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Ubiquitin-mediated degradation of Rpn4 is controlled by a phosphorylation-dependent ubiquitylation signal

Donghong Ju, Haiming Xu, Xiaogang Wang, Youming Xie *

Barbara Ann Karmanos Cancer Institute, Wayne State University School of Medicine, 110 E Warren Avenue, Detroit, MI 48201, USA

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Abstract

A ubiquitylation signal of a protein substrate is defined as a short primary sequence or a structural feature recognized by a specific E3. Our previous work has mapped the ubiquitylation signal of Rpn4, the transcription activator for the *Saccharomyces cerevisiae* proteasome genes, to an N-terminal acidic domain (NAD) consisting of amino acids 211–229. However, the molecular mechanism by which Ubr2, the cognate E3, recognizes NAD remains unclear. Here we show that phosphorylation of either Ser-214 or Ser-220 enhances the binding of NAD to Ubr2. However, phosphorylation of Ser-220 but not Ser-214 plays a predominant role in Rpn4 ubiquitylation and degradation. Interestingly, NAD does not constitute the major Ubr2-binding site of Rpn4 even though it serves as the ubiquitylation signal essential for Rpn4 degradation. By contrast, the stable binding with Ubr2 conferred by other domains of Rpn4 is not required for Rpn4 degradation. Our results indicate that ubiquitin-mediated degradation of Rpn4 is controlled by a phosphorylation-dependent ubiquitylation signal. This study also suggests that binding to E3 may be only a part of the function of a ubiquitylation signal.
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Keywords: Ubiquitin; Proteasome; Protein degradation; Protein phosphorylation; Rpn4; Ubr2

1. Introduction

The ubiquitin (Ub) system plays a crucial role in marking abnormal proteins and regulatory proteins for degradation by the proteasome [1–3]. Protein ubiquitylation is a consecutive process involving multiple enzymes. 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 a Ub-conjugating enzyme (E2). With the participation of an E3 enzyme (also called Ub-ligase), the Ub moiety of the E2 ~ Ub thioester is conjugated, via an isopeptide bond, to the ε-amino group of a lysine residue of a substrate or a preceding Ub molecule attached to the substrate, the latter reaction resulting in a substrate-linked poly-Ub chain. A Ub-ligase also denotes an E2/E3 complex. Most E3s are grouped into two families (HECT E3s and RING E3s) based on their catalytic modules and features of sequence and structure [3,4]. 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 mechanism by which a RING E3 catalyzes the transfer of Ub from E2 ~ Ub thioester to substrate remains speculative. The current hypothesis is that a RING E3 acts as an adaptor to optimize the positioning of the bound substrate to the E2 ~ Ub thioester [5]. The specificity of protein ubiquitylation is mainly controlled by the substrate's degradation signal (degron) that consists of a ubiquitylation site and a ubiquitylation signal. The ubiquitylation site is a lysine residue accessible for Ub conjugation, whereas the ubiquitylation signal is usually a short primary sequence or a structural feature recognized by a specific E3. The E3–substrate interaction is often modulated through covalent modification of the ubiquitylation signal. The known modifications include phosphorylation, acetylation, hydroxylation and glycosylation [6–11]. In line with the large

* Corresponding author. Tel.: +1 313 833 0715; fax: +1 313 831 7518.
E-mail address: xiey@karmanos.org (Y. Xie).

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number of E3s, the substrates of the Ub-system are extremely diverse, and the ubiquitylation signals recognized by different E3s seldom share consensus sequences. So far, relatively few ubiquitylation signals have been characterized.

The *Saccharomyces cerevisiae RPN4* gene (also named *SON1* and *UFD5*) was originally isolated as a suppressor of *sec63-101*, a temperature-sensitive mutant of *SEC63*, which encodes an essential component of the endoplasmic reticulum translocation channel [12–15]. Recent studies demonstrated that the *RPN4*-encoded protein, Rpn4, is a transcription activator that regulates the expression of a large number of genes, including many of the Ub-proteasome system [16–18]. Rpn4 has also been shown to play an important role in response to a variety of environmental stresses [19–26]. The involvement of Rpn4 in stress response is directly related to its role in regulating proteasome homeostasis through a negative feedback circuit [18,21]. On the one hand, Rpn4 upregulates the expression of the proteasome genes; on the other hand, Rpn4 is extremely short-lived and degraded by the proteasome. Rpn4 can be degraded by two distinct pathways, Ub-dependent and Ub-independent [27]. The Ub-dependent degradation of Rpn4 is mediated by the Ubr2/Rad6 Ub-ligase [28]. Ubr2 is a sequence homolog of Ubr1, the RING E3 component of the N-end rule pathway [28,29]. Rad6 (also named Ubc2) is an E2 enzyme involved in the N-end rule pathway and DNA repair [29–32]. In dissecting the Ub-dependent degron of Rpn4, we found that Ub-dependent degradation of Rpn4 can be mediated by six different lysines, of which K187 is the preferred ubiquitylation site , whereas the other five lysines act as alternative ubiquitylation sites in the absence of K187 [33]. We also located the ubiquitylation signal of Rpn4 to the N-terminal acidic domain (NAD) including amino acids 211–229. However, the molecular mechanism through which Ubr2 recognizes NAD has not been elucidated.

In this study we demonstrate that phosphorylation of either S214 or S220 enhances the NAD–Ubr2 interaction. However, it is phosphorylation of S220 but not S214 that plays a predominant role in Rpn4 ubiquitylation and degradation. In addition to NAD, other domains of Rpn4 also interact with Ubr2. This interaction, while rather stable, is not required for ubiquitylation at K187. Instead, it supports the use of the less efficient alternative ubiquitylation sites. Our results indicate that ubiquitin-mediated degradation of Rpn4 is controlled by a phosphorylation-dependent ubiquitylation signal. The current work also suggests whether or not an E3-binding site can function as a ubiquitylation signal is not necessarily determined by the E3-binding stability.

