated that Lys-187 and a proximal acidic domain constitute a portable Ubr2-dependent degradation signal.

EXPERIMENTAL PROCEDURES

Yeast Strains and Plasmids—The yeast strains used were JD52 (MATa his3–Δ200 leu2–3, 112 lys2–801 trp1–Δ63 ura3–52) and YXY274 (a urb2Δ::HIS3 derivative of JD52; see Ref. 18). Details of plasmid constructs are available upon request. All mutants carrying point mutations and/or deletions were generated by PCR-mediated mutagenesis and confirmed by DNA sequencing.

Pulse-Chase and Immunoprecipitation Analysis—S. cerevisiae cells from 10-ml cultures (A600 of 0.8–1.0) in SD medium containing 0.1 mM CuSO4 and essential amino acids were harvested. The cells were resuspended in 0.3 ml of the same medium supplemented with 0.15 mCi of
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[35S]methionine/cysteine (Expr35S35S labeling mix, PerkinElmer Life Sciences) and incubated at 30 °C for 5 min. The cells were then pelleted and resuspended in the same SD medium with cycloheximide (0.2 mg/ml) and excessive cold l-methionine/l-cysteine (2 mg/ml l-methionine and 0.4 mg/ml l-cysteine) and chased at 30 °C. An equal volume of sample was withdrawn at each time point. Labeled cells were harvested, lysed in an equal volume of 2X SDS buffer (2% SDS, 30 mM dithiothreitol, 90 mM Na-HEPES, pH 7.5), and incubated at 100 °C for 3 min. The supernatants were diluted 20-fold with buffer A (1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 50 mM Na-HEPES, pH 7.5) before being applied to immunoprecipitation with anti-HA antibody (Sigma) or anti-β-galactosidase antibody (Promega) combined with protein A-agarose (Calbiochem) or anti-FLAG M2 affinity-agarose (Sigma). The volumes of supernatants used in immunoprecipitation were adjusted to equalize the amounts of 10% trichloroacetic acid-insoluble 35S. The immunoprecipitates were washed three times with buffer A and resolved by SDS-PAGE followed by autoradiography and quantitation with a Phosphorimagor (Amersham Biosciences).

Pulldown-Ubiquitination Assay—The pulldown-ubiquitination assay was carried out as described previously (18). Specifically, ubr2Δ cells harboring plasmid 425GAL1FLAGUBR2 overexpressing N-terminally FLAG-tagged Ubr2 from the GAL1 promoter in a high copy vector were grown in synthetic selective medium containing 2% galactose to an OD600 of 1. Cells were spun down and manually ground to fine powder with a pestle. The pellet being ground was kept frozen by liquid nitrogen. Crude extracts were prepared by incubation of the powder in buffer B (0.2% Triton X-100, 150 mM NaCl, 50 mM HEPES, pH 7.5) plus protease inhibitor mix (Roche Diagnostics). Approximately, 0.2 mg of crude extract was used for each pulldown. After a standard pulldown, the beads were washed three times with buffer C (25 mM HEPES, pH 7.5, 25 mM KCl, 5 mM MgCl2, 2 mM ATP, and 0.1 mM dithiothreitol) and then subjected to ubiquitination reactions containing 100 mM Uba1 and 1 μM Rad6 with or without 7 μM ubiquitin in buffer C at 30 °C for 45 min. The GST fusion proteins were resolved by SDS-PAGE followed by immunoblotting with anti-GST antibody (Sigma). Rad6 was overexpressed and purified from vector pET-11d in bacteria, whereas N-terminally hexahistidine-tagged Uba1 was overexpressed from YEplac181 in S. cerevisiae and purified by nickel-nitrilotriacetic acid chromatography according to the manufacturer’s instructions (Qiagen).

RESULTS

Ub-dependent Degradation of Rpn4 Mediated by Different Lysines with Lys-187 Being the Most Efficient—Our early work has shown that proteasomal degradation of Rpn4 can be mediated by two distinct mechanisms, Ub-dependent and -independent (17). Taking advantage of the observation that deletion of the N-terminal 10 amino acids substantially inhibits the Ub-independent degron but displays no effect on the Ub-dependent degradation of Rpn4 (17, 18), we adopted Rpn441–10 to study the molecular mechanism underlying the Ub-dependent degradation of Rpn4.

