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Award Number: W81XWH-05-1-0310

TITLE: Role of Rad23 and Dsk2 in Nucleotide Excision Repair and Spindle Pole Body Duplication

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REPORT DATE: March 2006

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
               Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
                        Distribution Unlimited

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20070314133

REPORT DOCUMENTATION PAGE

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
01-03-2006	Annual Summary	28 Feb 2005 - 27 Feb 2006

4. TITLE AND SUBTITLE
Role of Rad23 and Dsk2 in Nucleotide Excision Repair and Spindle Pole Body Duplication

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-05-1-0310

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Minnesota
Minneapolis, MN 55455

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The three yeast UBL-UBA proteins, Rad23, Ddi1 and Dsk2 bind both ubiquitin and the proteasome. They are not essential for viability and some redundancy in terms of stabilization of ubiquitinated substrates has been shown, suggesting that they may have overlapping functions. Here we showed that Rad23 is indeed redundant with both Ddi1 and Dsk2 for cell cycle related roles. Surprisingly, Ddi1 and Dsk2 do not show any redundancy but the triple deletion shows an synthetic defect, suggesting that Rad23 has at least two different roles in cell cycle progression during G2/M. In addition, we found that these putative roles do not include a role in SPB duplication, which contradicts a previously reported study (3). We do not know at the time the nature of this discrepancy. In addition, we show that a tetra-ubiquitin chain is able to bind several UBL-UBA proteins at once, which might explain the redundancies observed, as well as suggesting that these multiple interactions might be relevant for efficient but regulated delivery of ubiquitinated substrates to the proteasome.

15. SUBJECT TERMS
Rad23, Ddi1, Dsk2, Ubiquitin-Proteasome System, Protein Degradation, Cell Cycle, Spindle Pole Body

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U	UU	17	19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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Introduction
Genetic instability and aneuploidy are hallmarks of and might initiate cancers. Failures in the Nucleotide Excision Repair (NER) pathway as well as chromosome misegregation due to centrosome abnormalities are two known causes of genetic instability and aneuploidy respectively, and have been found in most breast tumours [1,2].

There are three UBL-UBA proteins in budding yeast: Ddi1, Dsk2 and Rad23. Rad23 and Dsk2 have been reported to be involved in duplication of the Spindle Pole Body (SPB), the yeast equivalent of mammalian centrosomes [3]. A role for Rad23 and Ddi1 in cell cycle control [4] as well as their homo- and hetero-dimerization has also been reported [5]. In addition, these proteins have been shown to bind to ubiquitin [6] and the proteasome, leading to the hypothesis that they shuttle ubiquitinated substrates to the proteasome for degradation [7].

This proposal aimed to gain a better understanding of the role of UBL-UBA proteins in cell cycle control, SPB duplication and NER. By using strains deleted for the UBL-UBA proteins in different combinations we have observed overlapping roles for these proteins in cell cycle control. In addition, we have shown that Rad23 and Ddi1 interact through their UBL/UBA domains and that several UBL-UBA proteins can bind to a common tetra-ubiquitin chain [8], a process that might influence the efficiency of substrate delivery to the proteasome and explain the basis of their redundancy.

Rad23, Ddi1 and Dsk2 have partially redundant roles in cell cycle progression
The ability of UBL-UBA containing proteins to interact both with the proteasome and with poly-ubiquitin chains has led to the hypothesis that these proteins shuttle ubiquitinated substrates to the proteasome for degradation [7,9,10]. Ddi1, Dsk2 and Rad23 interact both with the proteasome and with ubiquitin chains, although with different affinities [10,11]. Both *DSK2, DDI1* and *RAD23* are non-essential genes. Single deletion of *RAD23* or *DSK2* induces partial stabilization of a model substrate targeted for degradation via the ubiquitin proteasome pathway, but the *rad23Δdsk2Δ*
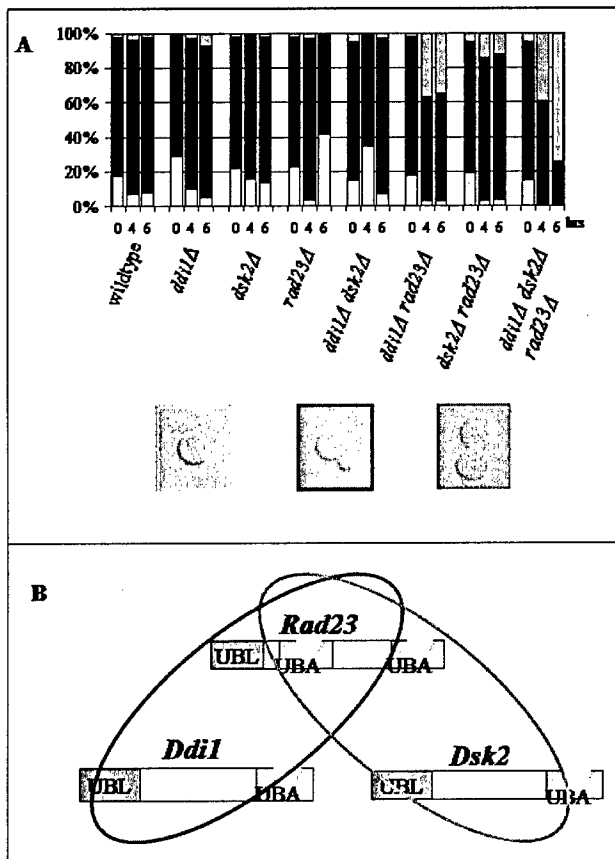
Figure 1. Deletion of *DDI1*, *DSK2* and *RAD23* have a synthetic effect in cell cycle progression at high temperatures.
(A) Cells were grown to mid-log phase in liquid YEPD at 30°C then shifted to 37°C for 0, 4 and 6 hrs. Cell cycle distribution was determined by bud morphology: cells in G1 are unbudded (yellow), S-phase cells with small buds (blue) and G2/M cells with large buds (red). Large buds are defined as cells where the bud is as big as the mother cell, that is dumbbells.
(B) Schematic showing the redundancies observed within the yeast UBL-UBA proteins. Rad23 is redundant with Ddi1 and Dsk2 but Ddi1 and Dsk2 are not redundant.

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double deletion completely inhibits degradation of the model substrate [10] and accumulates polyubiquitinated proteins at high temperatures [11] suggesting redundancy for Rad23 and Dsk2 roles in the ubiquitin-proteasome pathway. In addition, double deletion of *RAD23* and *DSK2* has been reported to induce accumulation of cells with large buds (dumbbells) due to a failure in SPB duplication [3]. On the other hand, Rad23 and Ddi1 have been shown to be dosage suppresors of the checkpoint-defective *pds1-128* allele [4]. These results suggest a certain level of redundancy within UBL-UBA containing proteins.

