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Molecular Biophysics: The Ubiquitin-associated (UBA) 1 Domain of Schizosaccharomyces pombe Rhp23 Is Essential for the Recognition of Ubiquitin-proteasome System Substrates Both in Vitro and in Vivo

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The ubiquitin-proteasome system is essential for maintaining a functional cell. Not only does it remove incorrectly folded proteins, it also regulates protein levels to ensure their appropriate spatial and temporal distribution. Proteins marked for degradation by the addition of Lys48-linked ubiquitin (Ub) chains are recognized by shuttle factors and transported to the 26 S proteasome. One of these shuttle factors, Schizosaccharomyces pombe Rhp23, has an unusual domain architecture. It comprises an N-terminal ubiquitin-like domain that can recognize the proteasome followed by two ubiquitin-associated (UBA) domains, termed UBA1 and UBA2, which can bind Ub. This architecture is conserved up to humans, suggesting that both domains are important for Rhp23 function. Such an extent of conservation raises the question as to why, in contrast to all other shuttle proteins, does Rhp23 require two UBA domains? We performed in vitro Ub binding assays using domain swap chimeric proteins and mutated domains in isolation as well as in the context of the full-length protein to reveal that the Ub binding properties of the UBA domains are context-dependent. In vivo, the internal Rhp23 UBA2 domain provides sufficient Ub recognition for the protein to function without UBA1.

Ubiquitin (Ub) is a conserved 76-amino acid protein that is utilized by eukaryotic cells as a dynamic signaling molecule to regulate many intracellular pathways. However, by far the most studied Ub-dependent process is its use as a signal to target proteins for degradation by the 26 S proteasome, a multisubunit protease. An enzyme cascade involving activating, conjugating, and ligase enzymes transfers Ub to a lysine residue of a specific target protein (1–4). Ub itself has seven lysine residues, all of which can nucleate chain formation. However, biochemical and genetic studies have implicated only Lys29-, Lys48-, and Lys63-linked chains as having a role in protein degradation with Lys48 chains being by far the most important (5, 6). A Ub chain of at least four molecules in length is required for the protein substrate to be efficiently recognized by the 26 S proteasome (5, 7). In addition, Lys11-linked chains have recently been shown to be specifically targeted by the cell cycle regulator E3 ligase anaphase-promoting complex/cyclosome (8).

Protein substrates are recognized at the proteasome by mult ubiquitin receptors. Two subunits of the proteasome, Rpn10 (also called Pus1 in fission yeast) and Rpn13, have the ability to recognize mult ubiquitin chains via their C-terminal ubiquitin-interacting motif (UIM) and N-terminal Pru domain (9–11), respectively. Members of another class of Ub receptors only interact transiently with the proteasome and are not subunits of the multiprotein protease. This class of receptors has been termed “shuttle proteins” as they are thought to transport ubiquitinated substrates destined for degradation from the E3 ligase to the proteasome. Functionally, each of the shuttle proteins shares similar biochemical properties. They each have a mult ubiquitin binding domain to recognize UPS substrates and a proteasome-interacting domain to transiently interact with the proteasome (12, 13).

The best characterized of these receptors in Schizosaccharomyces pombe are Dph1 (equivalent to Dsk2 in budding yeast), Rhp23 (Rad23), and Pus1 (Rpn10). Pus1 uniquely appears to localize at the spindle pole body and interacts transiently with the proteasome. Rhp23, a member of the UBA family, shares similar biochemical properties.
function both as a subunit of the proteasome and as a shuttle protein (10, 14). It contains an N-terminal von Willebrand factor-type A domain that recognizes the proteasome (12) and a C-terminal UIM (10, 15). Dph1 and Rhp23 both contain a ubiquitin-like domain at the N terminus to dock onto the proteasome (16–18) and a ubiquitin-associated (UBA) domain at the C terminus that binds Ub (19). Unusually, Rhp23 contains an additional internal UBA domain that is conserved in all of its eukaryotic orthologues and therefore must be important for the in vivo function of the Rhp23 protein (20, 21). We call the internal Rhp23 UBA domain UBA1 and the C-terminal domain UBA2.

