Ubiquitin-associated (UBA) domains are found in a large number of proteins with diverse functions involved in ubiquitination, DNA repair, and signaling pathways. Recent studies have shown that several UBA domain proteins interact with ubiquitin (Ub), specifically p62, the phosphotyrosine-independent ligand of the SH2 domain of p56 

The lifespan of proteins inside and outside a cell is tightly regulated by the ubiquitin-proteasome system, and numerous studies show that protein degradation is tightly interlocked with cell cycle progression and is therefore an integral part of transduction pathways and other cellular processes (1–4). For protein degradation by the Ub/proteasome system, the target proteins need to be tagged with a poly-Ub chain. These covalent complexes are then recognized and degraded by the 26 S proteasome (1, 2). The principle mechanism of this covalent modification has been identified: an enzyme cascade known as E1-E2-E3 is responsible for activation and transfer of Ub onto the target protein in a linkage-specific manner (1, 5).

The 26 S proteasome is formed by a 20 S cylindrical proteolytically active subunit and two 19 S regulatory subunits (1, 2, 6, 7). The 19 S particles represent the lid of the proteasome and regulate the access to the proteolysis (8). Although the polyubiquitinated substrate seems to be recognized by the S5a subunit in the 19 S particle (9–11), additional contacts between poly-Ub chains and parts of the 19 S regulatory subunit have been identified (12). Deletion studies indicate that other polyubiquitin-binding sites must exist (13).

Monoubiquitination is not sufficient for targeting proteins to the proteasome, however; assembly of a poly-Ub chain of at least four Ub moieties is required to create a degradation signal (11, 14, 15). Although Ub contains seven lysine residues, they are not used with the same frequency in poly-Ub chain assembly. The predominant linkages observed are Lys48-Gly76 (16), Lys63-Gly76 (17), and Lys11-Gly76 (18), of which Lys48-linked chains appear to be the most frequent degradation signal. Poly-Ub assembly via Lys29 and Lys63 is less common, and chain formation via Lys63 seems to be involved in nondegradation signal events, e.g. DNA repair (18).

Although key steps of Ub activation and transfer to a subunit as well as the structure of the 20 S subunit of the proteasome are known, the question of how proteins are targeted to the proteasome remains unanswered. It is not known whether there is an additional mechanism to regulate the time point of degradation. One possibility is that monoubiquitination leads to a “point of no return,” which proceeds to substrate destruction in a defined time span. Recently, several groups reported that proteins containing a UBA motif can bind directly to Ub and/or poly-Ub, leading to an inhibition of the degradation of target substrates through the proteasome (19–21). UBA domains are a common motif in a variety of protein families involved in protein degradation, cell cycle control, or DNA repair. In vitro and in vivo assays have revealed that UBA domains of the DNA damage-inducible proteins, RAD23 (22) as well as the fission yeast homolog Rhp23 and DDI1, as well as proteins with no function in DNA repair, p62 and Mud1p, interact specifically with Ub. Furthermore, poly-Ub chain formation is inhibited by RAD23 in vitro in a concentration-dependent manner (20, 21). In addition, it has been shown that poly-Ub chain extension stops at a length of three Ub moieties and that the inhibition of chain extension of RAD23 is specific for Lys48-linked chains (22). As a consequence of the UBA-Ub interaction, Clarke et al. (23) concluded that RAD23 and DDI1 are involved in the checkpoint control of the cell cycle. They proposed that the UBA domains of RAD23 and DDI1 could bind to the nascent poly-Ub chain of the Pds1 substrate, inhibiting chain extension and thereby increasing the lifetime of Pds1, which would otherwise be rapidly degraded.

In this report we provide a structural basis for the interaction of UBA domains with monomeric Ub based on an NMR chemical shift mapping study as well as Ub mutagenesis. Ub binds specifically to both UBA(1) and UBA(2) of HHR23A. The binding interface for both UBA domains is almost identical despite the low overall sequence similarity. Both UBA domains bind to the same region of monomeric Ub, which is also in-
volved in the binding to the proteasome subunit S5a. Models for the UBA(1)-Ub and UBA(2)-Ub complexes were generated from the chemical shift mapping data by de novo docking as well as by homology modeling with the solution structure of the closely related CUE domain in complex with Ub (24). These models revealed very different orientations of the UBA domains on the surface of Ub. Our results suggest that UBA domains may interact with Ub as well as other proteins, e.g. the HHR23A-binding proteins HIV-1 (25), methyladenine DNA glycosylase (26), p300/cyclic AMP-responsive element-binding protein (27), and peptid:N-glycanase (Png1) (28), in more than one way while utilizing the same binding surface.