To test whether the three yeast UBL-UBA proteins have a redundant role in cell cycle progression we took a genetic approach, obtaining strains deleted for each one of the UBL-UBA genes (Table 1) as well as all the double deletion combinations and the triple deletion, and asked whether any redundancy in terms of cell cycle progression could be observed. The experiments were performed at 37°C because it is the temperature at which the *rad23Δdsk2Δ* double-deletion strain has been shown to accumulate ubiquitinated substrates [11], as well as have a cell cycle phenotype [3]. We found that *RAD23* is redundant with *DDI1* and *DSK2*, since the double deletions show a 40% and 20% of cells accumulated at the G2/M stage respectively after 6h at the non-permissive temperature. On the other hand, DDI1 and DSK2 are not redundant (Figure 1A). Interestingly, the triple deletion shows an additive defect in cell cycle progression, reaching 75% of G2/M arrested cells after 6 hours at 37°C, which suggests an additive effect, that is, *RAD23* redundancy with *DDI1* is different than that with *DSK2* and the effect seen in the triple deletion is the result of both defects combined (Figure 1).

G2/M arrest is not due to a failure in SPB duplication

Since defects in SPB duplication have been reported in the *rad23Δdsk2Δ* double deletion [3], we decided to look at SPB duplication in the triple deletion by using both a SPB component fused to GFP (SPC42:GFP) or a tubulin-GFP fusion (TUB1:GFP) to look at spindle morphology (strains listed in Table 1). Surprisingly, the kinetics of SPB duplication in the triple deletion, as well as the *rad23Δdsk2Δ* double deletion were indistinguishable from the wildtype strain, except that the triple deleted cells arrested as large budded cells with two SPBs (G2/M arrest, data not shown). At present we do not know whether these contradictory results are due to a difference in genetic background between our strain and the one used in the published study [3].

Several UBL-UBA proteins can bind to a single ubiquitin chain

Yeast-two-hybrid experiments have previously indicated that homo- and hetero-dimerization of Rad23 and Ddi1 might occur [5]. But these studies did not rule out the possible existence of bridging molecules. By NMR analysis of the interactions between different Rad23 and Ddi1 protein fragments, we have found that Ddi1 and Rad23 interact in isolation through UBL-UBA domain interaction (see attached manuscript [8]). Rad23 homodimerization, on the other hand, occurs through its C-terminus while Ddi1 homodimerization does not require either the UBL nor the UBA domains [8] supporting previous yeast-two-hybrid results [5].

Since Rad23-Ddi1 interaction involves both their UBL and UBA domains, and the UBA domains are required for ubiquitin binding, we tested whether ubiquitin can disrupt the heterodimer. We found that mono-ubiquitin can successfully disrupt the heterodimer but that tetra-ubiquitin chains can actually form a ternary complex with Rad23 and Ddi1, suggesting that tetra-ubiquitin chains are able to bind more than one UBL-UBA protein at once (see attached manuscript [8]).

Identification of UBA-binding proteins

Since the UBL-UBA proteins are proposed to shuttle ubiquitinated substrates into the proteasome [7,9,10]. We decided to identify potential substrates by looking for UBA-interacting proteins using TAP-purification followed by mass spectrometry. Since the sequence surrounding the UBA domains might play an important role in the specificity of the UBA domains, identification of the UBA-binding proteins will be determined by comparing proteins that bind to the full length proteins against those that bind to the same protein lacking the UBA domain. I have so far obtained strains expressing TAP:Rad23 and TAP:Ddi1ΔUBA (Figure 2) and I am in the process of integrating the expression vectors harboring the TAP:RAD23ΔUBA, TAP:DSK2 and TAP:DSK2ΔUBA genes.

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Figure 2. Expression of TAP-tagged proteins. Expression of TAP:Rad23 (lft) or TAP:Ddi1ΔUBA (right) was induced by growing the strains in galactose containing medium. Total protein was extracted and western blots were performed using anti-Rad23 or anti-Ddi1 polyclonal antibodies.

Table 1. List of yeast strains obtained	
#	**genotype**
L162	a bar1Δ ddi1Δ dsk2Δ
L166	a BAR1+ rad23Δ ddi1Δ dsk2Δ
L167	α rad23Δ ddi1Δ dsk2Δ
L168	a bar1Δ ddi1Δ rad23Δ pds1-128
L169	a bar1Δ spc42:GFP[TRP]
L174	a bar1Δ rad23Δ dsk2Δ
L175	a bar1Δ ddi1::KAN dsk2::KAN TUB1:GFP[URA3]
L176	a bar1Δ ddi1::KAN dsk2::KAN TUB1:GFP[URA3]
L181	a bar1Δ dsk2::KAN spc42:GFP[TRP]
L182	a bar1Δ dsk2::KAN spc42:GFP[TRP]
L183	a bar1Δ rad23::KAN dsk2::KAN
L184	a bar1Δ rad23::KAN dsk2::KAN SPC42:GFP[TRP]
L185	a bar1Δ rad23::KAN dsk2::KAN SPC42:GFP[TRP]
L186	a bar1Δ ddi1::KAN dsk2::KAN
L187	a bar1Δ ddi1::KAN dsk2::KAN SPC42:GFP[TRP]
L189	a bar1Δ rad23::KAN ddi1::KAN dsk2::KAN
L192	a bar1Δ rad23::KAN dsk2::KAN TUB1:GFP[URA]
L194	a bar1Δ rad23::KAN SPC42:GFP[TRP]
L196	a bar1Δ ddi1::KAN rad23::KAN SPC42:GFP[TRP]
L200	a bar1Δ ddi1::KAN rad23::KAN dsk2::KAN TUB1:GFP[URA]
L202	a bar1Δ dsk2::KAN rad23::KAN SPC42:GFP[TRP] GAL:DSK2[LEU]
L204	a bar1Δ dsk2::KAN rad23::KAN SPC42:GFP[TRP] GAL:DSK2[LEU]
L206	a bar1Δ dsk2::KAN rad23::KAN SPC42:GFP[TRP] GAL:DSK2ΔUBL[LEU]
L211	a bar1Δ dsk2::KAN rad23::KAN SPC42:GFP[TRP] GAL:DSK2ΔUBA[LEU]
L214	a bar1Δ dsk2::KAN rad23::KAN SPC42:GFP[TRP] GAL:DSK2ΔUBLΔUBA[LEU]
L216	a bar1Δ dsk2::KAN rad23::KAN SPC42:GFP[TRP] GAL:DSK2ΔUBLΔUBA[LEU]
L218	a bar1Δ ddi1::KAN dsk2::KAN rad23::KAN SPC42:GFP[TRP]
L221	a bar1Δ ddi1::KAN SPC42:GFP[TRP]
L225	a bar1Δ pre1-1 pre4-1 pep4::URA3 YTAP:RAD23[LEU]
L228	a bar1Δ pre1-1 pre4-1 pep4::URA3 YTAP:DDI1ΔUBA[LEU]
L233	alpha bar1Δ rad23::KAN leu2::YIPG:RAD23ΔUBL[LEU] ARG4
L240	a bar1Δ rad23::KAN PDS1-HA[KAN] leu2::YIPG:RAD23ΔUBL[LEU] DDI1-6XHIS[TRP]
L242	alpha bar1Δ leu2::YIPG:RAD23ΔUBL[LEU] DDI1-6XHIS[TRP]
L244	alpha bar1Δ rad23::KAN PDS1-HA[KAN] leu2::YIPG:RAD23ΔUBL[LEU]
L245	alpha bar1Δ rad23::KAN PDS1-HA[KAN] leu2::YIPG:RAD23ΔUBL[LEU] DDI1-6XHIS[TRP]
L248	a bar1Δ leu2::YIPG:RAD23ΔUBL[LEU] DDI1-6XHIS[TRP]
L249	alpha bar1Δ rad23::KAN PDS1-HA[KAN] leu2::YIPG:RAD23ΔUBL[LEU]
L258	ddi1::KAN dsk2::KAN TUB1:GFP::URA3 RAD23ΔUBA2-MYC[TRP]
L259	a bar1Δ ddi1::KAN dsk2::KAN TUB1:GFP::URA3 RAD23ΔUBA2-MYC[TRP]
L262	TUB1:GFP::URA3 RAD23ΔUBA2-MYC[TRP] SPC42:GFP[TRP] DDI1ΔUBA-6HIS[LEU2]
L263	a bar1Δ ddi1::KAN dsk2::KAN TUB1:GFP::URA3 RAD23ΔUBA2-MYC[TRP] SPC42:GFP[TRP]