2. Materials and methods

2.1. Strains and plasmids

The yeast strains used were JD52 (*MAT*a *his3-Δ200 leu2-3, 112 lys2-801 trp1-Δ63 ura3-52*), and YXY274 (a *ubr2Δ::HIS3* derivative of JD52). *Escherichia coli* strain BL(DE3) was used to express GST fusion proteins. The details of plasmid construction are available upon request. All mutants carrying point mutations and/or deletion were generated by PCR-mediated mutagenesis and confirmed by DNA sequencing.

2.2. β-galactosidase assay

The enzymatic activity of β-galactosidase in liquid yeast culture was determined as described [36], using the chromogenic substrate *o*-nitrophenyl-β-D-galactopyranoside (ONPG). For induction of the *CUP1* promoter, CuSO4 was added to the final concentration of 0.1 mM.

2.3. Pulse-chase and immunoprecipitation analysis

S. cerevisiae cells from 10 ml cultures (OD_{600} of 0.8–1.0) in SD medium containing 0.1 mM $CuSO_4$ supplemented with essential amino acids were harvested. The cells were resuspended in 0.3 ml of the same medium supplemented with 0.15 mCi of [^{35}S]-methionine/cysteine (EXPRESS [^{35}S] Protein Labeling Mix, PerkinElmer), and incubated at 30 °C for 5 min. The cells were then pelleted and resuspended in the same 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. Equal volume of sample was withdrawn at each time point. Labeled cells were harvested and lysed in equal volume of 2× SDS buffer (2% SDS, 30 mM dithiothreitol, 90 mM Na–HEPES, pH 7.5) by incubation 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 applied to immunoprecipitation with anti-ha antibody (Sigma) or anti-βgal antibody (Promega) combined with Protein A agarose (Calbiochem). The volumes of supernatants used in immunoprecipitation were adjusted to equalize the amounts of 10% trichloroacetic acid-insoluble [^{35}S]. The immunoprecipitates were washed 3 times with buffer A, and resolved by SDS-PAGE, followed by autoradiography and quantitation with a PhosphorImager (Molecular Dynamics).

2.4. GST pulldown-immunoblotting assay

ubr2Δ cells overexpressing N-terminally FLAG-tagged Ubr2 (FLAG-Ubr2) from the *GAL1* promoter in a high copy vector (425GAL1FLAGUBR2) were grown in synthetic selective medium containing 2% galactose to OD_{600} of 1.8. Cells were spun down and manually grounded 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 (150 mM NaCl, 50 mM HEPES, pH 7.5, 0.2% Triton X-100) plus protease inhibitor mix (Roche Diagnostics). For preparation of purified FLAG-Ubr2, supernatants were incubated with 1/5 volume of anti-FLAG M2 affinity agarose (Sigma) at 4 °C for 3 h. The beads were washed 3 times with buffer B, and eluted with buffer B plus 100 μg/ml FLAG peptide at 4 °C for 2 h. For each pulldown, approximately 0.1 μg purified FLAG-Ubr2 was incubated with glutathione-agarose beads pre-loaded with GST fusion proteins at 4 °C for 2 h. The beads were then washed 3 times with buffer B, and the retained proteins were separated by SDS-PAGE, followed by immunoblotting with anti-FLAG M2 antibody (Sigma) and detection with the SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology) or the Odyssey infrared imaging system according to the manufacturer's instruction (Li-Cor Biosciences).

2.5. In vitro phosphorylation assay

Approximately 1 μg GST fusion proteins were pulled down from *E. coli* extracts by glutathione–agarose beads and then incubated with 20 μg crude yeast extract in the presence or absence of 0.2 mM ATP at 30 °C for 30 min. After washed with buffer B, the GST fusion proteins bound on the beads were resolved on 10% SDS-PAGE, followed by Coomassie blue staining. For in vitro CK2 phosphorylation assay, the preloaded GST fusion proteins were treated with recombinant CK2 (New England BioLabs) in the presence of [γ-^{32}P]-ATP at 30 °C for 10 min. A 25-μl kinase reaction consisted of 10 units of CK2, 0.1 mM cold ATP and 4 μCi [γ-^{32}P]-ATP (PerkinElmer) in CK2 kinase buffer (20 mM Tris–Cl, pH 7.5, 50 mM KCl, 10 mM $MgCl_2$). After washing with buffer B, the phosphorylated GST fusion proteins were separated on SDS-PAGE (10% gel), and subjected to autoradiography.

3. Results

3.1. S220 is an important determinant of the Ub-dependent degron of Rpn4

Rpn4 carries two domains rich in acidic amino acids (Fig. 1A). The N-terminal acidic domain (NAD) consists of residues 211–229, whereas the C-terminal acidic domain (CAD) lies between residues 300 and 312. Although both NAD and CAD are rich in acidic residues, NAD but not CAD acts as the ubiquitylation signal recognized by Ubr2 E3 [33]. NAD is in a proximal position to K187, the preferential ubiquitylation site of Rpn4, which may functionally distinguish itself from CAD. On the other hand, NAD has a different amino acid composition from CAD (Fig. 1A). We wanted to determine if the two Ser residues in NAD (S214 and S220) are important for the Ub-dependent degron of Rpn4. Our early work has shown that residues 172–229 of Rpn4 constitute a sufficient and portable degron and that the $Rpn4_{172-229}$–βgal fusion protein is degraded in a Ubr2-dependent manner [33]. We therefore replaced S214, S220, or both with alanine and examined the effect of these substitutions on the stability of $Rpn4_{172-229}$-βgal. We first measured the βgal enzymatic activity, which is a sensitive indicator of the in vivo metabolic stability of the βgal fusion substrates. $Rpn4_{172-229/K187R}$-βgal, a stable version of $Rpn4_{172-229}$-βgal with K187 mutated to arginine [33], was used as a control (Fig. 1B). While S214A mutation slightly decreased the turnover of $Rpn4_{172-229}$-βgal, S220A substitution significantly increased the stability of $Rpn4_{172-229}$-βgal. The S214/220A double mutation exhibited a modestly stronger effect than the S220A single mutation. Consistent with the βgal enzymatic assay, pulse-chase analysis demonstrated that S220A mutation substantially stabilized $Rpn4_{172-229}$-βgal even though the effect of the S214/220A double mutation was slightly greater (Fig. 1C, compare lanes 10–15 and 4–6). By contrast, the influence of S214A substitution was marginal (Fig. 1C, lanes 7–9). Thus, while both S214 and S220 contribute to the Ub-dependent degron of Rpn4, S220 appears much more important than S214.

