Solution Conformation of Lys\(^{63}\)-linked Di-ubiquitin Chain Provides Clues to Functional Diversity of Polyubiquitin Signaling\(^\text{a}^\text{b}\)

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Diverse cellular events are regulated by post-translational modification of substrate proteins via covalent attachment of one or a chain of ubiquitin molecules. The outcome of (poly)ubiquitination depends upon the specific lysine residues involved in the formation of polyubiquitin chains. Lys\(^{48}\)-linked chains act as a universal signal for proteasomal degradation, whereas Lys\(^{63}\)-linked chains act as a specific signal in several non-degradative processes. Although it has been anticipated that functional diversity between alternatively linked polyubiquitin chains relies on linkage-dependent differences in chain conformation/topology, direct structural evidence in support of this model has been lacking. Here we use NMR methods to determine the structure of a Lys\(^{63}\)-linked di-ubiquitin chain. The structure is characterized by an extended conformation, with no direct contact between the hydrophobic residues Leu\(^8\), Ile\(^{44}\), and Val\(^{70}\) on the ubiquitin units. This structure contrasts with the closed conformation observed for Lys\(^{48}\)-linked di-ubiquitin wherein these residues form the interdomain interface (Cook, W. J., Jeffrey, L. C., Carson, M., Zhijian, C., and Pickart, C. M. (1992) J. Biol. Chem. 267, 16467–16471; Varadan, R., Walker, O., Pickart, C., and Fushman, D. (2002) J. Mol. Biol. 324, 637–647). Consistent with the open conformation of the Lys\(^{63}\)-linked di-ubiquitin, our binding studies show that both ubiquitin domains in this chain can bind a ubiquitin-associated domain from HHR23A independently and in a mode similar to that for mono-ubiquitin. In contrast, Lys\(^{48}\)-linked di-ubiquitin binds in a different, higher affinity mode that has yet to be determined. This is the first experimental evidence that alternatively linked polyubiquitin chains adopt distinct conformations.

An increasing number of cellular processes has been found to be regulated by ubiquitin (Ub\(^3\))-mediated signaling events (1, 2). In all such events, a specific lysine residue on the surface of the substrate protein is covalently attached to the C terminus of a ubiquitin molecule via tightly regulated enzymatic steps (3). Chains of Ub are assembled on the substrate via formation of isopeptide bonds between a lysine on the surface of the first Ub and the C-terminal Gly\(^{76}\) of the next. There are seven different lysine residues in Ub, five of which (Lys\(^{6}\), Lys\(^{11}\), Lys\(^{29}\), Lys\(^{48}\), and Lys\(^{63}\)) have been observed to be involved in the formation of polyUb chains in vitro or in vivo (for review, see Ref. 4). A recent analysis revealed that all seven of the theoretical Ub-Ub linkages are detectable in the yeast proteome (5). At least in some cases the lysine site used for Ub-Ub conjugation is preserved throughout the chain (3, 6, 7).

Interestingly, the consequence of (poly)ubiquitination has been found to depend upon which lysine residue forms the isopeptide bonds in the polyUb chain. The best characterized of these outcomes is the proteasomal degradation of substrate proteins tagged by Lys\(^{48}\)-linked chains (8, 9). PolyUb chains assembled via Lys\(^{29}\) have been implicated in degradation of certain model proteasome substrates (10). Lys\(^{63}\)-linked polyUb chains, on the other hand, function in post-replicative DNA repair in the RAD6 pathway (11, 12), IкB kinase activation (13), translational regulation (6), and some instances of endocytosis (14), although their mechanistic role in these processes is still unclear. Mono-Ub, like Lys\(^{63}\)-linked polyUb chains, is not involved in proteasome proteolysis and appears to act as a trafficking signal in a host of processes, including endocytosis, endosome internalization, and viral budding (for reviews, see Refs. 15 and 16). It is remarkable that different polyUb chains, assembled from identical Ub units, are able to function as distinct signals in the cell. Because the only obvious chemical difference between the functionally distinct chains is in their linkage via alternative lysine residues, the answer is likely to lie in different topologies/conformations of the alternatively linked polyUb chains, although direct recognition of the isopeptide bond cannot yet be excluded. Elucidation of the conformation of alternatively linked polyUb chains would greatly aid in distinguishing between these models and in understanding the basis of structural diversity in polyUb signaling.

