Ubiquitin Recognition by the Ubiquitin-associated Domain of p62 Involves a Novel Conformational Switch

Jed Long†, Thomas R. A. Gallagher‡, James R. Cavey§, Paul W. Sheppard¶, Stuart H. Ralston¶, Robert Layfield∥1, and Mark S. Searle†‡2

From the †School of Chemistry, Centre for Biomolecular Sciences, University of Nottingham, Nottingham NG7 2RD, ‡School of Biomedical Sciences, Queen’s Medical Centre, University of Nottingham, Nottingham NG7 2UH, ¶BIOMOL International LP, Palatine House, Matford Court, Exeter EX2 8NL, and §Rheumatic Diseases Unit, University of Edinburgh, Western General Hospital, Edinburgh EH2 2XU, United Kingdom

The p62 protein functions as a scaffold in signaling pathways that lead to activation of NF-κB and is an important regulator of osteoclastogenesis. Mutations affecting the receptor activator of NF-κB signaling axis can result in human skeletal disorders, including those identified in the C-terminal ubiquitin-associated (UBA) domain of p62 in patients with Paget disease of bone. These observations suggest that the disease may involve a common mechanism related to alterations in the ubiquitin-binding properties of p62. The structural basis for ubiquitin recognition by the UBA domain of p62 has been investigated using NMR and reveals a novel binding mechanism involving a slow exchange structural reorganization of the UBA domain to a “bound” non-canonical UBA conformation that is not significantly populated in the absence of ubiquitin. The repacking of the three-helix bundle generates a binding surface localized around the conserved Xaa-Gly-Phe-Xaa loop that appears to optimize both hydrophobic and electrostatic surface complementarity with ubiquitin. NMR titration analysis shows that the p62-UBA binds to Lys48-linked di-ubiquitin with ~4-fold lower affinity than to mono-ubiquitin, suggesting preferential binding of the p62-UBA to single ubiquitin units, consistent with the apparent in vivo preference of the p62 protein for Lys63-linked polyubiquitin chains (which adopt a more open and extended structure). The conformational switch observed on binding may represent a novel mechanism that underlies specificity in regulating signal-induced protein recognition events.

The p62 protein (encoded by the sequestosome 1 (SQSTM1) gene) functions as a scaffold in signaling pathways downstream of the interleukin-1, tumor necrosis factor-α, nerve growth factor, and receptor activator of NF-κB receptors, which ultimately lead to activation of the NF-κB transcription factor, with receptor activator of NF-κB signaling being a critical determinant in the regulation of osteoclast formation (1–3). Mice that are deficient in p62 show no obvious skeletal phenotype under normal conditions but exhibit defective osteoclastogenesis when challenged with bone-resorbing factors (4). Moreover, mutations affecting p62 are a common cause of Paget disease of bone (PDB), a condition associated with increased osteoclast and osteoblast activity (5–8). PDB is characterized by excessive bone turnover leading to bone expansion, structural weakness, deformity, and pain (9, 10).

The p62 protein has a domain structure consistent with its participation in multiple signaling complexes, although p62 also appears to be multifunctional, not least in controlling protein recruitment to endosomes (3) and proteasomal proteolysis (11). Within the p62 sequence, an N-terminal PB1 domain has been identified that binds atypical protein kinase C. In addition, a ZZ motif is evident, a binding site for the RING finger protein TRAF6, and two PEST sequences that lie adjacent to the C-terminal ubiquitin-associated (UBA) domain (Fig. 1), a motif that occurs in enzymes of the ubiquitin (Ub) conjugation pathway and in regulatory proteins involved in Ub-dependent proteolysis (12–14). Accordingly, p62 has been shown to bind noncovalently via its UBA domain to mUb and Lys48- or Lys63-linked polyUb chains (11, 15, 16). All of the PDB mutations identified to date cluster within or close to the UBA domain of the p62 protein, suggesting that the disease may involve a common mechanism related to alterations in the Ub-binding properties of p62 (17–19).

To rationalize the structural basis for mUb/polyUb chain recognition by p62, we report NMR structural and binding studies of a recombinant polypeptide corresponding to the C-terminal UBA domain of the human protein (Fig. 1). The isolated 50-residue polypeptide sequence forms a compact three-helix bundle with a structure analogous to the UBA domains of hHR23A and the related CUE domain of the yeast

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1 The abbreviations used are: NF-κB, nuclear factor-κB; UBA, ubiquitin-associated domain; mUb, mono-ubiquitin; diUb, di-ubiquitin; PDB, Paget disease of bone; CSP, chemical shift perturbation; HSQC spectrum, heteronuclear single quantum coherence spectrum; RDC, residual dipolar coupling; r.m.s., root mean square; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; h, human.
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Cue2 protein, but with differences in the loop regions connecting the helices (20). Other UBA and CUE domains have been shown to bind noncovalently with low affinity to mUb through a conserved hydrophobic patch (21–28), but in some cases with much higher affinity to Lys48-linked diUb and longer chains (29, 30). We describe NMR complexation studies with the 15N-labeled p62-UBA domain and have mapped the interaction surface of the p62-UBA domain with both mUb and Lys48-linked diUb. The UBA domain in complex with mUb undergoes global chemical shift perturbations on binding, which we show result from the p62-UBA polypeptide undergoing a novel structural reorganization and repacking of the three-helix bundle. The structural rearrangement results in a large activation barrier to complexation that leads to atypical slow exchange kinetics between free and bound forms on the NMR chemical shift time scale, followed by a secondary fast Ub binding step that enables the identification of the binding surface on the rearranged UBA domain. Upon extending our NMR investigations to study the interaction of the UBA domain with Lys48-linked diUb, we find no evidence for linkage-dependent binding specificity, suggesting preferential binding to single Ub units, which may rationalize the apparent in vivo preference of p62 for the more open and extended Lys48-linked polyUb chains (11).

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—The cDNA encoding the UBA domain of human p62 was amplified from the IMAGE clone 29006264 by PCR and cloned between the BamHI and XhoI sites of plasmid pGEX-4T-1 (Amersham Biosciences). All mutations were introduced by site-directed mutagenesis (QuikChange mutagenesis kit, Stratagene) and the constructs were confirmed by 12% SDS-PAGE, and protein concentration estimated by absorbance at 280 nm. Ub mutants was confirmed by 15N-HSQC analysis, were purified from soluble cell lysates by precipitation with 1% trichloroacetic acid, 2 mM EDTA, pH 4. A gradient was run to 2 M NaCl with 50 mM potassium phosphate, 100 mM NaCl, pH 7. The fractions containing the pure protein of interest, as determined by SDS-PAGE analysis, were concentrated by lyophilization and buffer exchanged into water using a desalt column (Amersham Biosciences) before lyophilization. Uniformly 15N-labeled UBA and mUb were obtained by growing the bacteria in M9 minimal medium containing 1 g/liter of 15N-labeled ammonium chloride (Isotec) and purified as described above. Doubly 13C/15N-labeled UBA domain was prepared similarly with [13C]glucose as the sole source of carbon in the same minimal medium containing 2 g/liter of [13C]glucose (Isotec).