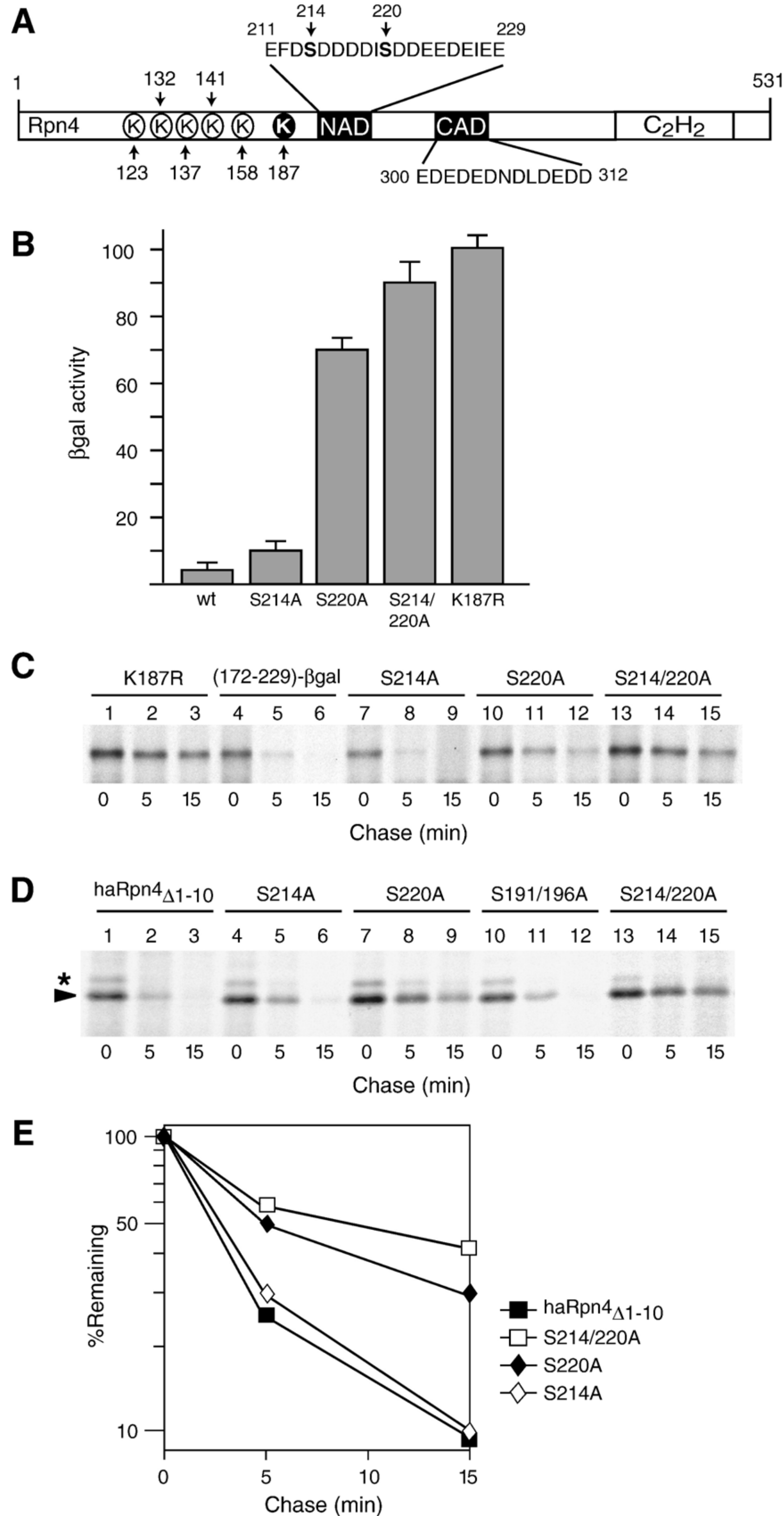
We then introduced S-to-A mutation at S214 or S220 or both in the context of $haRpn4_{\Delta 1-10}$, an Rpn4 mutant with the N-terminal 10 residues replaced by an ha tag [28,33], and measured the stability of the resulting mutants, $haRpn4_{\Delta 1-10/S214A}$, $haRpn4_{\Delta 1-10/S220A}$ and $haRpn4_{\Delta 1-10/S214/220A}$, by pulse-chase assays. Our early work has shown that deletion of the N-terminal 10 amino acids substantially inhibits the Ub-independent degradation of Rpn4 but has no effect on the Ub-dependent degron [21,28,33]. As shown in Fig. 1D and E, the degradation of $haRpn4_{\Delta 1-10/S220A}$ was significantly slower than that of $haRpn4_{\Delta 1-10}$, whereas $haRpn4_{\Delta 1-10/S214A}$ was degraded almost as rapidly as $haRpn4_{\Delta 1-10}$. Again, the turnover of $haRpn4_{\Delta 1-10/S214/220A}$ was slightly slower than that of $Rpn4_{\Delta 1-10/S220A}$ (Fig. 1D, E). The Ub-dependent degron of Rpn4 contains two other serine residues (S191 and S196) in addition to S214 and S220. We found that a double mutation of S191 and S196 to alanines did not affect the turnover of $haRpn4_{\Delta 1-10}$ (Fig. 1D, lanes 10–12). Together, these data demonstrate that S220 is an important element of the Ub-dependent degron of Rpn4. S214 plays a relatively minor role compared to S220.