In fission yeast, single deletion of the pus1, dph1, or rhp23 genes results in viable cells with a modest proteolytic phenotype. In addition, dph1Δrhp23Δ and dph1Δpus1Δ double mutants display a mild synthetic phenotype for growth. In contrast, the rhp23Δpus1Δ double mutant shows a dramatic synthetic growth phenotype. At 25 °C, the double mutant growth rate is severely compromised compared with wild type, whereas at 36 °C, the double mutant strain is not viable. The triple mutant dph1Δrhp23Δpus1Δ cannot be constructed, demonstrating that shuttle factors are essential (13).

Surface plasmon resonance experiments have shown that the isolated human hRAD23A UBA1 domain binds preferentially to Lys63-linked chains over Lys48, whereas the UBA2 domain binds Lys48-linked chains better than those linked through Lys63 (22, 23). This result suggests a model in which the two domains may allow Rhp23 to recognize a greater variety of substrates because Lys63 can also signal for degradation.

However, work carried out by Heessen et al. (24) has shown that a single point mutation in the UBA2 domain of Rad23 in Saccharomyces cerevisiae results in a 75% decrease in its half-life. Subsequent removal of the ubiquitin-like domain to prohibit Rad23 from binding to the proteasome restabilized the level of the protein, leading the authors to conclude that the UBA2 domain protects Rad23 from being degraded by the proteasome during substrate transport (24). More recent work by the same group using domain swap experiments in which UBA1 was replaced with UBA2 and vice versa demonstrated that only UBA2 at the C terminus had a protective effect (25).

Rhp23 is also involved in nucleotide excision repair where it forms a complex with Rph41 (Rad4) to photoactivate and help initiate DNA repair. Within this complex, the role of Rhp23 again seems to be to confer stability because a lack of the homologue Rad23 causes degradation of Rad4 and Xeroderma pigmentosum Group C protein. However, neither UBA domain seems to be involved in the nucleotide excision repair pathway (26–28).

In this study, we prepared a series of Rhp23 mutants in which either (a) point mutations were introduced in both UBA domains to disrupt Ub binding or (b) the UBA domains were interchanged. Chimeric proteins were also constructed to test whether the Pus1 UIM could be replaced with a UBA. Using a combination of in vitro biochemical and biophysical binding assays as well as phenotypic characterization of the in vivo rhp23Δpus1Δ phenotype, we demonstrate that the Rhp23 UBA domains differ in their affinities for Ub chains when in isolation compared with the context of the full-length Rhp23 or Pus1 proteins. We also show that the UBA1 domain is primarily responsible for binding Ub conjugates for the UPS.

**EXPERIMENTAL PROCEDURES**

**Strains and Media**—This study used strains derived from 972h+ and 975h− using the standard background leu1-32, ura4-D18, and ade6-M216. For the in vivo assay, we used pus1Δ::NatR− (this study) rhp23::ura4Δ h+ (13).

All strains were grown in either liquid or solid yeast extract with supplements or PMG with the appropriate antibiotic or nutritional selection. Crosses and lithium acetate transformations with pREP81 plasmid were performed via standard protocols (29).

**General DNA Methods**—Primers used for site-directed mutagenesis of rhp23 in pBS were as follows: Rhp23 M157A: Fw 5′-GGC GAT CCT TAA TAG TGT AGT AAG CCG AGC CAG CGC; Rev 5′-CGC TGC GTT GTC ATC CCC CTT CTA CCA ATT TTC AC; Rhp23 L183A: Fw 5′-GCC AGT GGA ATA CTT AGC TGG TAT TCC CGA AG; Rev 5′-CTT CG G GAA TAG TGG CTA AGT ATT CCT ACA CTG CC; Rhp23 L332A: Fw 5′-TAG ATT ATG TCA AGT GCG CTT CTA CCA GAG AAA; Rev 5′-TTT CTG TCG AAG CCA GCA GTC GTA CAT AAT CTA; and Rhp23 F358A: Fw 5′-GCT GAT GCT AAT ACC TTG CCC AGC ATG GAC ATG; Rev 5′-CAT GTC CAT GCT CGG CAA GGT AAT TAG CAC G.

Mutations were made using the QuickChange® site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions. Mutated and authentic rhp23+ were cloned into the pREP81 and pGEX6P1 plasmids in the Sall and BamHI sites using Fw 5′-ACG CGT CCA CTG ATG AAT TTG ACA TTC AAA AAT CTA CAG CAG and Rev 5′-CGC GAA TGA TGG TAT TCC AAA ATT ACT CAA GAA GAA TCT G and Rhp23 FL Rev. 

