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, 16477–16471; 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)^1-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), IκBα 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...
adopt a different conformation that could result in a different 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 Ub2 adopts a closed conformation in which these functionally important hydrophobic residues (Leu6, Ile4, Val3) are sequestered at the interdomain interface (19). This indicates that for this Ub pair forming this 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 Ub4 (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 Ub2 differs strongly from the structure of Lys48-linked Ub2.

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 in previous studies (21, 22). The aligned liquid crystalline phase. The medium was composed of 1:1 Mls2 (21) in overnight reaction properties. Residual dipolar couplings (RDCs) were measured in a weakly aligned liquid crystalline phase. The medium was composed of 1:1 5:1 increments were used. The RDCs were obtained from the difference in the 1H,15N couplings observed in the oriented (25 °C) and isotropic phases (23). The alignment tensor was calculated using the in-house program ALTENS (19); the atom coordinates were from PDB code 1D3Z (19). 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 Flossmann and Cottur (24). These extra residues do not detectably affect the Ub2-binding properties of UBA2, and the notations used throughout the text are as follows. Iso- ene ubiquitin (Ub) are sequestered at the interdomain interface (19). This indicates that for this Ub pair forming this 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 Ub4 (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 Ub2 differs strongly from the structure of Lys48-linked Ub2.

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Structure of Lys$^{63}$-linked Di-ubiquitin

where Perturbations observed in UBA upon Ub$_2$ titration do not discriminate to search for the interdomain interface in Lys63-linked Ub$_2$.

— We used the chemical shift mapping approach Shift Mapping between the two Ub domains; therefore, the 1:1 model gives the same measurements on the distal or proximal domain in Ub$_2$, the 1:1 model gives

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

(Eq. 1)

Here $[P]_1$ and $[L]_1$ are the total molar concentrations of the protein (Ub$_2$) and ligand (UBA). For a 2:1 model, considerations based on partitioning between the free and various ligand-bound states of Ub$_2$ give

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

(Eq. 2)

Perturbations observed in UBA upon Ub$_2$ titration do not discriminate between the two Ub domains; therefore, the 1:1 model gives the same equation as for UBA-monoUb binding,

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

(Eq. 3)

where $P$ and $L$ in this case represent UBA and Ub$_2$, respectively. The 2:1 binding model gives

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

(Eq. 4)

RESULTS AND DISCUSSION

Segmentally isotope-labeled Lys$^{63}$-linked Ub$_2$ 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 Ub$_2$-D and in Lys$^{63}$ and adjacent residues in Ub$_2$-P (Fig. 1).

Search for Interdomain Interactions in Ub$_2$ Using Chemical Shift Mapping—We used the chemical shift mapping approach to search for the interdomain interface in Lys$^{63}$-linked Ub$_2$.

Any difference in chemical shifts observed between monomeric Ub and Ub$_2$ 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 Ub$_2$. However, under our experimental conditions no significant backbone amide chemical shift perturbations were observed in the Ub$_2$ (Fig. 1) except for the residues involved in the linkage. This suggests that 1) both Ub units remain unperturbed in Ub$_2$ and 2) no specific interaction exists between the two Ub moieties in Lys$^{63}$-linked Ub$_2$. The latter conclusion contrasts with our results for the Lys$^{48}$-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 Lys$^{63}$-linked Ub$_2$—We next determined the conformation of the Lys$^{63}$-linked Ub$_2$. The relative orientation of the two domains in Lys$^{63}$-linked Ub$_2$ 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 Ub$_2$ 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 Ub$_2$ 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 Ub$_2$ 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 T$_2$ values are inversely proportional to the molecular weight of the
constructed, 26 ms for Ub2 and 50 ms for monoUb. A similar behavior was observed for the average levels of 15N T2 values, 88 ± 6 ms (Ub2-D) and 83 ± 7 ms (Ub2-P) versus 166 ± 10 ms for monoUb. The overall rotational correlation times derived for each Ub unit in Ub2 (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 molecular weight dependence of the overall rotation of a molecule, expected from the Stokes-Einstein-Debye equation. All molecular weight dependence of the overall rotation of a molecule, expected from the Stokes-Einstein-Debye equation.

The alignment tensor S was defined as $S = \Sigma_{i,j} S_{ij} \delta_i \delta_j$, where D is the measured RDC and $\delta_i$ and $\delta_j$ are the direction cosines of the principal axes frame of the alignment tensor of Ub2 with respect to 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).

