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Tetrameric Assembly of Monoubiquitin Accurately Mimics the Lys11 Polyubiquitin Chain Structure

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ABSTRACT: Specific lysine residues on the ubiquitin surface were selected during the course of evolution to form different polyubiquitin chain structures that signal diverse cellular processes. A vast number of ubiquitin receptors specifically recognize and decode the signals conferred by these polyubiquitin chains. The mechanisms of formation and the structure of Lys11-linked ubiquitin, which signals for cell-cycle and innate immune control, have been elucidated. Here, we present a new crystal structure of monomeric ubiquitin that accurately mimics one of the structures of Lys11-linked ubiquitin. Analysis of the ubiquitin:ubiquitin interface demonstrates structural fitness and specificity. The interaction is exclusively hydrophilic, leaving the Ile44 hydrophobic patch, a major recognition site for ubiquitin receptors, exposed. These noncovalent ubiquitin:ubiquitin interactions are nearly identical to those reported for Lys11-linked ubiquitin and seem to play a significant role in stabilizing the crystal structure without the isopeptide bond. *In vitro* cross-linking analysis with wild-type ubiquitin or its mutants partially mimics the interactions in the crystal. We suggest that these interactions may play a biological role in transmitting Lys11-linked ubiquitin chain-type cellular signals.

Ubiquitylation is a post-translational modification in which the carboxyl terminus of ubiquitin is covalently attached to free amino groups of lysine side chains or the N-termini of target proteins.[1,2] The versatility of the ubiquitin signal is increased by the ubiquitylation of ubiquitin, which forms different lengths and topologies of polyubiquitin chains. Seven lysine residues and the N-terminus of ubiquitin can undergo ubiquitylation, resulting in a variety of polyubiquitin chains with different topologies. Ubiquitylation changes the molecular landscape of a protein and can therefore control the protein's interactions with ubiquitin receptors. Sequence analysis and a literature search reveal that ubiquitin receptors usually harbor three modules: (i) ubiquitin-binding module(s), (ii) a targeting (or context) module, and (iii) a response module. Thus, by binding to ubiquitylated proteins, ubiquitin receptors function as sensors that decode and convert the ubiquitin signal into a specific response in a given cellular location (or context). For example, the yeast ubiquitin receptor Vps9p decodes mono- or short Lys63 polyubiquitylation signals on transmembrane cargo into a guanine nucleotide exchange of the Rab5 family, an essential event en route to vacuolar degradation.[3−5] Proteins marked with Lys48 polyubiquitin are recognized by proteasomally associated ubiquitin receptors, such as Rpn10 and/or Rpn13, which subject them to proteasomal degradation.[6−9] Recently, attention has been drawn to the unconventional structures of polyubiquitin chains and their signaling pathways.[10] It has been reported that Lys11 polyubiquitin chains are mainly synthesized during mitosis by the E2s Ube2C and Ube2S and their cognate E3 anaphase-promoting complex (APC/C), suggesting that this unique chain formation is involved in cell-cycle control.[11−13] The levels of Lys11 conjugates are low in asynchronous cells or cells synchronized in mitosis with nocodazole; however, they dramatically increase during anaphase and mitotic exit.[12] APC/C modifies the mitotic regulators, including securin, cyclin B, and HURP.[14,15] Indeed, inhibition of Lys11 polyubiquitin chain formation blocks mitotic progression in *Xenopus*, *Drosophila*, and humans.[11,14,16] However, a yeast ubiquitin K11R mutant does not present any growth phenotype.[17,18] Lys11-linked ubiquitin chains are also involved in the nuclear factor κB (NF-κB) signaling pathway.[19,20] The structures of Ube2S and its product, Lys11-linked ubiquitin, were determined.[12,21−23] Moreover, *in silico* modeling followed by *in vitro* assessment of the Ube2S:ubiquitin transient complex has been presented.[24] In this model, one ubiquitin molecule is transiently linked to the catalytic Cys residue of the E2, while the other ubiquitin molecule specifically interacts with a patch of residues on the E2 surface, orienting its Lys11 in the proximity of the Gly76−catalytic Cys thioester intermediate bond. In this structure, however, despite the proximity near the linkage site, no physical protein−protein interactions between the two ubiquitin molecules are seen. On the other hand, examination of the structure of Lys11 diubiquitin [Protein Data Bank (PDB) entry 2XEW] reveals a highly juxtaposed and compact formation of C_4-symmetric ubiquitin-like chains. While the input for the crystallization was Lys11 diubiquitin, because of the compact