**EXPERIMENTAL PROCEDURES**

**Preparation of Proteins—**UBA(1) and UBA(2) of HHR23A were prepared as previously described (29, 30). 15N- and 13C-labeled proteins were prepared by growing cells on M9 minimal medium using 15NH4Cl and 13C-glucose as the sole nitrogen and carbon sources. For the titration of the UBA domain proteins, bovine Ub (amino acid sequence identical to human Ub) was purchased from Sigma and purified by gel filtration. Purity was subsequently checked by SDS-PAGE and analytical reversed phase HPLC. For the NMR chemical shift mapping of Ub, yeast Ub was prepared from the expression plasmid pET11c-Ub (gift from A. Varshavsky). The protein was purified by reverse phase HPLC. Alanine mutants of yeast Ub were generated by site-directed mutagenesis using QuikChange (Stratagene). The CUB and CUE domains were verified by sequencing using the Dyex-Q Terminator method (PerkinElmer). Ub mutant proteins were prepared similar to wild type Ub. Protein concentrations were determined using an extinction coefficient at λmax of 1280 M⁻¹ cm⁻¹ for Ub and 1520 M⁻¹ cm⁻¹ for UBA(1), based on their respective amino acid compositions.

**Chemical Shift Mapping Experiments—**Proteins for titration experiments were dialyzed against identical buffer (50 mM sodium phosphate, pH 6.5, 100 mM sodium chloride, 2 mM deuterated dithiothreitol (Cambridge Isotope Laboratories CIL) to avoid chemical shift changes because of differences in buffer conditions. NMR samples for titration studies contained 0.25–0.5 mM 15N-labeled protein. Unlabeled protein was added stepwise up to a final ratio of 1:10. Unlabeled Ub was concentrated as far as possible to minimize volume changes throughout the titration. The “reverse” titration of Ub with UBA proteins was performed similar to the experiment described above. In addition, the UBA domains were tested for homo- and heterodimerization with NMR mapping experiments. 15N-Labeled UBA(1) (0.7 mM) was mixed with unlabeled UBA(2). All of the measurements were performed at 37 °C using a Bruker DRX-500 or DRX-600. Two-dimensional 15N-1H HSQC experiments with Watergate water suppression (60) and water flip-back pulses were used for monitoring the chemical shift changes. All of the titration data sets were processed identical using the software Quanta98. The structures were then subsequently refined by energy minimization and short (20 ps) molecular dynamics simulation in explicit solvent to allow for a correct implementation of the electrostatic contribution.

**RESULTS**

**Ubiquitin Binds Specifically to HHR23A UBA(1) and UBA(2) Domains—**High resolution structures of both UBA domains of HHR23A have been determined showing that overall the three-helical bundles are largely identical, despite a low (∼20%) sequence identity (29, 30, 36). The main structural differences are slightly different packing of side chains in the hydrophobic core and the conformation of the N and C termini. Both domains have an unusually large hydrophobic surface patch that was predicted to be a protein-protein interface (29, 30). Based on the structures and sequence analysis of UBA domains, we predicted that a conserved portion of this hydrophobic patch would be the binding region for Ub (29).

To map the binding site of Ub on UBA domains, NMR chemical shift perturbation experiments were performed on both UBA(1) and UBA(2) from HHR23A. Samples of 15N-labeled UBA(1) and UBA(2) were mixed with bovine Ub, and two-dimensional HSQC spectra were acquired to monitor the changes in the chemical shifts of the backbone amides induced by the binding to Ub (Fig. 1, a and b). Changes in chemical shifts are observed for several residues in both UBA(1) and UBA(2) starting at about 0.6 molar equivalents, indicating the formation of specific complexes in fast exchange on both the 500 and 600 MHz NMR time scale. Assuming a binary interaction between the UBA domains and Ub, a Kₚ in the range of 500–600 μM can be determined from nonlinear fitting of the titrations with either UBA(1) or UBA(2) (see also Fig. 3, a and b). However, a binding constant of about 10 μM was reported for full-length Rad23 (19), which may indicate cooperative binding of both UBA domains (37). Plots of the chemical shift changes versus residue (Fig. 1, c and d) show that the absolute magni-
FIG. 1. Chemical shift mapping of UBA(1) and UBA(2). a and b, 500 MHz ¹H-¹⁵N two-dimensional HSQC spectra of UBA(1) and UBA(2) free and bound to Ub. A, ¹³C-,¹⁵N-labeled UBA(1) free (black contour levels) and with 1:10 Ub (red); B, ¹⁵N-labeled UBA(2) free (black) and with 1:10 Ub (red) at 27°C. Blue contours are used for side chain amide protons. c and d, chemical shift change versus sequence of UBA(1) and UBA(2). Graph representing the average chemical shift change of the amide proton and nitrogen/¹H and ²H of UBA(1) (c) and UBA(2) (d) upon the addition of 10 equivalent Ub. The dashed lines indicate the thresholds chosen for the color coding used in e and f. Chemical shift changes of Δδ < 0.05 ppm were considered insignificant. e and f, binding interface of Ub on UBA(1) and UBA(2). Residues of UBA(1) (e) and UBA(2) (f) shifting by more than 0.05 ppm upon the addition of Ub are marked in orange. A representation of the molecular surface is shown on the left, and a ribbon sketch is shown on the right.
tudes of the chemical shift changes are similar for UBA(1) and UBA(2).