**Construction of Chimeric Domain Swap Constructs**—Chimeric proteins of Pus1ΔUIM (amino acids 1–195) fused to either Rhp23 UBA1 (amino acids 146–190) or UBA2 (amino acids 314–368) domains were created by homologous PCR. In the case of Pus1ΔUIM, the chimeric protein was amplified using Fw 5′-ACG CGT CCA CAT GGT GGT ATG AGC AAG AAG GAT GA and Rev 5′-TCG ACA GCA ACA TTT GTT GGT GAG CAA CTA CAC CTT GTC CAA, whereas the UBA1 domain was amplified by Fw 5′-TTG GAC AAC ATG TGG TAG TGG CTT CTA AAC AAC GAA ATG TGG TTG TCG A and Rev 5′-CGG GAT CTA CAC GGT TCA GCA AAG GAT CAA ATT ACT CAA GAA GAT TCT G and Rhp23 FL Rev.
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purified by gel extraction, and both Pus1ΔUIM and UBA PCR products were used as template DNA to amplify the new domain swap construct. For Pus1ΔUIM+UBA1, the Pus1ΔUIM Fw primer was used with the UBA1 Rev primer. Pus1ΔUIM+UBA2 was amplified using the Pus1ΔUIM Fw and UBA2 Rev primers. Products were then inserted into pGEX6P1. Rhp23UBA1mutΔUBA2+UBA1 was made in the same way using Fw 5’-AGC CGT CGA CAT GGT GTT AGA AGC AAC GTG A and Rev 5’-TTC ACA GCA ACT TTTC GCT GCT GAA TTC CAC CAG AAG GTA to amplify Rhp23M157A/L183AUBA2, whereas Fw 5’-TTC CTT ACC TTC TGG TGG ATT GTA CCA GAA AAA TGT TGC TGT and Rev 5’-AGC CGT CGA CAT GGT GTT AGA AGC AAC GTG A were used to amplify UBA1. Rhp23UBA1mutΔUBA2+UBA1 Rev primers were then used to produce the chimeric construct in pGEX6P1.

In Vitro Binding Assay—GST fusion proteins were produced from recombinant Escherichia coli BL21 (DE3) pLysS cells containing the various pGEX6P1 constructs grown at 37 °C to an OD of 0.4–0.8. Isopropyl-β-d-galactopyranoside was added to 0.1 mM, and cells were incubated at 25 °C for 4 h. Cells were lysed by sonication in a GST binding buffer (50 mM Tris, pH 7.5, 100 mM NaCl, 10% glycerol, and 0.1% Triton X-100 supplemented with 1 mM PMSF and one Complete protease inhibitor tablet (Roche Applied Science)/50 ml of buffer). The fusion proteins were then purified on glutathione-Sepharose 4B beads (GE Healthcare) following the manufacturer’s protocol. 5–30 μl of beads were incubated with 100 μl of either Lys48- or Lys63-linked Ub chains (final concentrations of 6.25 ng/μl for Lys48-linked Ub and 12.5 ng/μl for Lys63-linked Ub) and 0.5 mM EDTA (supplied by Boston Biochem) in TBS buffer supplemented with one complete EDTA-free inhibitor tablet (Roche Applied Science)/50 ml. Mixtures were incubated for at least 2 h at 4 °C, and beads were washed five times with TBS buffer to remove unbound chains. After the final wash, the beads were boiled in SDS-PAGE gel loading buffer for 2 min to release bound proteins, which were then separated by SDS-PAGE and visualized by Coomassie staining and Western blot analysis using anti-Ub antibody (Dako) at 1:1000 in 5% BSA.

Protein Purification and Identification—Recombinant full-length Rhp23 protein with and without mutations as well as the isolated domains were purified from crude extracts of recombinant E. coli using glutathione-Sepharose 4B beads as described above. The samples used for fluorescence anisotropy measurements were further purified by size exclusion chromatography using a Sephadex S-200 column (GE Healthcare) equilibrated in 50 mM Tris, pH 7.5, 100 mM NaCl, and 0.5 mM EDTA. Prior to this step, GST was removed by incubating the protein samples with PreScission protease 3C at a 1:200 molar ratio for at least 6 h at 4 °C. The purity of the samples was confirmed by SDS-PAGE. Circular dichroism (CD) spectroscopy was performed (see supplemental Experimental Procedures) to confirm the structural integrity of the full-length Rhp23 mutants. The Rhp23 molar extinction coefficient was calculated experimentally by amino acid analysis.

