Protein Modification

DOI: 10.1002/ange.201402642

Nonenzymatic Rubylation and Ubiquitination of Proteins for Structural and Functional Studies**

*Rajesh K. Singh, Adithya Sundar, and David Fushman**

Abstract: *Uncovering the mechanisms that allow conjugates of ubiquitin (Ub) and/or Ub-like (UBL) proteins such as Rub1 to serve as distinct molecular signals requires the ability to make them with native connectivity and defined length and linkage composition. A novel, effective, and affordable strategy for controlled chemical assembly of fully natural UBL–Ub, Ub–UBL, and UBL–UBL conjugates from recombinant monomers is presented. Rubylation of Ub and Rub1 and ubiquitination of Rub1 was achieved without E2/E3 enzymes. New residue-specific information was obtained on the interdomain contacts in naturally-occurring K48-linked Rub1–Ub and Ub–Rub1, and K29-linked Rub1–Ub heterodimers, and their recognition by a K48-linkage-specific Ub receptor. The disassembly of these heterodimers by major deubiquitinating enzymes was examined and it was discovered that some deubiquitinases also possess derubylase activity. This unexpected result suggests possible crosstalk between Ub and Rub1/Nedd8 signaling pathways.*

Post-translational modification of many cellular proteins by covalent attachment of the 76 amino acid protein ubiquitin (Ub) or other Ub-like (UBL) modifiers controls a wide range of vital functions in eukaryotes, including cellular trafficking, DNA repair, ribosomal biogenesis, cell cycle progression, and antigen processing.[1] This modification involves a cascade of enzymatic reactions catalyzed by E1, E2, and E3 enzymes, and results in the formation of an isopeptide bond between the carboxylate group of the C-terminal glycine (G76 in Ub and Rub1) and the ε-amine of a lysine residue in the target protein.[2] Ubiquitination can result in the attachment of a single Ub or a polymeric chain of Ubs (polyUb) and the same chemistry is involved in the formation of Ub–Ub linkages through any of the seven Ub lysine residues or through the α-amine of the N-terminal M1 in Ub.[3] Rub1 (Nedd8 in mammalian cells) is the most closely related to Ub of the UBL family, with ca. 53% sequence identity and ca. 76% sequence similarity to Ub.[4] The key surface residues and the 3D structures of the two proteins are also incredibly similar.[4] Despite these striking similarities, Ub, but not Rub1, is well established as a chain-forming protein.[5] The modification (rubylation) of cullin proteins with Rub1 and the subsequent activation of a multisubunit SCF ligase is the most prevalent and the best studied biological function of Rub1 reported to date.[6] Understanding of the outcomes of rubylation and the signalling properties of Rub1 requires the ability to attach it to other proteins at will.

We and others have recently shown that Rub1 and Ub can form heterologous (Rub1–Ub and Ub–Rub1) conjugates in vivo.[4,7] Interestingly, mass spectrometry analyses of cellular proteins revealed that, similar to Ub, all of the lysine residues of Rub1 can be used to form isopeptide bonds (either with another Rub1 or with Ub).[8] Three types of heterologous conjugates are biologically evident under diverse stress conditions:[4,7] Ub–^{48}Rub1, in which G76 of Ub is linked to K48 of Rub1, and Rub1–^{48}Ub and Rub1–^{29}Ub, in which G76 of Rub1 is linked to K48 and K29 of Ub, respectively (see Ref. [9] for chain/linkage nomenclature). These findings indicate that both rubylated Ub and ubiquitinated Rub1 (and possibly rubylated Rub1) are biologically relevant building blocks of heterologous polymeric chains. Despite their presence within cells, the physiological importance of these conjugates remains poorly understood. Recently, we showed that an enzymatically assembled Rub1–^{48}Ub heterodimer can signal proteasomal targeting (i.e., it is recognized by the proteasomal shuttles and Ub receptors) and is disassembled by the proteasome essentially like the "canonical" Ub–^{48}Ub.[4] These results point to a possible role for Rub1 as part of the polyUb signaling system. However, investigation of the structural and functional properties of the conjugates of Rub1 and Ub of various linkages has been hindered by the lack of unavailability of the corresponding linkage-specific conjugating E2 enzymes. Remarkably, the Ub-conjugation machinery allowed us to rubylate Ub but not to ubiquitinate Rub1.[4] Moreover, neither Rub1 E2 enzymes that form chains nor K29-selective Ub E2 enzymes are currently known. All this necessitates the development of chemical methods for rubylation and ubiquitination that circumvent the need for E2 and E3 enzymes.

