Differential ubiquitin binding of the UBA domains from human c-Cbl and Cbl-b: NMR structural and biochemical insights

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Abstract

The Cbl proteins, RING-type E3 ubiquitin ligases, are responsible for ubiquitinating the activated tyrosine kinases and targeting them for degradation. Both c-Cbl and Cbl-b have a UBA (ubiquitin-associated) domain at their C-terminal ends, and these two UBA domains share a high sequence similarity (75%). However, only the UBA from Cbl-b, but not from c-Cbl, can bind ubiquitin (Ub). To understand the mechanism by which the UBA domains specifically interact with Ub with different affinities, we determined the solution NMR structures of these two UBA domains, cUBA from human c-Cbl and UBAb from Cbl-b. Their structures show that these two UBA domains share the same fold, a compact three-helix bundle, highly resembling the typical UBA fold. Chemical shift perturbation experiments reveal that the helix-1 and loop-1 of UBAb form a predominately hydrophobic surface for Ub binding. By comparing the Ub-interacting surface on UBAb and its counterpart on cUBA, we find that the hydrophobic patch on cUBA is interrupted by a negatively charged residue Glu12. Fluorescence titration data show that the Ala12Glu mutant of UBAb completely loses the ability to bind Ub, whereas the mutation disrupting the dimerization has no significant effect on Ub binding. This study provides structural and biochemical insights into the Ub binding specificities of the Cbl UBA domains, in which the hydrophobic surface distribution on the first helix plays crucial roles in their differential affinities for Ub binding. That is, the amino acid residue diversity in the helix-1 region, but not the dimerization, determines the abilities of various UBA domains binding with Ub.

Keywords: c-Cbl; Cbl-b; UBA; ubiquitin; binding; NMR; structure

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The Cbl proteins usually function as negative regulators in several receptor protein tyrosine kinase signaling pathways. They mediate the ubiquitination of activated tyrosine kinases, such as EGFR, and target them for degradation. So far, three members of the Cbl protein family have been extensively studied in mammals, namely c-Cbl, Cbl-b, and Cbl-3 (Thien and Langdon 2005). All the three proteins share a highly conserved

N-terminal region containing a tyrosine-kinase-binding domain (TKB) and a RING finger domain (Joazeiro et al. 1999; Levkowitz et al. 1999). The C-terminal regions of them are less well conserved; however, they do share common roles with abilities to interact with a range of SH2- and SH3-domain-containing proteins, such as CIN85 (Petrelli et al. 2002; Soubeyran et al. 2002). Interestingly, c-Cbl and Cbl-b, but not Cbl-3, have a ubiquitin-associated (UBA) domain at their C-terminal ends (see Fig. 1A).

The two C-terminal UBA domains of Cbl proteins were previously thought to be leucine-zipper motifs (Blake et al. 1991; Keane et al. 1995), for they contain leucines and isoleucines every seven amino acid residues and mediate the homodimerization of c-Cbl and heterodimerization of c-Cbl and Cbl-b (Bartkiewicz et al. 1999; Liu et al. 2003). Later, these two domains were predicted to be UBA domains through bioinformatics approaches (Robertson et al. 2000). The core sequences (Fig. 1B) of these two UBA domains share 48% identities and 75% similarities. However, a recent study by biochemical approaches showed that only the UBA from Cbl-b, but not from c-Cbl, could significantly interact with ubiquitin (Ub) (Davies et al. 2004).

The UBA domain is a conserved sequence found in a variety of proteins having connections to the ubiquitination pathway: (1) E2-25K and several other Ub-conjugating enzymes (E2); (2) several Ub ligases (E3); (3) a series of deubiquitin enzymes, such as isopeptidase T (isoT) (USP5 and USP13); and (4) many Ub-like proteins, such as hHR23A, Dsk2, and BMSC-UbP, that usually contain both UbL (ubiquitin-like) and UBA domains (Hofmann and Bucher 1996; Chang et al. 2006; Kang et al. 2006, 2007). Most of these UBA domains can bind with Ub, while some cannot. Interestingly, Some UBAs can bind with both Ub and UbL domains, such as isoT-UBA2, hHR23A-UBA2,

and ubiquitin-UBA (Raasi et al. 2005). There is a minority of the UBA domains that can only bind with UbL domains, but not with Ub. For example, the UBA domain of HOIP protein binds directly to the UbL domain of HOIL-1L but not to Ub, and the interaction is critical for stable complex formation (Kirisako et al. 2006). In addition to the well-known Ub/UbL domain binding ability, some UBA domains can form homo- or heterodimers, such as Dsk2 UBA and Cbl UBA domains, in regulating biological activities (Sasaki et al. 2005; Kozlov et al. 2007; Peschard et al. 2007).