### Table I

<table>
<thead>
<tr>
<th>Ub unit</th>
<th>Sxx</th>
<th>Syy</th>
<th>Szz</th>
<th>a</th>
<th>b</th>
<th>c</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ub2-P</td>
<td>$-5.5$ (0.6)</td>
<td>$-7.7$ (0.5)</td>
<td>$12.3$ (0.7)</td>
<td>$233$ (2)</td>
<td>$28$ (2)</td>
<td>$233$ (3)</td>
<td>$0.09$</td>
</tr>
<tr>
<td>Ub2-D</td>
<td>$-4.1$ (0.5)</td>
<td>$-6.7$ (0.7)</td>
<td>$10.8$ (0.7)</td>
<td>$272$ (3)</td>
<td>$38$ (2)</td>
<td>$148$ (11)</td>
<td>$0.11$</td>
</tr>
</tbody>
</table>

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Note that the solution structures shown in Fig. 2 represent time-averaged conformations of Ub2. Our relaxation data suggest that the interdomain linker (the four C-terminal residues in Ub2-D) is flexible, although to a significantly lesser extent than the free C terminus of Ub2-P. The interdomain mobility in the Lys63-linked Ub2 would be consistent with the lack of a well defined interface, as inferred from our chemical shift data. It is interesting to note that the diffusion tensor reported by the distal domain appears more axially symmetric than for the proximal domain (hence, greater error in the c angle). This might be a consequence of the dynamics of the system associated with the nature of the linkage between the two Ub domains.

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 Ub2-D). The attachment of the spin label was confirmed by an increase in the mass of the Ub2 by 183 Da (185 Da expected) recorded by MALDI-TOF mass spectrometry and by signal attenuation observed in the residues close to Cys63 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 15N-labeled proximal Ub that come in close proximity to the modified Cys should show relaxation rate enhancements. 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 Lys63-linked Ub2 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 Lys63-linked chain does not adopt a conformation that would result in the close proximity of the proximal domain to Cys63 on the distal domain.

**Comparison with Lys63-linked Chains—Lys63-linked Ub2 was previously shown to predominantly adopt a closed conformation (Fig. 2d) under physiological conditions (19). In this conformation hydrophobic residues (Leu4, Ile44, and Val70) implicated in recognition of polyUb chains by the proteasomal machinery are sequestered at the interface between the Ub units. The average conformation of the Lys63-linked Ub2 presented in this study is in striking contrast with that of Lys63-linked Ub2. Under identical buffer/experimental conditions, the Lys63-linked Ub2 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 Lys63-linked polyUb chains in strong preference to Lys63- and Lys29-linked chains (22). However, this preference has only been demonstrated for Ub chains. We note that the conformation adopted by Lys63-linked Ub2 should be readily accessible to longer chains. To directly test if the distinct conformations of Lys63- versus Lys63-Ub2 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 Ub2 chains. Pull-down assays with GST-fused UBA2 (versus a control of GST alone) were performed in triplicate for Lys63- and Lys63-linked Ub4 and Ub2.
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-Ile14-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 clustered around Leu8, Ile14, and Val70, consistent with the expectation that the hydrophobic patch mediates the interaction with the UBA domain. The somewhat greater magnitudes of chemical shift changes in the distal Ub could suggest that this unit is more involved in the UBA binding than the proximal domain. On the other hand several amides in the proximal domain (marked in Fig. 4b) exhibit significant line broadening, indicative of the onset of intermediate exchange on the NMR time scale (37), which could be caused by slower off-rates, hence somewhat tighter binding. Considering both frequency shift
FIG. 3. Linkage specificity of UBA binding to Ub₂ and Ub₄ chains. The solid and open bars show the percent of the input radioiodinated chain that bound to GST-fused UBA₂ or GST (control), respectively. Each binding experiment was performed in triplicate. The measurements for Ub₂ chains were performed twice, yielding identical results.

FIG. 4. Chemical shift mapping of the interactions between Lys⁶³-linked Ub₂ 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 Ub₂ and in UBA (c). The sites perturbed upon binding were mapped on the surface of Ub₂ (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 Ub₂ (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 Ub₂ 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 Ile₄₄ and Val₇₀-Arg₇₂ (distal Ub) and Ile₁⁴, Thr₁⁵, Lys₄₈, and His₆₈-Arg₇₂ (proximal Ub), exhibiting significant (>30%) signal attenuation due to extensive kinetic broadening of their resonances upon UBA binding. The sites perturbed in Ub₂ upon UBA binding comprise Thr₂, Leu₂, Lys¹⁵, Ile¹⁷, Thr¹⁹, Arg²⁶, Lys₆₄, Gln⁶₈, and His⁸⁸-Leu¹³ in the distal domain and Gln⁶, Leu⁶, Lys¹⁵-Leu¹⁷, Arg²⁶, Lys₆₄, Gln⁶₈, Glu⁸₁, and His⁸⁸-Leu¹³ 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 UBA 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 seen by NMR (Fig. 2).

The positions of amide cross-peaks shifted in the course of titration along a straight line on the $\text{H}_{3},\text{N}$ 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 $\text{H}_{3},\text{N}$ coordinates reflects changes in the local electron environment of both $\text{H}$ and $\text{N}$ 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 Ub$_2$ 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 Ub$_2$ and to monoUb.