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structure and the noncrystallographic symmetry, the authors could not assign the two isopeptide bonds linking Lys11 to Gly76 to particular residues in the tetrameric structure but instead modeled all the possible options.[21] Here, we present a novel crystal structure of monomeric ubiquitin, in which four molecules in the asymmetric unit form a compact C_4-symmetric-like structure essentially identical to one of the Lys11-linked ubiquitin structures. Structural analyses of the contact interfaces suggest that they represent a specific binding mode rather than crystallization coincidence.

EXPERIMENTAL PROCEDURES

Plasmid Construction. Histidine tag human ubiquitin was subcloned by polymerase chain reaction (PCR) into the *Bam*HI/*Eco*RI restriction endonuclease sites of the pHis$_6$-Parallel.2 plasmid, in which the β-lactamase gene was replaced with a kanamycin resistance cassette and the affinity tag was removed.[5]

Protein Purification. Untagged ubiquitin was purified as described by Pickart and Raasi,[25] followed by size exclusion chromatography with a Sephadex-75 column in a 150 mM NaCl, 50 mM Tris-HCl, pH 8.0 buffer. The protein was concentrated, flash-frozen in liquid nitrogen, and kept at −80 °C.

Protein Crystallization. Initial hits of crystals were obtained using the sitting-drop vapor diffusion method. Ubiquitin crystals were grown in drops containing an equal volume of protein and reservoir [1 μL of a 10 mg/mL ubiquitin sample with 1 μL of 3.4 M sodium malonate (pH 5.0) and 100 mM thiocyanate] and incubated at 18 °C. Ubiquitin crystals were obtained after ∼3 weeks. Crystals were harvested, cryo-protected by immersion in paratone-N, and frozen by being dipped into liquid nitrogen.

Data Collection and Structure Determination. Data collection was performed using an in-house X-ray source at λ = 1.5418 Å. Indexing and data reduction were performed with HKL2000. Matthews coefficient calculation provided four molecules in the asymmetric unit (V_m = 2.23; 44.8% solvent). The structure was determined by molecular replacement (MR), using "Phaser" with four copies of ubiquitin (1UBQ) as an initial search model.[26] To facilitate the MR search, the flexible carboxyl terminus (residues 73−76) was removed from the initial model. The structure was refined using Refmac5 with loose NCS parameters. The stereochemistry and Ramachandran bond analysis were validated using Procheck and the RCSB validation server.

Cross-Linking Assay. Cross-linking assays with the fast protein liquid chromatography-purified wild type or the indicated ubiquitin mutants were conducted according to the method described by Azem and co-workers.[27] Reaction mixtures (360 μL for each sample) contained 57 μM indicated proteins, 10 mM glutaraldehyde in 20 mM Hepes, pH 7.4 buffer, 100 mM KCl, and 5 mM MgCl. The reaction mixtures were incubated at room temperature for 15 min. Boiling the samples in sodium dodecyl sulfate loading buffer terminated the reactions. Samples were resolved via 17% sodium dodecyl sulfate−polyacrylamide gel electrophoresis and detected by Coomassie blue staining.