3.2. S214 and S220 are phosphorylation sites

Phosphorylation is a frequent involvement in ubiquitylation. We found that substitution of S220 with phosphor-mimetic glutamate had less effect on the turnover rate of $haRpn4_{\Delta 1-10}$ compared to other mutations including S220A, S220V and S220L (data not shown), suggesting that S220 may be a phosphorylation site important for Rpn4 ubiquitylation. Note that a phosphorylated species of $haRpn4_{\Delta 1-10}$ with slower mobility (Fig. 1D, marked with *asterisk*, also see ref. 33) survived the S214/220A double mutation. This observation suggests that Rpn4 may contain multiple phosphorylation sites and that phosphorylation of S220 and/or S214, if indeed occurs, results in no obvious mobility shift. We therefore decided to examine if S214 and S220 could be phosphorylated using the $Rpn4_{172-229}$ degron fused to the C-terminus of glutathione *S*-transferase (GST). The molecular mass of the GST-$Rpn4_{172-229}$ fusion is much lower than Rpn4, so that phosphorylation of S220 and/or S214 may cause an appreciable mobility change on SDS-PAGE. GST-$Rpn4_{172-229}$ and GST-$Rpn4_{172-229/S214/220A}$, a S214/220A double mutant, were pulled down by glutathione-conjugated agarose beads from *E. coli* extracts, and incubated with yeast extract in the presence or absence of ATP. We repeatedly observed that GST-$Rpn4_{172-229}$ migrated slightly slower after treated with yeast extract in the presence but not in the absence of ATP (Fig. 2A, compare lanes 1 and 2). By contrast, the same treatment did not cause mobility shift for GST-$Rpn4_{172-229/S214/220A}$ (Fig. 2A, lanes 3 and 4). These observations suggest that GST-$Rpn4_{172-229}$ is phosphorylated at S214 and/or S220. We then generated another 2 GST-$Rpn4_{172-229}$ mutants bearing S214A or S220A single mutation.

Fig. 1. S220 is a critical element of the Ub-dependent degron of Rpn4. (A) Diagram of Rpn4 with the preferential (K187) and alternative (K123–K158) ubiquitylation sites, 2 acidic domains (NAD and CAD) and a putative C_2H_2 DNA binding site. (B) Comparison of the steady-state levels of $Rpn4_{172-229}$-βgal (wt) and its derivatives with K187R mutation or Ser→Ala substitution(s) through measurement of βgal activity. The βgal fusions were expressed from the *CUP1* promoter in a low-copy vector. The βgal activity in the transformants expressing $Rpn4_{172-229/K187R}$-βgal was set at 100%. Values are the means of duplicate experiments of three independent transformants. Standard deviations are indicated. (C) Pulse-chase analysis of $Rpn4_{172-229}$-βgal and its derivatives as used in (B). Cells were labeled with [^{35}S]-methionine/cysteine for 5 min, chased for 0, 5 or 15 min. Cell extracts were immunoprecipitated with anti-βgal antibody, resolved by SDS-PAGE (6% gel), and subjected to autoradiography. (D) The metabolic turnover rates of $haRpn4_{\Delta 1-10}$ and its derivatives with Ser→Ala substitution(s) as indicated were measured by pulse-chase experiments. All mutants were expressed from the *CUP1* promoter in a low copy vector. Anti-ha antibody was used in the immunoprecipitation. The precipitated proteins were separated on SDS-PAGE (6% gel). Arrowhead indicates $haRpn4_{\Delta 1-10}$ and its derivatives, whereas asterisk marks a phosphorylated species of these proteins. (E) Quantitation of data in (D) by PhosphorImager to show the decay curves. The intensity of the signals at each time point was plotted as a percentage of the same protein at time zero (before chase), which was set at 100%. Note that the decay curve of the S191/196S mutant, which is similar to that of $haRpn4_{\Delta 1-10}$, was not included here to make the figure more viewable.

As shown in Fig. 2B, both S214A and S220A mutants migrated slightly but reproducibly slower upon treatment with yeast extract in the presence but not in the absence of ATP. The mobility shift was abolished after the fusion proteins were further treated with calf intestinal phosphatase (data not shown). These data demonstrate that both S214 and S220 can be phosphorylated.



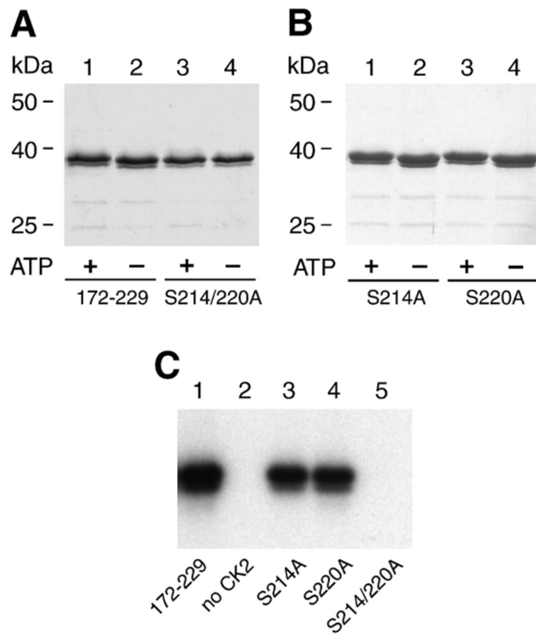


Fig. 2. S214 and S220 are phosphorylation sites. (A) Phosphorylation of NAD by yeast extract. Glutathione-conjugated agarose beads pre-loaded with GST-Rpn4$_{172–229}$ (lanes 1, 2) or GST-Rpn4$_{172–229/S214/220A}$ (lanes 3, 4) were incubated with yeast extract in the presence or absence of ATP. The GST fusion proteins were then separated on SDS-PAGE (10% gel), followed by Coomassie blue staining. A subtle mobility shift of GST-Rpn4$_{172–229}$ was observed after treatment in the presence of ATP (lane 1). (B) Phosphorylation of S214 and S220 by yeast extract. The same procedure as in (A) was applied to detect phosphorylation of GST-Rpn4$_{172–229/S214A}$ and GST-Rpn4$_{172–229/220A}$ by yeast extract. (C) In vitro phosphorylation of S214 and S220 by CK2. GST-Rpn4$_{172–229}$ and various mutants with S→A substitution(s) were pulled down from bacterial extracts, treated with CK2 in the presence of [γ-^{32}P]-ATP, separated on SDS-PAGE (10% gel), and subjected to autoradiography. GST-Rpn4$_{172–229}$ incubated with [γ-^{32}P]-ATP without CK2 was included as control (lane 2).