Toward this goal, we and others have recently developed various chemical methods for making Ub–Ub conjugates[10–14] (reviewed in Ref. [15]). Some of the methods use total chemical synthesis combined with native/isopeptide chemical ligation to generate dimers[10] and longer chains of Ub.[16] Although a remarkable achievement, the need to use total chemical synthesis has limited their utility in biochemical laboratory settings. To circumvent this problem, we recently developed a nonenzymatic method for the controlled assembly of unbranched or branched Ub chains from bacterially

[*] Dr. R. K. Singh, A. Sundar, Prof. D. Fushman
Department of Chemistry and Biochemistry, University of Maryland
College Park, MD 20742-3360 (USA)
E-mail: fushman@umd.edu

[**] This work was supported by the NIH grants GM065334 and GM095755 to D.F. We thank M. H. Glickman for plasmids of USP2 and UBP6, R. E. Cohen for the AMSH plasmid, B. Nicholson for the Cezanne plasmid, C. A. Castañeda for Ub(K29Boc), and M. A. Nakasone and A. Chaturvedi for some of the deubiquitinases used.

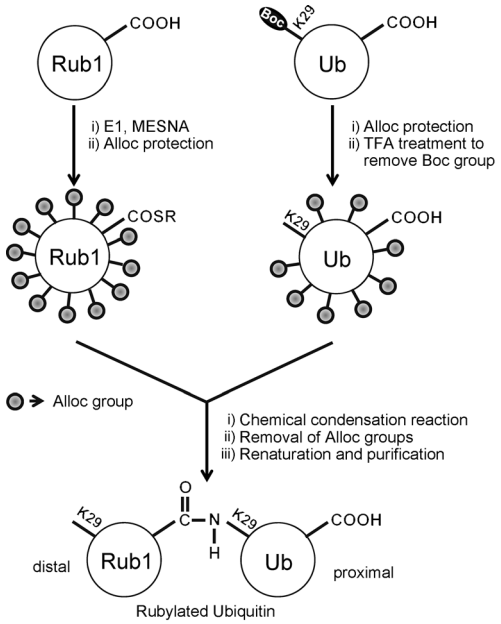
Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201402624.

expressed Ub monomers[12,13] that 1) yields entirely natural polyUb chains of any desired length and linkage composition, 2) can be used readily in any biochemical laboratory, and 3) allows independent isotopic labeling of any monomer in the chain, thus making it amenable to atomic-resolution studies by NMR spectroscopy. While inspired by the GOPAL approach,[11] our method employs mutually orthogonal protecting groups, which provide the advantage of allowing the assembly of chains longer than dimers with full control of chain length and linkage. However, a methodology has yet to be developed for the conjugation of UBLs, as well as for the ubiquitination of proteins other than Ub (i.e. to bypass the E3 enzymes).

Herein, we describe a nonenzymatic method that allowed us to achieve, for the first time, rubylation of both Ub and Rub1 and ubiquitination of Rub1 at desired lysine positions. We made all the above-mentioned naturally occurring heterodimers of Rub1 and Ub. This allowed us to obtain previously unavailable structural insights into interdomain interactions and receptor recognition of these chains. Moreover, since we recently attributed a derubylase function to the 26S proteasome,[4] we used the heterologous conjugates of Rub1 and Ub to examine the derubylase/deubiquitinase activity of several deubiquitinating enzymes (DUBs). Surprisingly, we found that some of the DUBs (USP5, OTUB1) can act as derubylases.

Our method for isopeptide bond formation uses a chemical condensation reaction between the thioesterified C terminus of one monomer (distal-to-be unit, e.g., Rub1 in Rub1–^{29}Ub) and the ε-amine of a specific lysine residue of the other monomer (proximal-to-be unit, e.g., Ub in Rub1–^{29}Ub; Scheme 1). Critically, in order to direct the chemical reaction to a specific lysine residue on the proximal unit, we use a combination of two mutually orthogonal removable protecting groups, Boc and Alloc. The former is introduced as a Lys(Boc) at the desired conjugation site in the proximal-to-be unit by genetic incorporation of unnatural amino acids and is removed prior to the conjugation reaction, while the latter group is introduced as a chemical modification of each unit (reactant) post-purification to protect all of the remaining amines. The mutual orthogonality of the two protecting groups and of the thioester allows the attachment and removal of each one at desired steps without affecting the others.[12,13]