We first tried to define the lysine(s) required for Rpn441–10 degradation. Rpn4 possesses 35 lysines throughout its 531-amino acid sequence. Lys-9 was already deleted in Rpn441–10. Previous study and current pulse-chase analysis showed that the turnover of Rpn441–10/10R, a mutant of Rpn441–10 with 10 N-terminal lysines (Lys-47 to Lys-187, referred to the positions in wild type Rpn4, see Fig. 1A) mutated to arginine, was severely inhibited (Fig. 1, B and E), indicating that the major ubiquitination site or sites are among the 10 N-terminal lysines. Note that the band just above the position of Rpn441–10 and Rpn441–10/10R represents a phosphorylated form of these two proteins, which is sensitive to treatment with calf intestinal alkaline phosphatase (data not shown). To determine whether all 10 N-terminal lysines are required for Rpn441–10 degradation, we constructed two Rpn441–10 mutants that bear Lys-to-Arg mutations for the first four lysines (N-4R) and for the fifth to tenth lysines (C-6R), respectively. D, the turnover rates of various Rpn441–10 mutants carrying one of the N-terminal lysines were measured by pulse-chase experiments. These mutants were derived from N-terminally HA-tagged Rpn441–10 derivatives carrying Lys→Arg substitutions for the first four lysines (N-4R) and the next six lysines (C-6R), respectively. The decay curves of various Rpn441–10 derivatives carrying one of the N-terminal lysines were measured by pulse-chase experiments. These mutants were derived from N-terminally HA-tagged Rpn441–10/10R by adding back individual lysines as indicated. E, quantitation of representative results from B and D by Phosphorimagor to show the decay curves of Rpn441–10, Rpn441–10/10R, and Rpn441–10/10R, respectively. The decay curves of Lys-123, Lys-137, Lys-141, and Lys-158, which are similar to that of Lys-123, were not included here to make the figure more viewable.

FIGURE 1. Lys-187 is more efficient than other lysines in mediating Ub-dependent degradation of Rpn4. A, diagram of Rpn4 with the N-terminal lysines ( ), two acidic domains (NAD and CAD), and a putative C2H2 DNA binding site. B, the 10 N-terminal lysines are required for Ub-dependent degradation of Rpn441–10. Pulse-chase analysis was carried out to compare the degradation of Rpn441–10 and Rpn441–10/10R, a mutant carrying Lys→Arg substitutions for the 10 N-terminal lysines of Rpn441–10. An HA tag was added to the N-termini of Rpn441–10 and Rpn441–10/10R expressed from the CUP1 promoter in a low copy vector. Rpn441–10 and Rpn441–10/10R are indicated by an arrow, whereas the asterisk marks a phosphorylated form of Rpn441–10 and Rpn441–10/10R. C, pulse-chase analysis of N-terminally HA-tagged Rpn441–10 derivatives carrying Lys→Arg substitutions for the first four lysines (N-4R) and the next six lysines (C-6R), respectively. D, the turnover rates of various Rpn441–10 mutants carrying one of the N-terminal lysines were measured by pulse-chase experiments. These mutants were derived from N-terminally HA-tagged Rpn441–10/10R by adding back individual lysines as indicated. E, quantitation of representative results from B and D by Phosphorimagor to show the decay curves of Rpn441–10, Rpn441–10/10R, and Rpn441–10/10R, respectively. Pulse-chase experiments were carried out to measure the stability of these two proteins. Although C-6R remained unstable, its turnover was much slower than that of N-4R, which was degraded as rapidly as Rpn441–10 (Fig. 1, C). The decay curves of Lys-123, Lys-137, Lys-141, and Lys-158, which are similar to that of Lys-123, were not included here to make the figure more viewable.

Ub-dependent Degradation of Rpn4 Mediated by Different Lysines with Lys-187 Being the Most Efficient—Our early work has shown that proteasomal degradation of Rpn4 can be mediated by two distinct mechanisms, Ub-dependent and -independent (17). Taking advantage of the observation that deletion of the N-terminal 10 amino acids substantially inhibits the Ub-independent degron but displays no effect on the Ub-dependent degradation of Rpn4 (17, 18), we adopted Rpn441–10 to study the molecular mechanism underlying the Ub-dependent degradation of Rpn4.