RESULTS

Overall Structure of Ubiquitin in the Asymmetric Unit. Using a high concentration of sodium malonate, we crystallized ubiquitin in the *C*2 space group (Table 1). Malonate is a strong kosmotropic ion that stabilizes native protein interactions.[28] One crystal diffracted to 2.3 Å resolution in a laboratory X-ray source and was used for data set collection. We determined the structure by molecular replacement (MR) using the coordinates of ubiquitin.[29] Unambiguous maps were obtained, facilitating structural refinement. To demonstrate the quality of the data and the model, we present an omit map that covers residues 41−47 (Figure 1A). Matthews coefficient calculation suggests that the asymmetric unit contains four ubiquitin molecules.[30] Indeed, in our new crystal form, we found four ubiquitin molecules that were compactly juxtaposed in the asymmetric unit (Figure 1B). Structural comparison of these four molecules showed that they all assume a nearly identical structure with a root-mean-square deviation (rmsd) of 0.15−0.26 Å (Figure 1C). The largest differences were found at the β3−β4 connecting loop and at the beginning of β4, where the Cα atoms of Ala46 from chains A and D are at a distance of 1.0 Å and the Cα atoms of Lys48 from chains C and D are at distance of 0.8 Å. The common class 2 two-residue β-hairpins,[31] which connect β1 to β2, were also present with a somewhat greater deviation with a distance of two Cα atoms of 0.5 Å. Naturally, the relatively large deviation at the molecule backbone near Lys48 is also reflected in the deviation at the location of the side chain atoms. Figure 1C shows that Lys residues 6, 11, 27, 29, and 33, from the four ubiquitin molecules, essentially assume the same conformation (rotamers). However, Lys48 and Lys63 seem to possess much

Table 1. Data Collection and Refinement Statistics

Data Collection	
space group	*C*2
cell dimensions	
a, *b*, *c* (Å); α, β, γ (deg)	81.3, 81.3, 58.6; 90.0, 129.3, 90.0
wavelength (Å)	1.5418
resolution (Å)	49.75−2.3 (2.36−2.30)[a]
R_{merge}	0.047 (0.147)
$\langle I/\sigma(I)\rangle$	16.6 (7.3)
completeness (%)	100 (100)
redundancy	8.0
Refinement	
resolution (Å)	49.75−2.3 (2.38−2.30)
no. of unique reflections	13141 (1292)
no. of reflections used for R_{free}	651 (5%)
R_{factor}/R_{free}	0.186/0.223
no. of atoms	
protein	2736
ligand	81
water	147
average *B* factor (Å^2)	
protein	27.8
ligand	38.8
water	29.9
rmsd	
bond lengths (Å)	0.006
bond angles (deg)	1.08
Ramachandran plot (%)	
favored	100
additional	0.0
disallowed	0.0

[a]Values in parentheses are for the highest-resolution shell.

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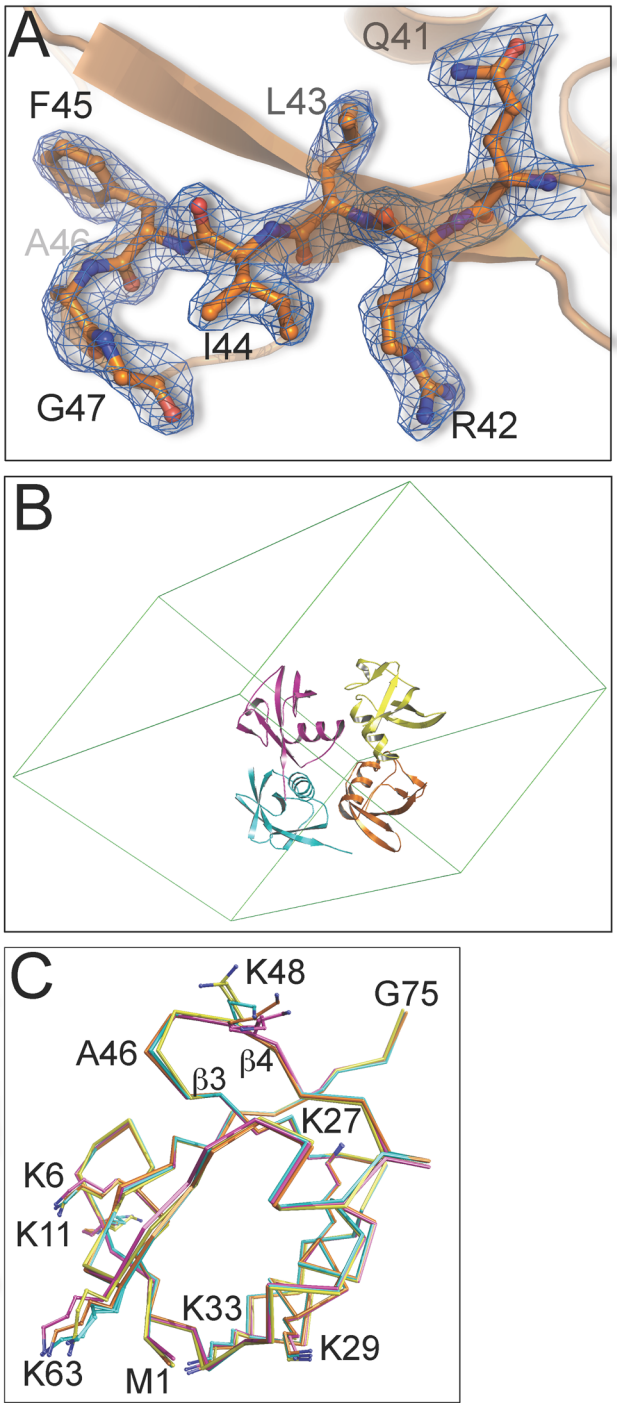