Using this method, we successfully assembled uniformly ^{15}N-enriched Rub1–^{29}Ub, Rub1–^{48}Ub, and Ub–^{48}Rub1 heterodimers (Figure 1A). Since each monomer is bacterially expressed separately, our method allows unit-specific isotopic labeling, which is critical for NMR studies of polyUb chains.[12,17] However, because most of the NMR signals of Ub and Rub1 do not overlap, it was possible to perform NMR characterization of the heterodimers containing both units isotopically enriched at the same time. All of the steps in the assembly of the heterodimers were closely monitored by ESI-MS. Consistent with the expected mass, the ESI-MS results confirmed the complete attachment of SR and Boc groups to their respective monomers (Figure 1B–D) prior to the condensation reaction. The observed mass (ca. 17322 Da) of the assembled Ub–^{48}Rub1, Rub1–^{48}Ub, and Rub1–^{29}Ub heterodimers clearly indicates that the resulting products are fully natural (Figure 1B–D). It should be emphasized that by successfully rubylating Ub and Rub1 (see below), we demonstrate for the first time that a protein other than Ub can be conjugated to other proteins without E2/E3 enzymes. Furthermore, by successfully ubiquitinating Rub1, we show that Ub can be attached to a protein other than Ub without E2/E3 enzymes by using our method.



Scheme 1. Nonenzymatic rubylation of Ub. Similar schemes are used for the ubiquitination or rubylation of Rub1. The basic steps involved in this method are as follows: 1) the C terminus of either Rub1 or Ub (distal-to-be unit) is thioesterified (SR) by incubating the protein with its respective cognate E1 activating enzyme; 2) to direct the reaction to a specific lysine residue (e.g., K29) on the proximal-to-be unit (Ub or Rub1), the ε-amine of that lysine is protected with a Boc group, which is incorporated in the form of Lys(Boc) into the proximal-to-be unit through the use of a genetically encoded unnatural amino acid (UAA); 3) all remaining free amines of both the distal-to-be and proximal-to-be monomers are protected with Alloc. Note that there are 12 such groups in distal-to-be Rub1 (8 lysines, 3 histidines, and the N terminus) and 8 such groups in proximal-to-be Ub (6 lysines, 1 histidine, and the N terminus) that need to be protected with Alloc; 4) the Boc group is removed to make the ε-amine of the lysine of interest the sole site for the ligation reaction; 5) the two monomers are ligated through an Ag-mediated condensation reaction; 6) all of the Alloc groups are removed from the product; and 7) the dimer is renatured and separated from the unreacted monomers to yield a fully natural product, in this case Rub1–^{29}Ub. Boc = *tert*-butoxycarbonyl, Alloc = allyloxycarbonyl, MESNA = sodium 2-mercaptoethanesulfonate.

Our success with the assembly of the above-mentioned heterodimers (and previously with various Ub–Ub homodimers and longer chains[12]) has motivated us to extend our method to the assembly of a fully natural Rub1–^{48}Rub1 homodimer. A polymeric chain of Rub1 has been reported to modify some of the cullin proteins,[18] thus suggesting that a Rub1–^{48}Rub1 conjugate may be a biologically relevant signal. However, such a conjugate has not been made to date. We succeeded in the assembly of the Rub–^{48}Rub1 homodimer (Figure S1 in the Supporting Information) by following the

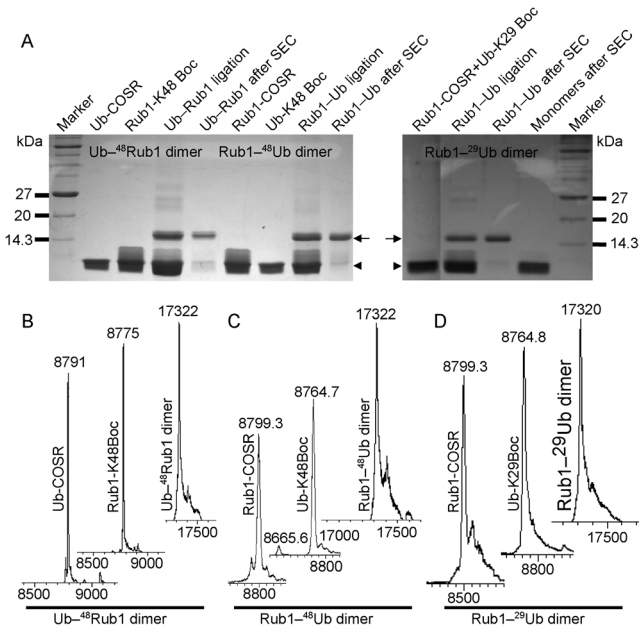


Figure 1. Chemical assembly of Ub–^{48}Rub1, Rub1–^{48}Ub, and Rub1–^{29}Ub dimers. A) 2 mg of each of the reacting monomers were incubated in a condensation reaction for 36 h. 15% SDS-PAGE was performed with 2 μL of each of the reacting monomers, ligated product mix (nonpurified), and the SEC-purified dimers/monomers. The running positions of the monomers and dimers are indicated by the arrowhead and arrow, respectively. B–D) ESI-MS spectra of various intermediate and final steps during the assembly of the Ub–^{48}Rub1 (B), Rub1–^{48}Ub (C), and Rub1–^{29}Ub (D) heterodimers. The molecular masses of ^{15}N-enriched Ub and Rub1 are 8665 Da and 8675 Da, respectively. Protection with the Boc group increases the mass by 100 Da, while attachment of the SR group increases the mass by 125 Da.

above-described procedure (Scheme 1) up to step 6, and the product was confirmed by SDS-PAGE. However, when we attempted to renature the product (step 7), the dimer precipitated. We are currently testing various renaturing approaches to solubilize and study Rub1–^{48}Rub1.