The location of Lys\(^{48}\) with respect to the C terminus of the Ub molecule should cause an almost 90° turn in the direction of the Lys\(^{48}\)-linked chain with every added Ub unit and could result in a “compact” conformation of the polyUb chain. Given that Lys\(^{63}\) is located on a different face of the ubiquitin molecule compared with Lys\(^{48}\), it is conceivable that Lys\(^{63}\)-linked chains

methyl methanesulfonate; HSQC, heteronuclear single-quantum coherence; RDC, residual dipolar coupling; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; GST, glutathione S-transferase; E1, ubiquitin activating enzyme; IPAP, in-phase/anti-phase.

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‡ The abbreviations used are: Ub, ubiquitin; monoUb, mono-ubiquitin; polyUb, polyubiquitin; UBA, ubiquitin-associated domain; PDB, Protein Data Bank; MTSL, 1-oxyl-2,2,5,5-tetramethyl-3-pyrroline-3-
adopt a different conformation that could result in a differential display of the hydrophobic residues that have been implicated in several Ub-dependent signaling events (17, 18).

Recently we have shown that under physiological conditions Lys63-linked Ubα adopts a closed conformation in which these functionally important hydrophobic residues (Leu9, Ile8, Val23) are sequestered at the interdomain interface (19). This is important to that Ub, the formation of closed conformation, however, is in dynamic equilibrium with one or more open conformations (19), thus allowing the exposure of these hydrophobic residues to the solvent and, presumably, to specific interacting proteins. The same hydrophobic residues are involved in interdomain contacts in Ubβ (19), although the solution conformation of this chain is yet to be elucidated. Here we present the results of our NMR studies indicating that the solution conformation of Lys63-linked Ubβ differs strongly from the structure of Lys48-linked Ubβ.

MATERIALS AND METHODS

Biochemicals were from Sigma unless otherwise stated. E1 was from BostonBiochem Inc., and ethyleneimine was from Chemservice. Yeast Mms2 and Ubc13 were expressed and purified as previously described (21, 22). The alignment of the C-terminus of Ubc13/Mms2 (21) in overnight reactions at 37 °C was performed. The reaction tube was flushed with N2–37°C for 12 h. The heterodimeric complex of Ubc13/Mms2 (21) in overnight reactions at 37°C was performed. The reaction tube was flushed with N2–37°C for 12 h. The solution conformation of Lys63-linked Ub2 differs strongly from the structure of Lys48-linked Ub2.

Design and Synthesis of Segmentally Isotope-enriched polyUb Chains—To overcome the expected spectroscopic equivalence of all monomers in a homopolymeric chain we employed chains in which only one Ub unit was isotope-enriched in each NMR sample, as described earlier (19). The notations used throughout the text are as follows. Isotope-enriched Ub moieties in Ubαβ are labeled P for the proximal (with respect to a possible substrate) or D for the distal location in the chain. Lys48-linked Ub2 molecules were synthesized using E1 and the heterodimeric complex of Ubc13/Mms2 (21) in overnight reactions at 37 °C. The alignment of the C-terminus of Ubc13/Mms2 (21) in overnight reactions at 37°C was performed. The reaction tube was flushed with N2–37°C for 12 h. The heterodimeric complex of Ubc13/Mms2 (21) in overnight reactions at 37°C was performed. The reaction tube was flushed with N2–37°C for 12 h. The solution conformation of Lys63-linked Ub2 differs strongly from the structure of Lys48-linked Ub2.