Figure 1. Crystal structure of ubiquitin in space group *C*2. The structure of ubiquitin in the *C*2 space group crystals was determined by MR. (A) A $2mF_o - DF_c$ omit map covering the region around Ile44 of ubiquitin (chain B) and contoured at 2.0σ. Residues 41−47 of ubiquitin were omitted, and the structure was subjected to simulated annealing and individual *B* factor refinement before map calculation. The refined structure is shown with atoms colored as follows: orange, carbon; red, oxygen; blue, nitrogen. (B) Four determined ubiquitin molecules of the asymmetric unit in the *C*2 unit cell. (C) Superimposition of the individual ubiquitin molecules. Lysine residues are shown in ball-and-stick representation (chain A, green; chain B, cyan; chain C, magenta; chain D, orange).

more mobile structures, with each of the ubiquitin chains providing a different rotamer. This result agrees with the versatile functional role of ubiquitylation of residues Lys48 and Lys63 as signals that may be interpreted by many different ubiquitin receptors and therefore may provide the high flexibility of the conjugates.

Tetrameric Assembly Mimicking Lys11 Polyubiquitin Chains. Careful examination of the molecules packing in the noncrystallographic symmetry (NCS) structure shows that their C-termini are in the proximity of the amide ε groups of Lys11 of the neighboring molecules. Moreover, the overall structure is reminiscent of the architecture of the Lys11-linked diubiquitin structure, recently determined by Komander and co-workers,[21] which prompted us to compare these two structures. We were surprised to note that these two structures are accurately superimposed (Figure 2A). All four molecules are superimposed using their backbone atoms (i.e., 300 amino acids) with a rmsd of 0.6 Å. Only the C-terminal Gly76 residues, whose electron density was not observed, were absent from the alignment.

The evidence that these two structures are largely identical suggests that the Lys11-linked ubiquitin structure strengthened noncovalent ubiquitin:ubiquitin interactions that are possibly formed *in vivo*. In these two structures, each of the ubiquitin molecules has two interfaces that interact with its two neighbors to form a tetracyclic (C_4-symmetric) closed and compact structure (Figure 2B). These interfaces are centered at Glu34 and Arg74 of the two adjacent ubiquitin molecules (Figure 2C). In this C_4-symmetrical architecture, the interfaces provided by each of the ubiquitin molecules are nearly identical in a circular manner, with very minor differences that may reflect the flexibility and dynamics of this noncovalent "tetrameric" assembly. The contacts at the noncrystallographic symmetry (NCS) are exclusively polar, leaving the Ile44 hydrophobic surface, known to interact with most of the ubiquitin receptors, entirely exposed. Careful analysis of these contact interfaces revealed that the orientations of the ubiquitin molecules in the Lys11 diubiquitin structure and the current structure are identical and most of the residues also presented the same rotamers, particularly those residues located at the binding interfaces, which presented a highly similar binding mode (Figure 2B). The fact that these two nearly identical structures belong to two different crystallographic space group systems (*C*121 versus *P*2221) indicates that the forces holding these C_4-assemblies are not related to crystal packing.