As Cbl proteins are important E3 ligases in ubiquitination, it is of interest to investigate the structural basis for the differential binding properties of these two UBA domains to Ub. Here, we report the NMR structural and biochemical studies of the UBA domains from human c-Cbl (cUBA) and Cbl-b (UBAb). A combination of structural analysis, chemical shift perturbation, mutagenesis, and fluorescence titration reveals that the hydrophobic surface distribution on the first helix play crucial roles in their differential affinities for Ub binding.

Results

Structure determination of cUBA and UBAb

The structures of the dimeric forms of both cUBA and UBAb have been solved by crystallography (Peschard et al. 2007) and NMR (RIKEN Structural Genomics/Proteomics Initiative). To further explore the nature of the different Ub binding abilities, structurally and biochemically, we cloned two UBA domains from c-Cbl and Cbl-b and solved their 3D structures in solution by an NMR approach (Fig. 2). The sequences for both cUBA and UBAb contain an N-terminal five-residue extension compared to those in the literature (PDB: 2D9S for cUBA and 2DO6 for UBAb). It appears that both cUBA and UBAb in crystal and probably in solution form symmetric homodimers (Peschard et al. 2007). We have only ambiguously identified several intermolecular NOEs in solution on the NMR spectra of cUBA and UBAb, which are not enough for computing an accurate structure in the dimeric form. Therefore, in this study, both cUBA and UBAb were calculated as monomers from intramolecular NOEs. The experimental restraints and structural statistics for these two UBA domains in monomeric form are summarized in Table 1.

cUBA and UBAb share the same fold, a highly compact three-helix bundle

The overall fold of these two UBA domains is similar and consists of three α -helices connected by two loops,

Figure 1. Multiple alignments of the UBA domains from Cbls. (A) Schematic domain representation of c-Cbl, Cbl-b, and Cbl-3. The UBA domain is located at the C terminus. (B) Sequence alignment of the UBA domains from human c-Cbl (cUBA) and Cbl-b (UBAb). Residues 1–56 of cUBA correspond to 851–906 of c-Cbl, and residues 1–57 of UBAb correspond to 926–982 of Cbl-b. Residues, which are same in these two domains, are labeled on a dark background and those with high similarity are highlighted by boxes. The secondary structures of cUBA are shown at the top. The sequences were aligned using ClustalW and represented using ESPript.

Figure 2. cUBA and UBAb adopt the same fold of highly compact three-helix bundle. (A) Ensemble of the C α traces representing a bundle of 15 representative structures of cUBA. (B) Ribbon representation of the cUBA structure. The three helices are labeled as α 1, α 2, and α 3. (C) Ensemble of the C α traces representing a bundle of 15 representative structures of UBAb. (D) Ribbon representation of UBAb. The figures were prepared with MOLMOL.

flanked by coils at the two far ends, highly resembling the typical UBA fold (Mueller and Feigon 2002; Chang et al. 2006). The structure of cUBA is stabilized by a hydrophobic core comprising residues Leu7, Ile11, Leu14, Ile24, Leu28, Ile35, Ala38, and Leu42 (Fig. 3A). There is a type I turn near loop-1 (residues 16–20) and a type II turn within loop-2 (31–35). Its C-terminal tail (46–57), different from those of the known UBA domains, is not totally random. Residues 46–49 fold back to loop-1 through hydrophobic interactions and adopt an extended conformation. The side chains of residues Val46 and Ile48 face toward the hydrophobic core, and those of residues Ser47 and Ser49 are exposed to the solvent. This is also coincident with the results from the secondary chemical shifts of the backbone residues, and is evidenced by the NOE connections between Ile48 and the residues within loop-1.