We recently showed that the enzymatically assembled Rub1–^{48}Ub adopts a conformation in which Rub1 and Ub form an interface mediated by the hydrophobic surface patches (comprising L8, I44, and V70) of both monomers.[4] To determine whether the chemically assembled Rub1–^{48}Ub adopts a similar conformation, we recorded its ^{1}H-^{15}N NMR spectrum (at pH 6.8) and quantified the chemical shift perturbations (CSPs) for each residue in Rub1–^{48}Ub versus the corresponding monomer (Figure S2 A, B). The strong similarity between the CSPs observed in the chemically (Figure S2 B) and the enzymatically[4] assembled Rub1–^{48}Ub indicates that the heterodimers made by the two different methods are structurally identical. Moreover, mapping the significant CSPs and signal attenuations on the 3D structures of Rub1 and Ub (Figure S2 C, D) revealed that the surfaces involved in the interdomain interface are essentially the same as in the enzymatically assembled heterodimer.[4]

Next, to examine whether Rub1 and Ub in the other two heterodimers, Ub–^{48}Rub1 and Rub1–^{29}Ub, form an interface, we recorded ^{1}H-^{15}N NMR spectra of ^{15}N-enriched dimers (Figure 2 A, B) and quantified the CSPs (versus the corresponding monomers) for each residue (Figure 2 C, F). The NMR signals are well dispersed and the close similarity with the spectra of monomeric Rub1 and Ub indicates fully folded heterodimers (Figure 2 A, B). Significant CSPs clustered around residues L8, I44, and V70 of Ub clearly indicate the formation of an interdomain interface in Ub–^{48}Rub1 (Figure 2 C). Mapping the CSPs and signal attenuations on the surface of both Ub and Rub1 revealed that the largest perturbations are located on one side of each unit (Figure 2 D, E), thus suggesting a specific interdomain contact in Ub–^{48}Rub1, mediated by the hydrophobic surface patches of both Ub and Rub1. By contrast, no significant CSPs (except for the C terminus, which is conjugated to Ub) were detected for Rub1 in Rub1–^{29}Ub (Figure 2 F, G), thus indicating the absence of a specific noncovalent contact between Rub1 and Ub in this heterodimer. Consistent with this observation, residues perturbed in Ub are located primarily on the opposite side of its surface from the hydrophobic patch and are clustered around residue K29 (Figure 2 H), thus suggesting that the observed CSPs are mainly due to the isopeptide bond formation. These results indicate that there is no defined interdomain interface in Rub1–^{29}Ub.

To verify that chemically assembled Rub1–^{48}Ub is not only structurally but also functionally similar to the enzymatically conjugated heterodimer, we examined its interactions with the UBA2 domain of the proteasomal shuttle protein hHR23a by using NMR titration assays. Interestingly, the CSP patterns (Figure 3 A) and the mapped UBA2-interacting surfaces (Figure 3 B, C) are essentially same as those observed for the enzymatically conjugated Rub1-^{48}Ub,[4] thus confirming that our chemically assembled rubylated Ub is both structurally and functionally indistinguishable from the heterodimer synthesized by the enzymatic machinery. Next, to examine whether the Ub–^{48}Rub1 heterodimer is recognized by the UBA2 domain of hHR23a, unlabeled UBA2 was titrated into ^{15}N-labeled Ub–^{48}Rub1. The observed strong CSPs and signal attenuations (Figure 3 D) clearly indicate that the ubiquitinated Rub1 binds to UBA2. As in Rub1–^{48}Ub (and Ub–^{48}Ub[19]), this interaction is residue specific and mediated by the hydrophobic-patch surfaces of both Ub and Rub1 (Figure 3 E, F), thus suggesting that Ub–^{48}Rub1 can be a signal for proteasomal targeting.