Fluorescence Anisotropy Assay—The K63C mutation was introduced into Ub by site-directed mutagenesis. Lys48- and Lys63-linked Ub were prepared as described (30, 31). Mono-Ub, Lys48-, and Lys63-linked Ub2 were fluorescently labeled according to the manufacturer’s instructions with the thiol-reactive maleimide derivative of Oregon Green 488 (Invitrogen). The reaction mixture contained a 100 nM concentration of fluorescent probe (mono-Ub, Lys48-, or Lys63-Ub2) and 0–375 μM concentrations of various Rhp23 constructs in a total volume of 20 μl. The reaction mixtures were prepared in triplicate, and fluorescence polarization was recorded in black low protein-binding 384-well plates (Corning) using the PHERAstar FS plate reader (BMG Labtech) equipped with a fluorescence polarization module. The binding curves were analyzed in Prism 5 (GraphPad) using a one-site binding model with nonspecific binding (NS), starting anisotropy level (BG), and ligand depletion by binding to the fluorescent probe (P) calculated according to the following equation.

\[
\gamma = \frac{B_{\text{max}} \times (P + x + K_d)}{P + x + K_d} - \frac{(P + x + K_d)^2 - (4P \times x)}{2P} + NS \times \frac{x}{P} (\text{Eq. 1})
\]

In Vivo Assay—pREP81 plasmids were stably integrated into rhp23::ura and crossed to produce an rhp23::ura pus1::natR double mutant using standard protocols. For each cross, 1 ml of water was mixed with 5 μl of β-glucuronidase (Sigma, reference number G0876) before sterilizing through a filter and aliquoting. A loop full of the crossed cells taken from the middle of the cross was suspended in the β-glucuronidase solution and incubated overnight at 25 °C. The produced spores were washed twice in 1 ml of water, and 5000 spores were plated on PMG-Ura-Leu+NAT and incubated at 36 °C to detect rescue.

RESULTS

Mutation of Rhp23 UBA Domains—To dissect the role of the Rhp23 UBA1 and UBA2 domains in the UPS, we constructed mutant versions of each domain that had lost the ability to interact with Ub chains. Care was taken in the design of these mutant versions to avoid causing a gross loss of structural integrity. The authenticity of the mutant folds was verified by circular dichroism (supplemental Fig. S1). To predict key residues important for Ub interaction, we used the data reported by Ryu et al. (17) that characterized the interaction between human RAD23B and Ub. Using these structural findings and a sequence comparison of the S. cerevisiae and S. pombe homologues, we identified two residues in each UBA domain that are important for Ub interaction. The authenticity of the mutant folds was verified by circular dichroism (supplemental Fig. S1). To predict key residues important for Ub interaction, we used the data reported by Ryu et al. (17) that characterized the interaction between human RAD23B and Ub. Using these structural findings and a sequence comparison of the S. cerevisiae and S. pombe homologues, we identified two residues in each UBA domain that are predicted to be important for Ub binding (17). These residues are Met157 and Leu183 within UBA1 and Leu332 and Phe358 within the UBA2 domain. All of these residues were mutated to alanine to disrupt the binding of the UBAs to Ub. These mutant versions of Rhp23 were cloned into the S. pombe expression vector pREP81 and the bacterial expression vector pGEX6P1 to produce WT Rhp23, Rhp23 M157A/L183A (Rhp23UBA1mut), Rhp23 L332A/F358A (Rhp23UBA2mut), and Rhp23 M157A/L183A L332A/F358A (Rhp23UBA1&UBA2mut). In addition, individual pGEX6P1 UBA1 (residues 146–190), UBA1 M157A/L183A (UBA1mut), UBA2 (residues 314–368), and UBA2 L332A/F358A (UBA2mut) were constructed (Fig. 1).