The hydrophobic core of UBAb is composed of Val7, Ile11, Leu14, Val24, Leu28, Val35, Ala38, and Leu42 residues (Fig. 3B). The orientations of these hydrophobic residues, relative to the three helices, are nearly identical to those of cUBA. Dissimilar to that of cUBA, the C-terminal segment of UBAb is largely flexible, for most resonances of the nonproline residues in this region are missing. The angles between the axes of helix-1 and helix-2 for cUBA and UBAb are 132° and 120°, respectively, those between helix-2 and helix-3 are 123° and

131°, and those between helix-1 and helix-3 are 17.4° and 42.5° (Fig. 3C). In general, except for a little variation of the angles between helix-1 and helix-3, the secondary structure elements and the overall fold of these two UBA domains are roughly similar.

UBAb but not cUBA binds to the hydrophobic surface of Ub

To map the UBA recognition surface onto Ub, we performed [^1H , ^{15}N]-HSQC experiments with ^{15}N -labeled Ub alone and with an increasing amount of cUBA or UBAb domain. The titration data show that no significant chemical shift change occurs upon addition of cUBA at a cUBA/Ub molar ratio of 3:1 (Fig. 4A). But when Ub titrated with UBAb, some peaks such as Leu8, Leu43, Lys48, His 68, Val70, and Leu71 disappear gradually due to line broadening, and some peaks such as Gln40, Gln41, Leu50, Ile61, and Ser65 largely shift at a molar ratio of 2:1 (Fig. 4B). This indicates that the exchange rate between free and bound Ub is in an intermediate to fast exchange regime on the NMR timescale. The titration results for UBAb binding are mapped onto the surface diagram of the Ub molecule (Fig. 4C, PDB ID: 1UBQ) (Vijay-Kumar et al. 1987). As usual, the UBAb-binding site on Ub is formed by β -strands and a largely hydrophobic patch, which is the typical surface for Ub binding to UBA and other domains (Hurley et al. 2006).

The Ub-binding surface on UBAb is different from the counterpart on cUBA

We also performed the [^1H , ^{15}N]-HSQC experiments with ^{15}N -labeled cUBA or UBAb alone and with an increasing amount of Ub for mapping the Ub-interacting surfaces onto the UBA domains. Similarly, no significant chemical shift change in the spectra of cUBA domain is observed even when Ub is in twofold excess (Fig. 5A). However, when unlabeled Ub is titrated into ^{15}N -labeled UBAb, some resonance peaks of the residues in helix-1 (Asn6, Asp8, Ala9, Lys10, and Ala12), loop-1 (Gly16), and loop-2 (Ala31) disappear immediately, while some peaks of the residues in helix-1 (Leu14, Met15) and helix-2 (Glu22) undergo large peak shifting (Fig. 5B). The chemical shift perturbation data are also mapped onto the surface of UBAb (Fig. 5C,D). In this manner, the Ub-binding surface on UBAb is composed of the hydrophobic residues in helix-1 and part of loop-1 and helix-2. This binding mode is slightly different from the reported UBA binding in which the hydrophobic residues in helix-3 also contribute to the Ub binding (Chang et al. 2006). The residues involved in the UBAb-Ub interaction are mainly located at helix-1 and loop-1. Note that residue Ala31 is located

Table 1. *Experimental restraints and structural statistics of the cUBA and UBAb domains*

	cUBA (1–56)	UBAb (3–46)
No. of experimental restraints		
Total unambiguous distance restraints	754	569
Intra-residual	353	311
Sequential ($ i - j = 1$)	179	122
Medium ($2 \leq i - j \leq 4$)	151	87
Long range ($ i - j \leq 5$)	71	49
Hydrogen bond restraints	20	16
Dihedral angle restraints		
Φ	37	31
Ψ	37	31
Structural statistics		
Coordinate precisions ($\text{\AA}$) ^a , residues 6–45		
Backbone (N, C α , CO)	0.44 ± 0.13	0.76 ± 0.17
Heavy atoms	1.26 ± 0.19	1.72 ± 0.23
RMSD from experimental restraints		
NOE distances ($\text{\AA}$)	0.0222 ± 0.0011	0.0180 ± 0.0013
Dihedral angles ($^\circ$)	0.2804 ± 0.0170	0.0466 ± 0.0603
RMSD from idealized geometry		
Bond lengths ($\text{\AA}$)	0.0024 ± 0.0001	0.0016 ± 0.0001
Bond angles ($^\circ$)	0.4485 ± 0.0093	0.3142 ± 0.0058
Impropers	0.3731 ± 0.0099	0.2264 ± 0.0116
Energies (kcal/mol)		
E _{total}	-1947.7 ± 16.9	-1595.9 ± 48.6
Restraint violations		
Distances ($>0.1 \text{ \AA}$)	0	1
Dihedral angles ($>5^\circ$)	0	0
Hydrogen bonds	0	0
Ramachandran analysis ^b		
Residues in most favored regions	84.7	83.0
Residues in additionally allowed regions	14.2	14.2
Residues in generously allowed regions	0.6	0.8
Residues in disallowed regions	0.5	2.0

^aThe coordinate precision is defined as the average RMS deviation between the 15 refined structures and their mean coordinate positions.

