Published in final edited form as:

Biomol NMR Assign. 2016 October ; 10(2): 291–295. doi:10.1007/s12104-016-9686-7.

¹H, ¹⁵N, ¹³C resonance assignments for *Saccharomyces cerevisiae* Rad23 UBL domain

Xiang Chen¹ and Kylie J. Walters^{1,*}

¹Protein Processing Section, Structural Biophysics Laboratory, Center for Cancer Research, National Cancer Institute, Frederick, MD 21702, USA

Abstract

Rad23 functions in nucleotide excision repair and proteasome-mediated protein degradation. It has four distinct structural domains that are connected by flexible linker regions, including an N-terminal ubiquitin-like (UBL) domain that binds proteasomes. We report in this NMR study the ¹H, ¹⁵N and ¹³C resonance assignments for the backbone and side chain atoms of the Rad23 UBL domain (Rad23^{UBL}) with BioMagResBank accession number 25825. We find that a Rad23 proline amino acid (P20) located in a loop undergoes isomerization. The secondary structural elements predicted from the NMR data fit well to that of the Rad23^{UBL} when complexed with E4 ubiquitin ligase Ufd2, as reported in a crystallographic structure. These complete assignments can be used to study the protein dynamics of the Rad23^{UBL} and its interaction of with other ubiquitin receptors or proteasome subunits.

Keywords

Ubiquitin-proteasome pathway; UV excision repair protein Rad23; Ubiquitin-like domain; NMR

Biological context

The radiation sensitivity abnormal 23 (Rad23) protein was first characterized in nucleotide excision repair (NER), a pathway used to remove helix-distorting DNA lesions caused by genotoxic agents such as ultraviolet (UV) radiation (Dantuma et al. 2009). By forming a complex with the lesion sensor protein Rad4, Rad23 recognizes bulky lesions in DNA (Guzder et al. 1998) and triggers downstream repair events (Dantuma et al. 2009). Rad23 also serves as a proteasome-associated ubiquitin receptor that can concurrently (Kang et al. 2007a) recognize ubiquitinated substrates with its two ubiquitin-associated (UBA) domains (Bertolaet et al. 2001) and the proteasome with an N-terminal ubiquitin-like (UBL) domain (Elsasser et al. 2002; Hiyama et al. 1999; Husnjak et al. 2008; Shi et al. 2016). Rad23 also has a Rad4-binding domain between its two UBA domains, and in hHR23a, a human ortholog of Rad23, the four functional domains are well defined and connected by flexible linker regions (Walters et al. 2003). The hHR23a UBL domain interacts dynamically with the two UBA domains and this interaction is broken when hHR23a binds to either proteasome component S5a or ubiquitin (Walters et al. 2003; Wang et al. 2003). The Rad23/

*To whom correspondence should be addressed: kylie.walters@nih.gov.

hHR23 UBL domain binds to the proteasome at surfaces provided by Rpn1, Rpn10 or Rpn13 (Elsasser et al. 2002; Gomez et al. 2011; Husnjak et al. 2008; Mueller and Feigon 2003; Rosenzweig et al. 2012; Shi et al. 2016). It can also bind to the other UBL/UBA ubiquitin receptor proteins, Ddi1 and a Dsk2 ortholog hPLIC2 by using their UBA domains (Kang et al. 2006; Kang et al. 2007b). Although the structures of hHR23a/b have been extensively studied (Fujiwara et al. 2004; Mueller and Feigon 2003; Ryu et al. 2003; Walters et al. 2003), the Rad23^{UBL} has only been resolved in a complex structure with the E4 ubiquitin ligase Ufd2 (Hanzelmann et al. 2010). Here, we report the chemical shift assignments for Rad23^{UBL} in its free state. These assignments may serve as a foundation for NMR studies of Rad23^{UBL} dynamics or its binding interactions with proteasome subunit Rpn1 or other ubiquitin receptors, such as Rpn10, Rpn13, Ddi1 or hPLIC2.