Ubiquitin Binding Assays of Isolated UBA Domains—Recombinant GST fusion proteins of the individual authentic and
mutant UBA1 and UBA2 domains were bound to glutathione-Sepharpse 4B beads. Visualization by Coomassie staining following SDS-PAGE confirmed the presence of a single species of the expected size for all four proteins. These fusion proteins were tested for their ability to bind Lys48- and Lys63-linked Ub chains. WT full-length Rhp23 and Pus1 as GST fusions and GST were used as positive and negative controls, respectively, on a Western blot with anti-Ub antibodies (Fig. 2). The results showed that both UBA domains bind comparably to Lys48- and Lys63-Ub chains but with a lower affinity when compared with full-length Rhp23 and Pus1. Under these experimental conditions, the subsequent introduction of the point mutations M157A and L183A into UBA1 and L332A and F358A into UBA2 expressed as isolated domains resulted in no detectable binding to either Lys48 or Lys63 chains.

To quantify these results and to avoid potential artifacts arising from the GST tag, the binding of untagged and fluorescently labeled mono-Ub, Lys48-Ub2, and Lys63-Ub2 to full-length Rhp23 and to the individual authentic and mutated UBA domains was measured using fluorescence anisotropy (Fig. 3). Under these experimental conditions, full-length Rhp23 showed an approximately 15-fold preference for binding to Lys48-Ub2 compared with Lys63-Ub2 or mono-Ub and produced affinities of 7.0 ± 0.8, 103 ± 36, and 138 ± 47 μM, respectively (Fig. 3A and Table 1). Authentic UBA1 also showed a preference for Lys48-Ub2 (Fig. 3B and Table 1). Binding ofUb to UBA2 was significantly weaker, displaying non-appreciable affinities (>200 μM) for mono-Ub and Lys63-Ub2 but significant binding to Lys48-Ub2 (48 ± 13 μM; Fig. 3C and Table 1). As expected, the introduction of the M157A and L183A mutations into the UBA1 domain and the L332A and F358A mutations into the UBA2 domain resulted in a considerable loss of binding to Lys48-Ub2 in both cases (Fig. 3E).

Ubiquitin Binding Assays of Rhp23 with Mutated UBA Domains—GST fusion Rbp23UBA1mut, Rbp23UBA2mut, and Rbp23UBA1&UBA2mut were also tested for their ability to bind Ub chains by Western blot analysis. Rbp23UBA1mut could not bind Lys63 chains and had only a limited residual ability to bind Lys48-Ub2 (34 ± 7 μM) and Lys63-Ub2 but significant binding to Lys48-Ub2 (48 ± 13 μM; Fig. 3C and Table 1). As expected, the introduction of the M157A and L183A mutations into the UBA1 domain and the L332A and F358A mutations into the UBA2 domain resulted in a considerable loss of binding to Lys48-Ub2 in both cases (Fig. 3E).
UBA1 of Rhp23 Binds Ubiquitin Chains for the UPS

Again consistent with the results of the Western blot analysis. Taken together, these results suggest that the UBA1 domain is responsible for the majority of the interaction of Rhp23 with Lys^{63}-linked Ub.

**Ubiquitin Binding Assays of Pus1 and Rhp23 Chimeric Proteins**—To test the ability of each Rhp23 UBA domain to recognize Ub chains independently of the protein context, Pus1∆UIM, Pus1∆UIM+UBA1, and Pus1∆UIM+UBA2 were produced. In the Pus1∆UIM+UBA1 and Pus1∆UIM+UBA2 constructs, the Pus1 UIM was replaced with either the Rhp23 UBA1 or the UBA2 domain. In this context, only UBA1 restored the Pus1 function lost by the removal of the UIM motif (Fig. 5). Unlike Pus1∆UIM+UBA1, the chimeric protein containing the UBA2 domain was unable to recognize either Lys^{48} or Lys^{63} chains. This finding further highlights the role of UBA1 as the major Ub binding unit of Rhp23 (Fig. 5). To explore further whether it is an intrinsic property of the UBA1 domain or its position within the Rhp23 protein that confers its Ub binding properties, another chimeric version of Rhp23 was constructed in which the authentic UBA2 domain of the Rhp23UBA1mut construct, which shows reduced binding to Ub chains (see Fig. 4), was replaced by the UBA1 domain (see Fig. 1). When tested as a GST fusion, the Rhp23UBA1mut∆UBA2+UBA1 mutant protein bound both Lys^{48} and Lys^{63} chains to levels comparable with WT Rhp23 (Fig. 6). This result clearly demonstrated that UBA1 function is independent of the position of the domain within the protein.