^bException of Pro and Gly residues.

at loop-2 that connects helix-2 and -3; therefore the disappearance of this residue might be caused by the conformational change upon binding, rather than directly by the interaction with Ub itself. As for Lys10, though the amide resonance disappears during titration, it is impossible that this residue is involved in the direct interaction with Ub, because the side chain of Lys10 is directed toward the opposite side of the interaction surface. This result is also confirmed by the following experiments of mutagenesis and fluorescence titration (see below). Interestingly, the largely perturbed residues of UBAb, such as Ala9, Lys10, Ala12, and Gly16, upon Ub binding are located at helix-1 and these residues are different in cUBA (Fig. 1B).

The overall fold of these two UBA domains and the secondary structure arrangement are nearly the same. To investigate the differences in their affinities for Ub, we compared the electrostatic potential around the Ub-binding site of UBAb and the counterpart on cUBA (Fig. 6A,B).

The Ub-binding surface on UBAb is composed of predominately hydrophobic residues with some negatively charged residues at the surface edges, while the hydrophobic surface of the counterpart on cUBA is interrupted by an acidic residue Glu12 and lacks the negatively charged edges. Another key feature of the cUBA domain is that it contains a relatively ordered C-terminal stretch formed by residues Val46–Ser49 that extends toward loop-1. This extended stretch might, to some extent, contribute to the inability of cUBA to bind Ub.

Ala12 is crucial for specific binding of UBAb with Ub

The chemical shift perturbation data show that the binding of UBAb to Ub is predominately through hydrophobic interactions, and the differential binding abilities of cUBA and UBAb to Ub come from the different electrostatic potential distributions on the first helix. To confirm

Figure 3. Both cUBA and UBAb domain structures are stabilized by a hydrophobic core. (A) Hydrophobic core of cUBA. The side chains of the residues in the hydrophobic core are displayed in neon style (orange); these residues include Leu7, Ile11, Leu14, Ile24, Leu28, Ile35, Ala38, and Leu42. (B) Hydrophobic core of UBAb. The core residues are Val7, Ile11, Leu14, Val24, Leu28, Val35, Ala38, and Leu42. (C) Overlay of the secondary structure elements of cUBA (cyan) and UBAb (red).

this observation, we constructed several mutants for UBAb. The residues largely perturbed in Ub titration, which are assumed to play roles in binding to Ub, are mutated to their counterparts on cUBA (Fig. 1B). These mutants include K10E, A12E, G16S, A9S/K10E, A12E/G16S, and A9S/K10E/A12E/G16S. The Ub binding affinities of wild-type cUBA and UBAb and their several mutants were determined by fluorescence titration experiments. For the sake of fluorescence titration, we produced F45W, a fluorescent-containing mutant of Ub, which was thought to be virtually identical to the wild-type protein in terms of thermodynamic stability, solution structure, and biological activity (Khorasanizadeh et al. 1993; Laub et al. 1995). As expected, cUBA does not bind with Ub under the experimental condition, while UBAb binds with Ub relatively strongly (Fig. 7A). By fitting the titration curves, the dissociation constant (K_D) for UBAb binding with Ub is 53.1 μ M, being similar to that from ITC determination (Peschard et al. 2007). The Ub binding affinities for K10E, G16S, and A9S/K10E mutants of UBAb are roughly unchanged (Fig. 7B; Table 2). This suggests that the affinity difference for Ub binding between UBAb and cUBA is not chiefly caused by these residues (A9S, K10E, and G16S). However, Ala12-to-Glu mutations (A12E and other Glu12-containing mutants) completely abolish the binding ability of UBAb to Ub (Fig. 7B; Table 2). This finding strongly suggests that