Methods and experiments

Expression and purification of Rad23^{UBL}

Plasmid pET23b including *Saccharomyces cerevisiae* Rad23 amino acid 1–78 and a C-terminal His₆ tag was transformed into *Escherichia coli* strain BL21(DE3). The transformed colonies were grown in 10 mL of LB medium (ampicillin 100 µg/ml) overnight and spun down at 2,000g for 10 minutes. The pellets were re-suspended and diluted 1:100 into 1 L of M9 media supplemented with ¹⁵N ammonium chloride and ¹³C glucose (Sigma-Aldrich). The cells were grown at 37 °C to OD₆₀₀ of 0.5–0.6 and protein expression induced by 0.4 mM IPTG at 37 °C for 4 hours. The cells were harvested by spinning down at 5,000g for 30 minutes and the harvested pellets frozen in liquid nitrogen and stored at –80 °C for ~ 4 hours. The pellets were re-suspended in lysis buffer (20 mM NaPO₄, 300 mM NaCl, 15 mM β-mercaptoethanol at pH 7.0) supplemented with EDTA-free protease inhibitor cocktail tablets (Roche Diagnostics) and lysed by sonication. Cell lysates were spun down at 27,000g for 30 minutes. The supernatant was incubated with pre-washed TALON metal affinity resin (Clontech) for 1 hour, and the beads washed extensively in lysis buffer containing 20 mM imidazole. Rad23^{UBL} was eluted from the TALON resin with elution buffer (20 mM NaPO₄, 100 mM NaCl, 250 mM imidazole, 15 mM β-mercaptoethanol at pH 7.0). The fractions containing Rad23^{UBL} were further purified by size exclusion chromatography on an FPLC ÄKTA pure system (GE Healthcare) equipped with a HiLoad 16/600 Superdex 75 prep grade column in FPLC buffer (20 mM NaPO₄, 50 mM NaCl, 2 mM DTT at pH 6.5). Rad23^{UBL} was concentrated by an Amicon Ultra-15 filter with a 3 kDa cutoff (EMD Millipore) to ~ 0.7 mM.

NMR experiments

NMR experiments were performed with 0.7 mM of ¹⁵N-, ¹³C-labeled samples on Bruker Avanced III 600 and 700 MHz spectrometers equipped with cryogenically cooled probes at 25 °C. For the backbone assignments, 2D ¹H-¹⁵N HSQC, 3D HNCACB, CBCA(CO)NH, HNCO and HN(CA)CO spectra were recorded. For aliphatic side chain assignments, 2D ¹H-¹³C HSQC, 3D HCCH-TOCSY, ¹⁵N-dispersed NOESY (200 ms mixing time), and ¹³C-edited NOESY-HSQC (80 ms mixing time) spectra were recorded. All experiments were conducted in 20 mM NaPO₄ at pH 6.5, 50 mM NaCl, 2 mM DTT, 0.1% NaN₃ and 5% ²H₂O/95% ¹H₂O, except for 2D ¹H-¹³C HSQC, 3D HCCH-TOCSY and ¹³C-edited

NOESY-HSQC experiments, which were acquired on samples dissolved in $^2\text{H}_2\text{O}$. All NMR data processing was performed with NMRpipe (Delaglio et al. 1995), and spectra were visualized and analyzed with XEASY (Bartels et al. 1995). Secondary structure was assessed by comparing chemical shift values of C_α and C' atoms to random coil positions to generate a chemical shift index (CSI) (Wishart and Sykes 1994) and also by the TALOS+ program (Shen et al. 2009).

Extent of assignments and data deposition

Fig 1 shows an assigned 2D ^{13}C -decoupled ^1H - ^{15}N HSQC spectrum of ^{15}N -, ^{13}C -labeled Rad23^{UBL} with a C-terminal His-tag at pH 6.5 and 25 °C. Excluding the C-terminal His-tag, 99% of backbone HN atoms (missing M1), 96% of backbone N atoms (missing M1, P15 and P20), and 100% of backbone C_α , C_β , C' and H_α atoms were assigned. 92% of aliphatic protons and 100% of aliphatic carbon atoms were assigned. All Asn and Gln side chain amide groups were assigned, but none of the exchangeable side chain protons of Lys residues were identified. The assignments have been deposited in the Biological Magnetic Resonance Data Bank (www.bmrb.wisc.edu) with accession code 25825.

Three sets of signals were identified for the δ atoms of P20 (Fig 2A, left panel), two of which were in a trans configuration and one showing hallmarks of a cis isomerization state (Fig 2B), including NOE interactions between the H_α atoms of E19 and P20 (Fig 2B, right panel). By using relative peak intensities in the 2D ^1H - ^{13}C HSQC spectrum, we estimated that the two trans configurations present for the δ atoms represent 81 and 12 % of the population, whereas the cis δ atoms comprise 7 % of the population (Fig 2A, left panel). The P20 α atoms present only one trans and one cis state, similarly at an estimated 93 and 7 %, respectively (Fig 2A, right panel). Two sets of resonances were similarly assigned for residues S3, L4, T5, D17, L18, E19, S21, N22, S58, D64, G65, D66, and V69 (Fig 1, italic); these amino acids are spatially close to P20 (Fig 2C), suggesting that isomerization of this proline may play a role in their presentation of two states.