The mobility shift induced by phosphorylation of S214 and S220 is indeed rather subtle.

S214 and S220 are surrounded by acidic amino acids, suggesting that they may be phosphorylated by casein kinase 2 (CK2), an extremely conserved pleiotropic serine/threonine protein kinase [34]. To directly test this possibility, we performed an in vitro phosphorylation assay using recombinant CK2 expressed and purified from *E. coli*. GST-Rpn4$_{172–229}$ was pulled down from *E. coli* extract and treated with or without CK2 in the presence of [γ-^{32}P]-ATP. As shown in Fig. 2C, GST-Rpn4$_{172–229}$ was phosphorylated by CK2 (compare lanes 1 and 2). The in vitro kinase assay also showed that the S214A and S220A single mutants but not the S214/220A double mutant of GST-Rpn4$_{172–229}$ were phosphorylated by CK2 (lanes 3–5). Thus, both S214 and S220 can be phosphorylated by CK2.

3.3. S220 phosphorylation is crucial for Rpn4 ubiquitylation

To assess the involvement of phosphorylation of S214 and S220 in Rpn4 ubiquitylation, we examined the effect of S214A and S220A mutations on Rpn4 ubiquitylation in vivo. To facilitate the detection of Ub-conjugated substrates, we co-overexpressed myc-Ub or a void vector (as control) with haRpn4$_{Δ1–10}$, haRpn4$_{Δ1–10/S214A}$, haRpn4$_{Δ1–10/S220A}$ and haRpn4$_{Δ1–10/S214/220A}$, respectively, in yeast cells. These co-transformants were metabolically labeled with [^{35}S]-methionine for 5 min. [^{35}S]-labeled cell extracts were incubated with anti-ha antibody and the precipitated substrates were separated by SDS-PAGE. Whereas no ubiquitylated substrates were observed in the absence of myc-Ub overexpression, mono- and di-ubiquitylated (Ub1 and Ub2) species of the substrates were readily detected in the presence of myc-Ub overexpression (Fig. 3). Moreover, the ratio of ubiquitylated (Ub1 plus Ub2) to non-ubiquitylated (Ub0) species was much lower for haRpn4$_{Δ1–10/S220A}$ and haRpn4$_{Δ1–10/S214/220A}$ compared to haRpn4$_{Δ1–10}$ and haRpn4$_{Δ1–10/S214A}$. The difference in ubiquitylation efficiency is consistent with the different turnover rates of these substrates (Fig. 1). Together, this in vivo analysis demonstrates that S220 phosphorylation is crucial for efficient Rpn4 ubiquitylation, whereas the effect of S214 phosphorylation is relatively minor.

3.4. Multiple domains of Rpn4 interact with Ubr2

Modification of ubiquitylation signals by phosphorylation often increases substrate binding to E3s. To test if phosphorylation of NAD enhances the binding of Rpn4 with Ubr2, we used the GST pulldown assay, which has successfully demonstrated the Rpn4–Ubr2 interaction in our early work [28]. N-terminally FLAG-tagged Ubr2 (FLAG-Ubr2) purified from yeast cells by anti-FLAG affinity column was incubated with agarose beads pre-loaded with GST-Rpn4 and CK2-treated GST-Rpn4, respectively. Retained proteins were resolved by SDS-PAGE and analyzed by immunoblotting analysis with anti-FLAG antibody. As shown in Fig. 4A, ~5% FLAG-Ubr2 was pulled down by either GST-Rpn4 or CK2-treated GST-Rpn4 (upper panel, lanes 1–3), suggesting that phosphorylation of

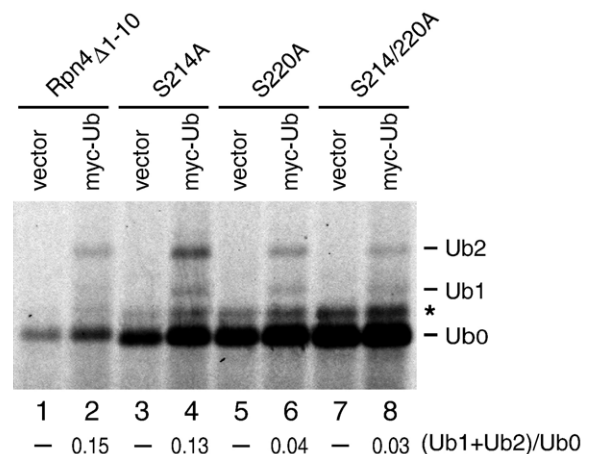


Fig. 3. Phosphorylation-dependent ubiquitylation of Rpn4. Cells co-expressing myc-Ub or a void vector with haRpn4$_{Δ1–10}$, haRpn4$_{Δ1–10/S214A}$, haRpn4$_{Δ1–10/S220A}$ and haRpn4$_{Δ1–10/S214/220A}$, respectively, were labeled with [^{35}S]-methionine/cysteine for 5 min. Cell extracts were immunoprecipitated with anti-ha antibody, followed by SDS-PAGE (6% gel) and autoradiography. The ratios of ubiquitylated (Ub1 plus Ub2) to nonubiquitylated (Ub0) species were assessed by PhosphorImager. Asterisk marks a phosphorylated species of the substrates.