The disassembly of Ub conjugates and subsequent recycling of Ub is critical for maintaining Ub homeostasis in the cell.[20] A variety of DUBs play a central role in the disassembly of different types of Ub conjugates. Among them, OTUB1,[21] UBP6[22] (USP14 in humans), and USP2[23] are mainly K48-linkage specific, while Cezanne and AMSH show specificity for K11 and K63 linkages, respectively. USP5 (isopeptidase T) has been shown to recycle Ub by hydrolyzing the isopeptide bond in a variety of unanchored polyUb linkages.[24] The remarkable similarity between Rub1 and Ub[4] raises the question of whether DUBs can recognize Rub1 and treat it as Ub. To study the recognition pattern of these DUBs, as well as their enzymatic activity towards the heterodimers of Ub and Rub1, we performed an in vitro deubiquitination assay (Figure 4; see controls in Figure S3).

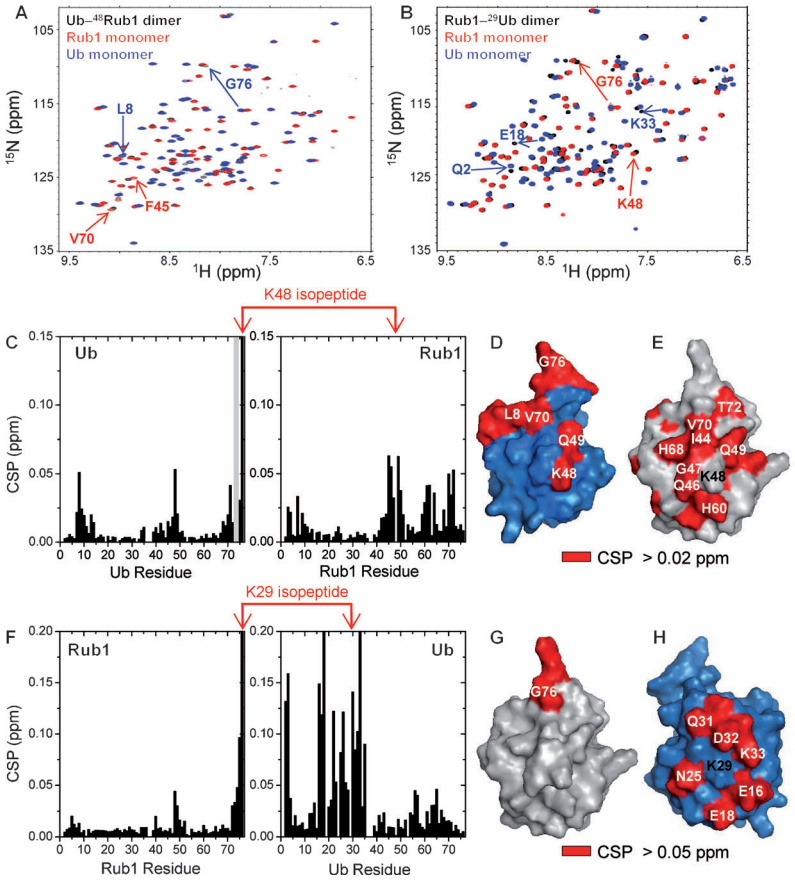


Figure 2. Analysis of the interdomain interface in the heterodimers of Ub and Rub1. ^{1}H-^{15}N SOFAST-HMQC spectra (black) of Ub–^{48}Rub1 (A) and Rub1–^{29}Ub (B) overlaid with the spectra of the Rub1 (red) and Ub (blue) monomers are shown. Selected residues showing significant signal shifts are marked with numbers and indicated by arrows. Note that residue K48 in the proximal units was not ^{15}N labeled. Chemical shift perturbations (CSPs, black bars) and significant signal attenuations (> 75%; grey bars) of backbone amides in Ub–^{48}Rub1 (C) and Rub1–^{29}Ub (F) were plotted as a function of the residue number. Isopeptide-forming residues are connected by red arrows. The perturbed residues (red) in Ub–^{48}Rub1 (D, E) and Rub1–^{29}Ub (G, H) are mapped onto the 3D structures of Ub (blue) and Rub1 (grey), oriented such that the hydrophobic-patch surfaces face the reader except for in (H), where the opposite side of Ub surface is shown.

have both deubiquitinase and derubylase activity. Neither Cezanne nor AMSH disassembled any of the dimers, a result consistent with their specificity for K11 and K63 linkages, respectively.

