A Mechanism for Protein Monoubiquitination Dependent on a trans-Acting Ubiquitin-binding Domain*

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Background: The mechanisms by which ubiquitin chain length is regulated remain largely unknown.

Results: A ubiquitin-binding domain (UBD) acts in trans to direct substrate monoubiquitination in vitro.

Conclusion: UBD binding to the first conjugated ubiquitin prevents further polyubiquitination.

Significance: We report a new mechanism to control ubiquitin chain elongation.

The length of the ubiquitin chain on a substrate dictates various functional outcomes, yet little is known about its regulation in vivo. The yeast arrestin-related protein Rim8/Art9 is monoubiquitinated in vivo by the Rsp5 ubiquitin ligase. This also requires Vps23, a protein that displays an ubiquitin-E2 variant (UEV) domain. Here, we report that binding of the UEV domain to Rim8 interferes with ubiquitin chain elongation and directs Rim8 monoubiquitination. We propose that Vps23 UEV competes with Rsp5 HECT N-lobe for binding to the first conjugated ubiquitin, thereby preventing polyubiquitination. These findings reveal a novel mechanism to control ubiquitin chain length on substrates in vivo.

Ubiquitin ligases (E3) of the HECT (homologous to the E6-AP C terminus) family are enzymes that transfer ubiquitin to a substrate through an E3-ubiquitin thioester intermediate (1). Yeast Rsp5 is a member of the Nedd4 family of HECT E3 ubiquitin ligases, characterized by a common domain architecture, with a C-terminal catalytic HECT domain, an N-terminal phospholipid-binding C2 domain, and several WW domains responsible for substrate recognition via PY (PYX or PPXY) motifs (2). Rsp5 substrates containing such motifs are preferentially modified with long Lys-63-linked polyubiquitin chains in vitro, likely following a simple sequential-addition mechanism (3). However, some of these substrates are monoubiquitinated in vivo (4, 5), suggesting that additional mechanisms control ubiquitin chain length in the cell. It has been proposed that Ubp2, a deubiquitinating enzyme that antagonizes Rsp5 activity, promotes the monoubiquitination of certain targets by trimming Lys-63-linked ubiquitin chains (6, 7). Additionally, a process termed “coupled monoubiquitination” is responsible for the monoubiquitination of ubiquitin-binding domain (UBD)3-containing proteins by Nedd4/Rsp5 (8–10). These proteins use their UBD, instead of PY motifs, to interact with a self-ubiquitinated E3 ligase. The proposed model suggests that monoubiquitinated substrates cannot be further polyubiquitinated because the UBD folds back on the conjugated ubiquitin attached in cis, thus disrupting the association of the substrate with the ubiquitinated E3 enzyme. Nevertheless, it remains to be clarified how Rsp5 monoubiquititates proteins that do not have a UBD and use a PY motif for E3 recognition.

We previously found that Rsp5 binds to a PY motif in the arrestin-related protein Rim8/Art9 and catalyzes its monoubiquitination in vivo (11). Rim8 is a component of the ambient pH-signaling pathway and acts as an adaptor that mediates the association of the putative pH sensor Rim21 with the endosomal sorting complex required for transport (ESCRT). Data indicate that the monoubiquitinated lysine residue in Rim8 contributes to binding to the ESCRT-I subunit Vps23 (11). Intriguingly, Rim8 monoubiquitination requires Vps23 and is strongly induced upon overexpression of the Vps23 ubiquitin-binding UEV domain. In this study, we demonstrate, both in vitro and in vivo, that binding of Vps23 UEV to Rim8 favors its monoubiquitination by inhibiting its polyubiquitination. We suggest a model in which interaction of Vps23 UEV with the first conjugated ubiquitin prevents ubiquitin binding to Rsp5 HECT N-lobe and impairs further polyubiquitination.

Experimental Procedures

Strains and Genetic Methods—Saccharomyces cerevisiae strains were: SUB280 (MATa ubi1-1:TRP1 ubi2-Δ:ura3,ubi3-Δ:sub-2 ubi4-Δ2:LEU2 (pUB39-Ub) (pUB100) lys2-801 leu2-3112 ura3-52 his3-Δ200 trp1-1(αam)) and SUB413 (MATa ubi1-1:TRP1 ubi2-Δ2:ura3,ubi3-Δ:ub-2 ubi4-Δ2:LEU2 (pUB39-UbK63R) (pUB100) lys2-801 leu2-3112 ura3-52 his3-Δ200 trp1-1(αam)), and the isogenic vps23::KanMX6 derivatives, SUB280-vps23Δ and SUB413-vps23Δ (12, 13). Standard genetic methods were followed, and yeast cultures were grown in synthetic dextrose medium lacking appropriate amino acids to maintain selection for plasmids (14).