A recent chemical shift mapping study of the closely related HHR23B reported that the Ubl and UBA domains interact with each other with a reported $K_D$ of $2 \text{mM}$, which is $10$-fold higher than their calculated $K_D$ for the HHR23B-UBA interactions of $390 \text{ nM}$ (38). The Ubl domain is structurally very similar to Ub (39, 40) and exhibits a high sequence identity. Weak binding between the HHR23A Ubl and UBA domains was also detected in the context of the full-length protein as well as isolated domains (41) under slightly different buffer conditions and at higher field strength, with a binding affinity at least $10$-fold lower compared with the already weak interaction between the UBA domains and Ub.

Ubiquitin Binds to a Conserved Surface Epitope of UBA Domains—The results of the chemical shift perturbation study were mapped onto the structures of UBA(1) and UBA(2) (Fig. 1). Although UBA(1) and UBA(2) have a relatively low sequence identity, the binding interfaces revealed by the chemical shift mapping are remarkably similar. Residues exhibiting the largest chemical shift changes upon binding to Ub, Gly$^{174}$ and His$^{192}$ for UBA(1) and Leu$^{200}$, Gly$^{201}$, and Glu$^{248}$ for UBA(2), respectively, are in similar locations. A large cluster of residues in UBA(1) involved in binding is located at the C-terminal end of the first helix, Ile$^{170}$, Met$^{171}$, Ser$^{172}$, and the first three residues in the short and highly conserved loop 1, Met$^{173}$, Gly$^{174}$, and Tyr$^{175}$ (Fig. 1, a, c, and e). Several positions on the third helix of UBA(1) also show large changes upon binding, specifically His$^{192}$ and Arg$^{193}$ at the N terminus of helix 3 and Tyr$^{197}$ at the second turn of helix 3. Together, these residues form a consecutive patch of about $520 \text{ Å}^2$ on the surface of UBA(1) (Fig. 1c). The residues with the largest chemical shift changes, His$^{192}$, Met$^{173}$, and Gly$^{174}$, are in the center of the epitope.

Residue Tyr$^{197}$ also exhibits a significant change in chemical shift upon the addition of Ub but is located on the “back face” of the binding interface. The change for the amide proton and nitrogen amide frequencies of Tyr$^{197}$ might be attributed to small structural changes in the hydrophobic core because of interactions of the side chain of Tyr$^{197}$ with Arg$^{193}$, which is part of the binding interface. Residues in the C terminus of UBA(1) do not exhibit chemical shift changes when Ub is added (Fig. 1c) and do not seem to be part of the binding interface. This was surprising, because the C terminus is close to the hydrophobic patch involved in binding and is of relatively rigid nature (29).

Analysis of the binding of UBA(2) to Ub reveals that the general location of the epitope remains the same compared with UBA(1) (Fig. 1). Identical positions in the C-terminal end of helix 1 (Arg$^{326}$, Leu$^{327}$, and Ala$^{329}$) as well as the first three residues of the hydrophobic loop 1 (Leu$^{330}$, Gly$^{331}$, and Phe$^{335}$) form a large part of the binding interface to Ub. In addition, residues at similar positions at the N terminus of helix 3 (Glu$^{348}$ and Asn$^{349}$) are also among the residues with the highest chemical shift changes upon binding. The structurally equivalent residue of Glu$^{348}$ in UBA(1) is Pro$^{191}$; therefore no information about whether or not Pro$^{191}$ is involved in the binding of Ub can be deduced from this analysis. A larger number of residues at the C-terminal end of helix 3 of UBA(2) are involved in binding than observed for UBA(1). On the last turn of helix 3, residue Leu$^{356}$ is affected by the binding, whereas residues of UBA(1), Leu$^{199}$ and Thr$^{200}$, do not exhibit significant changes. Overall, with a binding area of $650 \text{ Å}^2$, UBA(2) appears to have about a $25\%$ larger interface than UBA(1), for which the residues within the binding epitope yield an area of about $520 \text{ Å}^2$.

UBA Domains Bind on the Five-stranded $\beta$-Sheet Surface of Ubiquitin—To investigate the binding surface for UBA domains on Ub, NMR chemical shift mapping was performed under the identical conditions as reported above using yeast Ub. Yeast Ub differs in three amino acids compared with human Ub, S21P, D24E, and S28A, none of which is close to the binding site for UBA domains determined in this study. The residue Pro$^{191}$ in human Ub is located in a tight turn in the loop between the second $\beta$-strand and the first $\alpha$-helix. The two other residues Asp$^{274}$ and Ser$^{285}$ are located on the $\alpha$-helix, facing in the opposite direction of the determined binding interface. We therefore concluded that these mutations do not interfere with or modulate the binding of the UBA domains to Ub. However, to confirm that the recognition process is not influenced by indirect effects, we repeated the titration using $^{15}$N-labeled human Ub purchased from VLI research (Mavern) and UBA(2); no differences in the chemical shift changes compared with the study using yeast Ub were detected (data not shown).