**In Vivo Function of the Different Rhp23 Mutant Constructs**—Previous work has established that the ts phenotype of the S. pombe rhp23 Δ/sps1Δ mutant results from a failure to deliver substrates to the proteasome (13). Therefore, the rescue of this strain at a restrictive temperature by expression of an Rhp23 mutant would indicate the presence of an intact UBA domain able to mediate this role of Rhp23 in the UPS. The fission yeast expression vector pREP81 containing either authentic or mutated rhp23 was stably integrated into an rhp23-Δ/sps1Δ null strain containing the puro marker. Asci were digested with β-glucuronidase, and 5000 spores were crossed to a ura selectable marker. These strains were then stably integrated into an ura marker. Asci were digested with β-glucuronidase, and 5000 spores were

### Table 1

<table>
<thead>
<tr>
<th>Binding affinities of wild-type and mutant Rhp23 for mono-Ub, Lys^{48}-, and Lys^{63}-linked Ub.</th>
<th>( K_d ) (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mono-Ub</td>
<td>Lys^{48}-Ub</td>
</tr>
<tr>
<td>Rhp23 WT</td>
<td>138 ± 47</td>
</tr>
<tr>
<td>Rhp23UBA1mut</td>
<td>34 ± 7</td>
</tr>
<tr>
<td>Rhp23UBA2mut</td>
<td>—</td>
</tr>
<tr>
<td>Rhp23UBA1&amp;UBA2mut</td>
<td>126 ± 80</td>
</tr>
<tr>
<td>UBA1 WT</td>
<td>126 ± 80</td>
</tr>
<tr>
<td>UBA1mut</td>
<td>ND</td>
</tr>
<tr>
<td>UBA2 WT</td>
<td>&gt;200</td>
</tr>
<tr>
<td>UBA2mut</td>
<td>—</td>
</tr>
</tbody>
</table>

ND, not detected; —, not determined.

FIGURE 3. Interaction of Rhp23 with mono- and di-Ub. The binding of mono-Ub, Lys^{48}, and Lys^{63} linked Ub, to full-length Rhp23 and to the isolated UBA1 and UBA2 domains was determined using fluorescence polarization. A, full-length authentic Rhp23 binding to Lys^{48}-Ub (green), mono-Ub (black), and Lys^{63}-Ub (brown). Rhp23 bound Lys^{48}-Ub with 7.0 µM affinity but shows a more than 10-fold lower affinity (138 and 103 µM, respectively) for both mono-Ub and Lys^{63}-Ub. B and C, UBA1 (B) and UBA2 (C) binding to mono-Ub and Lys^{48}- and Lys^{63}-Ub. Binding curves for the association of the isolated authentic UBA1 and UBA2 domains with mono-Ub (black), Lys^{48}-Ub (green), and Lys^{63}-Ub (brown) are shown. UBA1 has an ~6-fold lower affinity for mono-Ub compared with Lys^{63}-Ub (18 versus 126 µM) but shows less ability to discriminate Lys^{63}-Ub (51 µM), UBA2 is a weaker binder of Ub. Its measured affinities for both mono-Ub and Lys^{63}-Ub were >200 µM. Lys^{63}-Ub bound more strongly (48 µM) but much weaker than UBA1. D, binding of full-length authentic and mutant Rhp23 to Lys^{63}-Ub. In the context of full-length Rhp23 (authentic Rhp23; black), mutation of UBA2 (blue) had almost no effect, reducing the affinity for Lys^{63}-Ub, from 7.0 to 11.9 µM. However, when UBA1 was mutated (orange), the affinity decreased about 4.5-fold to 34 µM. Mutation of both UBA2 (red) resulted in almost complete loss of binding with an affinity of >200 µM. E, binding of the isolated mutant UBA1 and UBA2 domains to Lys^{48}-Ub. Mutation of UBA1 (red) results in the loss of Lys^{48}-Ub binding, but the mutated UBA2 domain (orange) retains some residual affinity (>200 µM). Authentic unmutated UBA1 (black) and UBA2 (blue) are the same as in B and C. mP, millipolarization units. Error bars represent standard deviation of multiple measurements.