Plasmids—Plasmid for the expression of GST-Vps23UEV and HA-Rim8 were described in Ref. 11. The sequences encoding Rsp5, Mms2, Vps23, and Vps23 UEV domain (codons 1–161) were amplified from genomic DNA of S. cerevisiae and

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cloned into a PET19b (Novagen)-derived expression plasmid. The genes encoding Rim8 or the C-terminal fragment Rim8CT (codons 461–542) were designed to encode the cAMP-dependent kinase recognition site (RRASV) at the 5′ end and were cloned into pGE X6p1 (GE Healthcare). Vps23 mutation resulting in a S55A,D56A,G57A (∆SDG)4 triple substitution and Rim8 mutations resulting in K507R, K513R, K521R, or K527R substitutions and in the V505A,P506A,K507A,Y508A (∆APKY) or E533A,S534A,D535A,P536A (∆ASDP) quadruple substitutions were obtained by mutagenic PCR.

Protein Expression and Purification—GST- and His-tagged expression plasmids were transformed into *Escherichia coli* BL21, and bacterial cells were collected after a 2–3-h induction of protein expression in the presence of 0.1 mM isopropyl-D-1-thiogalactopyranoside. Cell pellets were resuspended in STE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 150 mM NaCl) containing 1% Triton X-100, 5 mM DTT, and Complete protease inhibitor mixture (Roche Applied Science) and lysed with a French press. His-tagged Rsp5, Ubc4, Mms2, Vps23, and Vps23 UEV domain were purified using Talon metal affinity resin (GE Healthcare). For labeling of proteins with32P, GST fusions were affinity-purified on glutathione-SepharoseTM 4B resin (GE Healthcare). The genes encoding Rim8 or the C-terminal fragment Rim8CT (1K) were grown to mid-log phase in synthetic dextrose medium. Protein extracts were prepared by alkaline lysis as reported previously (11), and 1 × 107 cell equivalents were analyzed by 7.5% SDS-PAGE and immunoblotting with anti-HA (3F10, Roche Applied Science). Antibodies were detected with ECL Plus reagents (Amersham Biosciences).

**RESULTS**

Vps23 UEV Directs Rim8 Monoubiquitination in Vitro—Previous *in vivo* studies showed that monoubiquitination of Rim8 by the E3 ubiquitin ligase Rsp5 is strongly enhanced by overexpression of the Vps23 ubiquitin-binding UEV domain (11). To gain insight into the mechanisms that underlie this process, we set up an *in vitro* ubiquitination assay to test whether Vps23 UEV affects Rim8 ubiquitination by Rsp5 *in vitro*. Strikingly, although Rim8 was efficiently polyubiquitinated by Rsp5 *in vitro* (Fig. 1A), the amount of monoubiquitinated Rim8 was significantly increased upon the addition of Vps23 UEV.

Rim8 contains two arrestin domains and a C-terminal region containing the *in vivo* ubiquitination site (Lys-521) as well as an Rsp5-binding PKY motif (Fig. 1B) (11). To further analyze the effect of Vps23 UEV on Rim8 ubiquitination, and because *in vitro* ubiquitination can occur at multiple sites and cause difficulties in the analysis of reaction products, we used as substrate a Rim8 C-terminal fragment (Rim8CT(1K)) containing Lys/Arg substitutions that leave only Lys-521 intact (Fig. 1B). Additionally, and to avoid potential ubiquitination of the terminal amino group of the substrate (3), ubiquitination assays were performed with a GST fusion to Rim8CT(1K), radiolabeled with 32P at a cAMP-dependent kinase recognition site (RRASV), and the GST tag was removed by protease cleavage after the ubiquitination reaction was terminated (Fig. 1B). Rim8CT(1K) was polyubiquitinated *in vitro* and was only modified at Lys-521 because no ubiquitinated products were detected with the lysine-less derivative Rim8CT(0K) carrying the K521R substitution (Fig. 1C). In agreement with previous *in vivo* data (11), *in vitro* ubiquitination was not observed with the Rim8CT(1K)∆PKY mutant derivative, in which the Rsp5-binding PKY motif has been substituted with alanines (Fig. 1C). Strikingly, the addition of Vps23 or the Vps23 UEV domain to the single-lysine substrate Rim8CT(1K) inhibited the formation of polyubiquitinated species, yielding a substrate ligated to only one ubiquitin (Fig. 1, D and E). This effect was only seen above a certain concentration of Vps23 UEV (2 μg/ml) (Fig. 1E), near the approximate stoichiometric equivalence with Rsp5 (UEV = 1.2 μg/ml). In contrast, increased Rim8CT(1K) monoubiquitination was not observed with equivalent or even larger amounts of the UEV domain containing protein Mms2 (Fig. 1F), thus indicating that this effect is not simply due to the presence of a ubiquitin-binding domain in the reaction mixture. To determine whether the effect of the Vps23 UEV domain on Rim8CT(1K) ubiquitination was only due to an inhibition of multiubiquitin chain assembly or also to an increase in the rate of the initial ubiquitination reaction, we performed a time course of ubiquitination of Rim8CT(1K) with K0 ubiquitin, which cannot form ubiquitin chains. In these conditions, even a large amount of Vps23 UEV (8 μg/ml) had no effect on Rim8 monoubiquitination.
Thus indicating that the Vps23 UEV domain does not affect the conjugation of the first ubiquitin.