Preparation of Di-ubiquitin, [K48/(K48C)-ubiquitinyl], D77Ubiquitin—Ub mutants [K48C] and [D77] (plasmids kindly provided by the late Prof. Cecile Pickart, The Johns Hopkins University, Baltimore) were expressed in BL21 (DE3) E. coli (Novagen) by growing transformed cells at 37 °C until A600 = 0.6, followed by induction with 400 mM isopropyl 1-thio-β-d-galactopyranoside and incubation for a further 4 h. Proteins were purified from soluble cell lysates by precipitation with 1% (v/v) of 70% perchloric acid, SP-Sepharose cation exchange chromatography (Amersham Biosciences), and concentration using centrifugal filtration (Millipore UF-4 devices). Presence of purified Ub mutants was confirmed by 12% SDS-PAGE, and protein concentration was estimated by absorbance at 280 nm.

Lys48-linked diUb was synthesized by linking [K48C]Ub and [D77]Ub (1 mM) in the presence of 20 mM Ub-conjugating enzyme E2-25K (UbcH1) and 0.1 μM human Ub-activating enzyme in 50 mM Tris-Cl, pH 8.0 buffer, containing 5 mM MgCl2, 10 mM creatine phosphate, 0.6 units/ml inorganic pyrophosphatase, 0.6 units/ml creatine kinase, 2 mM ATP, and 0.5 mM dithiothreitol (31). Reactions were performed at 37 °C for 4 h, and the diUb was purified by Q-Sepharose (removal of Ub-activating/Ub-conjugating enzymes) and SP-Sepharose (separation of differing length Ub chains) ion exchange chromatography. Presence of purified diUb was confirmed by 10% SDS-PAGE and protein concentration estimated by absorbance at 280 nm.

NMR Experiments—The NMR assignment of the unbound UBA domain of p62 was achieved using a 1 mM 13C/15N-labeled protein sample in NMR buffer (50 mM potassium phosphate, 50 mM NaCl, 10% D2O, 0.04% sodium azide, pH 7.0) at 298 K and subsequently in the complexed form in the presence of 6 mM Ub. For the free and bound states of the UBA domain 15N-HSQC, 13C-HSQC, HNCO, HN(CO)CA, CBCANH, CBCA-(CO)NH, HCCH-TOSY, 13C-HSQC-NOESY (mixing time 100 ms), 15N-HSQC-TOCSY, and 15N-HSQC-NOESY (mixing time 150 ms) two- and three-dimensional spectra were collected and the spectra processed using XWINNMR version 2.5 (Bruker) and NMRPipe (32). The assignment was carried out in CCPNMR using standard methodologies (33). Backbone 1D NH residual dipolar couplings were obtained from the difference in 1J scalar couplings measured from 1H-15N IPAP-HSQC spectra.
(34). The solutions were soaked into predried 6-mm 5% polyacrylamide gels (35, 36), which were then compressed into a 5-mm NMR tube (Worldwide Glass Resource Ltd., Cambridge, UK). Two-dimensional spectra were collected with 64 scans and a resolution of 2048 by 1024 data points on a 1 mM 15N-UBA sample with or without 6 eq of ubiquitin. The spectra for the free UBA domain gave RDC values in the range −8.3 to 12.2 Hz. Spectra for the complex, collected using the same alignment conditions, showed a narrower range of values (−7.7 to 4.6 Hz), which appears to correlate with small changes in the apparent size or intrinsic flexibility of the UBA domain in the bound state that affects the degree of alignment (37). These observations are consistent with analytical gel filtration results (not shown), which also suggest changes in the average molecular dimensions of the UBA domain between the free and bound conformations.

Structure Determination from NMR Restraints—The structure determination was based upon NOE distance restraints and dihedral restraints, the former derived from the 13C-HSQC-NOESY and 15N-HSQC-NOESY spectra. The NOEs were classified based upon their intensity into very strong (1.8–2.5 Å), strong (1.8–2.8 Å), medium (1.8–4 Å), weak (1.8–5 Å), and very weak (1.8–6 Å). In addition, 45 backbone 1D NH RDC parameters from the 47 HSQC cross-peaks in spectra of both the free and bound UBA domain were used as restraints using the ISAC method (38) and visualized using MODULE (39). TALOS (40) dihedral restraints with ±30° limits were also used in the calculations. An initial 100 structures were produced using the standard three-step XPLOR-NIH 2.14 protocol (40). In the first stage, high temperature Cartesian dynamics was performed at 1000 K with a time step of 0.005 ps, for 20,000 steps, using the Verlet integrator. During the second cooling phase of the protocol, the temperature was reduced from 1000 to 100 K in steps of 50 K, with a time step of 5 fs, over 40,000 steps during which the relative weighting of nonbonded energy terms was increased from 10% of their default values to their force field default (41, 42). These initial structures were then refined with another 10,000 cooling steps. The structures were selected based upon NOE violations <0.5 Å and dihedral violations <2°. As a further cross-validation step, we attempted to refine the structure of the bound UBA domain with RDC restraints derived from the free UBA domain. This led to significant distortions in the relative orientations of the helices and structures with much higher energies. All structures were displayed using MOLMOL (43). The structural statistics are presented in Table 1. Hydrophobic surface area measurements were performed using the program Naccess (version 2.1.1) (68).

UBA Ub Binding Studies by NMR—A 1 mM sample of 15N p62-UBA in NMR buffer was titrated in 20 increments with ratios of mUb to UBA in the range 0.1–6.0 and 15N-HSQC spectra collected at each step. A second “reverse” titration was performed by titrating unlabeled p62-UBA into a 10 mM 15N-labeled sample of mUb in NMR buffer at the following ratios of UBA to mUb 0.25, 0.5, 0.75, 1, 1.25, 1.5, 2, 4, 6. All data were collected at 298 K, and changes in chemical shift and cross-peak intensity were monitored by acquisition of 15N-HSQC spectra. The 13N-HSQC spectra were acquired with a resolution of 2048 complex points and 112 t1 increments over a spectral width of 9000 Hz by 2400 Hz in the direct (1H) and indirect dimensions (15N) with 8 scans per t1 increment. The spectra were processed using a sine-squared apodization function shifted by π/2 in both dimensions and zero-filled to 2048 by 1024 real points (F2 by F1).