Site-directed Spin Labeling of Ub1—The spin label MTSL was covalently linked to the side chain of Cys175, a single cysteine residue in the Ubbc13 construct. In a control reaction, the corresponding reaction of Ub (or Ubβ) was performed. The probe was attached to the Cys175 residue at urea (15N-Ub/D) and 57 of 71 (Ub2-P) observed NH groups were used for the analysis. Distances from flexible regions, 8–11 and 72–76, were excluded from the analysis due to high uncertainty in their amide NH–vector orientations. Amides involved in conformational exchange (Ile32, Asn173) were not included because of the strong effect of this motion on the chemical shift data (23). The alignment tensor was calculated using the alignment tensor program ALTENS (19); the atom coordinates were from PDB code 1D3Z (29). 59 of the observed 71 NH groups were used for the analysis. Backbone NH groups of residues in loop regions 8–11 and 71–76 were excluded from the analysis due to the uncertainty in their orientations. The quality of the derived alignment tensors was assessed using the quality factor representing the agreement between the experimental and predicted values of the RDCs (33).

Alignment of Ub Moieties in Ub2 Based on NMR Data—The relative orientation of the two domains in Ub2 was determined by aligning the principal axes of the alignment or rotational diffusion tensor of the whole molecule experienced by each of the domains using the method of Fushman and Courtoy (23). The spin label MTSL was covalently linked to the side chain of Cys175, a single cysteine residue in the Ubbc13 construct. In a control reaction, the corresponding reaction of Ub (or Ubβ) was performed. The probe was attached to the Cys175 residue at urea (15N-Ub/D) and 57 of 71 (Ub2-P) observed NH groups were used for the analysis. Distances from flexible regions, 8–11 and 72–76, were excluded from the analysis due to high uncertainty in their amide NH–vector orientations. Amides involved in conformational exchange (Ile32, Asn173) were not included because of the strong effect of this motion on the chemical shift data (23). The alignment tensor was calculated using the alignment tensor program ALTENS (19); the atom coordinates were from PDB code 1D3Z (29). 59 of the observed 71 NH groups were used for the analysis. Backbone NH groups of residues in loop regions 8–11 and 71–76 were excluded from the analysis due to the uncertainty in their orientations. The quality of the derived alignment tensors was assessed using the quality factor representing the agreement between the experimental and predicted values of the RDCs (33).
where Perturbations observed in UBA upon Ub2 titration do not discriminate to search for the interdomain interface in Lys63-linked Ub2.

We used the chemical shift mapping approach—equation as for UBA-monoUb binding, between the two Ub domains; therefore, the 1:1 model gives the same measurements on the distal or proximal domain in Ub2, the 1:1 model gives

\[ p_B = \frac{([P]_1 + [L]_1 + \frac{1}{2} K_d - \sqrt{([P]_1 + [L]_1 + \frac{1}{2} K_d)^2 - 4[P]_1[L]_1})}{4[P]_1} \]

(Eq. 1)

Here \([P]_1\) and \([L]_1\) are the total molar concentrations of the protein (Ub2) and ligand (UBA). For a 2:1 model, considerations based on partitioning between the free and various ligand-bound states of Ub2 give

\[ p_B = \frac{(2[P]_1 + [L]_1 + K_d - \sqrt{(2[P]_1 + [L]_1 + K_d)^2 - 8[P]_1[L]_1})}{4[P]_1} \]

(Eq. 2)

Perturbations observed in UBA upon Ub2 titration do not discriminate between the two Ub domains; therefore, the 1:1 model gives the same equation as for UBA-monoUb binding,

\[ p_B = \frac{([P]_1 + [L]_1 + K_d - \sqrt{([P]_1 + [L]_1 + K_d)^2 - 4[P]_1[L]_1})}{4[P]_1} \]

(Eq. 3)

where \(P\) and \(L\) in this case represent UBA and Ub2, respectively. The 2:1 binding model gives

\[ p_B = \frac{([P]_1 + 2[L]_1 + K_d - \sqrt{([P]_1 + 2[L]_1 + K_d)^2 - 8[P]_1[L]_1})}{2[P]_1} \]