The secondary structure of the Rad23^{UBL} was predicted by plotting the difference between the chemical shift values of the Rad23^{UBL} C_α and carbonyl atoms relative to those of randomly coiled values (Wishart and Sykes 1994), or by the TALOS+ program from the HN, H_α , C_α , C_β , C' and N chemical shift values (Shen et al. 2009) (Fig 3). Five β strands (2–8, 13–18, 41–45, 49–51 and 67–73) and two α helices (24–35 and 57–59) were predicted, consistent with the crystal structure of Rad23^{UBL} complexed with Ufd2 (PDB 3M62). Secondary structure prediction from the chemical shift values of the second set of resonances similarly fit the crystal structure (grey bars in Fig 3), indicating that P20 isomerization does not cause disruption of the Rad23^{UBL} fold. All secondary structure was further confirmed by NOE interactions from ^{15}N -edited NOESY-HSQC and ^{13}C -edited NOESY-HSQC spectra.

Acknowledgments

This research was funded by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research to K.J.W. We thank Janusz Koscielniak for his technical assistance with the NMR facility..

References

- Bartels C, Xia T-H, Billeter M, Güntert P, Wüthrich K. The program XEASY for computer-supported NMR spectral analysis of biological macromolecules. *J Biomol NMR*. 1995; 6:1–10. [PubMed: 22911575]
- Bertolaet BL, Clarke DJ, Wolff M, Watson MH, Henze M, Divita G, Reed SI. UBA domains of DNA damage-inducible proteins interact with ubiquitin. *Nat Struct Biol*. 2001; 8:417–422. DOI: 10.1038/87575 [PubMed: 11323716]
- Dantuma NP, Heinen C, Hoogstraten D. The ubiquitin receptor Rad23: at the crossroads of nucleotide excision repair and proteasomal degradation. *DNA Repair (Amst)*. 2009; 8:449–460. DOI: 10.1016/j.dnarep.2009.01.005 [PubMed: 19223247]
- Delaglio F, Grzesiek S, Vuister GW, Zhu G, Pfeifer J, Bax A. NMRPipe: a multidimensional spectral processing system based on UNIX pipes. *J Biomol NMR*. 1995; 6:277–293. [PubMed: 8520220]
- Elsasser S, et al. Proteasome subunit Rpn1 binds ubiquitin-like protein domains. *Nat Cell Biol*. 2002; 4:725–730. DOI: 10.1038/ncb845 [PubMed: 12198498]
- Fujiwara K, et al. Structure of the ubiquitin-interacting motif of S5a bound to the ubiquitin-like domain of HR23B. *J Biol Chem*. 2004; 279:4760–4767. DOI: 10.1074/jbc.M309448200 [PubMed: 14585839]
- Gomez TA, Kolawa N, Gee M, Sweredoski MJ, Deshaies RJ. Identification of a functional docking site in the Rpn1 LRR domain for the UBA-UBL domain protein Ddi1. *BMC Biol*. 2011; 9:33.doi: 10.1186/1741-7007-9-33 [PubMed: 21627799]
- Guzder SN, Sung P, Prakash L, Prakash S. Affinity of yeast nucleotide excision repair factor 2, consisting of the Rad4 and Rad23 proteins, for ultraviolet damaged DNA. *J Biol Chem*. 1998; 273:31541–31546. [PubMed: 9813069]
- Hanzelmann P, Stinglee J, Hofmann K, Schindelin H, Raasi S. The yeast E4 ubiquitin ligase Ufd2 interacts with the ubiquitin-like domains of Rad23 and Dsk2 via a novel and distinct ubiquitin-like binding domain. *J Biol Chem*. 2010; 285:20390–20398. DOI: 10.1074/jbc.M110.112532 [PubMed: 20427284]
- Hiyama H, et al. Interaction of hHR23 with S5a. The ubiquitin-like domain of hHR23 mediates interaction with S5a subunit of 26 S proteasome. *J Biol Chem*. 1999; 274:28019–28025. [PubMed: 10488153]
- Husnjak K, et al. Proteasome subunit Rpn13 is a novel ubiquitin receptor. *Nature*. 2008; 453:481–488. DOI: 10.1038/nature06926 [PubMed: 18497817]
- Kang Y, Chen X, Lary JW, Cole JL, Walters KJ. Defining how ubiquitin receptors hHR23a and S5a bind polyubiquitin. *J Mol Biol*. 2007a; 369:168–176. DOI: 10.1016/j.jmb.2007.03.008 [PubMed: 17408689]
- Kang Y, Vossler RA, Diaz-Martinez LA, Winter NS, Clarke DJ, Walters KJ. UBL/UBA ubiquitin receptor proteins bind a common tetraubiquitin chain. *J Mol Biol*. 2006; 356:1027–1035. DOI: 10.1016/j.jmb.2005.12.001 [PubMed: 16405905]
- Kang Y, Zhang N, Koepp DM, Walters KJ. Ubiquitin receptor proteins hHR23a and hPLIC2 interact. *J Mol Biol*. 2007b; 365:1093–1101. DOI: 10.1016/j.jmb.2006.10.056 [PubMed: 17098253]
- Mueller TD, Feigon J. Structural determinants for the binding of ubiquitin-like domains to the proteasome. *EMBO J*. 2003; 22:4634–4645. DOI: 10.1093/emboj/cdg467 [PubMed: 12970176]
- Rosenzweig R, Bronner V, Zhang D, Fushman D, Glickman MH. Rpn1 and Rpn2 coordinate ubiquitin processing factors at proteasome. *J Biol Chem*. 2012; 287:14659–14671. DOI: 10.1074/jbc.M111.316323 [PubMed: 22318722]
- Ryu KS, Lee KJ, Bae SH, Kim BK, Kim KA, Choi BS. Binding surface mapping of intra- and interdomain interactions among hHR23B, ubiquitin, and polyubiquitin binding site 2 of S5a. *J Biol Chem*. 2003; 278:36621–36627. DOI: 10.1074/jbc.M304628200 [PubMed: 12832454]
- Shen Y, Delaglio F, Cornilescu G, Bax A. TALOS+: a hybrid method for predicting protein backbone torsion angles from NMR chemical shifts. *J Biomol NMR*. 2009; 44:213–223. DOI: 10.1007/s10858-009-9333-z [PubMed: 19548092]
- Shi Y, et al. Rpn1 provides adjacent receptor sites for substrate binding and deubiquitination by the proteasome. *Science*. 2016; 351:831.doi: 10.1126/science.aad9421