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plated onto PMG-Leu-Ura+NAT to select for double mutants that carry the plasmid and grow at 36 °C. The results shown in Fig. 7 reveal that, as expected, the empty pREP81 vector did not rescue the phenotype, whereas expression of WT Rhp23 produced viable cells. Expression of both the Rhp23UBA1mut and the Rhp23UBA2mut constructs was unable to rescue the double mutant lethal phenotype. In contrast, the Rhp23UBA2mut construct rescued the lethal phenotype as did the WT Rhp23. Although differences in the levels of expression of the authentic and mutant Rhp23 proteins from the pREP81 promoter might obscure subtle differences in the efficiencies with which they can rescue the *rhp23*/*pus1* mutant strain at a restrictive temperature, these results support the in vitro studies and indicate that the UBA1 domain of Rhp23 is sufficient for the recognition of ubiquitinated substrates in vivo for the UPS.

**DISCUSSION**

It is now recognized that Ub has important roles in many aspects of cellular regulation (32, 33) and in disease (34—37). A more complete understanding of Ub signaling will require determination of how mono-Ub and the various Ub chains are recognized by different domains that differ not only in sequence but also in architecture. In this regard, the two UBA domains of Rhp23 that can be distinguished by their Ub binding properties are an example. These domains differ in sequence but adopt a very similar three-dimensional structure composed of three consecutive α-helices and are both able to bind Lys48 and Lys63 chains (21).

Although extensive work has been carried out to dissect the roles of the UBA domains, this is the first study where the function of these domains has been directly compared both in isolation and in the context of full-length protein. Our results show that the Ub binding properties of the individual domains and full-length Rhp23 differ. The isolated Rhp23 UBA1 and UBA2 both bind to Lys48 and Lys63 chains albeit with different affinities. However, in the context of full-length protein, both in vitro and in vivo, Lys48 and Lys63 chain binding is primarily a function of UBA1. We have demonstrated that a functional UBA1 domain is sufficient to rescue the ts phenotype of the *rhp23Δpus1Δ S. pombe* strain, suggesting that the UBA2 domain is redundant. This result was unexpected given that previous studies had reported that UBA2 displays a preference for Lys48 chains, whereas UBA1 better recognizes Lys63-linked chains (22). However, our results do concur with similar studies in *S. cerevisiae* that concluded that expression of Rad23 with a mutated UBA1 could not fully rescue the cs phenotype of a *rad23Δrpn10Δ* strain (38). In this context, it should be noted that the cs phenotype is not as severe as the ts phenotype of *rhp23Δpus1Δ*. The authors of this work also showed that...
UBA1 is responsible for the majority of the Ub binding capability of *S. cerevisiae* Rad23 (28).

The chimeric proteins shed further light on the role of the UBA1 domain as the major Ub binding factor of Rhp23. We have shown that the Rhp23 UBA1 domain can restore in vitro Ub binding to a form of Pus1 from which the UIM has been deleted (39, 40). Furthermore, we demonstrated that Ub binding can be restored to a version of Rhp23 in which UBA1 is functionally compromised by mutation by replacing UBA2 with the authentic UBA1 sequence.

Although UBA1 makes the most substantial contribution to the observed affinity and selectivity of Rhp23 for Ub chains, chain binding to the full-length protein both in vitro and in vivo is enhanced when UBA2 is present. UBA2 may not only slightly increase the observed affinity but may also tune the discrimination between Lys48 and Lys63 chains. Consistent with this model, whereas isolated UBA1 shows a 7-fold selectivity for Lys48-Ub2 against mono-Ub and a 3-fold selectivity against Lys63-Ub2, the equivalent selectivities for WT Rhp23 are 20- and 15-fold, respectively. Raasi *et al.* (22) have reported a similar phenomenon whereby isolated Rad23 UBA1 bound preferentially to Lys63 chains, but the preference of a ubiquitin-like domain-UBA1 fragment protein was switched to Lys48 chains.

We conclude from these studies that the functionally significant binding properties of Ub binding domains can only be fully appreciated when studied in their authentic context. Our studies also suggest that although the UBA1 domain of Rhp23 is sufficient for its Ub binding function in the UPS the UBA2 contributes to discrimination between Lys48 and Lys63 chains. This function may be in addition to previously suggested roles of the UBA2 in protecting the protein from being degraded (24, 25) or facilitating Rhp23 dissociation from the proteasome (41).
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