(Eq. 4)

RESULTS AND DISCUSSION

Segmentally isotope-labeled Lys63-linked Ub2 molecules were synthesized as described under “Materials and Methods.” The correct mass of the purified product was confirmed by MALDI-TOF mass-spectrometry (17,304 Da versus 17,307 Da expected). The correct chain assembly is confirmed by the observed chemical shift perturbations in the C-terminal residues in Ub2-D and in Lys63 and adjacent residues in Ub2-P (Fig. 1).

Search for Interdomain Interactions in Ub2 Using Chemical Shift Mapping.—We used the chemical shift mapping approach to search for the interdomain interface in Lys63-linked Ub2. Any difference in chemical shifts observed between monomeric Ub and Ub2 under identical experimental conditions would indicate a change in the microenvironment of the residue as a result (direct or indirect) of the interactions between Ub domains in Ub2. However, under our experimental conditions no significant backbone amide chemical shift perturbations were observed in the Ub2 (Fig. 1) except for the residues involved in the linkage. This suggests that 1) both Ub units remain unperturbed in Ub2 and 2) no specific interaction exists between the two Ub moieties in Lys63-linked Ub2. The latter conclusion contrasts with our results for the Lys48-linked chains where a well defined interface was observed (Fig. 1; Ref. 19). Thus, the conformations of the two chains are different.

Solution Conformation of Lys63-linked Ub2.—We next determined the conformation of the Lys63-linked Ub2. The relative orientation of the two domains in Lys63-linked Ub2 was obtained from the molecular alignment in a dilute liquid crystalline medium (“Materials and Methods”). The characteristics of the alignment tensors for Ub domains in Ub2 derived from residual dipolar coupling measurements are presented in Table I. Both Ub domains exhibited good alignment in the medium, confirmed by the low values of the quality factor (Table I; see also the supplemental material). The Ub2 structure resulting from aligning the principal axes of the alignment tensors is shown in (Fig. 2, a and b). It should be noted that the distance between the two Ub units in this structure is arbitrary, since no direct distance constraints can be derived from the method. Consistent with our chemical shift perturbation data, the conformation of these chains appears to be extended with no clear interface between the two Ub domains.

Despite the absence of a clear interface, the two Ub units in Ub2 tumble together as one entity (i.e. as a dumbbell) rather than as independent beads on a flexible string. This conclusion is supported by our NMR relaxation data. The measured \(^1\)H T2 values are inversely proportional to the molecular weight of the...
The hydrodynamic parameters (rotational diffusion tensors) derived from RDCs and relaxation measurements in the presence of the alignment medium we also used the two independent, physically different methods (alignment tensors) to assess the quality of the derived alignment tensor (33). The validity of the structural data obtained here is justified by the low values of $R$, indicating good agreement between the measured RDCs and those predicted from the structure. The correlation coefficient between the measured and predicted RDCs was 0.982 versus 0.979 for linkage-specific protein-protein interactions, we tested the PDB coordinate frame for each domain.

Quality factor $R$ was used to assess the quality of the derived alignment tensor (33). The validity of the structural data obtained here is justified by the low values of $R$, indicating good agreement between the measured RDCs and those predicted from the structure. The correlation coefficient between the measured and predicted RDCs was 0.982 (distal) and 0.979 (proximal).

To verify that the observed conformation of Ub$_2$ is not biased by interactions with the alignment medium we also used the hydrodynamic parameters (rotational diffusion tensors) derived from $^{15}$N relaxation data to determine the average relative orientation of the two Ub units in Lys$_{63}$-linked Ub$_2$ (56). The overall rotational correlation times derived for each Ub unit in Ub$_2$ (8.5 ns for the distal and 8.9 ns for the proximal) indicate a 2-fold slower molecular tumbling compared with that of monoUb (4.2 ns), in accordance with the molecular weight dependence of the overall rotation of a molecule, expected from the Stokes-Einstein-Debye equation. All these data and the good alignment of the domains in the liquid crystalline medium confirm that the two Ub units in Lys$_{63}$-linked Ub$_2$ indeed reorient together in solution, which then justifies the use of the domain orientation approaches applied here. A similar behavior characterized by well defined relative orientation of individual domains in a molecule despite the absence of specific interdomain contacts was observed in the Src homology 3-2 domain construct system (28).