**Synergistic Binding of Vps23 UEV to Ubiquitin and an SDP Motif**—Previous work indicated that the ubiquitin moiety added to Rim8 contributes, together with an SDP motif near the ubiquitinated residue, to binding to the Vps23 UEV domain (Fig. 2A) (11). To test whether these two binding sites act synergistically, we carried out a pulldown assay with GST-Vps23UEV as bait and *in vitro* ubiquitinated Rim8CT(1K) or its mutant derivative Rim8CT(1K)/H9004, in which the SDP motif has been substituted with alanines (Fig. 2B). Under these conditions, binding of Vps23 UEV to either ubiquitin or the SDP motif was not sufficient to produce a stable complex because neither the nonubiquitinated form of Rim8CT(1K) nor the ubiquitinated form of Rim8CT(1K)/H9004 was retained by Vps23 UEV (Fig. 2B, lanes 2 and 5). In contrast, a strong interaction was detected between Vps23 UEV and the monoubiquitinated or polyubiquitinated forms of Rim8CT(1K) (Fig. 2B, lane 2), thus indicating that simultaneous binding of the monoubiquitinated residue and the SDP motif to Vps23 UEV strengthens the overall binding.

**Molecular Determinants for Rim8 Monoubiquitination**—To further characterize the mechanisms underlying Rim8 monoubiquitination *in vitro*, we tested whether Vps23 UEV binding to ubiquitin and SDP motif is required for the inhibition of Rim8 polyubiquitination. Fig. 2C shows a time course of ubiquitination of Rim8CT(1K) with K0 ubiquitin, in the absence or presence (8 µg/ml) of Vps23 UEV (Fig. 2C, compare panel b with panel a) was not observed with the Rim8CT(1K)ΔSDP or Vps23 UEVΔSDG mutant derivatives carrying an S55A,D56A,G57A substitution in the β-hairpin tongue (15), which impairs ubiquitin binding without affecting binding to the SDP motif (11). Inhibition of Rim8 polyubiquitination was strictly dependent on binding of Vps23 UEV to both ubiquitin and SDP motif because the accumulation of monoubiquitinated Rim8CT(1K) upon the addition of Vps23 UEV (Fig. 2C, compare panel b with panel a) was not observed with the Rim8CT(1K)ΔSDP or Vps23 UEVΔSDG mutant proteins (Fig. 2C, panels c and d). These findings, together with previous *in vivo* data (11), support the idea that binding of the UEV domain to both the SDP motif and the monoubiquitinated residue prevents its further polyubiquitination by Rsp5.

Although the Rim8 Lys-521 residue is the physiological site of ubiquitination (11), we were able to detect *in vitro* ubiquitina-
nation, albeit at a lower efficiency, of a Rim8 C-terminal fragment (Rim8CT(3K)) lacking Lys-521 but containing the other three lysine residues present at the C terminus of the protein (Fig. 2D, lane 3). However, monoubiquitination of this substrate, in contrast to that of Rim8CT(1K), was not increased upon the addition of the Vps23 UEV domain (Fig. 2D), thus suggesting that the precise location of the Lys-521 residue plays an important role in this process.

**Vps23 Prevents Rim8 Polyubiquitination in Vivo**—Previous in vivo studies showed that monoubiquitinated Rim8 is undetectable in a vps23/H9004 mutant (11). In light of our present results, it is likely that monoubiquitinated Rim8 is further polyubiquitinated and possibly degraded in the absence of Vps23. This assumption predicts that inhibition of multiubiquitin chain assembly in a vps23/H9004 mutant should restore normal levels of monoubiquitinated Rim8. Because Rsp5 preferentially catalyzes the formation of Lys-63-linked chains in vivo (6, 16), we examined Rim8 monoubiquitination in a vps23Δ mutant expressing the UbK63R point mutant as the sole source of ubiquitin. Fig. 3A shows that expression of UbK63R in a vps23Δ mutant restores near normal levels of monoubiquitinated Rim8, which migrates as a doublet due to protein phosphorylation. We noted that levels of monoubiquitinated Rim8 were reproducibly higher in wild type cells expressing UbK63R (Fig. 3A, compare lane 2 with lane 4), which may be due to the formation of ubiquitin chains linked through lysine residues other than Lys-63 in the absence of Vps23. In summary, these results

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5 A. Herrador and O. Vincent, unpublished observations.
indicate that the absence of detectable levels of monoubiquitinated Rim8 in a vps23Δ mutant is mainly due to its polyubiquitination via Lys-63 linkage, thus supporting our in vitro data that the Vps23 UEV domain prevents mult ubiquitin chain assembly. The lack of detection of polyubiquitin ladder ing in the vps23Δ mutant may simply be caused by the low abundance of ubiquitinated Rim8, which is barely detectable as a single band in the wild type strain, or may result from either the degradation or the concurrent deubiquitination of the polyubiquitinated protein.