Ub Binding Assays—WT-UBA domain (residues 387–436) and UBA mutants were assayed for Lys48-linked and Lys63-linked polyUb binding as described previously (11, 20), using 1 μg of polyUb chains, in 50 mM Tris, 0.1% (w/v) bovine serum albumin, pH 7.5, with all reagents maintained at the 37 °C throughout the binding/washing stages. After washing, bound proteins were detected by Western blotting (anti-Ub). All experiments were repeated on at least three independent occasions and representative examples of blots are presented.

RESULTS

Structure of the UBA Domain of p62—The NMR structure of the C-terminal UBA domain of p62 (residues 387–436 in full-length p62) has been determined previously by NMR (20), and further refined using a 13C/15N doubly labeled construct and additional 1H-15N residual dipolar coupling (RDC) restraints derived from two-dimensional 1H-15N HSQC spectra (see structural statistics in Table 1). The RDC data led to an improvement in secondary structure definition and a more precise relative alignment of the three helices (44–47). The structure has an overall topology similar to other UBA and CUE domains. The structure is well defined between Pro392 and Ile431 (with an average backbone r.m.s. deviation to the mean structure of 0.75 Å). NMR relaxation measurements (R1, R2, and 1H-15N NOE), and calculated order parameters, suggest that the helical secondary structure forms a compact fold with rapid dynamics confined to the few disordered residues at the N and C termini and in the first loop (48). The three helical segments are defined by residues Pro392–Met404, Leu413–Lys420 and Ile424–Ile431. The domain is compact with a well defined hydrophobic core composed primarily of the buried side chains of Met401, Leu417, Ala427, and Ile431 (Fig. 1E), which is further consolidated by Leu438, Phe406, Trp412, Leu413, Ile424, and Leu428. The first loop contains the highly conserved Xaa-Gly-Phe-Xaa motif (Met-Gly-Phe-Ser in p62-UBA; residues 404–407), which together with Asp408 and Gly409 form an extended single β-strand. However, a major difference between the p62-UBA and other UBA domains is the expansion of this connecting loop through the insertion of two glycines (Gly410 and Gly411; see Fig. 1B). The lack of NOE contacts from the Hαs of these two glycines to any adjacent residues, together with the analysis of 15N relaxation rates (R1 and R2), heteronuclear NOE data, and fluctuations evident in the ensemble of structures sampled by molecular dynamics simulations, shows that Gly410 and Gly411 undergo enhanced backbone dynamics and conformational exchange (48). All of the UBA domains so far characterized in structural detail lack the di-glycine segment found in the p62-UBA domain, making loop 1 of other UBA domains considerably shorter and more highly restrained. The di-glycine insertion appears to be structurally important in the p62-UBA because a UBA mutant lacking Gly410 and Gly411 appears to be unfolded in solution (not shown). The presence of a proline residue in loop 1 of hHR23A UBA2 (Leu-Gly-Phe-Pro) (22)
and in the CUE domain (Met-Phe-Pro) (24) forces a sharper turn. Analysis of surface hydrophobicity identifies a hydrophobic patch in the vicinity of the Met-Gly-Phe loop of the p62-UBA domain, and this appears to be common to many of the UBA domains so far characterized (23–25).

**UBA Interaction with mUb from Chemical Shift Perturbation Mapping**—The $^1$H-$^1$N HSQC spectra of isotopically labeled mUb and the UBA domain were assigned using established procedures (49). In particular, we examined NMR parameters for the UBA domain over a wide range of concentrations (0.1–1.2 mM). The ratio of spin–spin ($R_s$) to spin–lattice ($R_l$) relaxation rates and chemical shifts showed no significant dependence on protein concentration at 298 K up to 1.2 mM. Consequently, all structural studies of the UBA domain were performed below this concentration. Initially, unlabeled UBA domain was titrated into a sample of $^{15}$N-labeled mUb to characterize the chemical shift perturbations associated with binding. The free and bound forms of $^{15}$N-mUb were in fast exchange on the chemical shift time scale (Fig. 2A), characteristic of both fast on- and off-rates for complex formation and dissociation. The weighted average chemical shift perturbations up to saturating concentrations of UBA domain are shown in Fig. 2B with no further changes in shifts evident above a 6:1 excess ratio. The smooth change in chemical shifts was used to construct binding curves from 12 well resolved residues. Fitting these data to a 1:1 binding model yields a $K_d = 540(±45) \mu M$ with the error representing the standard deviation over the 12 data sets (Fig. 2C). This value is comparable with those reported for Mud1 (390 μM), for hHR23A (500–600 μM) and for the CUE domain (155 μM), demonstrating a relatively weak interaction in all cases (29). The pattern of chemical shift perturbations identifies a Ub binding epitope involving residues Leu$^8$, Ile$^{13}$, Arg$^{42}$, Ile$^{44}$, Ala$^{46}$, Leu$^{69}$, Val$^{70}$, Leu$^{71}$, and Arg$^{72}$ ($\Delta \delta > 0.1$ ppm) largely located on the surface of the $\beta$-sheet in a predominantly hydrophobic patch (Fig. 3A), with Arg$^{42}$ and Arg$^{72}$ flanking this patch. A number of adjacent residues such as Gln$^{69}$ and Gly$^{73}$ are also perturbed, whereas Lys$^{48}$ is significantly exchange broadened and undetected. This pattern of mUb shifts is very similar to that reported previously for the binding of the UBA domains of hHR23A (30) and Mud1 (29) and also highlighted in studies of the key determinants for endocytosis and proteasomal degradation (50, 51), suggesting that mUb uses a highly conserved binding epitope in protein recognition. Many much smaller shift perturbations (<0.1 ppm) are also apparent, which

| Table 1 | NMR structural statistics for the p62-UBA domain in its unbound and ubiquitin-bound state |
|-----------------|---------------------------------------------|
|                | Unbound p62-UBA | Bound p62-UBA |
| NOE distance restraints (Å)*  |                |                |
| Intraresidue     | 360            | 341            |
| Sequential       | 0.008 ± 0.001  | 0.012 ± 0.0004 |
| Short ($l (l - j)$)  | 259            | 204            |
| Long ($l (l - j) > 5$) | 0.058 ± 0.003  | 0.045 ± 0.006  |
| Residues in disallowed regions (%) | 0.011 ± 0.37   | 0.020 ± 0.006  |
| Torsion angle restraints      |                |                |
| Angles (°)        | 36             | 31             |
| r.m.s. deviations of secondary structure  |                |                |
| Backbone (Å)     | 0.75 ± 0.17    | 1.38 ± 0.38    |
| Heavy atoms (Å)  | 1.51 ± 0.25    | 2.11 ± 0.42    |
| Ramachandran statistics  |                |                |
| Residues in core regions (%) | 69.4         | 72.3           |
| Residues in generously allowed regions (%) | 30.6        | 27.7           |
| Residues in disallowed regions (%) | 0.0        | 0.0            |
| PDB codes        | 2jy7           | 2jy8           |