The Euler angles ($\alpha$, $\beta$, $\gamma$) in (degrees) characterize the orientation of the principal axes frame of the alignment tensor of Ub$_2$ with respect to the PDB coordinate frame for each domain.

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We also confirmed the structures we determined above by monitoring the effect of a spin-label (MTSL) attached at residue 63 in the distal domain (a K63C mutant containing a single Cys residue was used as Ub$_2$-D). The attachment of the spin label was confirmed by an increase in the mass of the Ub$_2$ by 183 Da (185 Da expected) recorded by MALDI-TOF mass spectrometry and by signal attenuation observed in the residues close to Cys$_{63}$ in the distal Ub, which also indicated correct positioning of the spin label. According to the structure shown in Fig. 2, residue 63 in the distal domain is at the distant end from the proximal domain. If the two Ub domains adopt a more compact structure (e.g., like that in Fig. 2d) instead of an extended conformation (Fig. 2, a–c), specific residues on the 185 Da labeled proximal Ub that come in close proximity to the modified Cys should show relaxation rate enhancement. However, no such effect was observed on the proximal Ub (data not shown). In a control experiment, an attachment of MTSL to the distal domain (K48C mutant) in Lys$_{48}$-linked Ub$_2$ resulted in strong attenuation of NMR signals from several residues in the proximal domain (data not shown). Thus, although the Ub domains display a significant degree of conformational freedom, the Lys$_{63}$-linked chain does not adopt a conformation that would result in the close proximity of the proximal domain to Cys$_{63}$ on the distal domain.

Comparison with Lys$_{48}$-linked Chains—Lys$_{63}$-linked Ub$_2$ was previously shown to predominantly adopt a closed conformation (Fig. 2d) under physiological conditions (19). In this conformation hydrophobic residues (Leu$_6$, Ile$_{3}$, and Val$_{75}$) implicated in recognition of polyUb chains by the proteasomal machinery are sequestered at the interface between the Ub units. The average conformation of the Lys$_{63}$-linked Ub$_2$ presented in this study is in striking contrast with that of Lys$_{48}$-linked Ub$_2$. Under identical buffer/experimental conditions, the Lys$_{63}$-linked Ub$_2$ chains adopt an extended conformation with the hydrophobic patch on the surface of each Ub fully exposed. This is the first experimental evidence that alternatively linked ubiquitin chains adopt distinct conformations in solution.

It has been demonstrated earlier that Rad23 UBA domains bind Lys$_{63}$-linked polyUb chains in strong preference to Lys$_{63}$- and Lys$_{29}$-linked chains (22). However, this preference has only been demonstrated for Ub$_2$ chains. We note that the conformation adopted by Lys$_{63}$-linked Ub$_2$ should be readily accessible to longer chains. To directly test if the distinct conformations of Lys$_{48}$ versus Lys$_{63}$-Ub$_2$ chains (Fig. 2) could be relevant for linkage-specific protein-protein interactions, we tested whether Rad23-UBA2 domain retains the previously described linkage specificity with Ub$_2$ chains. Pull-down assays with GST-fused UBA2 (versus a control of GST alone) were performed in triplicate for Lys$_{48}$- and Lys$_{63}$-linked Ub$_2$.
chains and for mono-Ub (Fig. 3). The data indeed show that the UBA2 domain of Rad23 binds Lys63-linked Ub2 in strong preference to the Lys63-linked Ub2. To verify the reproducibility of the observed differences, binding experiments for the Ub2 chains were repeated, with identical results. Note that Lys63-linked Ub2 still binds better than monoUb (Fig. 3), indicating that the weak binding of the atypically linked Ub2 is not due to the Lys63 linkage blocking interaction with monoUb, as discussed further below.