Chemical shift mapping of the amide resonances of Ub as a function of added UBA(1) or UBA(2) up to a ratio of 1:10 Ub:UBA again revealed complexes in fast exchange on the NMR timescale. Upon the addition of UBA(1) to Ub, significant changes in chemical shift are observed for 23 residues (Fig. 2, a, c, and e). Most of these residues are located on the $\beta$-strands or the connecting loops of the five-stranded $\beta$-sheet of Ub forming a consecutive patch. Leu$^{171}$–Leu$^{173}$ are located in the C terminus close to the Gly-Gly motif required for poly-Ub chain extension (Fig. 2c). When Ub was titrated with UBA(2), equivalent residues showing a significant change in their proton and nitrogen amide frequencies were almost identical with those for UBA(1) (Fig. 2).

These results suggest that both UBA domains are recognized by and bind to Ub using a similar binding epitope. The binding epitopes of UBA(1) and UBA(2) on Ub overlap perfectly, and the differences in several of the amino acids, e.g. His$^{192}$ versus Glu$^{348}$ and Arg$^{193}$ versus Asn$^{349}$, might account for the chemical shift differences observed for the titration of Ub with either UBA(1) or UBA(2). However, another possibility is that the two UBA domains are oriented differently on Ub, as discussed below.

Differential Chemical Shift Mapping of UBA Domains—To further address the question of how the UBA domains of HHR23A bind to Ub, especially whether the binding mechanism differs between the two UBA domains, we tried differential chemical shift mapping (42). Five Ub mutants (L8A, R42A, K48A, H68A, and R72A) with single amino acid substitutions were chosen for study. All of the mutated residues have side chains oriented toward the binding interface and show significant chemical shift changes upon binding to the UBA domains. Differences in the chemical shift changes between a Ub mutant and wild type Ub should be observed if the residue pair is in close proximity in the UBA-Ub complex. The binding affinities were determined by nonlinear fitting of the chemical shift changes of three residues located within the binding epitope (Ile$^{170}$, Gly$^{174}$, and His$^{192}$ for UBA(1); Gly$^{201}$, Glu$^{248}$, and Leu$^{356}$ for UBA(2)).

Surprisingly, the mutant Ub L8A exhibits much smaller absolute chemical shift changes upon binding to UBA(1) and UBA(2), suggesting a lower binding affinity. However, quantitative analysis of the binding curves (Fig. 3, a and b) shows that the affinity of UBA(1) and Ub L8A is not changed significantly (490 and 570 nM, respectively). Similar small changes in $K_D$ (less than 2-fold) were observed for the other Ub mutants (K48A, H68A, and R72A). Interestingly, the binding affinities of the Ub mutant R42A for UBA(1) and UBA(2) seem to be increased 3.5- and 2-fold, respectively.

Comparison of the UBA-Ub titration studies with those of
FIG. 2. Chemical shift mapping of Ub. a and b, \(^{1}H,^{15}N\) HSQC of free Ub and Ub bound to UBA(1) and UBA(2). \(^{13}C,^{15}N\)-labeled Ub in free conformation (black) and in complex with UBA(1) (a) and UBA(2) (b) (red contour levels, ratio 1:4). Blue contour levels mark side chain amide resonances. c and d, chemical shift change versus sequence of Ub bound to UBA(1) and UBA(2). The average chemical shift change (combined amide proton and amide nitrogen chemical shift as for Fig. 1, c and d) is shown for Ub bound to UBA(1) (c) and UBA(2) (d). The dashed lines indicate the thresholds of chemical shift change used for e and f, c and f, binding area of UBA(1) and UBA(2) on Ub. The changes of amide proton and nitrogen chemical shift are displayed on the surface (left) and on a ribbon diagram (right) of human Ub as classified in c and d. Residues are marked in orange according to the chemical shift change (\(\Delta \delta_{\text{ave}}\)) on Ub upon the addition of UBA(1) (e) and UBA(2) (f). For Ub binding to UBA(1) (E) large changes (\(> 0.1 \text{ ppm.}\)) for the chemical shifts are found for the residues Leu\(^{8}\), Lys\(^{11}\) (\(\beta_{1}\)-\(\beta_{2}\) loop), Ile\(^{44}\) (\(\beta_{2}\)), Gly\(^{45}\), Lys\(^{48}\), Gln\(^{60}\) (\(\beta_{4}\)), His\(^{68}\), Leu\(^{69}\) (\(\beta_{5}\)), Leu\(^{73}\) (\(\beta_{6}\)).
the Ub mutants showed no significant differences in the course of the chemical shift changes for UBA(2). In contrast, for UBA(1), the chemical shift pattern of Ub mutants L8A and R42A is different compared with wild type Ub. For L8A significant changes are observed for residues Glu169 (helix 1) as well as Arg193, Ala194, and Glu196 in helix 3 (Fig. 3), indicating that these residues are in close proximity to each other in the complex. For Ub mutant R42A, the largest differences are observed for residues Gly169 (helix 1) and Ala194, Glu196, Leu199, Gly47, Phe45, Ala46, Asn349 UBA(2) is buried in the interface. This is in good agreement with the interface size determined by chemical shift mapping. The nature of the interaction is almost exclusively hydrophobic (>80%).