Key accomplishments:

- Construction of *rad23Δ, dsk2Δ, ddi1Δ* yeast strains harboring *SPC42:GFP* and/or *TUB1:GFP*, as well as all combinations of double and triple deletions.

- Evaluation of cell cycle progression in the different deletion strains, showing a partially redundant phenotype

- Evaluation of SPB duplication, showing no difference among wildtype, *dks2Δrad23Δ* and *ddi1Δdsk2Δrad23Δ*

- Design and integration of galactose-inducible vectors containing RAD23, DDI1 or DSK2 wildtype genes as well UBA and/or UBL deleted versions of these genes.

- Evaluation of the role of the UBL and/or UBA domains of these proteins in cell cycle progression and Spindle Pole Body duplication

- Construction and integration of different TAP-tagged version of RAD23, DDI1 and DSK2 for protein interaction analysis

- Construction of bacterial-expression plasmids harboring different domains of RAD23, DDI1 and DSK2 for NMR-analysis.

Reportable outcomes:

- Kang Y, Vossler R, **Diaz-Martinez LA**, Winter NS, Clarke DJ and Walters KJ (2006). UBL/UBA ubiquitin receptor proteins bind a common tetraubiquitin chain. *J Mol Biol* 356:1027-1035 (attached)
- **Díaz-Martínez LA**, Kang Y, Vossler R, Winter NS, Walters KJ and Clarke DJ. Yeast UBL-UBA proteins have redundant functions in cell cycle control. (Manuscript in preparation)

Conclusions
The three yeast UBL-UBA proteins bind both ubiquitin and the proteasome, suggesting that they may have overlapping functions. Here we showed that Rad23 is indeed redundant with both Ddi1 and Dsk2 for cell cycle related roles. Surprisingly, Ddi1 and Dsk2 do not show any redundancy but the triple deletion shows an additive defect, suggesting that Rad23 has at least two different roles in cell cycle progression during G2/M, one that is shared with Ddi and another one shared with Dsk2. In addition, we found that these putative roles do not include a role in SPB duplication, which contradicts a previously reported study [3]. We do not know at the time the nature of this discrepancy.
Furthermore, we show that a tetra-ubiquitin chain is able to bind several UBL-UBA proteins at once, which might explain the redundancies observed, as well as suggesting that these multiple interactions might be relevant for efficient but regulated delivery of ubiquitinated substrates to the proteasome.

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UBL/UBA Ubiquitin Receptor Proteins Bind a Common Tetraubiquitin Chain

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The ubiquitin–proteasome pathway is essential throughout the life cycle of a cell. This system employs an astounding number of proteins to ubiquitylate and to deliver protein substrates to the proteasome for their degradation. At the heart of this process is the large and growing family of ubiquitin receptor proteins. Within this family is an intensely studied group that contains both ubiquitin-like (UBL) and ubiquitin-associated (UBA) domains: Rad23, Ddi1 and Dsk2. Although UBL/UBA family members are reported to regulate the degradation of other proteins, their individual roles in ubiquitin-mediated protein degradation has proven difficult to resolve due to their overlapping functional roles and interaction with each other and other ubiquitin family members. Here, we use a combination of NMR spectroscopy and molecular biology to reveal that Rad23 and Ddi1 interact with each other by using UBL/UBA domain interactions in a manner that does not preclude their interaction with ubiquitin. We demonstrate that UBL/UBA proteins can bind a common tetraubiquitin molecule and thereby provide strong evidence for a model in which chains adopt an opened structure to bind multiple receptor proteins. Altogether our results suggest a mechanism through which UBL/UBA proteins could protect chains from premature de-ubiquitylation and unnecessary elongation during their transit to the proteasome.

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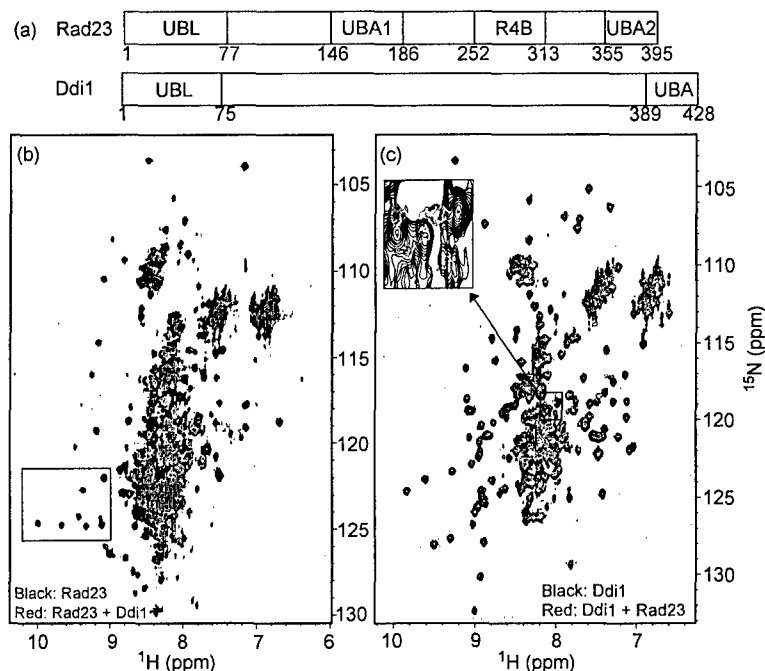
Corresponding author

Introduction

Ubiquitin signaling regulates an astounding array of cellular events and remains essential throughout the life cycle of a cell. In its most established role ubiquitylation targets proteins for degradation by the 26 S proteasome,[1] a process important for controlling the lifespan of regulatory proteins, removing misfolded proteins,[2] producing immunocompetent peptides,[3] activating and repressing transcription,[4,5] and regulating cell cycle progression.[6] In addition ubiquitylation can signal proteasome-independent events including

Abbreviations used: Rad23, radiation-sensitive mutant 23; Ddi1, DNA damage-inducible protein; hHR23, human homolog of Rad23; HSQC, heteronuclear single quantum coherence; R4B, Rad4 binding domain; UBA, ubiquitin-associated; UBL, ubiquitin-like; GST, glutathione-S-transferase.