Structural Insights into Ligand Binding Properties of Lys63-linked Chains: Mapping UBA-Ub2 Interactions—Our data presented above show that in the Lys63-linked chain the hydrophobic surfaces on the two Ub domains should be readily accessible for ligand binding. Thus, to a first approximation, each Ub unit could bind ligands independently and in a mode similar to monoUb. To test this hypothesis we investigated the mode of binding of Lys63-linked Ub2 to the UBA2 domain from HHR23A, which was shown to bind monoUb via the hydrophobic patch Leu8-Ile44-Val70 (36). Although the interactions between UBA2 and monoUb are of modest strength (Kd ~ 0.4 mM, (36), also see below) and binding to Lys63-Ub2 is also relatively weak (Fig. 3), binding of such low affinity can be reliably observed at the relatively high concentrations used in NMR (e.g. Ref. 37).

The chemical shift mapping approach was used to identify sites on the interacting molecules involved in the binding. Significant backbone amide chemical shift perturbations were observed in specific residues in both domains of Lys63-linked Ub2 and in the UBA2 domain, suggesting a specific association between the proteins (Fig. 4). The magnitude of these perturbations increases with UBA concentration and saturates at [UBA]/[Ub2] > 5. The perturbations in both Ub domains are weak (Fig. 3), binding of such low affinity can be reliably observed at the relatively high concentrations used in NMR (e.g. Ref. 37).

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FIG. 3. Linkage specificity of UBA binding to Ub$_2$ and Ub$_4$ chains. The solid and open bars show the percent of the input radiiodinated chain that bound to GST-fused UBA2 or GST (control), respectively. Each binding experiment was performed in triplicate. The measurements for Ub$_2$ chains were performed twice, yielding identical results.

FIG. 4. Chemical shift mapping of the interactions between Lys$_{63}$-linked Ub$_2$ and UBA2 domain from Rad23. The top panels depict combined amide chemical shift perturbations (“Materials and Methods”) observed in the distal (a) and proximal (b) domains of Ub$_2$ and in UBA (c). The sites perturbed upon binding were mapped on the surface of Ub$_2$ (d) and UBA domain (e). Panel f shows the amino acid sequence of the UBA2 domain construct, with residue numbers used here (top) and in the Rad23 sequence (bottom). Surface coloring in Ub$_2$ (d) is as follows. The unperturbed sites are shown in blue (distal Ub) and green (proximal Ub); the residues showing chemical shift perturbation and/or signal attenuation are colored orange (distal) and red (proximal). For the UBA surface (e) unperturbed sites are ivory, and those perturbed are violet. Numbers in d and e map surface location of the corresponding perturbed residues. The orientation of Ub$_2$ is the same as in Fig. 2a. UBA atom coordinates are from PDB code 1DV0 (24). Marked with triangles in panels a and b are Ile$_{44}$ and Val$_{70}$-Arg$_{72}$ (distal Ub) and Ile$_{13}$, Thr$_{14}$, Lys$_{48}$, and His$_{68}$-Arg$_{72}$ (proximal Ub), exhibiting significant (>30%) signal attenuation due to extensive kinetic broadening of their resonances upon UBA binding. The sites perturbed in Ub$_2$ upon UBA binding comprise Thr$_2$, Leu$_2$, Lys$_{11}$, Ile$_{13}$, Thr$_{14}$, Arg$_{22}$, Ile$_{44}$, Lys$_{48}$, Gln$_{49}$, Glu$_{64}$, and His$_{68}$-Leu$_{73}$ in the distal domain and Gln$_2$, Leu$_2$, Lys$_{11}$-Leu$_{13}$, Arg$_{22}$, Lys$_{48}$, Gln$_{49}$, Glu$_{64}$, and His$_{68}$-Leu$_{73}$ in the proximal Ub.
and signal attenuation as indicators of the perturbations, the sites involved directly or indirectly in UBA binding are very similar for both Ub domains. This suggests that the mode of UBA binding is similar for both Ub domains. Moreover, these perturbation maps are similar to that observed for monoUb binding to this UBA (data not shown, see also Ref. 36), suggesting that each of the two Ub domains binds the UBA domain in the same mode as does monoUb. This result is consistent with expectation based on the extended conformation of Lys63-linked Ub2 indicated by the ribbon (Fig. 5).