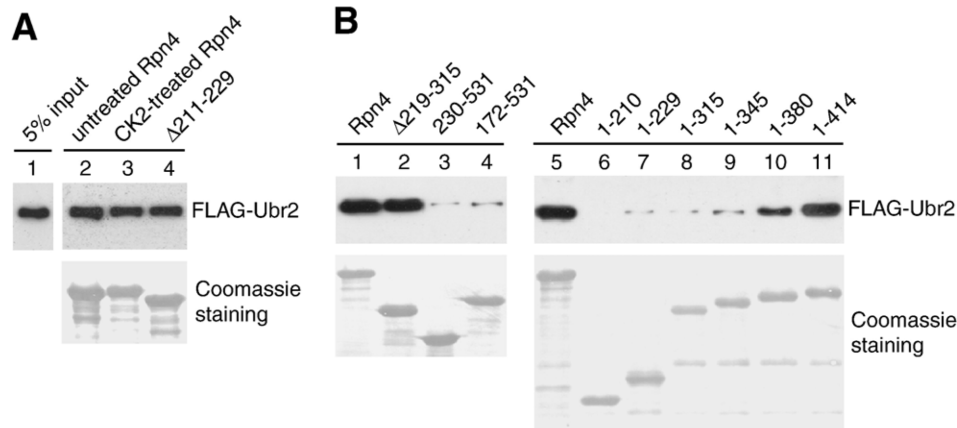


Fig. 4. Multiple domains of Rpn4 interact with Ubr2. (A) NAD is not required for physical association of Rpn4 with Ubr2. N-terminally FLAG-tagged Ubr2 (FLAG-Ubr2) was incubated with agarose beads pre-loaded with GST-Rpn4 (lane 2), CK2-treated GST-Rpn4 (lane 3) or GST-Rpn4$_{\Delta 211-229}$ (lane 4). Retained FLAG-Ubr2 was separated by SDS-PAGE (8% gel) and analyzed by immunoblotting with anti-FLAG antibody (upper panel). 5% of FLAG-Ubr2 input was included in the immunoblotting analysis to evaluate the pulldown efficiency (lane 1). Coomassie blue staining verified that comparable amounts of GST fusions were used in the pulldown assay (lower panel). (B) Multiple domains of Rpn4 involved in interaction with Ubr2. GST-Rpn4 and various truncated mutants as indicated were applied in pulldown assays with FLAG-Ubr2, following the same procedure as in (A).

NAD may not have a significant effect on Rpn4–Ubr2 interaction. However, it is also possible that NAD is not the only binding site for Ubr2, and, therefore, other binding sites may mask the effect of NAD phosphorylation. In support of the latter explanation, we found that FLAG-Ubr2 bound GST-Rpn4$_{\Delta 211-229}$, a mutant with NAD deleted, as efficiently as GST-Rpn4 (Fig. 4A, compare lanes 2 and 4). Coomassie blue staining verified that comparable amounts of GST fusions were used in the pulldown assay (Fig. 4A, lower panel).

We then decided to locate other Ubr2 binding site(s) on Rpn4. We first examined if CAD, a C-terminal acidic domain with residues 300–312 (Fig. 1A), could mediate the association with Ubr2 in the absence of NAD. FLAG-Ubr2 was incubated with agarose beads pre-loaded with GST-Rpn4 and GST-Rpn4$_{\Delta 219-315}$, respectively. GST-Rpn4$_{\Delta 219-315}$ carries an internal deletion covering CAD and large part of NAD (Fig. 1A). As shown in Fig. 4B, simultaneous deletion of NAD and CAD virtually had no effect on the stability of the Rpn4–Ubr2 association (compare lanes 1 and 2), suggesting that other Ubr2 binding site(s) involve N-terminal domain or C-terminal domain or both.

A series of truncated Rpn4 fragments from either the N-terminus or the C-terminus were fused to the C-terminus of GST, and applied in the pulldown assay. As shown in Fig. 4B, deletion of N-terminal 171 residues dramatically reduced the association of Rpn4 with Ubr2 (compare lanes 4 and 1). Our early work showed that deletion of the N-terminal 10 residues of Rpn4 exhibited no effect on binding with Ubr2 [28]. Thus, residues 11–171 are required for stable association with Ubr2. The association was further weakened by a larger deletion including residues 1–229 (lane 3). Interestingly, the sequences near the C-terminus are also important for stable association with Ubr2 (Fig. 4B, compare lanes 6–11 and 5). Removal of residues 381–531 dramatically reduced the binding stability (lane 10). Since deletion of residues 415–531 had a relatively minor effect (lane 11), residues 381–414 appear to be critical for stable association with Ubr2. The association was further weakened when the C-terminal deletion was extended to residue 346 (lane 9), suggesting that residues 346–380 are also important for Rpn4–Ubr2 interaction. The binding stability was further but only slightly decreased when deletion was extended to 316 and 230, respectively (lanes 7, 8). Interestingly, further deletion to residue 211 (removal of NAD) completely destroyed the association with Ubr2 (lane 6). Taken together, the pulldown assays demonstrated that multiple domains of Rpn4 participate in interacting with Ubr2. Stable association with Ubr2 requires residues 11–172 and 345–414, whereas the interaction between NAD and Ubr2 is relatively transient.