* Total number of restraints and the mean deviation from the experimental restraints ± S.D. are shown.
* r.m.s. deviations are from the average structure with standard deviation within the family of structures.
* Number of residual dipolar couplings are shown; mean deviation of experimental restraints were from calculated values ± S.D. (Hz). Asp$^{391}$ and Leu$^{416}$ were not resolved for the free UBA, and Gly$^{416}$ and Thr$^{50}$ were unresolved for the bound UBA due to spectral overlap or exchange-mediated line broadening.
* The values for $E_{	ext{NOE}}$ and $E_{	ext{DIHE}}$ are calculated from a square well potential with a force constant of 50 kcal mol$^{-1}$ Å$^{-2}$ and 200 kcal mol$^{-1}$ radian$^{-2}$. $E_{\text{REPEL}}$ is calculated with a force constant of 4 kcal mol$^{-1}$ Å$^{-1}$, and the final van der Waals radii were set to 0.80 times the value used in the CHARMM force field.
* The values for bonds, angles, and improper torsion angles show the deviation from ideal values based on ideal stereochemistry.
* Data were determined by the program PROCHECK.
* Protein Data Bank accession codes are for the RDC-refined structures.
suggest some small global structural readjustment occurs to optimize interactions with the UBA domain. Overall the magnitude of the shifts for mUb suggests that it is already largely preorganized for recognition and binding.

To map the binding surface of the UBA domain, we repeated the titration using 15N-labeled p62-UBA with the addition of up to 6 eq of unlabeled mUb. In contrast to the reverse titration where fast exchange was evident for 15N-mUb between free and bound forms, we observed HSQC cross-peaks for both the free and bound UBA domain indicative of slow exchange between the two species. The mid-point of the titration is shown in Fig. 4A. Separate cross-peaks could be identified for free and bound forms even where chemical shift differences are comparable with NMR line widths (see Asp421 and Asn423 in Fig. 4A), on the basis of which we estimated a slow rate of interconversion of <10 s⁻¹. The slow on- and off-rates for complexation contrast markedly with NMR data for the binding of other UBA domains, including Mud1 and the UBAs of hHR23A where typically fast-on/fast-off rates are evident in titration with mUb (21–23, 29). Furthermore, the magnitude of the chemical shift perturbations for 15N-p62-UBA appeared to be generally significantly larger than observed for mUb, such that assignment of the bound state was not possible without further detailed analysis. We assigned the spectra of the bound state of the UBA domain using a 13C/15N-labeled sample titrated to saturation with 6 eq of unlabeled mUb. The plot of weighted CSP for the UBA upon binding mUb reveals a wide distribution of perturbations with a significant number >0.5 ppm, including Ile431 which shows a free to bound shift of 3.14 ppm. The shift changes occur on such a global scale (Fig. 4B) that it was necessary to complete a full structural analysis of the bound state (Table 1; see “Experimental Procedures”).

Structure of the Bound UBA Domain of p62—The structures of the free and bound states of the UBA domain (Fig. 3, D and E) were generated using the same 13C/15N-labeled sample and assignment protocol and are of comparable quality (see Table 1). Loop 1 and the N and C termini show some flexibility in both the free and bound states. The analysis of the bound state reveals that the secondary structure content of the UBA domain is largely conserved; however, a novel realignment and packing of the three helices of the UBA domain is apparent that results in a noncanonical UBA conformation (Fig. 3, F–J). Given that the relative orientations of the helices are different in the two conformations, we used 1D_{NH} RDC measurements to provide further long range information to validate the change in structure (44–47). We were able to resolve the majority of 1D_{NH} couplings (45 of 47 residues in the highly structured part of the UBA domain in both the free and bound states) and observed significant differences in the magnitude and sign of RDC values from the two structures (Fig. 4C). Changes in a number of vectors in helix 1 suggested a structural tightening in the bound state. Intrinsic flexibility is consistent with the observation of rapid NH-ND exchange rates for amide groups in helix 1 of the unbound UBA (not shown). Residues in helix 2 showed a similar alignment of N-H vectors in the free and bound states. However, a quite different overall alignment was evident for residues in helix 3, which also correlated with some of the largest observed changes in chemical shifts between the free and bound states (Fig. 4B). Refinement of the structure of the bound UBA domain against the RDC data produced good agreement between experimental and calculated values (see Table 1 and supplemental Fig. 1). In other reported structural studies, RDC measurements have been used in an analogous fashion to demonstrate both similarities and differences in relative orientations of helices in solution and in x-ray structures (52), for identifying periodicities and conformational distor-
common contacts in both structures with residues close in sequence within loop 1 and helix 2. However, there are again some clear differences. The indole ring in the structure of the unbound UBA domain forms unique contacts with residues in loop 2 and helix 3 (Leu417, Asp423, Ala427, and Asp430), and in the bound state unique contacts with helix 1 (Leu398, Gln400, and Met401). The differences in the pattern of NOE interactions are summarized in the contact map shown in supplemental Fig. 2.

Furthermore, the 3.14 ppm shift in the proton resonance of the NH of Ile431 is also accounted for by the structural reorganization. The Ile431 NH is in close proximity to the indole ring of Trp412 and experiences a substantial ring current shift in the free UBA. However, this close contact is completely lost in the bound conformation, as are contacts between the side chain of the adjacent residue Gln432, which occur with Phe406. In both cases, large differences in H chemical shifts in the free and bound states are apparent. A schematic representation of the two structures is shown in Fig. 3H; the helices are represented as cylinders and the two structures are shown with a common alignment of helices 1 and 2. The overlaid structures shown in Fig. 3H show that the differences can be interpreted as the repacking of helix 3 against the other two helices.

Complex Kinetics between the Free and Bound States of the UBA Domain—The observation of slow exchange kinetics between free and bound states evident in titration experiments with the 15N-labeled UBA is consistent with a gross structural reorganization upon binding to mUb. The structural rearrangement of the UBA domain, which is atypical of UBA motifs in general, is associated with a large activation barrier to binding, as summarized in Reaction 1.

\[
\text{UBA} \overset{\text{slow exchange}}{\rightleftharpoons} \text{UBA}^* + \text{mUb} \overset{\text{fast exchange}}{\rightarrow} \text{UBA}^* \cdot \text{mUb} \tag{1}
\]

Here, UBA is the unbound conformation, and UBA* is the structure that we observe in the bound state with mUb. We were unable to detect a significant population of UBA* at equilibrium in HSQC spectra, suggesting that in the absence of mUb, [UBA]/[UBA*] > 10. To simplify the model, we have assumed that the UBA* in isolation has the structural characteristics found in the UBA*·mUb complex, with binding occurring through a process of conformational selection. However, we cannot rule out the possibility that UBA* in isolation may be partially unstructured, in which case the interaction with mUb may result in a process of induced fit to give the stable UBA*·mUb complex that we observed experimentally. The structural details of UBA* in isolation remain to be firmly established; however, the 1H-15N HSQC spectra of 13N-mUb titrate in a linear manner upon addition of the UBA domain, suggesting that a two-state fast exchange binding process occurs. This is consistent with UBA* possessing conformational features closely akin to those observed in the bound state.