The positions of amide cross-peaks shifted in the course of titration along a straight line on the $^1$H,$^15$N correlation map, consistent with a two-state fast exchange between the free and the bound states of a protein. The direction of a peak shift in the $^1$H,$^15$N coordinates reflects changes in the local electronic environment of both $^1$H and $^15$N nuclei, independently of the relative populations of the free and bound states and, therefore, is a sensitive indicator of local perturbations in protein structure as a result of binding. The directions of the peak shifts observed in the hydrophobic patch residues were practically identical between the distal and the proximal Ub as well as between Ub2 and monoUb. This finding further supports the idea that the same interactions are involved in UBA2 binding to each of the Ub domains in Lys63-linked Ub2 and to monoUb.

The UBA sites involved in Ub2 binding include residues Leu9, Ala11-Phe14 in the Gly-Phe-Pro loop (connecting helices 1 and 2) and Glu30-Ala34 and Leu38 in the C-terminal α-helix (helix 3), forming a binding epitope shown in Fig. 4g. In addition we observed a significant chemical shift perturbation in Phe24 and weaker but noticeable shifts in peak positions for Gln2, Thr21, and Val20 in both Ub units and most of perturbed residues in UBA2 (except Phe24 and Gln21). Also shown are side chains of residues Gln3, Thr3, and Glu3 (colored gold) in the proximal Ub (see “Results and Discussion”) to indicate their positioning facing the distal domain. Pink coloring of the ribbon indicates those sites that are specifically attenuated only in the proximal Ub domain (see “Results and Discussion”) when UBA is spin-labeled at the Cys89 side chain (shown using a stick representation, pink). The extended conformation of the Lys63-linked chain allows UBA domain binding to each of the Ub units.

To quantify UBA binding to Lys63-Ub2 we extracted $K_d$ values from the observed dependence of the chemical shift perturbations in Ub2 on UBA concentration. Assuming a 1:1 (UBA:Ub2) stoichiometry model (“Materials and Methods”) yields the microscopic dissociation constant of $1.40 \pm 0.28$ mM for the distal and $1.15 \pm 0.66$ mM for the proximal domain. These numbers are higher than the $K_d$ values observed for UBA binding to monoUb, $0.4 \pm 0.1$ mM in our study (not shown) and $0.36 \pm 0.1$ mM reported in Ryu et al. (36). However, if we assume that two UBA molecules can bind Lys63-Ub2 independently (1 per Ub unit), then the corresponding values are $0.28 \pm 0.10$ and $0.18 \pm 0.08$ mM, in good agreement with those for monoUb. These findings suggest that the stoichiometry of the UBA-Ub2 binding is 2:1, which is further supported by several lines of evidence. First, the 1:1 model predicts that Lys63-Ub2 (macroscopic dissociation constant $K_d/2 = 0.70–0.56$ mM) will bind more weakly than monoUb ($K_d = 0.36$ mM (35)). However, the GST pull-down data (Fig. 3) indicate a detectable preference for Lys63-Ub2 chains over monoUb. In contrast, the 2:1 model agrees with these data, as the predicted macroscopic dissociation constant ($\approx 0.14–0.09$ mM) is smaller than the $K_d$ observed for UBA- monoUb binding. Second, $^1$H T$_2$ values measured at high UBA:Ub2 molar ratios (6:1 and 10:1) were in the range of 14–16 ms, in good agreement with 15 ms expected for the 2:1 complex and noticeably shorter than 19 ms expected for a 1:1 complex, based on the inverse molecular weight dependence of T$_2$ ($T_2 \propto M^{-1}$) observed for Ub1, Ub2, and Ub4 at the same conditions (19). Finally, from the residuals of fit, the 2:1 model fits the experimental data better than the 1:1 model for the majority (e.g. 88% in Ub2-D) of analyzed residues. We also determined $K_d$ values by titrating the UBA domain with Ub2. The value of $0.21 \pm 0.1$ mM (2:1 stoichiometry) agrees well with those reported above for the Ub2 part of the complex. We conclude that two UBA domains can bind simultaneously to Lys63-Ub2, one per Ub unit. The similar $K_d$ values for each Ub domain in Ub2 and the shapes of the titration curves (Supplemental Fig. 3) indicate that the binding events are independent and non-cooperative.