3.5. Phosphorylation enhances the binding of NAD to Ubr2

Evidently, it is difficult to evaluate the effect of NAD phosphorylation on interaction with Ubr2 using full-length Rpn4. Taking advantage of the observation that NAD is the only detectable Ubr2 binding site in the N-terminal region of Rpn4 including residues 1–229 (Fig. 4B, compare lanes 6 and 7), we examined if phosphorylation of NAD could enhance the binding of Rpn4$_{1-229}$ to Ubr2. Purified FLAG-Ubr2 was incubated with agarose beads pre-loaded with GST-Rpn4$_{1-229}$ or CK2-treated GST-Rpn4$_{1-229}$. Retained FLAG-Ubr2 was resolved by SDS-PAGE and analyzed by immunoblotting analysis with anti-FLAG antibody. As shown in Fig. 5, CK2-treated GST-Rpn4$_{1-229}$ indeed pulled down FLAG-Ubr2 more efficiently than un-treated GST-Rpn4$_{1-229}$ (compare lanes 2 and 3). The increase in NAD-Ubr2 binding was specifically caused by phosphorylation of NAD in that the binding of FLAG-Ubr2 to GST-Rpn4$_{1-229/S214/220A}$, a S214/220A double mutant, was virtually not affected by CK2 treatment (lanes 8, 9). We also examined the binding of Ubr2 to GST-Rpn4$_{1-229/S214A}$ and GST-Rpn4$_{1-229/S220A}$ with or without prior treatment with CK2 (lanes 4–7). It appeared that phosphorylation at either S214 or S220 was able to increase the binding stability with Ubr2.

3.6. The potential role of the interaction between Ubr2 and the domains other than NAD in Rpn4 degradation

Our previous work has defined NAD as the ubiquitylation signal essential for Rpn4 degradation [33]. The above analysis demonstrated that the N- and C-regions of Rpn4 constitute another Ubr2-binding site (Fig. 4). However, this rather stable interaction is apparently not required for Rpn4 degradation. For instance, K187 and NAD (including flanking residues 172–229) constitute a sufficient Ubr2-dependent degron (Fig. 1B, C, and ref. 33). In addition, the N-terminally ha-tagged Rpn4 fragment of residues 11–229 (haRpn4$_{11-229}$) was rapidly degraded (Fig. 6A, lanes 1–3, B). The degradation of haRpn4$_{11-229}$ is Ub-dependent because haRpn4$_{11-229/K0}$, a lysine-free version of haRpn4$_{11-229}$ was stable (Fig. 6A, lanes 7–9, B). Moreover, haRpn4$_{11-229}$ was stabilized in *ubr2Δ* cells (data not shown). These results indicate that the interaction between NAD (phosphorylated) and Ubr2 is sufficient to mediate Ub-dependent degradation of Rpn4. So what is the physiological significance of the stable association between Ubr2 and the other binding site on Rpn4? Our early study has demonstrated that Ub-dependent degradation of Rpn4 can be mediated by six different lysines (K123, K132, K137, K141, K158 and K187) in the N-terminal region, of which K187 is preferred whereas the other five lysines serve as alternative ubiquitylation sites in the absence of K187 [33]. We suspected that K187 is such an efficient Ub-acceptor that the interaction between Ubr2 and NAD is sufficient for Ub conjugation at K187. By contrast, a more stable association with Ubr2 may be crucial for use of the alternative ubiquitylation sites that are less efficient than K187 [33]. If this hypothesis is correct, one would expect that lysines other than K187 are unable to mediate the degradation of haRpn4$_{11-229}$. To address this issue, we compared the kinetics of haRpn4$_{11-229}$ degradation mediated by K187 and K158, one of the alternative ubiquitylation sites. haRpn4$_{11-229/K187}$ and haRpn4$_{11-229/K158}$ were derived from haRpn4$_{11-229}$, carrying only one lysine at position 187 and 158, respectively. Pulse-chase analysis showed that, while haRpn4$_{11-229/K187}$ was degraded as rapidly as haRpn4$_{11-229}$, haRpn4$_{11-229/K158}$ was almost as stable as haRpn4$_{11-229/K0}$ (Fig. 6A, B). Thus, a stable association between Ubr2 and the domains other than NAD is important for use of the alternative ubiquitylation sites of Rpn4. Note that these domains, while stably interacting with Ubr2, cannot replace NAD as a ubiquitylation signal because deletion of NAD abolishes the Ub-dependent degradation of Rpn4 [33].

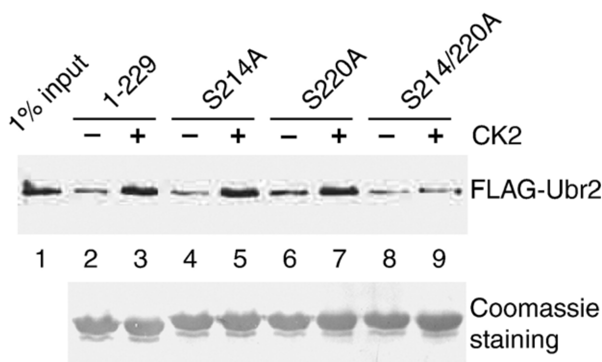


Fig. 5. Phosphorylation enhances the association of NAD with Ubr2. Agarose beads pre-loaded with GST-Rpn4$_{1-229}$, GST-Rpn4$_{1-229/S214A}$, GST-Rpn4$_{1-229/S220A}$, or GST-Rpn4$_{1-229/S214/220S}$ were treated with or without CK2 in the presence of ATP, then incubated with purified FLAG-Ubr2. Retained FLAG-Ubr2 was detected by immunoblotting analysis with anti-FLAG antibody (upper panel). 1% of FLAG-Ubr2 input was included in the immunoblotting analysis to estimate the pulldown efficiency. Comparable amounts of GST fusions were used as judged by Coomassie blue staining (lower panel).