The in vitro synthesis and subsequent functional studies of various types of chains composed of Ub and/or UBL proteins remained challenging for many years mainly owing to the lack of proper enzymes to form such conjugates. Herein, we present a chemical ligation method by which heterologous (Ub–Rub1 and Rub1–Ub) and homologous (Rub1–Rub1) conjugates can be made from recombinant monomers in a controlled manner, with fully natural connectivity, and in sufficient amounts for structural and biophysical studies. Moreover, our method can be readily used in any biochemical laboratory and yields fully natural products devoid of any chain-terminating mutations. By taking advantage of bacterial expression of the recombinant monomers, our method enables the cost-effective unit-specific isotopic labeling of the chains, which is required for high-resolution NMR studies. NMR characterization of the two novel heterodimers assembled with our method revealed that in Ub–^{48}Rub1 but not in Rub1–^{29}Ub, Ub and Rub1 form an interface that is very similar to the interface in Rub1–^{48}Ub and the corresponding Ub–^{48}Ub homodimer,[17] and provided site-specific information on the binding interaction with the UBA2 domain of the proteasomal shuttle protein hHR23a. Furthermore, the availability of heterodimers allowed us to examine, for the first time, the requirements for the recognition and cleavage of Ub units by several DUBs. Moreover, we discovered an unexpected derubylase activity of some of the deubiquitinases. All these results demonstrate that the method presented herein expands the repertoire of chemical-biology tools by opening previously unavailable opportunities to make polymeric chains containing various UBL modifiers and to study their structural and functional properties. We foresee a straightforward extension of our methodology to other UBLs, for example, SUMO, which forms both homologous (SUMO 2/3)[25] and heterologous (with Ub) chains.[26] It would be interesting to examine whether the structural properties of such chains correlate with their specific functional roles. Our ultimate goal is to be able to attach Ub or UBL, as a monomer or as a chain of any desired length and linkage composition, to a substrate protein at will, with no need for E2 or E3 enzymes. This would open endless possibilities for studying the structural and functional roles of the attachment of Ub/UBL monomers or chains to their physiological substrate proteins. The development of this method for the rubylation of Ub and Rub1 and the ubiquitination of Rub1 moves us closer to achieving this goal.

Interestingly, UBP6 and USP2 completely disassembled ubiquitinated Rub1 but did not cleave rubylated Ub (K48- or K29-linked). This indicates that Ub at the position distal to the isopeptide bond is required for the deubiquitinase function of UBP6 and USP2 and a distal Rub1 blocks this activity. By contrast, OTUB1 partially cleaved Rub1–^{48}Ub but not Ub–^{48}Rub1 or Rub1–^{29}Ub, thus indicating that the enzyme is particularly selective for the proximal Ub and for the K48 linkage. The results also suggest that Ubs on both sides of the K48 isopeptide bond are needed for optimal OTUB1-mediated cleavage. This is consistent with the role of UBP6 and USP2 in the removal of a (poly)Ub tag from substrates destined for degradation,[22] and with OTUB1 recognizing both the distal and proximal Ubs simultaneously.[21] Interestingly, USP5 cleaved all three heterodimers tested, thus confirming its ability to cleave different types of linkages. These results clearly show that USP5 and OTUB1

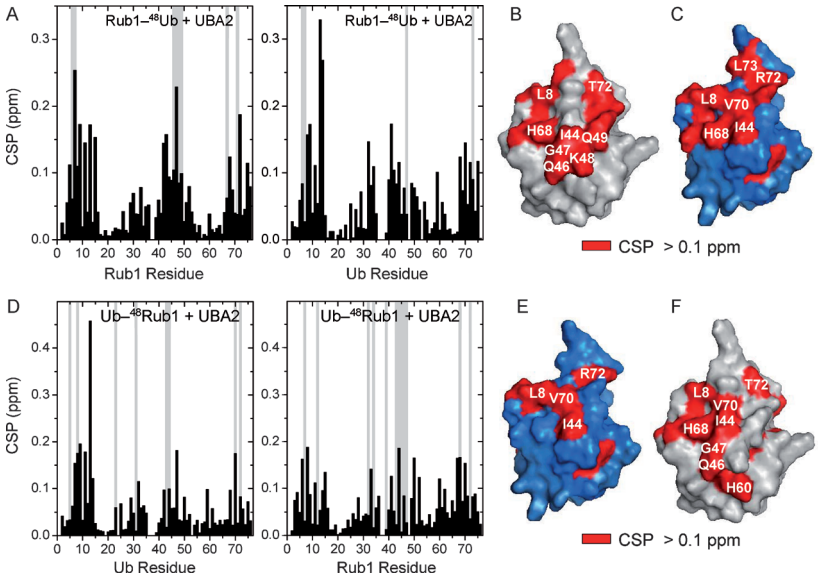


Figure 3. K48-linked heterodimers of Rub1 and Ub form residue-specific interactions with the UBA2 domain of hHR23a. CSPs (black bars) and significant signal attenuations (> 75%; grey bars) of backbone amides in Rub1–^{48}Ub (A) and Ub–^{48}Rub1 (D) at saturation with UBA2 are plotted as a function of the residue number. Residues with significant CSPs (> 0.1 ppm) and signal attenuations are mapped (red) on the surface of the distal and proximal units of Rub1–^{48}Ub (B, C) and Ub–^{48}Rub1 (E, F). Ub and Rub1 are shown in blue and grey, respectively.