In the Ub-UBA models the axis of helix 1 is oriented at an angle of 45° to the β-strand 5 of Ub, helix 3 is running at an angle of 50° across the β5, and the helical axis of helix 2 is running almost in parallel with β5 (Fig. 4a). Hydrophobic residues in loop 1 (Gly174-Tyr175 UBA(1) and Gly331-Phex132 UBA(2)) of the UBA domain interact with the β3-β4 loop residues of Ub (Fig. 4c). The N terminus of helix 3 of the UBA domains (Pro191-His192 UBA(1) and Gly348-Asn349 UBA(2)) is close to the C terminus of β-strand 5 and the β1-β2 loop of Ub. The important “hydrophobic triad” of Ub, residues Leu6, Ile44, and Val72, is buried in the interface. In the Ub-UBA(1) complex of the Cue2 CUE domain to Ub (KD = 150 µM) is more similar to the interaction of the UBA domains of HHR23A with Ub (KD = 500 µM). In contrast the binding of the Vps9p UBA domain is considerably tighter (KD = 20 µM). The rmsd for Cα positions of the three helices of UBA and CUE are 1.6 Å (CUE-UBA(2); 29 Cα positions) and 1.9 Å (CUE-UBA(1); 26 Cα positions). In the final models 410 Å2 (UBA(1)-Ub) and 440 Å2 (UBA(2)-Ub) surface area of the UBA domains are buried in the interface.
Fig. 4. Homology models for the UBA(1)-Ub and UBA(2)-Ub interaction. a, model for the interaction of HHR23A UBA(1) and Ub based on the structure of the complex of Cue2 CUE domain and Ub, in b for the interaction of UBA(2) and Ub. Left panels, ribbon sketches with the structure of UBA(1) (a) and UBA(2) (b) displayed by a color ramp blue to red (N terminus to C terminus). The helical axes of the UBA domains are displayed by dashed black lines, as a reference for the orientation the axis of β-strand 5 of Ub is shown as a dashed red line. Middle panels, interface between UBA(1) (a) or UBA(2) (b) and Ub, the surface of Ub is shown in atom colors. Residues of UBA(1) or UBA(2) in direct contact with Ub are shown and labeled. Right panels, as for middle panel but residues of Ub (gray) in direct contact with residues of UBA(1) (a) or UBA(2) (b) (dark gray) are shown and labeled.

Complex, Leu8 of Ub is surrounded by UBA(1) residues Glu169, Ile170 (helix 1), Pro191, and His192 (loop 2) (Fig. 4a). In the complex of Ub-UBA(2), residue Leu8 of Ub has van der Waals contacts to residues of UBA(2) occupying similar positions in the three-helical bundle (Ala123, Leu227 (helix 1), and Glu348 (loop 2)) (Fig. 4b). Ile44 of Ub is packed against Met173 (helix 1), Tyr175, and His192 (loop 3) of UBA(1); in Ub-UBA(2) residues Leu330 (helix 1), Ala352, and Leu356 (helix 3) of UBA(2) are close to Ile44 of Ub. Val70 of Ub contacts either His192 (loop 2) of UBA(1) or residues Ala123, Leu227 (helix 1), and Glu348 (loop 2) of UBA(2).

The differences in the environment of Ub Val70 bound to UBA(1) versus UBA(2) are due to slightly different interhelical angles of the three-helical bundle, which lead to helix 1 of UBA(1) pointing further away from the binding interface. Although different residues of both UBA domains interact with Leu8, Ile44, and Val70 of Ub, the hydrophobic nature in the contact area is preserved. Only a very small number of possible intermolecular hydrogen bonds can be identified in the binding interfaces of the model complexes: five for Ub-UBA(1) (Ub-UBA: Lys8–Glu169, Gly57–Tyr175, Gly57–Met173, His58–Glu169, and Leu7–His192) and two for Ub-UBA(2) (Ub-UBA: Thr1–Arg126 and His38–Ala229). Similarly, in the complex of Cue2 CUE domain and Ub, only two hydrogen bonds are observed. The dearth of polar interactions in addition to the relatively small interface area (~50 Å²) might explain the relatively low binding affinity between the monomeric CUE domain and Ub as well as for UBA domains and Ub.

De novo docking using the program HADDOCK and employing data from our chemical shift mapping (32). The chemical shift changes of UBA(1), UBA(2), and Ub were used together with surface accessibility data to define ambiguous distance constraints between the two molecules. The method relies on geometrical and electrostatic complementarity of the binding epitopes of the interacting molecules, and it has been applied successfully to other complexes (32). Much to our surprise, the results of the de novo docking of the Ub-UBA domain complexes revealed completely different complex structures compared with those obtained by homology modeling (Figs. 4 and 5). Additionally, the architecture of the complexes resulting from de novo docking for the Ub-UBA(1) and Ub-UBA(2) interaction (Fig. 5) is also different, indicating that the UBA domains of HHR23A might interact with Ub differently.