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To determine whether any one of the six lysines (Lys-123, Lys-132, Lys-137, Lys-141, Lys-158, and Lys-187) is sufficient to sustain the degradation of Rpn441–10 at a normal rate, an individual lysine was added back into Rpn441–10/10R, and the turnover rates of the resulting mutants were measured by pulse-chase analysis. As shown in Fig. 1D, all Rpn441–10/10R derivatives carrying one of the six lysines were short-lived...
(lanes 1–18). Remarkably, the mutant with Lys-187 (Rpn4Δ1–10/K187) was clearly degraded faster than others (Fig. 1D, compare lanes 4–6 with lanes 1–3 and 7–18). The half-life of Rpn4Δ1–10/K187 is ~3 min, which is similar to that of Rpn4Δ1–10 whereas the half-lives of other derivatives are ~8 min (Fig. 1, B, D, and E). Thus, the degradation of Rpn4Δ1–10 can be efficiently mediated by any one of the six lysines in the N-terminal region (Lys-123, Lys-132, Lys-137, Lys-141, Lys-158, and Lys-187). Lys-187, however, is the only one that can sustain wild type level degradation of Rpn4Δ1–10.

Lys-187 Is the Preferential Ubiquitination Site of Rpn4—The more efficient degradation of Rpn4Δ1–10 mediated by Lys-187 than by other lysines prompted us to examine if Lys-187 is a preferential ubiquitination site. We first sought to compare the ubiquitination efficiency of various Rpn4Δ1–10/10R derivatives that carry one of the lysines from Lys-132 to Lys-187. As the measurement of multiubiquitinated substrates formed in vivo can be complicated because of their degradation by the proteasome and rapid deubiquitination by the deubiquitinating enzymes, we decided to assess the efficiency of conjugation of the first Ub moiety to different lysines added back to Rpn4Δ1–10/10R. To this end, we took advantage of UbK48R/G76A, a ubiquitin mutant that inhibits the formation of a Lys-48-linked multi-Ub chain (21). Our early work has shown that the Ub-dependent degradation of Rpn4 is through Lys-48-linked mult ubiquitination (17). Moreover, UbK48R/G76A is a poor substrate for deubiquitinating enzymes (22) such that substrates conjugated with a moiety of UbK48R/G76A can be well preserved in a short pulse-labeling period. Therefore, the ratio of monoubiquitinated to nonubiquitinated species of a specific substrate largely reflects the ubiquitination efficiency at a defined lysine.

Cells co-overexpressing UbK48R/G76A with N-terminally HA-tagged Rpn4Δ1–10, Rpn4Δ1–10/10R, or Rpn4Δ1–10/10R derivatives carrying one of the lysines from Lys-132 to Lys-187 were metabolically labeled with [35S]methionine for 5 min. To facilitate the determination of monoubiquitinated substrates, a transformant co-overexpressing N-terminally HA-tagged Rpn4Δ1–10 and N-terminally His6-Myc-tagged UbK48R/G76A (His6-Myc-UbK48R/G76A) was used as control. Substrates conjugated with epitope-tagged Ub have been shown to migrate slower than those with untagged Ub (17, 23). [35S]-Labeled cell extracts were incubated with anti-HA antibody and the precipitated substrates were separated by SDS-PAGE. As expected, a characteristic difference in mobility was observed between the Rpn4Δ1–10 conjugates attached with His6-Myc-UbK48R/G76A and UbK48R/G76A (Fig. 2A, compare lanes 1 and 2).

Although no ubiquitinated species of Rpn4Δ1–10/10R was observed, monoubiquitinated species of the Rpn4Δ1–10/10R derivatives were readily detected (Fig. 2A, compare lanes 3 and 4–8), consistent with the observation that any one of these lysines can mediate Rpn4Δ1–10 degradation (Fig. 1D). Remarkably, the ratio of monoubiquitinated to nonubiquitinated Rpn4Δ1–10/K187 was comparable with that of Rpn4Δ1–10 and much higher (~3–5-fold) than those of other Rpn4Δ1–10/10R derivatives bearing a different lysine (Fig. 2A, lanes 2–8). This observation is in line with the expectation of Lys-187 as a preferential Ub acceptor. Interestingly, monoubiquitinated Rpn4Δ1–10/10R migrated noticeably faster than the monoubiquitinated species of other Rpn4Δ1–10/10R derivatives, whereas all nonubiquitinated substrates had a similar mobility (Fig. 2A, compare lanes 8 and 4–7), suggesting that the Lys-187-Ub conjugate exhibits a different configuration compared with the Ub conjugates at other lysines. Strikingly, only one type of monoubiquitinated species of Rpn4Δ1–10 was detected based on mobility, and this mobility was the same as that of the monoubiquitinated Rpn4Δ1–10/K187 (Fig. 2A, compare lanes 2 and 8). These observations reveal that Lys-187 is selected for ubiquitination from multiple lysines that could serve as Ub acceptors in Rpn4Δ1–10.