Upon titration of 15N-UBA with mUb, we see a shift in the equilibrium from UBA to UBA*·mUb characterized globally by slow exchange kinetics (slow-on and slow-off rates) (Fig. 4A).
However, a subset of resonances from the UBA domain showed clear evidence for further ligand-dependent chemical shift perturbations with a change in the position of HSQC cross-peaks for the bound state as the population of UBA-mUb increases (54, 55). These secondary shifts are associated with fast-on/fast-off rates that reflect the population-weighted average between UBA* and the UBA-mUb complex. This is illustrated in Fig. 4D for the cross-peaks of Gly405. Cross-sections are shown through two-dimensional HSQC spectra at different UBA:Ub ratios. The 1H chemical shift for the free UBA occurs at 7.85 ppm. The peak for the bound state first appears at 7.64 ppm and gradually shifts to higher field as the population of the UBA-mUb complex increases, reaching the fully bound shift at 7.58 ppm. In contrast, titration of 15N-labeled mUb with unlabeled UBA*plex increases, reaching the fully bound shift at 7.58 ppm. The secondary fast exchange shift perturbations for 15N-labeled UBA* identify the specific residues of UBA* that are involved in complexation with mUb. The perturbations are most significant for residues Gln400, Leu402, Ser403, Met404, Gly405, Phe406, Ser407, Asp408, Ala426, Ala427, Asp429, Thr430, Gln432, Tyr433, Ser434, and His436 with the side chains of Trp412 and Gln400 also affected. A significant line broadening effect is evident for Gly410 within the di-glycine insertion, which suggests conformational exchange processes taking place around this loop. When the location of all of these residues is mapped on to the UBA domain, they form a contiguous binding surface involving residues at the C terminus of helix 1, within loop 1, and along helix 3 (Fig. 3C). This structural reorganization appears to optimize both surface hydrophobicity and van der Waals complementarity between the two protein surfaces. Hydrophobic surface area measurements suggest that overall there is no great difference between the free (60%) and bound (59%) states, but the surface localization does change. Around loop 1 the side chains of Phe406 and Trp412 both become significantly more surface-accessible in the bound conformation, generating a slightly larger hydrophobic patch. In addition, the charged residues Glu396, Asp408, and Glu409 are brought together adjacent to the hydrophobic patch to allow for the possibility of electrostatic interactions with basic residues on the surface of mUb. Indeed, Arg42 and Arg72, which are co-localized on the surface of mUb (Fig. 3A), undergo chemical shift perturbations upon binding (Fig. 2B) consistent with an electrostatic component to the binding interaction. To guide modeling studies of the complex, we attempted to identify intermolecular NOES using 13C/15N-filtered NOESY experiments using 15N-p62-UBA and unlabeled mUb. However, definitive NOE cross-peaks proved elusive, despite variations in the relative concentrations ([mUb]/[UBA] ~0.5, 1, 2, 6), NOESY mixing times (100 and 200 ms), and temperature (298 and 288 K) (results not shown) (56).

**Ubiquitin Recognition by the p62-UBA Domain**

**Polyubiquitin Chain Recognition by p62-UBA**—The interaction properties of minimal isolated UBA domains show that they divide empirically into four classes that reflect linkage-selective chain recognition by binding Lys48-linked chains, by binding Lys63-linked chains, by showing no linkage-dependent discrimination, or by demonstrating an inability to bind Ub chains at all (57). It is well established that Lys48-linked chains are used to signal proteasomal proteolysis, whereas the Lys63 linkage is used in a number of nonproteolytic signaling pathways, and these linkages represent two of those most commonly found in vivo. Structural studies of polyUb chains (Ub, Ub1, and Ub2) show that there are linkage-dependent interactions between Ub motifs that are likely to have some bearing on chain recognition by other binding partners (58, 59). Lys48-linked chains form a more compact conformation in which the hydrophobic surface defined by Leu8, Ile44, and Val70, and identified as forming the UBA binding surface, are involved in interfacial contacts between both proximal and distal Ub molecules. This intramolecular self-association process must be competitive with binding other low affinity substrates, as illustrated schematically in Fig. 5A. Estimates from Varadan et al. (30) suggest that the equilibrium between open and closed states has a $K_{eq} (> 100)$ in favor of the closed state at physiological pH. In contrast, Lys63-linked chains appear to be more extended with Ub motifs presented as beads on a flexible string.

The minimal p62-UBA domain has been suggested to be nonselective for polyUb chain linkage (57), although one study in assessing the role of full-length p62 protein in polyUb chain aggregate formation and cell survival indicated a clear preference for Lys63-linked chains (11). Other studies have also implicated both intra- and intermolecular protein-protein interactions in modulating these recognition properties in full-length hHR23A (60, 61) and possibly p62 (11). We examined the binding properties of the 15N-UBA domain in the presence of Lys48-linked diUb. The slow exchange between free and bound forms of the UBA domain evident in the titration with mUb was again clearly apparent. The similarity in the CSPs was such that the assignments were readily transferred between complexes. The weighted CSPs for mUb and Lys48-diUb binding to the 15N-UBA are shown in Fig. 5B. The CSP difference between the two data sets shows a random distribution of small effects that demonstrate that the two titrations yield practically identical HSQC spectra of the complexes. This is in contrast to recent studies with the UBA domain of Mud1 (29), where differences between the binding of mUb and Lys48-diUb were clearly apparent with 0.1–0.3 ppm perturbations identifying further interactions with a second hydrophobic patch involving mainly residues within helix 2 but also Phe380 in helix 3. The latter is conserved in the UBA of Mud1 and hHR23A and both have been shown to form sandwich complexes with Lys48-diUb (29, 30). These secondary interactions appear to rationalize the marked enhancement in affinity (>100-fold increase in the case of Mud1) for Lys48-diUb and significant line broadening effects associated with slower on- and off-rates for complex formation and dissociation.