A structural alignment of the Ub molecules of the two models of UBA(2)-Ub (homology model versus de novo docking) shows the large difference. In the complex of UBA(2)-Ub obtained by de novo docking, helices 1 and 3 of UBA(2) run almost parallel to β-strand 5 of Ub, and UBA(2) helix 2 and Ub β-strand 5 are oriented at an angle of about 20° (Fig. 5b) relative to each other. In the homology model (and hence for the template complex Cue2 CUE domain-Ub), helices 1 and 3 of the UBA domain and β-strand 5 of Ub share an angle of almost 45°, and the helical axis of helix 2 of UBA(2) runs parallel to β-strand 5 of Ub (Fig. 4b). Consequently, the de novo docking model can be transformed into the homology model by a rotation of about 45° counterclockwise. A large positional movement is observed for the residues in the Gly-Phe-Pro loop of UBA(2), with the Cα positions of Leu330, Gly331, and Phe332 differing by 7–8 Å be-
De novo docking of UBA(1) and Ub resulted in a model complex that differs from the homology model to an even greater extent. The positional change of the residues in loop 2 (Glu\textsuperscript{348} and Asn\textsuperscript{349}) and helix 3 (Leu\textsuperscript{356}) with respect to the Ub binding interface is smaller (distances for C\textsubscript{α} positions: Glu\textsuperscript{348}, 3 Å; Asn\textsuperscript{349}, 4 Å; and Leu\textsuperscript{356}, 4 Å). Consequently a different residue pairing is observed in the interface of the de novo docking model. Leu\textsuperscript{6} of Ub is surrounded by Arg\textsuperscript{326}, Leu\textsuperscript{327}, Leu\textsuperscript{330}, and Glu\textsuperscript{348} of UBA(2), Ile\textsuperscript{14} of Ub is in van der Waals' contact with residues Ala\textsuperscript{352}, Asn\textsuperscript{353}, and Leu\textsuperscript{356} of UBA(2), and Val\textsuperscript{70} of Ub is in close proximity to Asn\textsuperscript{349} and Ala\textsuperscript{352} of UBA(2). In the complex of the Cue2-CUE domain and Ub (and thus in the homology model of UBA(2)-Ub), the residues located at the N terminus of the helix 1 are part of the binding interface, which is not the case for the de novo docking. In the homology model, Ala\textsuperscript{323} and Arg\textsuperscript{326} both contact residues Leu\textsuperscript{6} and Thr\textsuperscript{9} of Ub, whereas for the de novo docking model, Ala\textsuperscript{323} shares no contacts with any residues of Ub.

De novo docking of UBA(1)-Ub involved in the binding are conserved. In the homology model, Ala\textsuperscript{323} and Arg\textsuperscript{326} both contact residues Leu\textsuperscript{6} and Thr\textsuperscript{9} of Ub, whereas for the de novo docking model, Ala\textsuperscript{323} shares no contacts with any residues of Ub.

De novo docking models for the UBA(1)-Ub and UBA2-Ub interaction. Similar to Fig. 4, the interaction of UBA(1) (a) and UBA(2) (b) with Ub is shown. The complexes were obtained by computational docking using ambiguous restraints based on the chemical shift mapping results. Left panels, the helical axes of the UBA domains (UBA(1) in a and UBA(2) in b) are represented by dashed black lines, and the orientation of the UBA domain in respect to Ub can be determined from the axis of β-strand 5 of Ub shown as a red dashed line. Middle panels, the interface between UBA(1) (a) and UBA(2) (b) and Ub. The surface of Ub is shown in atom colors; the residues of either UBA domain in contact with Ub are shown and labeled. Right panels, as for the middle panel, but the residues of Ub in direct contact with UBA(1) (a) and UBA(2) (b) are shown in gray and labeled.
The hydrophobic surface patches that were predicted to be the site of protein-protein interactions for the UBA domains (29) comprise a large portion of the binding epitope for Ub. In addition, some charged and polar residues appear to be important based on the chemical shift mapping. These results agree very well with a mapping study of HHR23B UBA domains published very recently by Choi and co-workers (38), except for the equivalent residue to HHR23A UBA(1) Tyr197. Despite the relatively low binding affinity of 500 μM, the interaction of the UBA domains with Ub is specific. Furthermore, binding between HHR23A/B UBA and Ub domains is an order of magnitude weaker (38, 41).