It is noteworthy that small but noticeable amounts of Rpn4Δ1–10 and Rpn4Δ1–10/10R appeared to bear two Ub moieties (Fig. 2A, lanes 2 and 8). Given that Rpn4Δ1–10/10R was not ubiquitinated, Lys-187 was therefore the only Ub-acceptor in Rpn4Δ1–10/10R. It is likely that the diubiquitinated species of Rpn4Δ1–10/K187 was generated by attachment of an endogenous Ub molecule to Lys-187 followed by conjugation of a UbK48R/G76A moiety to Lys-48 of the preceding endogenous Ub. The detection of diubiquitinated species of Rpn4Δ1–10/K187 but not other species of Rpn4Δ1–10/10R derivatives carrying one of the lysines from Lys-132 to Lys-187 were metabolically labeled with [35S]methionine for 5 min. Therefore, the ratio of monoubiquitinated to nonubiquitinated species of a specific substrate largely reflects the ubiquitination efficiency at a defined lysine.

Cells co-overexpressing UbK48R/G76A with N-terminally HA-tagged Rpn4Δ1–10, Rpn4Δ1–10/10R, or Rpn4Δ1–10/10R derivatives carrying one of the lysines from Lys-132 to Lys-187 were metabolically labeled with [35S]methionine for 5 min. To facilitate the determination of monoubiquitinated substrates, a transformant co-overexpressing N-terminally HA-tagged Rpn4Δ1–10 and N-terminally His6-Myc-tagged UbK48R/G76A (His6-Myc-UbK48R/G76A) was used as control. Substrates conjugated with epitope-tagged Ub have been shown to migrate slower than those with untagged Ub (17, 23). [35S]-Labeled cell extracts were incubated with anti-HA antibody, and the precipitated substrates were separated by SDS-PAGE. As expected, a characteristic difference in mobility was observed between the Rpn4Δ1–10 conjugates attached with His6-Myc-UbK48R/G76A and UbK48R/G76A (Fig. 2A, compare lanes 1 and 2).

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Rpn4\textsubscript{11–210} derivatives carrying Lys-132, Lys-137, Lys-141, or Lys-158 reflects the higher ubiquitination efficiency at Lys-187, further supporting that Lys-187 is the preferential ubiquitination site of Rpn4.

To verify the results of the in vivo ubiquitination experiments, we performed a pulldown-ubiquitination assay, which has successfully been shown that Ubr2, pulled down by GST fusion of Rpn4\textsubscript{\Delta 1–10} (GST-Rpn4\textsubscript{\Delta 1–10}) from yeast extract, multiubiquitinated GST-Rpn4\textsubscript{\Delta 1–10} in the presence of Rad6 and Uba1 (18). Agarose beads preloaded with GST-Rpn4\textsubscript{\Delta 1–10}, GST-Rpn4\textsubscript{\Delta 1–10/187}, and GST-Rpn4\textsubscript{\Delta 1–10/158} were incubated with extract from \textalpha br\textalpha cells overexpressing Ubr2 from the GAL1 promoter in a high copy plasmid. After a standard pulldown, the beads were further incubated with purified Rad6 and Uba1 in the presence (lanes 1, 3, 5, and 7) or absence (lane 2, 4, 6, and 8) of ubiquitin (Fig. 2B). The GST fusions were then separated by SDS-PAGE and subjected to immunoblotting analysis with anti-GST antibody. As expected, the ubiquitination was specific to the N-terminus exclusive, degron of Rpn4 to its N-terminal 229-residue domain (17). To further define the minimal sequences required for the Ub-dependent degradation of Rpn4, we created a series of N-terminally truncated mutants (Fig. 3A). Pulse-chase analysis showed that deletion up to the N-terminal 148 amino acids had no effect on the turnover of Rpn4 (Fig. 3B, lanes 1–12), implying that the Ub-dependent degron was located within residues 149–229. Not surprisingly, further deletion to residue 190 or 205 inhibited the degradation (Fig. 3B, lanes 13–18) as the preferential and all alternative ubiquitination sites were removed. Interestingly, the sequence of residues 211–229 is rich in acidic amino acids, a characteristic of transcription activation domain (Fig. 3A).