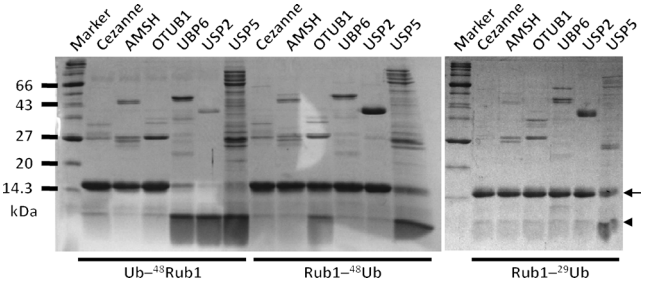


Figure 4. Cleavage of the synthesized heterodimers of Rub1 and Ub by deubiquitinases. The heterodimers were incubated for 16 hr in a cleavage reaction with the indicated deubiquitinases in a 10:1 molar ratio. The reactions were stopped by adding SDS loading buffer and loaded onto a 15% SDS-PAGE gel. The running positions of monomers and dimers are shown by the arrowhead and arrow, respectively.

Received: February 20, 2014
Published online: April 24, 2014

Keywords: deubiquitinases · nonenzymatic assembly · protein modifications · rubylation · ubiquitination

[1] a) M. D. Marmor, Y. Yarden, *Oncogene* **2004**, *23*, 2057 – 2070; b) S. P. Jackson, D. Durocher, *Mol. Cell* **2013**, *49*, 795 – 807; c) H. D. Ulrich, H. Walden, *Nat. Rev. Mol. Cell Biol.* **2010**, *11*, 479 – 489; d) D. Finley, B. Bartel, A. Varshavsky, *Nature* **1989**, *338*, 394 – 401; e) L. K. Teixeira, S. I. Reed, *Annu. Rev. Biochem.* **2013**, *82*, 387; f) J. Loureiro, H. L. Ploegh, *Adv. Immunol.* **2006**, *92*, 225 – 305.

[2] a) M. Hochstrasser, *Cell* **2006**, *124*, 27 – 34; b) C. M. Pickart, *Cell* **2004**, *116*, 181 – 190.

[3] D. Fushman, K. D. Wilkinson, *F1000 Biol. Rep.* **2011**, *3*, 26.

[4] R. K. Singh, S. Zerath, O. Kleifeld, M. Scheffner, M. H. Glickman, D. Fushman, *Mol. Cell. Proteomics* **2012**, *11*, 1595 – 1611.

[5] T. Hori, F. Osaka, T. Chiba, C. Miyamoto, K. Okabayashi, N. Shimbara, S. Kato, K. Tanaka, *Oncogene* **1999**, *18*, 6829 – 6834.

[6] a) G. Rabut, M. Peter, *EMBO Rep.* **2008**, *9*, 969 – 976; b) J. Merlet, J. Burger, J. E. Gomes, L. Pintard, *Cell. Mol. Life Sci.* **2009**, *66*, 1924 – 1938.

[7] O. Leidecker, I. Matic, B. Mahata, E. Pion, D. P. Xirodimas, *Cell Cycle* **2012**, *11*, 1142 – 1150.

[8] a) S. M. Jeram, T. Srikumar, X. D. Zhang, H. A. Eisenhauer, R. Rogers, P. G. Pedrioli, M. Matunis, B. Raught, *Proteomics* **2010**, *10*, 254 – 265; b) J. Jones, K. Wu, Y. Yang, C. Guerrero, N. Nillegoda, Z. Q. Pan, L. Huang, *J. Proteome Res.* **2008**, *7*, 1274 – 1287.

[9] M. A. Nakasone, N. Livnat-Levanon, M. H. Glickman, R. E. Cohen, D. Fushman, *Structure* **2013**, *21*, 727 – 740.

[10] a) K. S. Kumar, L. Spasser, L. A. Erlich, S. N. Bavikar, A. Brik, *Angew. Chem.* **2010**, *122*, 9312 – 9317; *Angew. Chem. Int. Ed.* **2010**, *49*, 9126 – 9131; b) F. El Oualid, R. Merkx, R. Ekkebus, D. S. Hameed, J. J. Smit, A. de Jong, H. Hilkmann, T. K. Sixma, H. Ovaa, *Angew. Chem.* **2010**, *122*, 10347 – 10351; *Angew. Chem. Int. Ed.* **2010**, *49*, 10149 – 10153; c) R. Yang, K. K. Pasunooti, F. Li, X. W. Liu, C. F. Liu, *Chem. Commun.* **2010**, *46*, 7199 – 7201.