The five-stranded β-sheet of Ubiquitin Is a Universal Binding Site for Ubiquitin-interacting Proteins—Despite the differences in the binding epitopes determined for the UBA domains of HHR23A, the position of the interface on Ub is practically identical for both UBA domains. This region has been identified to interact with several other protein domains, indicating that the hydrophobic surface patch on the five-stranded β-sheet of Ub is probably a general protein-protein interface that can facilitate various interactions. At least six ubiquitin-interacting domains have been described so far (44). The structures of five domains have been determined, and the site of their interaction with Ub has been mapped. The three-dimensional structures of these domains, UEV (ubiquitin E2 enzyme variant) (45), NZF (golub zinc finger domain) (46), UIM (ubiquitin-interacting motif) (39, 47, 48), UBA (29, 30, 36), and CUE (24, 43), vary greatly in architecture and size, being either a single helix (UIM), a pure β-strand structure (NFZ), three helical bundles (CUE and UBA), or a mixed α-β structure (UEV). Despite this large variety, all domains interact with Ub via the same hydrophobic patch on the five-stranded β-sheet of Ub. 

Comparing the binding to Ub for all domains reveals only very minor differences in the location of the binding sites. The center of the binding site, the hydrophobic patch around residue Ile44 of Ub, seems to be identical for all ubiquitin-interacting domains so far, although residues close to that patch have been mapped to different biological functions (49). Very little structural data for the interaction of monomeric Ub or ubiquitin-like domains in complex with an ubiquitin-interacting domain are available so far (24, 39, 43), probably because of the low to moderate binding affinities for such interactions ($K_d = -10$ to $500$ μM) (44). A comparison of the binding mechanism of
a single α-helix with an Ub-like domain (complex of HHR23A Ubl-UIM-2 of S5a) with that of a three-helical bundle bound to Ub (complexes of Cue2 CUE domain and Vps9p CUE with Ub) shows that complexes of Ub and Ub-interacting domains can adopt different architectures. The single α-helix of the UIM motif of S5a binds on top of β-strand 5 of HHR23A Ubl, with the axes of the α-helix of the UIM motif and of β-strand 5 of Ub running anti-parallel (39). In contrast, the three-helical bundle of the CUE domain of Cue2 binds via helices 1 and 3, which run across β-strands 1, 3, and 5 at an angle of about 30° (24). In the case of the Cue2 domain of Vps9p, the interaction is even more complex. In the x-ray crystal structure of the complex of the CUE domain of Vps9p with Ub, the CUE domain forms a domain-swapped dimer in which helices 1 and 3 interact with Ub in a similar manner to that observed for the Cue2 CUE domain-Ub complex, but here helix 2 has additional contacts with residues of Ub (43). Despite the differences in the architecture of the complexes, the interacting amino acids are conserved, with only hydrophobic amino acids taking part in the interaction. The absence of hydrogen bonds in the center of the interface probably explains the limited specificity of Ub, because there is no requirement to maintain the geometry of hydrogen bonding acceptors or donors, and therefore specificity for a binding partner is only generated by geometrical restrictions for the interacting hydrophobic side chains. It is also interesting to note that the binding affinity between Ub and the Ub-interacting domains of known structures correlates with the size of the interface. The Cue2 CUE domain Ub interface measures 450 Å² and has a binding affinity of about 150 μM (24), whereas the S5a UIM-2 HHR23A Ubl complex has a buried surface area of roughly 600 Å² (39) and a KD of ~10 μM. The interface between the CUE domain of Vps9p and Ub measures 520 Å² (because of additional interacting residues in the second helix) resulting in an affinity of 20 μM (43).

A Model for the Interaction of UBA Domains and Ubiquitin—One very surprising result of the modeling of the UBA-Ub interaction presented in this study is the large differences between the models obtained by homology modeling and de novo docking using the program HADDOCK. The homology models were built using the NMR structure of the Cue2 CUE-ubiquitin complex, with the CUE domain being replaced by the UBA domains of HHR23A. Residues of the Cue2 CUE domain that interact with residues of the five-stranded β-sheet of Ub are either conserved or replaced by homologous amino acids (Fig. 6) in the UBA domains of HHR23A. However, docking of UBA(2) to Ub using the program HADDOCK resulted in a model with the three-helix bundle rotated clockwise by about 45°. For the de novo docked complex involving UBA(1), the three-helix bundle is rotated by almost 90° but in a counterclockwise direction (Figs. 4 and 5). Thus, not only do the de novo docking results differ from the homology modeling approach, but the de novo docking even suggests different complex architectures for UBA(1) and UBA(2) bound to Ub. The differences in the complex of the two CUE domains of Cue2 and Vps9p with Ub provide experimental support for the idea that the interface of Ub allows for the binding of different helical bundle geometries. The CUE domain of Vps9p, being a domain-swapped dimer, binds to Ub not only via helices 1 and 3 but also via additional residues in helix 2 that likely contribute to affinity and specificity (43).