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endocytic sorting[7,8] and DNA repair.[9,10] Ubiquitylation is connected to proteasome-mediated protein degradation by an intricate network of ubiquitin recognition proteins. Elucidating this network remains a difficult albeit active area of research, as it is clouded by redundancy and cooperation between the large and growing ubiquitin receptor protein family. Among these proteins exists a group that harbors both ubiquitin-associated (UBA) and ubiquitin-like (UBL) domains (Figure 1(a)).

UBL/UBA proteins have attracted much attention for their ability to regulate the lifespans of other proteins. In *Saccharomyces cerevisiae*, Rad23 (hHR23a/b in humans), Dsk2 (hPLIC-1/2 in humans) and Ddi1 are UBL/UBA proteins that recruit ubiquitylated substrates to the proteasome for their degradation[11–16] *via* UBA domain interactions with ubiquitin[17–19] and UBL domain interactions with the proteasome.[20–23] Depending on their protein levels, UBL/UBA-containing proteins can also inhibit the degradation of ubiquitylated substrates.[16] Such inhibition occurs because UBA

Figure 1. Ubiquitin recognition proteins Rad23 and Ddi1 interact. (a) The sequence location of Rad23's and Ddi1's UBL and UBA domains as well as Rad23's Rad4/XPC binding domain is illustrated. (b) Comparison of the [^{1}H,^{15}N] HSQC spectrum of ^{15}N-labeled Rad23 alone (black) to that acquired in the presence of four-fold molar excess Ddi1 reveals that Ddi1 causes severe broadening of certain Rad23 amide resonances (boxed in black). (c) [^{1}H,^{15}N] HSQC spectra are displayed of Ddi1 alone (red) and with fourfold molar excess Rad23 (red). The zoomed region highlights chemical shift perturbations in Ddi1's [^{1}H,^{15}N] HSQC spectrum caused by Rad23 addition. Together (b) and (c) provide strong evidence for the direct interaction of Rad23 with Ddi1.

domains sequester K48-linked polyubiquitin chains to in turn prevent their elongation and de-ubiquitylation.[16,24,25] In a perhaps related role, C-terminal UBA domains are reported to protect Rad23/hHR23a, Ddi1 and Dsk2 from their own degradation via the proteasome.[26]

Adding to the complexity of the ubiquitin family network, Rad23 is reported to interact with Ddi1 and Dsk2, and yeast two-hybrid experiments implicate UBA/UBA domain interactions as essential for such dimerization.[27,28] However, in previous work on hHR23a we found no such UBA/UBA domain interactions but instead found that the UBL domain of hHR23a interacts dynamically with each of its UBA domains.[29] Furthermore, whereas Rad23 is reported to dimerize,[18,27,28] hHR23a does not.[29]

To resolve these ambiguities we used NMR spectroscopy to determine the mechanism by which Rad23 binds Ddi1 and itself. Yeast two-hybrid experiments are unable to discriminate between direct interactions and those that are mediated by other proteins, both of which, as we report here, are available to Rad23 and Ddi1. Here, we reveal that UBL/UBA and not UBA/UBA domain interactions result in heterodimerization of Rad23 and Ddi1. These findings demonstrate the first published example for UBL/UBA domain interactions mediating heterodimerization. According to its crystal structure, the ubiquitin moieties of K48-linked tetraubiquitin are packed against each other with only the most distal moiety available for binding a UBA domain.[30] This structure suggests that K48-linked tetraubiquitin, which is the smallest chain length that signals for proteasome degradation[31] is able to bind only one

ubiquitin receptor protein. In addition, Rad23 has two UBA domains, the C-terminal of which is reported to sandwich between the two ubiquitin subunits of diubiquitin.[32] Surprisingly, we have found that K48-linked tetraubiquitin can bind simultaneously to two Rad23 molecules as well as to Rad23 and Ddi1. This finding illustrates that K48-linked tetraubiquitin adopts an opened structure when bound to its receptors and leads to a working model for how ubiquitylated substrates are transferred to the proteasome.

Results

Rad23 heterodimerizes with Ddi1 via UBL/UBA domain contacts

Yeast two-hybrid experiments suggest that Rad23 interacts with itself, Dsk2 and Ddi1 by using UBA/UBA domain interactions.[27,28] These in vivo analyses, however, do not exclude the possibility of indirect associations through bridging molecules such as ubiquitin chains, which are known to interact with UBA domains.[18] To test whether Rad23 and Ddi1 interact in their purified forms we performed [^{1}H,^{15}N] heteronuclear single quantum coherence (HSQC) experiments on ^{15}N-labeled Rad23 or Ddi1 alone and in the presence of the other protein (Figure 1(b) and (c)). Such experiments detect amide nitrogen and proton atoms and their frequencies in spectra depend on their chemical environment; a phenomenon that makes them useful for detecting protein–protein interactions. The [^{1}H,^{15}N] HSQC spectrum of Rad23 resembles

that of its human homolog hHR23a, in that many sharp resonances appear in the region expected for randomly coiled residues (Figure 1(b)). This attribute originates from their long unstructured flexible linker regions that connect each of their small domains,[29] which for Rad23 comprise 42.7% of its amino acid residues.

In each set of [^{1}H,^{15}N] HSQC experiments direct interaction between Rad23 and Ddi1 was detected by spectral changes (Figure 1(b) and (c)). For Ddi1 the changes were confined to chemical shift perturbations (Figure 1(c)), whereas resonances derived from Rad23 experienced severe broadening and signal decay (Figure 1(b)). Although structural data and chemical shift assignments are available for the 40 kDa hHR23a protein,[29] neither Rad23 nor Ddi1 has been characterized structurally by NMR or X-ray crystallography.

Therefore, to identify the domains involved in forming the Rad23/Ddi1 protein complex we performed [^{1}H,^{15}N] HSQC experiments on single domain constructs of Rad23's UBL, UBA1 and UBA2 domains and on Ddi1's UBA domain. Comparisons between the truncated and full-length protein constructs allowed us to identify resonances derived from Rad23's UBL and UBA1 domains (Figure 2(a)) as well as Ddi1's UBA domain (data not shown). These resonance assignments were then used to interpret the results of the titration experiments recorded on the full-length proteins (Figure 1(b) and (c)). At equimolar protein concentration Rad23's UBL domain interacts with Ddi1's UBA domain and additional contacts are made between Ddi1's UBL domain and Rad23's internal UBA domain when Rad23 is present at twofold molar excess or greater (data now shown).

To test these UBL/UBA domain interactions more directly we performed analogous experiments in which we titrated the UBL domain of Rad23 into ^{15}N labeled full-length Ddi1 (Figure 3(a)) or its UBA domain (Figure 3(b)). We confirmed that the UBL domain of Rad23 binds the UBA domain of Ddi1 (Figure 3(b)), and found that the UBL domain of Rad23 induces identical chemical shift changes in Ddi1's [^{1}H,^{15}N] HSQC spectrum compared to equimolar concentrations of full-length Rad23 (Figure 3(a)). Finally, we tested directly whether either UBA domain of Rad23 can bind that of Ddi1 by performing [^{1}H,^{15}N] HSQC experiments on ^{15}N-labeled Ddi1 UBA domain alone and in the presence of either of Rad23's UBA domains. Even at eightfold molar excess Rad23's UBA domains do not cause chemical shift changes in that of Ddi1. Therefore, we conclude that Rad23 UBA domains do not interact with that of Ddi1 (data not shown).