The N-terminal Acidic Domain (NAD) Is Essential for the Ub-dependent Degradation of Rpn4—Our recent study has located the major, if not exclusive, degron of Rpn4 to its N-terminal 229-residue domain (17). To further define the minimal sequences required for the Ub-dependent degradation of Rpn4, we created a series of N-terminally truncated mutants (Fig. 3A). Pulse-chase analysis showed that deletion up to the N-terminal 148 amino acids had no effect on the turnover of Rpn4 (Fig. 3B, lanes 1–12), implying that the Ub-dependent degron was located within residues 149–229. Not surprisingly, further deletion to residue 190 or 205 inhibited the degradation (Fig. 3B, lanes 13–18) as the preferential and all alternative ubiquitination sites were removed. Interestingly, the sequence of residues 211–229 is rich in acidic amino acids, a characteristic of transcription activation domain (Fig. 3A).

We suspect that this domain might act as a ubiquitination signal because the ubiquitination signals of transcription activators are often overlapped with their activation domains (24). To test this hypothesis, we first compared the stability of two truncated Rpn4 mutants containing residues 11–229 (Rpn4\textsubscript{11–229}) and 11–210 (Rpn4\textsubscript{11–210}), respectively, using pulse-chase analysis (Fig. 3C). Whereas Rpn4\textsubscript{11–229} was extremely short-lived, Rpn4\textsubscript{11–210} was barely degraded, suggesting that residues 211–229 serve as a ubiquitination signal in Rpn4\textsubscript{11–229}.

We then examined the role of residues 211–229 in the context of Rpn4\textsubscript{\Delta 1–10}. Sequence analysis showed that Rpn4 carries two domains rich in acidic amino acids (Fig. 3A). NAD consists of residues 211–229, whereas the C-terminal acidic domain (CAD) lies between residues...
that NAD acts as the ubiquitination signal. We postulate that CAD inhibited the degradation of Rpn4172–229-3gal. Pulse-chase analysis revealed that Rpn4172–229-3gal was rapidly degraded, indicating that Lys-187 and NAD do not functionally distinguish itself from CAD. To examine whether NAD also plays a role in the Ub-independent degradation of Rpn4, we deleted NAD in the context of full-length Rpn4 and measured the turnover of Rpn4A31–10 via pulse-chase analysis. As shown in Fig. 3D (lanes 7–9), deletion of NAD displayed no effect on the Ub-independent degradation of Rpn4, indicating that NAD is specific for the Ub-dependent degradation of Rpn4.

Lys-187 and NAD constitute a portable Ubr2-dependent degron—A Ub-dependent degron is composed of a ubiquitination site and a ubiquitination signal, usually a short primary sequence or a structural feature recognized by a specific Ub ligase. The identification of Lys-187 as the preferential ubiquitination site and NAD as an essential sequence for the Ub-dependent degradation of Rpn4 prompted us to determine whether Lys-187 and NAD (including minimal flanking sequences) constitute a sufficient degron. To this end, we fused residues 172–229 of Rpn4 to the otherwise stable β-galactosidase reporter protein and measured the stability of the resulting fusion protein Rpn4172–229-β-galactosidase. As shown in Fig. 4 (lanes 1–4), Rpn4172–229-β-galactosidase Ub2 was rapidly degraded, indicating that Lys-187 and NAD do form a sufficient and portable degron. Interestingly, substitution of Lys-187 with arginine inhibited the degradation of Rpn4172–229-β-galactosidase (Fig. 4, lanes 9–12), indicating that the implanted Lys-187 functions as the ubiquitination site in the fusion protein. To rule out the possibility that a cryptic degron was created in the Rpn4172–229-β-galactosidase fusion, we measured the stability of Rpn4172–229-β-galactosidase in ubr2Δ, where Ubr2, the cognate Ub ligase for Rpn4, was absent. Pulse-chase analysis revealed that Rpn4172–229-β-galactosidase was stabilized in the ubr2Δ mutant (Fig. 4, lanes 5–8). Thus, Lys-187 and NAD constitute a portable degron specifically targeted by Ubr2. We postulate that NAD acts as the ubiquitination signal.