Taken together our results show that 1) the UBA domain binds to either of the two Ub domains in the same mode as to monoUb and 2) the binding epitope involves the hydrophobic...
residues on the UBA domain and the hydrophobic patch (Leu6- Ile64-Val70) on the Ub surface. Our data also suggest that one Lys48-linked Ub2 chain could interact with two UBA domains. To understand whether both binding sites on Ub2 can be occupied simultaneously, we modeled the structure of the Ub2-UBA complex. Our studies of UBA-Ub1 binding suggest that the orientation of the UBA domain on monoUb surface is similar to that recently observed for the CUE domain of the yeast Cue2 protein (38). Therefore, we used the CUE/monoUb structure to model the UBA/Ub interactions in Lys63-linked Ub2. As shown in Fig. 5, the extended conformation of the Lys48-linked Ub2 allows two Ub domains to bind Ub2 simultaneously, one per Ub unit. Interestingly, the structure shown in Fig. 5 is consistent with the shift perturbations observed in Glu2 and Glu64 (Fig. 4) and significant (>50%) line broadening in Ile13 and Thr14 that seem specific for the proximal domain in the presence of UBA. Given the location of these residues close to the ligand site and facing the UBA binding surface on Ub2-D, it is likely that these perturbations reflect UBA binding to the distal Ub. What we observe here is probably a dynamic effect of the proximal domain “bumping” into the UBA domain bound to the distal Ub as a result of the inter-domain flexibility in Ub2.

To validate the proposed structural model of the Ub2-UBA complex we attached a spin label (MTSL) to Cys63 of Ub2. A significant (>60%) signal attenuation was observed in residues 8–11, 44–49, and 68–75 in both Ub domains in Ub2. This is consistent with the proposed mode of UBA binding to individual Ub domains, as the same residues are attenuated when spin-labeled UBA binds to monoUb. In addition, unique signal attenuations were observed in Glu2-Thr14, Thr14-Leu15, and Asn8-Leu67 of the proximal Ub. As shown in Fig. 5, these groups are located on the surface of Ub2-P facing the distal domain and, therefore, are close in space to the UBA domain that is bound to Ub2-D. These results are in excellent agreement with the proposed structure of the Ub2-UBA complex. Interestingly, these data favor the Ub2 conformation shown in Fig. 2a over the one in Fig. 2b.

The interactions of Ub2 with UBA2 domain observed in this study emphasize the problem of multiple binding sites associated with ligand binding to polyUb chains. The extended conformation of the Lys48-linked Ub2 allows for independent interaction of each of the Ub units with UBA in a mode similar to that of the UBA-monoUb interaction. A comparison of this interaction to the mode of interaction of Ub2-D, it is likely that these perturbations reflect UBA binding. Elucidation of the mode of interaction of each of the Ub units with UBA in a mode similar to that of the UBA-monoUb interaction. A comparison of this interaction to the mode of interaction of Ub2-D, it is likely that these perturbations reflect UBA binding.

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