We note, in addition, that the differential chemical shift mapping employing Ub mutants showed differences for UBA(1) and UBA(2). These might indicate that both UBA domains bind to Ub by a different binding mechanism, supporting the results obtained by the de novo docking procedure. Analysis of the distribution of charged residues surrounding the hydrophobic patch, which is in the center of the binding interface for CUE and UBA domains, clearly shows that the electrostatic potentials are distinct for these domains. Biological data suggest differences in functions of the CUE and UBA domain (50–52), and sequence comparison clearly distinguishes between the CUB and UBA family (53). All of the CUE domains identified so far bind to monomeric Ub with low to moderate affinity (20–160 μM), and some but not all CUE domains seem to bind to poly-Ub in addition (52). It was not reported whether their binding affinity for poly-Ub is higher than for monomeric Ub; however, data from Shih et al. (52) suggest that at least the CUE domain of Vps9 has no preference for long poly-Ub chains over short poly-Ub chains. This binding preference is probably required for the maintenance of monoubiquitination (52), which is in turn a signal for trafficking and receptor endocytosis. However, for a more quantitative analysis the binding constants of several CUE domains for mono- and poly-Ub have to be determined.

The biological function of the UBA domain is, on the other hand, still in debate (20, 22, 23, 37, 54). Several groups have reported that UBA domains bind to monomeric Ub as well as to poly-Ub, although the data are contradictory in some cases (19, 54). Binding to monomeric Ub was associated with inhibition of further extension of the nascent Ub chain, which results in an inhibition of the degradation of a substrate (20–23). The binding to poly-Ub was explained with a possible shuttle function for the transport of a substrate to the proteasome (37, 54–57). Although the physiological “Ub target” of the UBA domains of RAD23 and other proteins has not been determined, all in vitro binding experiments have yielded an at least 1000-fold greater affinity of the UBA domains for tetra-Ub compared with mono-Ub (22, 54). Therefore it is very likely that in the presence of polyubiquitinated substrates, the main binding partner of UBA domains might be these poly-Ub chains rather than monoubiquitinated substrates or free Ub, unless the concentration of polyubiquitinated substrates is very low. The molecular nature of the tighter binding of UBA to poly-Ub is not yet clear; however, structure analysis (58) as well as mutagenesis data on poly-Ub (59) suggest that they do not form plain linear poly-protein chains like pearls on a string but rather adopt globular structures. Hence additional contacts between the linked Ub moieties and possibly other epitope(s) of the UBA domain might lead to the increase in affinity in comparison with monomeric Ub.

Because UBA domains are often associated together with Ub-like domains in modular proteins, the UBA domains could act as poly-Ub “receptors,” whereas the Ubl domain might interact directly with the proteasome. However, such a shuttle mechanism has not yet been confirmed in vivo. The differences for UBA and CUE domains in their biological function as well as in the probable binding target, monomeric Ub versus poly-Ub, make it plausible that the binding mechanisms of CUE and UBA domains do not need to be identical.

Interestingly, our de novo model was recently at least partially confirmed by results by Walters et al. (41). Based on chemical shift differences between the isolated Ubl and UBA domains and the domains in the context of the full-length HHR23A protein, it was concluded that in full-length HHR23A the Ubl domain interacts in a dynamic fashion with the individual UBA domains, and this interaction has a 1:1 stoichiometry, i.e. Ubl is exchanging between one or the other UBA domains. No interdomain NOEs were observed, consistent with very weak binding. Using residual dipolar couplings measurements, Walters et al. (41) defined the relative orientation of the domains in the modular protein. Although the coordinates for their models of the interaction between Ubl and the UBA
domains are not available, unless the figures of their publication we find a striking similarity between their models for Ub-UBA interaction and our Ub-UBA de novo models. Similar to the interaction with Ub, an identical surface epitope of Ub is involved in the binding to both UBA(1) and UBA(2) domains. Helix 1 of either UBA(1) (residues 191–199) or UBA(2) (residues 348–356) contacts the five-stranded β-sheet of either Ub or Ubl domain. For UBA(1) the relative orientation of the helix in the model is the same as observed for HHR23A Ubl-UBA(1). For UBA(2), the orientation of helix 1 is rotated by 180° in our de novo model compared with the results of Walters et al. (41). In contrast to our chemical shift mapping studies and those performed by Ryu et al. (38) on HHR23B UBA domains, Walters et al. (41) propose that only one helix of the UBA domains contributes to the interaction with the Ubl domain. We find that the binding of the UBA domains to Ub includes residues from both helices 1 and 3. This difference might explain the much lower affinity of UBA domains for Ub (KD = 2 μM) (38) than for Ub (KD = 300–500 μM). In summary, these results indicate that the interaction between Ub and UBA domains might be different from the binding to Ub found for the structurally homologous CUE domain in solution. However, further experimental data are required to understand how UBA domains interact with Ub on a molecular level and whether the binding mechanism is different from the CUE domains.

Finally, we note that RAD23D has been described as a binding partner through its UBA(2) domain for various proteins involved in DNA repair and cell cycle control, e.g., HIV-1 Vpr (25), methylenedine DNA glycosylase (26), p300/cyclic AMP-responsive element-binding protein (27), and Png1 (28). Because the UBA-binding epitopes of these proteins exhibit very different structures, the UBA domain of RAD23D must be able to bind to various structural architectures. Therefore the binding mechanism of UBA domains is also likely to vary for the diverse interacting proteins.

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