Figure 3A illustrates a representative interface (chains A:B). The interface is centered on Lys33 in one ubiquitin molecule and a surface around Arg74 of the other ubiquitin molecule. Several direct and solvent-mediated (including water and malonate) interactions were formed. Lys33 (of chain A) interacts simultaneously with two molecules of malonate (MLA). The electron density of these two molecules from the crystallization liquor is clearly seen (not shown). One molecule of MLA forms two additional interactions with the backbone nitrogen atoms of Glu16 and Gly53 in chains A and B, respectively. The second MLA is coordinated by a network of electrostatic interactions, including interaction with Asp52 of chain B and Lys33 of chain A. Asp32 of chain A forms two direct hydrogen bonds with Glu24 of chain B. The backbone oxygen of Glu34 in chain A directly interacts with the guanidinium group of Arg74 in chain B. The backbone oxygen of Asp32 in chain A directly interacts with the backbone nitrogen of Asp39 of chain B. Finally, Arg72 of chain B indirectly interacts with the backbone oxygen of Lys33 of chain A via a coordinated water molecule.

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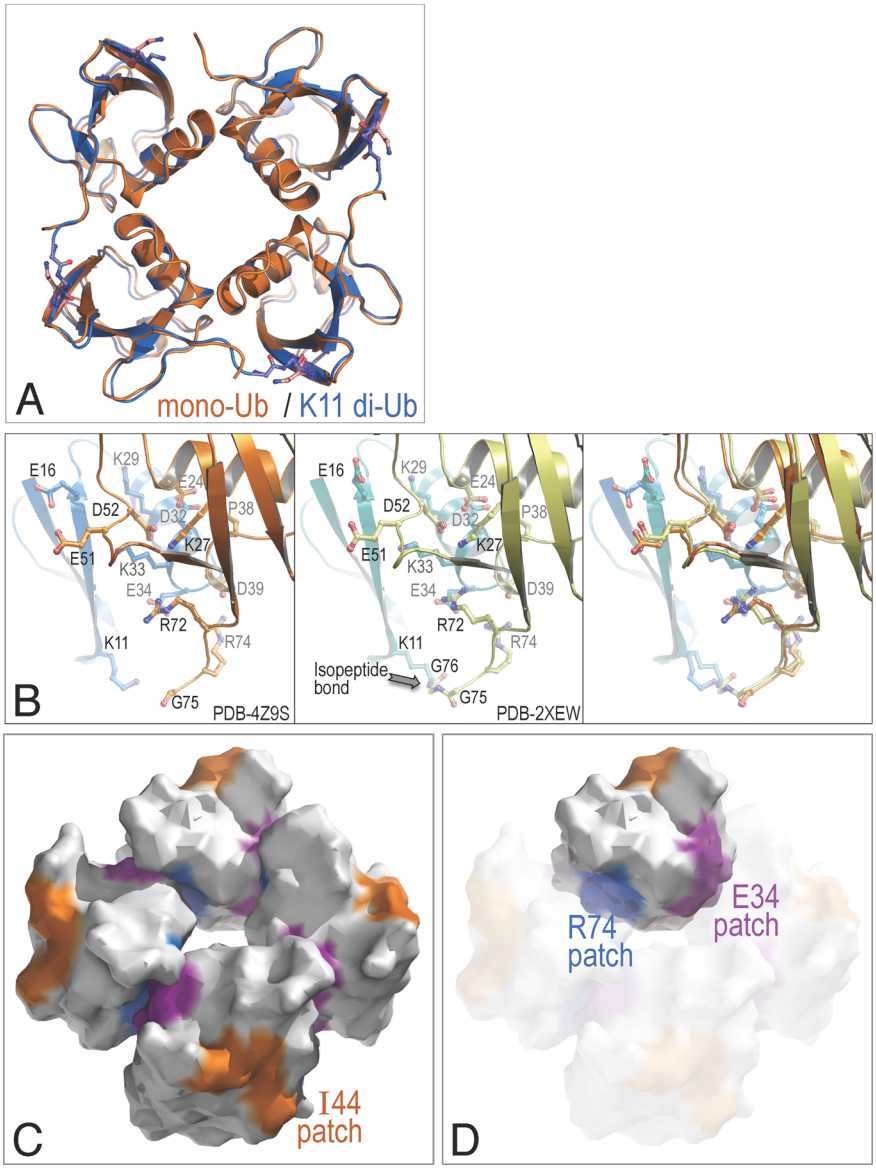