Rad23 and Ddi1 dissociate to bind ubiquitin

Since Rad23 and Ddi1 bind ubiquitin with their UBA domains, we tested whether the Rad23/Ddi1 heterodimer remains intact in the presence of monoubiquitin. To ^{15}N-labeled Rad23 mixed with fourfold molar excess Ddi1 (Figure 4(a)), we added monoubiquitin such that the molar ratio of Rad23/ Ddi1/monoubiquitin is equal to 1:4:10 (Figure 4(b)). At 1:4:10 molar ratio, the resonances derived from Rad23's UBL domain are restored, indicating that it is no longer bound to Ddi1's UBA domain. This loss of interaction is due to monoubiquitin successfully competing for Ddi1's UBA domain. In addition, resonances of Rad23's UBA1 and UBA2 domains shift upon ubiquitin addition, confirming that each
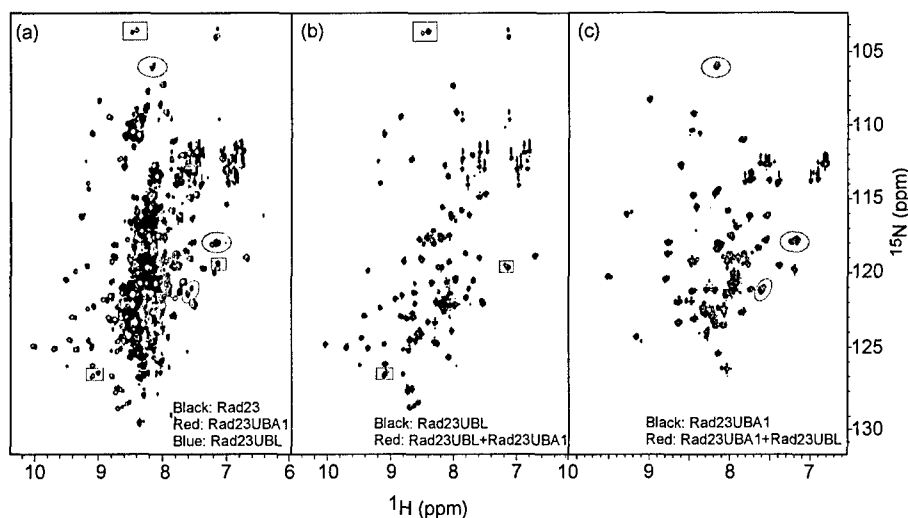
Figure 2. Rad23's UBA1 binds its own UBL domain. (a) The [^{1}H,^{15}N] HSQC spectrum of ^{15}N-labeled Rad23 (black) is superimposed onto that derived from single domain constructs of its UBA1 (red) and UBL (blue) domains. (b) Superimposed [^{1}H,^{15}N] HSQC spectra of ^{15}N-labeled Rad23's UBL domain alone (black) and at equimolar concentration with its UBA1 domain (red). (c) The converse experiment is shown: [^{1}H,^{15}N] HSQC spectra of ^{15}N-labeled Rad23's UBA1 domain alone (black) and with its UBL domain at equimolar ratio (red). Selected shifted UBL and UBA1 resonances are boxed by green rectangles and ovals, respectively.
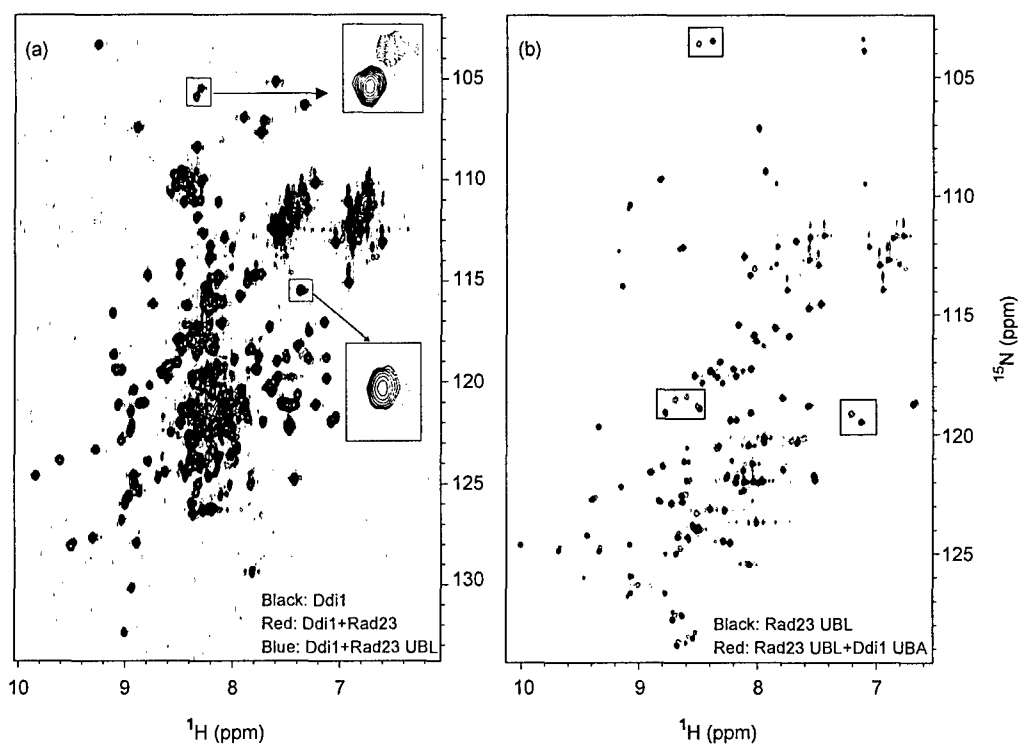
Figure 3. Rad23/Ddi1 heterodimerize *via* UBL/UBA domain interactions. (a) The [^{1}H,^{15}N] HSQC spectrum of ^{15}N-labeled Ddi1 alone (black) and with fourfold molar excess of either full-length Rad23 (red) or its UBL domain (blue). Rad23 and its UBL domain induce identical shifts in Ddi1's UBA domain. (b) The [^{1}H,^{15}N] HSQC spectrum of ^{15}N-labeled Rad23 UBL domain alone (black) and with Ddi1's UBA domain at equimolar concentration (red). Selected resonances of Rad23's UBL domain that shift by adding Ddi1's UBA domain are boxed.

of Rad23's UBA domains binds ubiquitin (Figure 4(b)).