[11] a) S. Virdee, Y. Ye, D. P. Nguyen, D. Komander, J. W. Chin, *Nat. Chem. Biol.* **2010**, *6*, 750 – 757; b) S. Virdee, P. B. Kapadnis, T. Elliott, K. Lang, J. Madrzak, D. P. Nguyen, L. Riechmann, J. W. Chin, *J. Am. Chem. Soc.* **2011**, *133*, 10708 – 10711.

[12] C. Castañeda, J. Liu, A. Chaturvedi, U. Nowicka, T. A. Cropp, D. Fushman, *J. Am. Chem. Soc.* **2011**, *133*, 17855 – 17868.

[13] E. K. Dixon, C. A. Castaneda, T. R. Kashyap, Y. Wang, D. Fushman, *Bioorg. Med. Chem.* **2013**, *21*, 3421 – 3429.

[14] a) S. Eger, M. Scheffner, A. Marx, M. Rubini, *J. Am. Chem. Soc.* **2010**, *132*, 16337 – 16339; b) E. M. Valkevich, R. G. Guenette, N. A. Sanchez, Y. C. Chen, Y. Ge, E. R. Strieter, *J. Am. Chem. Soc.* **2012**, *134*, 6916 – 6919.

[15] a) L. Spasser, A. Brik, *Angew. Chem.* **2012**, *124*, 6946 – 6969; *Angew. Chem. Int. Ed.* **2012**, *51*, 6840 – 6862; b) A. Brik, *Bioorg. Med. Chem.* **2013**, *21*, 3398 – 3399.

[16] a) K. S. Kumar, S. N. Bavikar, L. Spasser, T. Moyal, S. Ohayon, A. Brik, *Angew. Chem.* **2011**, *123*, 6261 – 6265; *Angew. Chem. Int. Ed.* **2011**, *50*, 6137 – 6141; b) T. Moyal, S. N. Bavikar, S. V. Karthikeyan, H. P. Hemantha, A. Brik, *J. Am. Chem. Soc.* **2012**, *134*, 16085 – 16092.

[17] R. Varadan, O. Walker, C. Pickart, D. Fushman, *J. Mol. Biol.* **2002**, *324*, 637 – 647.

[18] Y. Ohki, N. Funatsu, N. Konishi, T. Chiba, *Biochem. Biophys. Res. Commun.* **2009**, *381*, 443 – 447.

[19] R. Varadan, M. Assfalg, S. Raasi, C. Pickart, D. Fushman, *Mol. Cell* **2005**, *18*, 687 – 698.

[20] a) M. J. Clague, J. M. Coulson, S. Urbé, *J. Cell Sci.* **2012**, *125*, 277 – 286; b) D. Komander, M. J. Clague, S. Urbé, *Nat. Rev. Mol. Cell Biol.* **2009**, *10*, 550 – 563; c) D. Komander, *Subcell. Biochem.* **2010**, *54*, 69 – 87.

[21] T. Wang, L. Yin, E. M. Cooper, M. Y. Lai, S. Dickey, C. M. Pickart, D. Fushman, K. D. Wilkinson, R. E. Cohen, C. Wolberger, *J. Mol. Biol.* **2009**, *386*, 1011 – 1023.
[22] a) D. S. Leggett, J. Hanna, A. Borodovsky, B. Crosas, M. Schmidt, R. T. Baker, T. Walz, H. Ploegh, D. Finley, *Mol. Cell* **2002**, *10*, 495 – 507; b) A. Guterman, M. H. Glickman, *J. Biol. Chem.* **2004**, *279*, 1729 – 1738; c) M. Hu, P. Li, L. Song, P. D. Jeffrey, T. A. Chenova, K. D. Wilkinson, R. E. Cohen, Y. Shi, *EMBO J.* **2005**, *24*, 3747 – 3756.
[23] a) L. F. Stevenson, A. Sparks, N. Allende-Vega, D. P. Xirodimas, D. P. Lane, M. K. Saville, *EMBO J.* **2007**, *26*, 976 – 986; b) B. Oberfeld, D. Ruffieux-Daidie, J. J. Vitagliano, K. M. Pos, F. Verrey, O. Staub, *Am. J. Physiol. Renal. Physiol.* **2011**, *301*, F189 – 196.
[24] A. Amerik, S. Swaminathan, B. A. Krantz, K. D. Wilkinson, M. Hochstrasser, *EMBO J.* **1997**, *16*, 4826 – 4838.
[25] a) H. D. Ulrich, *Mol. Cell* **2008**, *32*, 301 – 305; b) A. Vertegaal, *Biochem. Soc. Trans.* **2007**, *35*, 1422 – 1423.
[26] T. Hunter, H. Sun, *Ernst Schering Found. Symp. Proc.* **2008**, 1 – 16.