- Walters KJ, Lech PJ, Goh AM, Wang Q, Howley PM. DNA-repair protein hHR23a alters its protein structure upon binding proteasomal subunit S5a. *Proc Natl Acad Sci U S A*. 2003; 100:12694–12699. DOI: 10.1073/pnas.1634989100 [PubMed: 14557549]
- Wang Q, Goh AM, Howley PM, Walters KJ. Ubiquitin recognition by the DNA repair protein hHR23a. *Biochemistry*. 2003; 42:13529–13535. DOI: 10.1021/bi035391j [PubMed: 14621999]
- Wishart DS, Sykes BD. The ¹³C chemical-shift index: a simple method for the identification of protein secondary structure using ¹³C chemical-shift data. *J Biomol NMR*. 1994; 4:171–180. [PubMed: 8019132]

Fig. 1.
2D ^{13}C -decoupled ^1H - ^{15}N HSQC spectrum of ^{15}N -, ^{13}C -labeled Rad23^{UBL} with a C-terminal His-tag (0.7 mM) in 20 mM NaPO_4 at pH 6.5, 50 mM NaCl , 2 mM DTT, 0.1% NaN_3 and 5% $^2\text{H}_2\text{O}$ /95% $^1\text{H}_2\text{O}$ collected at 25 °C on a Bruker Avance III 600 MHz spectrometer. Residue type and sequence position are included for the cross-peaks corresponding to backbone amides. The peaks from the second conformer are labeled in italics. A dashed box indicates an enlarged region in the upper left corner

Fig. 2.

(A) Expanded regions of a ^1H - ^{13}C HSQC spectrum of ^{15}N -, ^{13}C -labeled Rad23^{UBL} displaying P20 δ (left panel) or α (right panel) signals. The indicated percentage of P20 contributing to the three (left) or two (right) observed states was derived from the relative peak intensities. (B) Selected regions from a ^{13}C -edited NOESY-HSQC experiment acquired with ^{15}N -, ^{13}C -labeled Rad23^{UBL} in $^2\text{H}_2\text{O}$ displaying NOE interactions between E19 and P20; trans1 and trans2 refer to the signals at 81 and 12 % in (A), respectively. (C) Ribbon diagram of the Rad23^{UBL} from the crystal structure of it complexed with Ufd2 (PDB 3M62) with P20 side chain atoms colored in red, and amino acids that show a second set of resonances in NMR spectra (italics, Fig. 1) colored in orange. The image was generated by PYMOL (The PyMOL Molecular Graphics System, <http://www.pymol.org/>)

Fig. 3.

Secondary structure of the Rad23^{UBL} obtained from the crystal structure 3M62 in which it is complexed with Ufd2 (2nd panel) and as predicted (top panel) by $\delta C\alpha$ (3rd panel), $\delta C'$ (4th panel) and the TALOS+ program (bottom panel). Predicted order parameters S^2 (5th panel) are also shown, based on the chemical shift assignments and TALOS+. The predicted β strands and α helices are displayed by coloring the sequence blue and red, respectively. Plots derived from the second set of resonances are displayed as grey bars in $C\alpha$ -CSI, C' -CSI and TALOS+ predicted secondary structure.