Rad23 forms a homodimer through its C-terminal half

In addition to forming a heterodimer, Rad23 and Ddi1 each homodimerize. Ddi1 dimerization is independent of its UBL and UBA domain and occurs through residues located in the middle of its amino acid sequence.[27] These residues are absent from the [^{1}H,^{15}N] HSQC spectrum acquired on full-length Ddi1. Such absences are caused by line broadening due to chemical exchange or slow tumbling times (from bulkiness) and offer further support of an internal Ddi1 dimerization domain. That Ddi1's UBL and UBA domains are observable reflects their structural independence from its dimerization domain. [^{1}H,^{15}N] HSQC spectra reveal that the resonances of Ddi1's UBA domain superimpose well onto those derived from its full-length protein, as only two residues experience chemical shift changes due to this truncation (data not shown). These results support a model in which Ddi1's UBA and UBL domains are autonomous and connected to the rest of the protein by flexible linker regions.

In contrast to Ddi1, the mechanism by which Rad23 dimerizes is not well understood. Dynamic light-scattering experiments indicate that 99% of Rad23 species (at 23.6 μM) exist as a dimer in its purified form (data not shown). Interestingly, its human homolog hHR23a does not dimerize[29] and Rad23 dimerization was hypothesized to occur *via* UBA/UBL domain interactions.[27] In our [^{1}H,^{15}N] HSQC experiments chemical shift changes compared to full-length Rad23 were observed for its UBA1 domain when produced as a single domain construct (Figure 2(a)). Such changes suggest interactions with other regions of the protein and we performed titration experiments with single domain constructs to discover that it binds its own UBL domain (Figure 2(b) and (c)).

Most surprising, however, was that most of the resonances from the XPC/Rad4-binding and UBA2 domains were absent from the [^{1}H,^{15}N] HSQC spectrum recorded on full-length Rad23. These absences persisted even in an experiment performed for 14 h at 800 MHz with 128 increments in the ^{15}N dimension and 128 scans per increment on a ^{15}N-labeled 0.5 mM Rad23 sample. In contrast, all resonances derived from the XPC-binding and UBA2 domains are prominent in spectra recorded on hHR23a, even in experiments recorded with
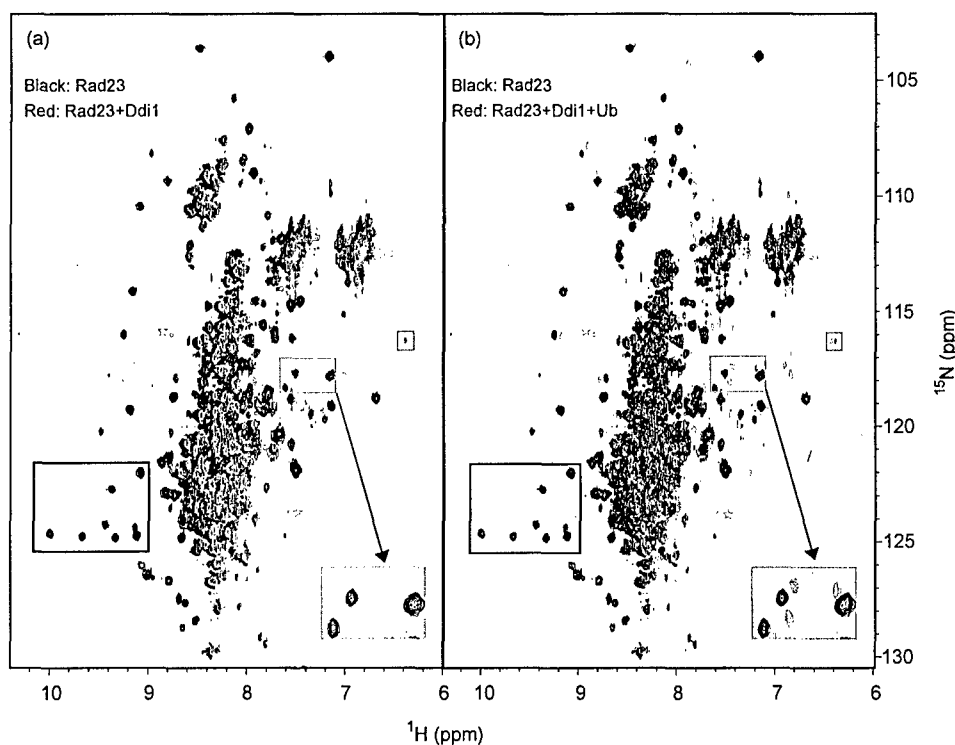
Figure 4. Rad23 and Ddi1 dissociate to bind ubiquitin. (a) [^{1}H,^{15}N] HSQC spectra of ^{15}N-labeled Rad23 alone (black) and with fourfold molar excess of Ddi1 (red) reveals that resonances of Rad23's UBL domain disappear upon binding Ddi1. (b) [^{1}H,^{15}N] HSQC spectra of ^{15}N-labeled Rad23 alone (black) and with Ddi1 and ubiquitin at 1:4:10 molar ratio, respectively, reveal ubiquitin to restore the broadened Rad23 resonances (boxed in black), and to cause chemical shift perturbations in Rad23's UBA1 (boxed in blue) or UBA2 (boxed in green) domains. Certain UBA2 resonances appear upon Ddi1 or ubiquitin addition (marked with an asterisk).
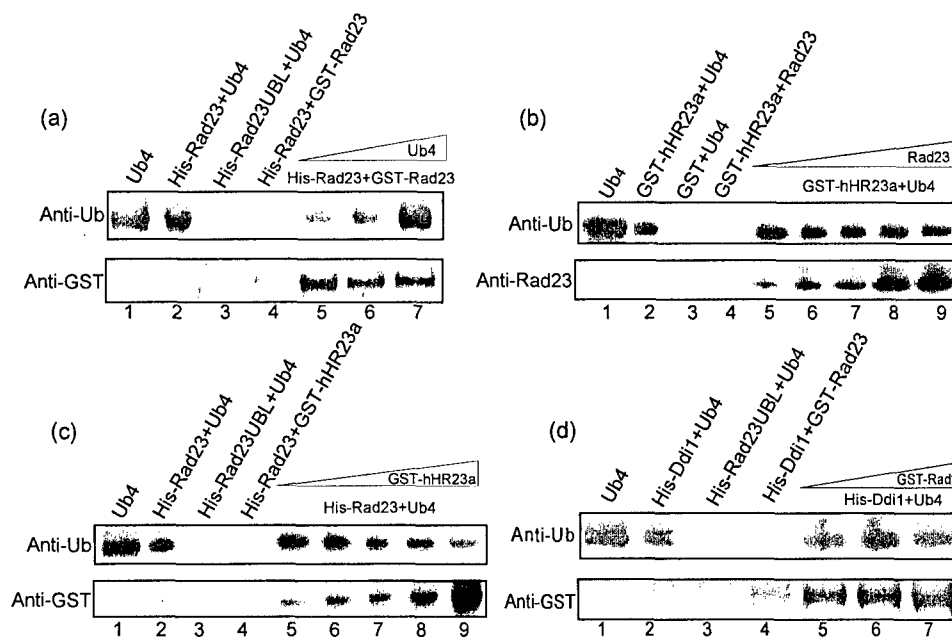
eight scans per increment and 0.1 mM sample concentration.[29] Interestingly, certain UBA2 resonances of Rad23 appear upon addition of Ddi1 (Figure 4(a)) or ubiquitin (Figure 4(b)); however, those of the Rad4-binding domain remain absent. These data support previous findings that Rad23 homodimerizes[18,27] and suggest a role for the Rad4-binding domain, which is consistent with the published finding that UBA2 is not sufficient for Rad23 dimerization.[28] The N-terminal half of Rad23 including its UBL and UBA1 domain does not appear to be in contact with its C-terminal half and they are most likely connected by a flexible linker region that allows them to move independently of one another, as the N-terminal but not the C-terminal half of Rad23 is observable.