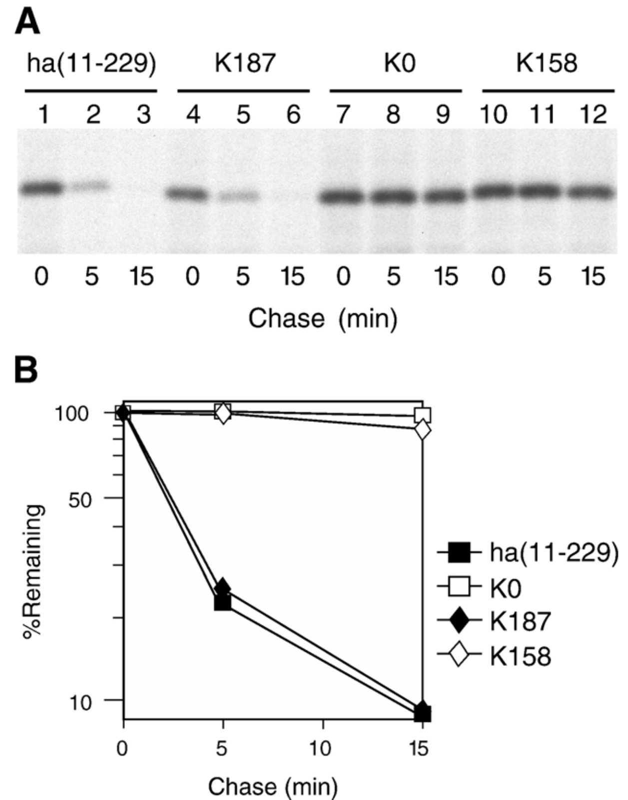


Fig. 6. Use of the alternative ubiquitylation sites of Rpn4 requires the interaction between Ubr2 and other Rpn4 domains. (A) Pulse-chase analysis was performed to determine the turnover rates of N-terminally ha-tagged Rpn4$_{11-229}$ (lanes 1-3), lysine-less haRpn4$_{11-229/K0}$ (lanes 7-9), haRpn4$_{11-229/K187}$ (lanes 4–6), and Rpn4$_{1-229/K158}$ (lanes 10–12). (B) Quantitation of the data in (A) by PhosphorImager to show the decay curves. The relative protein levels were normalized at time zero (before chase).

4. Discussion

We report two major findings in this study. First, Ub-dependent degradation of Rpn4 is controlled by a phosphorylation-dependent ubiquitylation signal. Second, multiple domains of Rpn4 interact with Ubr2, of which NAD functions as the ubiquitylation signal. The implications of our results are discussed below.

Phosphorylation-dependent ubiquitylation signals have been found in a number of substrates [8,9,35]. The exact role of phosphorylation in substrate recognition, however, is not fully understood. Presumably, phosphorylation provides a ubiquitylation signal with a negative charge that interacts with the cognate E3. For example, phosphorylation of S121 of Wee1, the

inhibitory kinase for cyclin-dependent kinase, allows its ubiquitylation signal to interact with the basic residues in the WD40 repeat domain of the F-box protein of the $SCF^{\beta\text{-TrCP}}$ Ub-ligase [35]. However, it seems unlikely that the increase in NAD–Ubr2 interaction by phosphorylation of S214 and S220 results from addition of a negative charge because NAD itself is already rich in acidic residues (Fig. 1A). We speculate that Ubr2 may directly recognize the phosphate moieties attached to S214 and S220. Alternatively, phosphorylation of S214 and S220 may cause a conformational change that makes NAD more accessible to Ubr2. It is interesting to note that phosphorylation at S214 and S220 has differential effect on Rpn4 ubiquitylation and degradation. While phosphorylation of S220 is virtually indispensable, the contribution of S214 phosphorylation is relatively minor. This differential effect is somewhat surprising, given that these two serine residues are closely spaced in NAD and phosphorylation of either one increases the binding stability with Ubr2. We propose that the effect of S220 phosphorylation may be twofold. First, it enhances the NAD–Ubr2 interaction. Second, it may also cause a local conformational change that optimizes the positioning of the Rpn4 ubiquitylation sites to the catalytic active site of the Ubr2/Rad6 Ub-ligase. By contrast, phosphorylation of S214 may lack the latter effect. In hindsight, this observation implies that binding to E3 is only a part of the function of a ubiquitylation signal (more discussion below). It will be of interest to determine whether Rpn4 is phosphorylated by CK2 in vivo and whether S220 phosphorylation is constitutive or subject to regulation.

Ub-ligases recognize substrates via their ubiquitylation signals. It is commonly perceived that a ubiquitylation signal equals an E3-binding site. We found that multiple domains of Rpn4 are able to interact with Ubr2. However, it is NAD but not other domains that functions as the ubiquitylation signal of Rpn4 [33]. These observations suggest that binding to E3 may be only one of the criteria for a ubiquitylation signal. The position of the E3-binding site in the substrate may be another important determinant. For example, a ubiquitylation signal should allow the bound Ub-ligase, more specifically the catalytic active site, to be placed in a suitable position such that the donor Ub can be efficiently transferred to the ubiquitylation site of the substrate. The primary sequence of Rpn4 does suggest that NAD is proximal to the preferred ubiquitylation site (K187), which may explain why NAD but not other Ubr2-binding domains serves as the ubiquitylation signal. More definitive study, however, is warranted for a better understanding of the molecular details of the ubiquitylation signal of Rpn4. Nevertheless, our results suggest that a ubiquitylation signal can be functionally distinct from an E3-binding site.

While the other Ubr2-binding domains do not function as a ubiquitylation signal and are not required for ubiquitylation at K187, their interaction with Ubr2 is crucial for use of the alternative ubiquitylation sites of Rpn4. This observation is in line with the current consensus that a decrease in substrate dissociation increases the chance of productive collision between the E3-bound substrate and the Ub-charged E2. K187 is likely a very efficient Ub-acceptor, perhaps due to its proximal position to the Rad6/Ubr2 active center, so that the interaction between Ubr2 and NAD, while relatively transient, is sufficient for Ub conjugation at K187. Compared to K187, the alternative ubiquitylation sites are presumably more distant from the active center of the Rad6/Ubr2 Ub-ligase, and, therefore, have less likelihood to be collided by the Ub-charged Rad6. A slower substrate dissociation rate, provided by the stable association between Ubr2 and other binding sites, would guarantee the encounter of the Ub-charged Rad6 to the alternative ubiquitylation sites before Rpn4 is released from Ubr2. Note that use of the alternative ubiquitylation sites remains NAD-dependent [33], further suggesting that an E3-binding site is not equal to a ubiquitylation signal. The current study highlights Rpn4 as an important model molecule for investigation of the molecular mechanism underlying protein ubiquitylation.

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