**DISCUSSION**

The results presented here address the molecular mechanism through which the ubiquitin-binding UEV domain of Vps23 directs the activity of the Rsp5 ubiquitin ligase toward the formation of monoubiquitinated Rim8. Our in vitro and in vivo data support the idea that Vps23 UEV promotes Rim8 monoubiquitination by preventing its further polyubiquitination. We propose the following models (Fig. 3B) for the mechanism of action of the Vps23 UEV domain. In the first step, Rsp5 binds to the PKY motif at Rim8 C terminus and catalyzes the conjugation of a single ubiquitin to the Lys-521 residue. In the absence of Vps23 (Fig. 3B, top), the chain is elongated by conjugation of an additional ubiquitin molecule via a Lys-63 linkage. Previous work showed that ubiquitin chain extension is dependent on an ubiquitin-binding site in the N-lobe of the Nedd4/Rsp5 HECT domain (Fig. 3B, top), which would orient the first ubiquitin to allow the addition of the next one and may also contribute to the interaction of the ubiquitinated substrate with the E3 (17, 18). Therefore, two models can be envisaged to explain how Vps23 prevents the second conjugation reaction. In the first model (Fig. 3B), interaction of the Vps23 UEV domain with ubiquitin could prevent further ubiquitination by restricting Lys-63 accessibility. However, previous findings do not support this hypothesis because Lys-63 is fully exposed in the Vps23 UEV-ubiquitin complex (Fig. 3C) (15) and SDP binding is not expected to produce any conformational changes in the UEV domain (19). Additionally, we showed that Rim8 polyubiquitination does not impair the ubiquitin-dependent interaction with Vps23 UEV in pulldown assays (Fig. 2B), thus strongly suggesting that the acceptor lysine residue in ubiquitin is located outside of the UEV-interacting surface. We therefore favor a second model (Fig. 3B) in which competition of the Vps23 UEV domain with the Rsp5 N-lobe for binding to the first conjugated ubiquitin prevents its correct positioning and impairs further polyubiquitination. Additionally, the inability of Rsp5 to bind ubiquitin may weaken its interaction with the monoubiquitinated substrate. The most compelling evidence for this competition model is that both the UEV domain and the Rsp5 N-lobe, which bind ubiquitin with low affinity, recognize the same hydrophobic patch of ubiquitin (15, 17). Thus, these two interactions are mutually exclusive, and binding of Vps23 UEV to the first conjugated ubiquitin should preclude ubiquitin chain extension (Fig. 3B). In this context, the Rsp5/Vps23 UEV stoichiometry could play an important role, as suggested by our in vitro data that Vps23 UEV prevents ubiquitin chain elongation above a minimum threshold value near the equimolar Rsp5/UEV concentration. Additionally, the relative position of the SDP motif with respect to the ubiquitinated lysine residue in Rim8 should also be critical in positioning the UEV domain to favor its interaction with the first conjugated ubiquitin. This observation is consistent with our findings that Vps23 UEV does not prevent the polyubiquitination of lysine residues other than Lys-521 in vitro (Fig. 2D) and with the high level of conservation of the distance between Lys-521 and the SDP motif in Rim8 homologs in other fungi (11).

In summary, our findings reveal a new mechanism by which the Rsp5 ubiquitin ligase, together with the UBD-containing protein Vps23, promotes the monoubiquitination of the ESCRT-I adaptor Rim8. In the process referred to as coupled monoubiquitination, some UBD-containing substrates of Nedd4/Rsp5 undergo monoubiquitination in a UBD-dependent manner (8–10). Here, we extend these findings and show that a UBD can also promote protein monoubiquitination when acting in trans, thus opening the possibility that other monoubiquitinated substrates of Rsp5, which bind to UBD-containing proteins, are regulated in a similar fashion. Interestingly, the Vps23 mammalian homolog, Tsg101, appears to promote the monoubiquitination of the apoptosis-antagonizing transcription factor by preventing its further polyubiquitination (20). Further work will be required to determine whether Tsg101 plays a similar role as Vps23 in the regulation of ubiquitin chain elongation.

**REFERENCES**


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