Tetraubiquitin bridges ubiquitin receptor proteins

We were interested in how the ability of Rad23 and Ddi1 to form homo- and heterodimers impacts their polyubiquitin-binding mechanisms and in particular whether one tetraubiquitin molecule can bind more than one ubiquitin receptor protein. We therefore tested whether tetraubiquitin can bind more than one Rad23 or Ddi1 molecule. To test

whether tetraubiquitin is capable of binding more than one Rad23 molecule we incubated K48-linked tetraubiquitin with Ni-NTA agarose resin containing pre-bound His-Rad23. After removing the unbound tetraubiquitin, glutathione-S-transferase (GST)-Rad23 was added and the beads washed again to remove unbound GST-Rad23. The resin-bound protein complex was fractionated by gel electrophoresis, transferred to a membrane, and probed with anti-GST or anti-ubiquitin antibody (Figure 5(a)). This experiment revealed the presence of a ternary complex containing GST-Rad23, His-Rad23 and tetraubiquitin. Since ubiquitin binding is reported to cause Rad23 homodimers to dissociate,[27] we hypothesized that this ternary complex must be formed by each Rad23 construct binding directly to tetraubiquitin. In addition, this experiment was performed at a temperature (4 °C) that does not permit monomer exchange in Rad23 homodimers as GST-Rad23 and His-Rad23 failed to interact in the absence of tetraubiquitin (Figure 5(a), lower panel, lane 4).

To test whether its human homolog hHR23a occupies all ubiquitin moieties of tetraubiquitin we examined whether the hHR23a–tetraubiquitin complex can also bind Rad23 (Figure 5(b) and (c)). Indeed, in experiments analogous to that described

(a) Anti-Ub / Anti-GST — lanes 1–7, Ub4 gradient, His-Rad23+GST-Rad23

(b) Anti-Ub / Anti-Rad23 — lanes 1–9, Rad23 gradient, GST-hHR23a+Ub4

(c) Anti-Ub / Anti-GST — lanes 1–9, GST-hHR23a gradient, His-Rad23+Ub4

(d) Anti-Ub / Anti-GST — lanes 1–7, GST-Rad23 gradient, His-Ddi1+Ub4

Figure 5. Ubiquitin receptor proteins Rad23 and Ddi1 bind a common tetraubiquitin molecule. In lanes 5–7 of (a), 20 µl of Ni-NTA resin pre-incubated with 0.1 nmol of His-Rad23 was mixed with 0.1 nmol of GST-Rad23 and increasing quantities of K48-linked tetraubiquitin, 0.008, 0.033, 0.132 nmol, respectively, and probed with anti-ubiquitin (top panel) and anti-GST (bottom panel). As a negative control, this experiment was performed on His-Rad23 UBL mixed with tetraubiquitin (lane 3), or in the absence of tetraubiquitin (lane 4). As a positive control, the experiment was performed without GST-Rad23 (lane 2). The 0.01 nmol of K48-linked tetraubiquitin (Boston Biochem.) used in these experiments and those in (b), (c) and (d) was loaded directly onto lane 1 of each gel. In lanes 5–9 of (b), 20 µl of glutathione S-Sepharose resin pre-incubated with 0.1 nmol of GST-hHR23a was mixed for 1 h at 4 °C with 0.033 nmol of tetraubiquitin and increasing quantities of untagged Rad23: 0.033, 0.067, 0.1, 0.2 and 0.4 nmol, respectively. After washing the resin the proteins were fractionated, transferred to a membrane and probed with anti-ubiquitin (top panel) or anti-Rad23 (lower panel). Indeed, the tetraubiquitin-Rad23-GST-hHR23a protein complex was observed. In lanes 5–9 of (c) the converse experiment was performed whereby 20 µl of Ni-NTA resin pre-incubated with 0.1 nmol of His-Rad23 was mixed for 1 h at 4 °C with 0.033 nmol of tetraubiquitin and increasing quantities of GST-hHR23a: 0.033, 0.067, 0.1, 0.2 and 0.4 nmol, respectively. The samples were treated as described for (a) and probed with anti-ubiquitin (top panel) or anti-GST (bottom panel). As a control, the analogous experiment was performed in the absence of tetraubiquitin to ensure that hHR23a and Rad23 do not interact directly (lane 4 of (b) and (c)). In addition, tetraubiquitin binding to GST-hHR23a (lane 2 of (b)) and His-Rad23 (lane 2 of (c)) but not to either tag or resin (lane 3 of (b) and (c)) was demonstrated. In lanes 5–7 of (d), 20 µl of Ni-NTA resin pre-incubated with 0.1 nmol of His-Ddi1 was mixed for 1 h at 4 °C with 0.132 nmol of tetraubiquitin and increasing quantities of GST-Rad23: 0.1, 0.5 and 1.0 nmol, respectively. The samples were treated as described above and probed with anti-ubiquitin (top panel) and anti-GST (bottom panel). Positive control with no GST-Rad23 (lane 2) and a negative control using His-Rad23 UBL with tetraubiquitin (lane 3) were also performed. Without tetraubiquitin, Rad23/Ddi1 interaction under these conditions is weak (lane 4, lower panel), whereas in the presence of tetraubiquitin, the Rad23/Ddi1/tetraubiquitin ternary complex forms (lanes 5–7).

above, tetraubiquitin was found to form a ternary, complex with Rad23 and hHR23a. Furthermore, the addition of hHR23a to resin-bound His-Rad23/ tetraubiquitin reduced the amount of tetraubiquitin that was retained on the resin (Figure 5(c)), a trend that was not observed when Rad23 was added to resin-bound GST-hHR23a/tetraubiquitin (Figure 5(b)). This finding suggests that hHR23a binds tetraubiquitin more strongly than Rad23, which most likely stems from the loss of UBA2-mediated homodimerization.

Altogether our results support a model in which the Rad23 dimer dissociates upon binding tetraubiquitin and each molecule is used to bind individual moieties. In addition, these experiments suggest that although the hHR23a UBA2 domain

sandwiches between the two ubiquitin moieties of diubiquitin,[32] the hHR23a/Rad23 UBA domains can also bind a single ubiquitin moiety of tetraubiquitin, as supported by the complexes that these UBA domains form with monoubiquitin.[19,33]

Analogously, since monoubiquitin is able to dissociate the Rad23/Ddi1 heterodimer, we hypothesized that these proteins could also form a ternary complex with tetraubiquitin. We therefore incubated tetraubiquitin with Ni-NTA agarose resin containing pre-bound His-Ddi1. After extensive washing, GST-Rad23 was added to the mixture, which was washed extensively again and probed for GST-Rad23 by performing Western blot analysis with anti-GST antibody (Figure 5(d)). This experiment supported our hypothesis that tetraubiquitin

can simultaneously bind two different ubiquitin receptor proteins as much more GST-Rad23 is retained on the resin in its presence.