Figure 2. Tetrameric assembly of ubiquitin mimics the Lys11 diubiquitin structure. (A) Superposition of the current monoubiquitin structure (orange) and Lys11 diubiquitin (PDB entry 2XEW, marine blue). The rmsd for 300 Cα atoms is 0.6 Å. (B) The residues in the vicinity of the C-terminus and Lys11 interface in the monomeric (left), diubiquitin (middle), and superimposed (right) structures are shown. (C) Surface of the four ubiquitin molecules in the asymmetric unit is presented. Each molecule buries a surface of ~550 Å^2 (magenta and blue areas). The Ile44 hydrophobic patch is fully exposed (orange). (D) Complete buried surfaces (magenta and blue areas), where the neighboring ubiquitin molecules are rendered as a transparent surface.

Buried Surfaces and Shape Complementarity. Buried/accessible surface calculations suggest specific protein−protein interactions among all four molecules in the asymmetric unit (Figure 3B). It has been suggested that a buried surface larger than ~400 Å^2 may indicate specific protein−protein interactions.[32,33] Each of the interfaces in the reported structure buries a solvent-accessible surface area larger than this benchmark, with an average value of 564 Å^2 (Figure 3B). Because ubiquitin is a relatively small protein (8.5 kDa, with a solvent-accessible surface area of ~9200 Å^2) we do not expect to see a large interaction surface. A clear relationship between protein mass and buried surface interface has been demonstrated.[34] Indeed, all known ubiquitin-binding domains present fairly small interaction surfaces, varying from 389 to 923 Å^2 for the two extreme cases of the Npl4-NZF:ubiquitin (PDB entry 1Q5W) and Vps9p-CUE:ubiquitin (PDB entry 1P3Q) complexes, respectively.[4,35]

To further assess whether the interfaces in the asymmetric unit represent specific protein−protein interactions, we performed shape complementarity (S_c) calculations (Figure 3B). The calculated S_c statistic scores were all above 0.72. Values equal to or greater than 0.7 are comparable to those of specific protein−protein/inhibitor complexes, consistent with the predicted functional role for these interfaces.[36] Similar values were shown for other ubiquitin-binding domains. For example, the GAT:ubiquitin and double-sided Hrs-UIM:ubiquitin complexes present S_c values of 0.72 and 0.73, respectively.[37,38] Both *in silico* procedures excluded water and other ligands from the analyses of the surfaces. These results support our hypothesis that the ubiquitin:ubiquitin interactions

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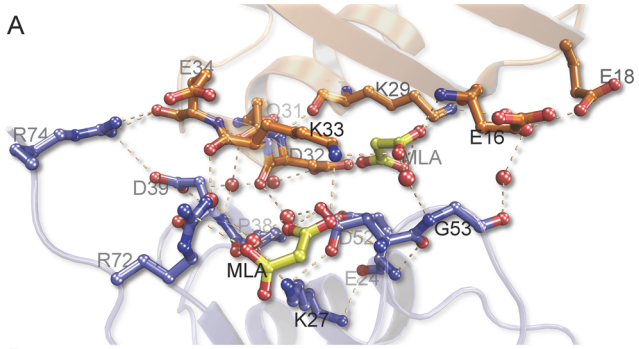
A



B

Interface (chains)	Buried surface (Å²)	S_c
A:B	530.8	0.73
B:C	536.6	0.72
C:D	579.2	0.73
D:A	570.2	0.72
Total	2216.8	

C

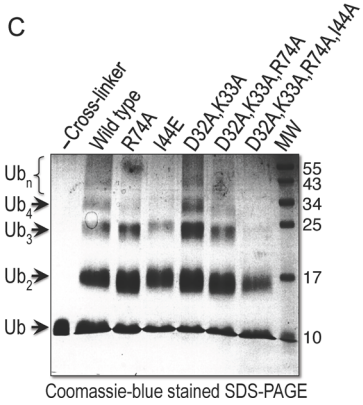


Figure 3. Structural analyses of the ubiquitin:ubiquitin binding interface. Zooming in on the ubiquitin:ubiquitin binding interface (chains A and B) reveals a complexed network of hydrophilic interactions. (B) Shape complementarity score (S_c) and buried surface area, calculated with the SC and AREAIMOL of the Collaborative Computational Program Number 4 (CCP4).[36] (C) Cross-linking analysis of the gel-filtered purified wild type and the indicated ubiquitin mutants.

at the presented interfaces are structurally specific and may possess a biological significance.