More significantly, our NMR titration studies with p62-UBA and Lys48-linked diUb reveal that the binding affinity for the
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latter is lower than for mUb. We were unable to reach a fully populated bound state of the UBA with diUb even though binding saturation was readily achieved under similar experimental conditions in the presence of mUb. With the free and bound forms in slow exchange, an estimate of the relative intensities of cross-peaks in the \(^{1}H\)–\(^{15}N\)-HSQC spectrum during the titration enabled us to calculate an apparent \(K_{d}\) value for the UBA-diUb interaction that is \(\sim4\) times higher than for UBA-mUb. The HSQC cross-peak intensities for the free and bound states for Leu\(^{23}\) during the titrations of \(^{15}N\)-UBA with mUb and with Lys\(^{48}\)-linked diUb are shown in Fig. 5C. In each case the ratio of UBA: mUb and UBA: diUb in the titration is 1:4. Under these conditions, the UBA domain is almost fully bound with mUb and UBA:diUb in the presence of 6 eq of mUb (298 K and pH 7.0). The mid-point of the titration is 7.58 ppm at a 1:6 ratio of UBA to mUb.

FIGURE 4. NMR analysis of the interaction of mUb with \(^{15}N\)-p62 UBA. A, portion of the \(^{15}N\)-HSQC spectrum of \(^{15}N\)-UBA domain in the presence of unlabeled mUb (298 K and pH 7.0). The mid-point of the titration is shown in which the free and bound states of the UBA are similarly populated and are in slow exchange, with saturation of the bound state achieved at a 6:1 ratio of mUb:UBA. Specific assignments are derived from a full data analysis (see “Experimental Procedures”). B, binding shifts (\(\Delta\delta\), ppm) for \(^{15}N\)-UBA at 298 K, pH 7.0, in the presence of 6 eq of unlabeled mUb calculated as the weighted average r.m.s. value of the \(^{15}N\) and \(^{1}H\) perturbations as follows: \(\Delta\delta = ((\Delta\delta)^{2} + (\Delta\delta^{r})^{2})^{1/2}\). C, \(\Delta\delta_{\text{NH}}\) RDC data for the free and bound forms of the \(^{15}N\)-labeled UBA domain collected at 298 K in a 5% polyacrylamide gel. D, cross-sections through \(^{1}H\)–\(^{15}N\) HSQC spectra during the titration of \(^{15}N\)-UBA with unlabeled mUb. The spectra show the resonances for Gly\(^{405}\) in the free and bound states. The shift of the bound signal becomes visible at 7.64 ppm (representing slow exchange between free and bound states) and increases in intensity while simultaneously undergoing a second fast exchange process between the UBA\(^{*}\) conformation of low population before reaching the shift for the fully bound UBA\(^{*}\)-mUb complex at 7.58 ppm at a 1:6 ratio of UBA to mUb.

FIGURE 3. Structural analysis of the p62 UBA domain and the binding surfaces of the UBA and mUb. Chemical shift perturbations are mapped to the surface of Ub on a linear scale of white to red (largest) (A), as described by the data in Fig. 2B. B, surface representation of the structure of the p62-UBA domain in the Ub-bound state showing the surface charge distribution (acidic, red; basic, blue) and hydrophobicity (white). The structure is viewed toward the Met-Gly-Phe-Ser loop region. C, identical orientation and representation of the UBA domain of p62 showing secondary fast exchange chemical shift perturbations (red) indicative of binding interactions with mUb. These binding perturbations correlate well with the hydrophobic surface shown in B, comprising residues within loop 1 and the C terminus of helix 3. Ribbon diagram shows NMR structures of the unbound UBA domain (D) and the bound form in the presence of 6 eq of mUb (E). The structural statistics are shown in Table 1. In the unbound state the environment of the side chain of Gin\(^{406}\) is defined by NOEs to mUb and polyUb chains (data not shown), suggesting the possibility that certain residues may have a role in negatively regulating binding specificity and affinity, as also recently proposed for a similar T134A mutation in the Edel UBA domain (62). The results from our helix 2 mutants contrast with those of Seibenhener et al. (11) who demonstrated in a similar pulldown assay that the conservative L417V mutation, which is sandwiched between our own R415A and Q418A helix 2 substitutions, largely eliminated binding, but L413V had little effect. Interestingly, in our structure of the bound state, Leu\(^{417}\) is sub-

still in excess. This reduction in UBA affinity is consistent with the competitive effects on binding of a significant population of the “closed” structure of Lys\(^{48}\)-linked diUb under the conditions of the titration (Fig. 5A). Our estimate of a 4-fold reduction in binding affinity for diUb by the p62-UBA is in good agreement with the self-association constant for Lys\(^{48}\)-linked diUb calculated by Varadan et al. (58), suggesting that the competitive self-association property of the distal and proximal Ub domains in Lys\(^{48}\)-linked diUb is able to account for the difference in affinities.

Effects of Mutations in the UBA Domain on Ubiquitin Binding Affinity—We were unable to detect a secondary binding site within helix 2, or elsewhere, on the basis of comparisons of binding-induced shifts for mUb versus diUb, and conclude that there are no Lys\(^{48}\)-linked diUb-specific interactions with the p62-UBA domain. We further examined our model on the basis of the effects of point mutations on Ub binding affinity. Mutations to Ala at four solvent-exposed sites within helix 2 (T414A, R415A, Q418A, and T419A; Fig. 6A) failed to produce any significant reduction in the affinity for polyUb chains in pulldown assays, consistent with the absence of primary or secondary binding sites within helix 2 in wt-UBA. However, the data for T414A at 4 °C provided evidence for enhanced binding to mUb and polyUb chains (data not shown), suggesting the possibility that certain residues may have a role in negatively regulating binding specificity and affinity, as also recently proposed for a similar T134A mutation in the Edel UBA domain (62). The results from our helix 2 mutants contrast with those of Seibenhener et al. (11) who demonstrated in a similar pulldown assay that the conservative L417V mutation, which is sandwiched between our own R415A and Q418A helix 2 substitutions, largely eliminated binding, but L413V had little effect. Interestingly, in our structure of the bound state, Leu\(^{417}\) is sub-

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The absence of any significant binding-induced differences in chemical shift perturbations in complexation studies with mUb and Lys^{48}-linked diUb suggests that the p62-UBA domain

stantially buried in the hydrophobic core of the UBA domain, suggesting the possibility that the effects of this mutation are mediated not by direct effects on interfacial interactions with mUb but by specific effects that destabilize the bound conformation (UBA*). Indeed, CD spectra of the L417V mutant show that the unbound structure is native-like (11) and unperturbed by the mutation. In contrast, mutations to T414A, R415A, Q418A, and T419A, which are also in helix 2, appear to have little effect on binding affinity and presumably the stability of the UBA* conformation. Selective destabilization of UBA* may also be a plausible explanation for the loss of binding affinity reported for the I431V helix 3 mutant. Although we see no secondary chemical shift perturbations to Ile^{431} for wt-UBA on binding mUb, a number of adjacent residues (Thr^{430} and Gln^{432}) are affected, suggesting that Ile^{431} is close to the primary binding site on the UBA domain but is involved in core packing interactions rather than intermolecular contacts.