DISCUSSION

Lysine selection by the RING E3s is a central question in the field of protein ubiquitination. Although several models exemplified by the “effective concentration” and the “hit-and-run” models have been proposed to explain the process of lysine selection (25–28), these models can only interpret the data of ubiquitination of one substrate but not the other. It is well known that many substrates of the Ub-proteasome system can be ubiquitinated at multiple lysines. However, in every case that we are aware of, it has never been determined if one lysine is preferred when all other lysines are also available. Extensive analysis of several substrates including Gcn4, p53, and c-Jun show that no single Lys-to-Arg substitution affects protein degradation, suggesting that it is rather nonselective with respect to which lysine of these substrates is ubiquitinated (11, 12, 29). However, strictly speaking, this type of analysis only demonstrates whether one of the lysines is essential for substrate ubiquitination but is unable to define a potential preferential ubiquitination site. Using an approach of adding back individual lysines to a lysine-free Sic1 allele, Petroski and Deshaies (10) recently demonstrated that each one of the six N-terminal lysines is ubiquitinated and is sufficient to mediate the degradation of Sic1. Although these authors showed that the Ub chains attached at different N-terminal lysines influence the rate of in vitro proteasomal proteolysis, the in vivo half-lives of the Sic1 mutants carrying different lysines are quite similar (10). It is unknown whether one of these N-terminal lysines of Sic1 behaves as a preferred ubiquitination site.

Using a similar “adding-back” approach, we identified six lysines in the N-terminal domain of Rpn4, each of which was able to sustain the degradation of Rpn4. Remarkably, among these six lysines, Lys-187 was ubiquitinated more efficiently than the others, and the turnover rate of Rpn4 via Lys-187 was also markedly higher than that by other individual lysines. Consistently, Lys-187 and NAD constitute an efficient and portable Ubr2-dependent degron. More importantly, both in vivo and in vitro ubiquitination assays demonstrated that Lys-187 is selected for ubiquitination when all other lysines are also available. To the best of our knowledge, this is the first demonstration of a preferential ubiquitination site that is selected from a group of lysines susceptible for ubiquitination in a substrate. It is currently unclear how the Ub ligase Ubr2/Rad6 selects Lys-187 over other lysines. One possibility is that Lys-187 is proximally positioned to the active center of the Ubr2/Rad6 Ub ligase, as the primary sequence of Rpn4 suggests that Lys-187 is closer to the NAD ubiquitination signal than other lysines. Further investigation of the molecular details of Rpn4 ubiquitination, e.g. deciphering the atomic structure of the Ubr2/Rad6/Rpn4 complex, will shed new light on the general mechanism of lysine selection.

It remains an open question whether the presence of multiple Ub chains on a protein substrate is essential for its degradation by the proteasome. Our current study demonstrates that the Ub-dependent degradation of Rpn4 can be mediated efficiently by six individual lysines. Consistent with our data, a recent report has shown that each one of the six N-terminal lysines is sufficient for the degradation of Sic1 (10). Therefore, it appears that a single multi-Ub chain is sufficient to sustain efficient degradation of at least a subset of proteasomal substrates. What would be the physiological meaning of having multiple Ub chains on a substrate? Given the presence of multiple Ub chain receptors including the intrinsic proteasome subunits (Rpn10 and Rpt5) and proteasome-associated UBA domain proteins in the cell (30), it is likely that multiple Ub chains may slow the dissociation of a substrate from the proteasome, providing the proteasome with sufficient time to unfold and translocate the substrate into the proteolytic chamber. This high affinity conferred by multiple Ub chains, although not an absolute requirement for the degradation of some substrates such as Rpn4 and Sic1, may be essential for others, especially those that are difficult to unfold. We reason that the structural features of protein substrates determine whether a single Ub chain is enough or multiple Ub chains are required for their degradation. For instance, a single multi-Ub chain may be sufficient for the degradation of substrates with loosely structured segments such as unfolding initiation sites.

Acknowledgment—We thank Michael Ellison for plasmids.
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