It is worth noting that a different crystal structure of Lys11-linked diubiquitin (PDB entry 3NOB) has been reported.[12] In this structure, the C-termini of the two ubiquitin molecules face each other and the Ile44 hydrophobic patches are in the proximity of each other, forming an elongated and continuous hydrophobic face. Importantly, the two ubiquitin molecules form hydrophobic interactions that bury some of the known Ile44-binding patch. Conversely, the calculated S_c value is significantly lower (0.51), suggesting that this ubiquitin:ubiquitin noncovalent interaction is not specific and may represent a transient binding possibly to hide the Ile44 patch.

***In Vitro* Analysis of Interacting Interfaces.** Ubiquitin is known to possess a monomeric structure in solution. However, it is possible that specific patches on its surface favor highly transient self-interactions that cannot be detected by the currently available biophysical methods. Cross-linking is a simple biochemical method that captures protein−protein interactions in solution. It may be useful for identifying specific patches that interact transiently. We therefore performed a cross-linking assay with purified wild-type and mutated ubiquitin proteins. We demonstrated that under the tested conditions wild-type cross-linked ubiquitin formed high-molecular weight species (Figure 3C). Intriguingly, mutations at the C_4-symmetric interfaces resulted in various effects on the cross-linked species. Specifically, the R74A mutation predicted to abrogate the Lys74−Glu34 interaction decreased the amount of high-molecular weight species. However, the R74A mutant clearly formed di- and triubiquitin species, suggesting that other patches contribute to the cross-linking pattern. Ala mutations at Asp32 and Lys33 did not present a phenotype. We also found that mutation at Ile44 (I44E) had a significant effect on the cross-linking reaction. These findings suggest that several interfaces contribute to the transient noncovalent ubiquitin:ubiquitin interactions.

DISCUSSION

Recently, Fushman and co-workers conducted a comprehensive and in-depth NMR analysis to study alternative structures of Lys11 diubiquitin.[23] They demonstrated a number of structures that fall between the two crystal structures of Lys11 diubiquitin, signifying the flexibility of the linkage and the loose ubiquitin:ubiquitin noncovalent interactions.

The new crystal form reported here shows that the hydrophilic noncovalent ubiquitin:ubiquitin interactions are sufficient to stabilize the structure of the Lys11-linked ubiquitin chain in a particular arrangement. Our analyses suggest that the structure represents a biologically relevant conformation that transmits one or more of the Lys11 chain signals. Moreover, because most ubiquitin receptors recognize the Ile44 patch, the current ubiquitin:ubiquitin configuration allows accessibility and recognition by ubiquitin receptors, such as NEMO, of the NF-κB signaling pathway.[19,20] We propose that the various structures of Lys11 ubiquitin chains conduct different biological roles. When the Ile44 patch is hidden, the signal cannot be read by most known ubiquitin receptors. Our cross-linking data demonstrate that Ile44:Ile44 patch interactions significantly contribute to this "unreadable configuration" of free ubiquitin molecules in solution. As the I44E mutant presents the strongest phenotype, we suggest that the closed structure (PDB entry 3NOB) is more stable and probably more abundant. The reported structure and the structure determined by Komander and co-workers (PDB entry 2XEW), in which the Ile44 patches are exposed, probably present cellular conditions that allow interactions with ubiquitin receptors. These ubiquitin receptors convert the signal into a cellular response. The crystal structure presented here provides a molecular insight into the non-covalent interactions between adjacent ubiquitin molecules in the Lys11-linked chains, which stabilize this "readable" chain configuration. It would be interesting to explore the complex of Lys11-linked chains with such a ubiquitin receptor.

ASSOCIATED CONTENT

Accession Codes

The atomic coordinates and structure factors have been deposited in the Protein Data Bank as entry 4Z9S.

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Notes

The authors declare no competing financial interest.

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