The effects of PDB mutations within the isolated UBA domain on Lys^{63}-linked polyUb chains (Fig. 6C). Although at best semi-quantitative in nature, pulldown assays do not reveal any significant linkage-specific differences (Lys^{68} versus Lys^{63}-linked Ub chains) in the apparent binding affinity for wt-UBA or for the mutant UBA domains (Fig. 6, B and C). Our results, and those reported by Seibenhener et al. (11), are summarized in Fig. 7. The M404V, M404T, and F406V mutations in loop 1 occur in the region where we observe the largest chemical shift perturbations on binding. Loss of affinity associated with these mutations is readily rationalized in terms of direct perturbations to (or close to) the Met-Gly-Phe-ser loop sequence. Mutations within the flexible N terminus and within helix 1 (P387L, P392L, L398V, and S399P) have little effect on binding, consistent with chemical shift mapping experiments that place this part of the structure away from the binding interface. Notably however, the P387L, P392L, and S399P mutations do exert effects on the ability of the full-length p62 to bind mUb (19), implicating other components of p62 in the subunits of Ub recognition in vivo.

We have shown that the G425R PDB mutation results in a significant loss of binding affinity in both the isolated UBA and the full-length protein (17–19). Located at the N terminus of helix 3, Gly^{425} appears to be peripheral to the UBA binding surface identified from NMR chemical shift mapping experiments. Interestingly, the substitution of a Gly residue for the bulky and highly polar guanidinium group of Arg increases the stability of the UBA domain toward thermal denaturation (ΔΔT_m ~ 9 °C) as measured from CD melting curves (data not shown). Thus, the G425R mutation presents the intriguing possibility of reducing the binding affinity for mUb by stabilizing the conformation of the unbound state of the UBA and thereby further diminishing the population of UBA* at equilibrium.

DISCUSSION

Ubiquitin Binding Specificity of the p62-UBA Domain—The UBA domain sequence of the p62 protein, and those derived from other UBA domains whose complexes have been characterized structurally, including Mud1, Ede1p, UBA1, and UBA2 of hHR23A and p47, are shown schematically in Fig. 7. In each case, residues whose NMR chemical shifts are significantly perturbed on binding mUb are shown (see Fig. 7, *). The consensus suggests that residues at the end of helix 1, those within the Met-Gly-Phe-ser loop 1 region (or equivalent), and within helix 3 form the primary binding site for mUb. In a number of cases, perturbations are observed within helix 2, which are attributed to indirect effects arising from some degree of structural rearrangement on binding. However, more recent structural investigations with the UBA2 domain of hHR23A and the UBA domain of Mud1 have demonstrated high affinity binding to Lys^{48}-linked diUb through formation of a sandwich complex (Fig. 5A), in which a single UBA domain uses different binding faces to form specific contacts simultaneously between both the distal and proximal Ub motifs, thereby enhancing the binding affinity ~100-fold compared with the interaction with mUb (29, 30).

The absence of any significant binding-induced differences in chemical shift perturbations in complexation studies with mUb and Lys^{48}-linked diUb suggests that the p62-UBA domain
does not simultaneously recognize the distal and proximal Ub motifs of Lys48-linked diUb using two binding faces. To rationalize this behavior, the conserved residue Phe330 in helix 3 is found in the UBAs of Mud1 and hHR23A and has been implicated in specific recognition of Lys48-linked diUb. However, Thr430 is found at the equivalent position in the p62-UBA and constitutes part of the primary mUb-binding site (see Fig. 7). More generally, only a subset of UBA domains show conservation of Phe330, suggesting that selective recognition of Lys48-linked polyUb chains may be confined to this group of UBAs (29), from which the p62-UBA domain is excluded.

The effects on binding affinity of a significant number of p62-UBA domain mutations, several of which cause PDB, have provided further probes for examining the conformational equilibrium between the free and bound states. The effects of a number of mutations appear to be rationalized on the basis of perturbations to specific intermolecular contacts in the bound state (M404V/M404T and F406V). However, others appear to have the potential to (i) stabilize the structure of the UBA domain in its nonproductive (free) conformation (G425R), or (ii) destabilize the productive (bound) UBA* conformation, because of differences in side chain packing interactions between the free and bound structures (L417V and I431V, from the work of Seibenhener et al. (11)). The observations suggest the possibility of both direct and indirect read-out mechanisms for the effects of mutations on Ub chain recognition that we are investigating further.

In this study we have shown that binding of the p62-UBA domain to mUb is achieved by a novel structural rearrangement of the three helices, which preserves essentially the same elements of secondary structure but optimizes the binding surface for interaction with mUb. By analogy, NMR and x-ray structures of the CUE domains bound to mUb have demonstrated the possibility of distinctly different mechanisms of Ub recognition, in one case through a monomeric compact helix bundle that is structurally homologous to the UBA domains (Cue2 from yeast) (24), and in the other (Vps9p exchange factor) through formation of a novel interlocked dimer involving reciprocal exchange of helix 3 (63). The latter achieves high affinity mUb binding by increasing the interaction surface through dimerization, demonstrating the versatile use of a small motif in achieving binding specificity. Analytical ultracentrifugation studies suggest a monomer-dimer equilibrium in solution with a $K_d$ for dimerization of $\times 10^{-11}$ M, with conformational selection in the presence of mUb favoring the dimer form (63).

It is possible however that the rearrangement of the p62-UBA described in this work is not a mechanism for optimizing Ub binding affinity per se but for achieving binding specificity. In the free form the basic residues on the UBA surface are disposed in different positions, and the burial of these charges in the bound form may be critical to achieving high affinity binding.