Discussion

Perhaps the most impressive attribute of ubiquitin receptor proteins and polyubiquitin is their adaptability. The ubiquitin moieties of K48-linked tetra-[30] and diubiquitin[34] pack against each other to form a closed structure. Recent literature, however, suggests a flexible model for polyubiquitin structure,[35,36] and diubiquitin forms an open structure to bind the C-terminal UBA domain of hHR23a.[32] Here, we demonstrate that tetraubiquitin can bind more than one ubiquitin receptor protein and thereby reveal that it too opens to bind UBA domains. Our rationale for this conclusion is that only the most distal subunit relative to an attached protein substrate is available for binding a UBA domain in its closed structure.

The structural changes that occur in polyubiquitin parallel those of Rad23's human homolog hHR23a. In an earlier study we revealed that hHR23a forms a closed structure via UBL/UBA domain interactions that opens to bind either ubiquitin[19] or the proteasome component S5a.[29] Here, we demonstrate such UBL/UBA domain interactions to similarly mediate Rad23/Ddi1 heterodimerization and be displaced by ubiquitin.

Our findings invite the question: why do UBL/UBA ubiquitin receptor proteins interact? An answer to this question is suggested by two of the results presented here: the Rad23/Ddi1 heterodimer dissociates to bind polyubiquitin and these two proteins can bind a common polyubiquitin chain. Such attributes enable Rad23/Ddi1 heterodimers to bind a greater number of ubiquitin moieties in a polyubiquitin chain than either protein alone. This feature is important because Rad23 binding to polyubiquitin can block its elongation[18,24,25] and de-ubiquitylation,[25] most likely by precluding chains from the enzymes required for these activities. We therefore propose that dimerization of UBL/UBA domain proteins plays an important role in preventing unnecessary and therefore wasteful chain elongation or premature chain disassembly during the transit of ubiquitylated substrates to the proteasome (Figure 6).

In summary, we reveal that UBL/UBA ubiquitin receptor proteins interact with each other in a manner that does not interfere with their binding to polyubiquitin. On the contrary, since these proteins bind a common polyubiquitin chain, we propose such heterodimerization to aid in their ability to bind a greater number of ubiquitin moieties, an attribute that has implications for the chemistry performed on chains during their transit to the proteasome. In humans, Rad23 can bind an additional ubiquitin receptor protein, S5a,[20] which contains two ubiquitin interaction motifs (UIMs) rather than UBA domains. The S5a homolog in
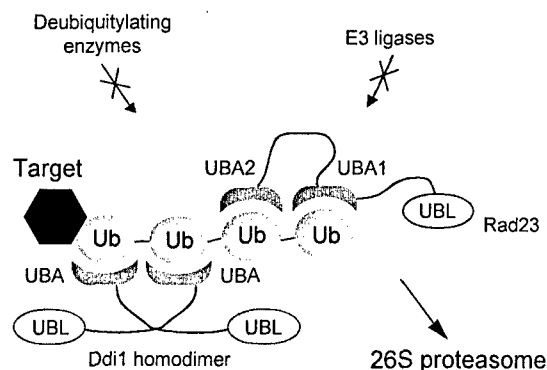
Figure 6. Proposed model for how UBL/UBA domain proteins prevent de-ubiquitylation and unnecessary chain elongation during the transit of substrates to the proteasome. A polyubiquitin chain is greeted with a complex containing multiple UBA domains that are available for binding individual moieties.

yeast (Rpn10) is truncated and lacks the C-terminal UIM to which hHR23a binds.[20] Consequently, it does not bind Rad23 and this example of an evolved interaction between two abundant ubiquitin receptor proteins offers further evidence for the importance of their collaborative relationships.

Materials and Methods

Sample preparation

For NMR spectroscopy Rad23, Ddi1 and single-domain constructs of the Rad23 UBL, UBA1 and UBA2 domains as well as of Ddi1's UBA domain were each cloned into the pET15b expression vector (Novagen) in-frame with the N-terminal histidine tag. The plasmids containing these genes were each transformed into Escherichia coli BL21 (DE3) cells and grown at 37 °C in M9 minimal medium or in Luria broth containing ampicillin (100 μg/ml). The cells were harvested 3 h after protein expression was induced with 0.4 mM isopropyl β-D-thiogalactoside (IPTG). The proteins were purified by using affinity purification on Ni-NTA resin as described.[2f] Further purification was achieved on an FPLC system (Pharmacia), by either Superdex 200 (for full-length Rad23 and Ddi1) or 75 (for UBL and UBA domains) preparative columns. We produced ^{15}N-labeled samples for NMR spectroscopy by growth and expression in M9 minimal medium with [^{15}N]NH$_4$Cl as the only source of nitrogen. Unlabeled monoubiquitin was purchased (Sigma-Aldrich).

NMR spectroscopy

All NMR samples were dissolved in 20 mM NaPO$_4$ (pH 6.5), 100 mM NaCl, 0.1% (w/v) NaN$_3$, and 10% ^{2}H$_2$O. Spectra were acquired at 25 °C on Varian NMR spectrometers operating at either 800 MHz or 600 MHz. Processing was performed in NMRPipe[37] and the resulting spectra were visualized in XEASY.[38] Protein concentrations were calculated by using extinction coefficients based on amino acid composition and absorbance at 280 nm for protein dissolved in 8 M urea.

Western blot analysis

GST-tagged Rad23 and hHR23a were produced from the pGEX-2T and pGEX-6P-1 expression vectors (Amersham Pharmacia), respectively, by cloning their cDNA in-frame with glutathione-S-transferase (GST). Each protein was expressed and purified as described[29] and Rad23 was separated from GST by cleaving with thrombin. The 0.1 nmol of purified His-tagged Rad23 or Ddi1 or of purified GST-tagged hHR23a was bound to 20 μl of pre-washed Ni-NTA resin or glutathione S-Sepharose resin, respectively. Each resin was allowed to mix at 4 °C overnight with K48-linked tetraubiquitin (Boston Biochem Inc.) and then washed extensively with buffer A (20 mM sodium phosphate (pH 6.5), 100 mM NaCl, 0.5% (v/v) Triton X-100). The Ni-NTA resins were then incubated with either GST-Rad23 or GST-hHR23a for 1 h at 4 °C as the glutathione S-Sepharose resin was mixed with untagged Rad23 under the same conditions. Each resin was pelleted and then washed extensively with buffer A or buffer B (50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 20 mM imidazole, 10% (v/v) glycerol). Proteins that were retained on the resin were fractioned by electrophoresis, transferred to a PVDF membrane, and probed with a polyclonal anti-ubiquitin (Boston Biochem Inc.), anti-Rad23,[18] or anti-GST (Santa Cruz Biotechnology) antibody. Visualization was performed using anti-rabbit-horseradish peroxidase and ECL.

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