The UBA sites involved in Ub$_2$ binding include residues Leu$^9$, Ala$^{11}$-Phe$^{14}$ in the Gly-Phe-Pro loop (connecting helices 1 and 2) and Glu$^{30}$, Ala$^{34}$ and Leu$^{38}$ in the C-terminal $\alpha$-helix (helix 3), forming a binding epitope shown in Fig. 4g. In addition we observed a significant chemical shift perturbation in Phe$^{24}$ and weaker but noticeable shifts in peak positions for Gln$^{32}$ and Leu$^{38}$, all located in helix 2, which is on the other side of the UBA molecule. These perturbations, also present in the monoUb-UBA complex (36), are probably caused by an indirect effect of the binding. In comparison with the chemical shift perturbations we observed for the UBA2 domain when it binds mono-Ub (data not shown), no additional residues appear to be involved in the UBA2-Ub$_2$ interaction. This observation rules out the possibility that one UBA domain binds to two Ub domains at the same time, e.g. forming a Ub-UBA-Ub “sandwich.”

To quantify UBA binding to Lys$^{63}$-Ub$_2$ we extracted $K_d$ values from the observed dependence of the chemical shift perturbations in Ub$_2$ on UBA concentration. Assuming a 1:1 (UBA:Ub$_2$) stoichiometry model (“Materials and Methods”) yields the microscopic dissociation constant of $1.40 \pm 0.28$ mM for the distal and $1.13 \pm 0.66$ 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 Lys$^{63}$-Ub$_2$ 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-Ub$_2$ binding is 2:1, which is further supported by several lines of evidence. First, the 1:1 model predicts that Lys$^{63}$-Ub$_2$ (macroscopic dissociation constant $K_d$ = $0.70–0.56$ 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 Lys$^{63}$-Ub$_2$ chains over monoUb. In contrast, the 2:1 model agrees with these data, as the predicted macroscopic dissociation constant ($0.14–0.09$ mM) is smaller than the $K_d$ observed for UBA-monoUb binding. Second, $1^H$ T$_2$ values measured at high UBA:Ub$_2$ 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 Ub$_1$, Ub$_2$, and Ub$_4$ 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 Ub$_2$-D) of analyzed residues. We also determined $K_d$ values by titrating the UBA domain with Ub$_2$. The value of $0.21 \pm 0.1$ mM (2:1 stoichiometry) agrees well with those reported above for the Ub$_2$ part of the complex. We conclude that two UBA domains can bind simultaneously to Lys$^{63}$-Ub$_2$, one per Ub unit. The similar $K_d$ values for each Ub domain in Ub$_2$ 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–Ile6–Val57) on the Ub surface. Our data also suggest that one Lys8–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 Lys63–linked Ub2 allows two UBA 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 Glu44 (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 ligation 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 interdomain flexibility in Ub2.

To validate the proposed structural model of the Ub2–UBA complex we attached a spin label (MTSL) to Cys36 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 and Glu44 (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 ligation 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 interdomain flexibility in Ub2.

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 Lys63–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, thus illustrating a simple case of independent multiple binding sites with no cooperativity. In contrast, UBA2 binding to Lys63–linked Ub2 has to compete with the Ub–UBA interaction (Fig. 2d), which involves the same hydrophobic patch of Ub that participates in Ub-UBA binding.

In conclusion, we have presented the first structural evidence that Lys8–linked Ub2 adopts a conformation in solution that is distinct from that of Lys48–linked Ub2. These results support the model that ubiquitination polyUb chain signaling depends on specific conformations adopted by alternatively linked chains. In contrast to Lys48–linked Ub2, the average conformation of Lys8–linked Ub2 is an extended chain, with no clear interface between Ub domains. This is most likely a result of the steric hindrance, due to a relatively short Ub–Ub linker, that prevents a direct contact between the hydrophobic patches on the surface of the two Ub units. These findings further suggest that the ability of adjacent Ub units to form a hydrophobic interface (or position the hydrophobic patches in a specific pattern) in Lys8–linked polyUb chains is critical for their function as the proteasomal recognition signal. It is possible that the hydrophobic interfaces involving remote Ub units might be formed in longer Lys63–linked chains, however, resulting in a pattern of Ub–Ub interactions distinct from that in the Lys48–linked chains. The extended conformation of the Lys8–linked chain allows UBA domains to interact with the Ub units in a mode similar to that of the UBA-monoUb interaction. A comparison of this interaction to the mode of interaction of UBA domains with Lys63–linked polyUb will shed light on the differential recognition of alternatively linked chains.

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REFERENCES
Solution Conformation of Lys63-linked Di-ubiquitin Chain Provides Clues to Functional Diversity of Polyubiquitin Signaling

Ranjani Varadan, Michael Assfalg, Aydin Haririnia, Shahri Raasi, Cecile Pickart and David Fushman

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