![Figure 6](image-url) **Figure 6.** In vitro binding assays for mixtures of polyUb chains with wild-type and mutant p62 UBA domains. Pulldown binding assays show the affinity of wt-UBA (WT) and the T414A, R415A, Q418A, and T419A UBA mutants for mixtures of Lys48-linked polyUb chains (A). UBA domains were immobilized on glutathione-Sepharose beads and used to precipitate polyUb chain mixtures. After washing, precipitated proteins were detected by Western blotting using an Ub-reactive antibody. None of the mutants show any significant loss of binding affinity compared with wt-UBA (WT). Identical binding assays with both Lys48-linked (B) and Lys63-linked polyUb chains (C) for the isolated wt-UBA (WT) and UBA domains with Paget disease point mutations, P387L, P392L, S399P, M404T, M404V, G411S, and G425R. The effects of the P392L, M404V, G411S, and G425R mutations on Lys48-linked polyUb binding affinity have been reported (17).
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A

| Ede1p   | TTPKSLAVELSSGMCPTE--EYHNLKATNWDELAAATFLDQA |
| Mud1    | PTDGPLNTAQILVSGMFDP--EAAQALDAANG德LVAASFFFF |
| hHR23A UBA1 | YEMLTLEISNYTTE--RVAQLRSAYNPHRAVTEIL |
| hHR23A UBA2 | EKEAIERKLGFPES--LVIQAYFACENELANNFLLS |
| p47     | RQDALREPAVAGGED--RRFPLESADMQIQLASYFTE |
| p62     | PPEAPRLLIESLSMGLSFSDEGWLTRLTIQRTNYDIAGALITDIQYSKH |

FIGURE 7. Analysis of the binding sites of UBA domains from chemical shift perturbations. A, a schematic representation showing the UBA domain sequence of the p62 protein and other UBA motifs that have been characterized by NMR in complexation studies with Ub, including Mud1, Ede1p, UBA1, and UBA2 of hHR23A and p47. In each case, residues whose NMR chemical shifts are significantly perturbed on binding Ub are shown (*). The consensus suggests that residues at the end of helix 1, within the helix 1-helix 2 connecting loop region and within helix 3, form the primary Ub-binding site for Ub. In many cases perturbations within helix 2 appear to correlate with indirect effects arising from structural rearrangement. B, secondary structure is shown for the p62-UBA sequence as determined by NMR. The p62-UBA mutation sites so far studied from our own work ( ■ ) and that of Seibenhener et al. (11) ( ● ) are shown. The corresponding open symbols ( □ ) represent the residues that appear to significantly reduce the affinity for binding polyUb chain mixtures in pulldown assays involving immobilized UBA domains (as shown in Fig. 6).

Persed, whereas in the bound form there is a basic patch formed by residues Arg<sup>303</sup>, Arg<sup>415</sup>, and Lys<sup>420</sup>. Similarly, an acidic surface evident in the free form of the UBA domain is further consolidated in the bound state, running from the N-terminal end of helix 2, across the whole of helix 3, and around to helix 1. It is a possibility that the rearrangement of the UBA domain serves to expose (or conceal) other binding sites. Indeed, there is a precedent for the interaction of the p62-UBA with non-ubiquitin targets, for example MuRF2, although the binding interface has not been characterized (64).

A recent structural analysis of the Par-1/MARK protein kinase has demonstrated interactions between the hMARK3 UBA domain and the kinase domain (65). The monomeric UBA domain is unusual in several intriguing ways. It adopts a non-canonical UBA fold, in which helix 3 is inverted, and appears to be in equilibrium with a partially disordered state that results in extensive line broadening associated with conformational exchange. The hMARK3 UBA binds Ub only weakly ($K_d \sim 2.35 \pm 0.5$ mM), despite having the conserved Met-Gly-Tyr-binding motif (α1–α2 loop) common to many Ub-binding UBA domains (23–25), including the p62 UBA. Helix 3 of the p62 UBA appears to remain largely disordered in the complex with Ub, implying that both its structure and conformational instability may be important in regulating the binding affinity for Ub and in promoting intramolecular interactions with its catalytic kinase domain (65). These studies, and those with the p62 UBA reported here, demonstrate that the UBA domain is a versatile structural motif for which molecular recognition and binding specificity may depend on very different binding properties.

Biological Significance of the Model.—We have demonstrated from NMR titration studies and from an analysis of chemical shift perturbations that the isolated UBA domain of p62 binds Lys<sup>48</sup>-linked diUb with marginally lower affinity than monomeric Ub units. However, this does not preclude lower affinity binding of p62 to Lys<sup>48</sup>-Ub chains as being physiologically relevant (15). Observations appear to suggest an apparent preference within cells for the binding of the full-length p62 to Lys<sup>63</sup>-linked chains (11), which adopt a more open extended conformation, analogous to Ub units assembled in a beads-on-a-string arrangement. Our NMR results qualitatively support this model by demonstrating marginally higher affinity for Ub units. The in vitro pulldown assays shown in Fig. 6 show little discernible difference in binding affinity for Lys<sup>48</sup>- versus Lys<sup>63</sup>-linked chains, which suggests that any selectivity is at best marginal. In contrast to the NMR titration studies, diUb and longer Ub chains are retained with higher affinity on UBA-loaded Sepharose beads in apparent contradiction of the NMR binding affinity experiments. A plausible explanation for this discrepancy is that a chelate effect operates on the surface of the bead to artificially enhance the affinity of ubiquitin chains through the interaction with multiple Ub binding partners. Any subtle differences in affinity of an individual UBA domain for Lys<sup>48</sup>- or Lys<sup>63</sup>-Ub chains are likely to be masked by this effect.

The full-length hHR23A provides a clear example where the intramolecular interactions of subdomains (UBL-UBA1) play a key role in modulating Ub linkage selectivity (60, 61). In in vitro pulldown assays with Sepharose-conjugated mUb, we were able to bind full-length p62 with higher affinity than the isolated UBA domain (19). These findings provide further indications that other non-UBA domains/sequences within the full-length p62 can influence Ub recognition. Indeed, when certain PDB mutations that do not affect Ub binding in the isolated UBA domain are introduced into the full-length protein, we see a common loss of ability to bind Ub relative to wild-type p62 UBA at physiological temperatures (19). The presence of flexible unstructured regions proximal to compact UBA domains appears to be a common feature of UBA-containing proteins, implying some regulatory role that we are currently investigating for p62. It has also been proposed that different oligomerization states of p62 could be influential in imparting specificity for Ub versus polyUb chains (11), with a possible chelate-like enhancement of binding affinity available to the latter through multiple p62-UBA domains displayed on a single binding surface. This mechanism would appear to have certain similarities to the binding of polyUb chains to UBA-loaded Sepharose beads where we see preferential retention of the longer chains. This is partially supported by evidence for formation of dynamic aggregates in multimeric signaling complexes (66, 67). It will be of particular interest to note whether the conforma-
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A functional switch in the p62-UBA domain reported in these studies is in any way influenced by signaling complex assembly. As the p62-UBA has been shown to have binding partners other than Ub (64), this rearrangement could be a way for achieving Ub modulation of p62 binding partners without the need for conformational switching. This would be an attractive biological mechanism for the explanation for the participation of p62 in multiple signaling complexes, with the possibility that other proteins may achieve specificity in binding to the UBA domain by recognizing different conformational states. There is also scope for binding to the p62-UBA domain to inhibit the Ub-induced rearrangement, perhaps serving as a negative regulator of p62-mediated signaling.

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