Ala12 is critical for the interaction between UBAb and Ub. Ala12 lies in the center of a hydrophobic patch formed by residues Ala9, Ala12, Leu14, and Met15, so the binding is predominately through hydrophobic interactions. This is consistent with the electrostatic potential mapping of the two UBA domains (Fig. 6A,B) that the surface of helix-1 on cUBA is a bit negatively charged while that of UBAb is largely hydrophobic. We also generated the E12A mutant of cUBA and its C-terminal deletion mutant E12A(1–47), and tested their Ub binding affinities. However, these two mutations still cannot improve the binding affinity of cUBA with Ub (Table 2), probably because Ala12 is crucially required but not sufficient to Ub binding. Other residues also contribute to the hydrophobic surface for the overall binding environment.

Homodimerization has little effect on Ub binding

Both cUBA and UBAb readily form symmetric homodimers in solution, and it seems that cUBA forms a dimer more strongly than UBAb (Davies et al. 2004). The dimeric interfaces on these two UBA domains have been reported (Fig. 6C,D; Kozlov et al. 2007; Peschard et al. 2007), suggesting that the Ub-binding surfaces of Cbl UBA domains are roughly opposite to their dimerization interfaces. We generated two mutants, I30E/I41E of UBAb and E12A/I30E/I41E of cUBA, as it was reported that these mutations can disrupt dimerization of UBA (Kozlov et al. 2007; Peschard et al. 2007). The result shows that I30E/I41E mutant of UBAb retains the Ub binding ability, while E12A/I30E/I41E of cUBA still possesses undetectable Ub binding (Fig. 7C; Table 2). Gel filtration analysis reveals that the apparent molecular weight of the I30E/I41E mutant is lower than that of wild-type UBAb (Fig. 7D), corroborating that wild-type UBAb predominantly forms a dimer whereas the I30E/I41E mutant is a monomer. Taken together, we conclude that dimerization of the UBA domains have no significant effect on the Ub binding properties.

Discussion

Cbl proteins are a family of Ub E3 ligases that function as negative regulators of many tyrosine kinases. A truncated form of c-Cbl missing its UBA domain has been identified from the HUT78 T-lymphoma cells (Blake and Langdon 1992). Deletion of the cUBA domain abolishes the interaction of c-Cbl with APS, a Lnk family of adaptor protein that is tyrosine-phosphorylated by the insulin receptor, as well as the APS-mediated tyrosine phosphorylation of c-Cbl in response to insulin

Figure 4. The UBA domain binds to the hydrophobic surface of ubiquitin. (A) Overlay of a representative region of the $[\text{}^1\text{H}, \text{}^{15}\text{N}]$ -HSQC spectra of ^{15}N -labeled ubiquitin alone (red) and at a molar ratio of 1:3 with unlabeled cUBA (blue). (B) Overlay of the $[\text{}^1\text{H}, \text{}^{15}\text{N}]$ -HSQC spectra of ubiquitin alone (red) and in the presence of an equimolar amount of UBA (blue). UBA titration causes severe line broadening and/or peak shifting of certain amide resonances of ubiquitin. (C) Mapping the perturbed resonances of ubiquitin to its ribbon diagram upon titration of UBA. The residues with broadened resonances are displayed in green (L8, I13, T14, R42, L43, F45, K48, Q49, H68, V70, L71, and R72), and those with chemical shift change are colored in yellow (Q40, Q41, L50, I61, S65, R74). The opposite surface that does not contain perturbed residues is not shown in the figure.

(Liu et al. 2003). A variant of Cbl-b without the UBA domain has also been identified (Keane et al. 1995). Deletion of UBA can partially alter the ability of Cbl-b to down-regulate the activated EGFR (Ettenberg et al. 2001) and to ubiquitinate Met RTK (Peschard et al. 2007). In addition, UBA, but not cUBA, can mediate the association with ubiquitinated proteins and the UBA preferentially binds to polyubiquitin chains (Davies et al. 2004). Although cUBA does not bind Ub, it cannot be excluded that cUBA binds with other special UbL-domain-containing proteins. Further studies will be needed to identify the proteins that interact with the cUBA domain.

We have determined and compared the solution structures of cUBA and UBA. The overall fold of these two UBA domains is very similar, except for a difference in the angle between helix-1 and -3. We presume that the small change in the angle is unlikely to result in the differential binding properties of cUBA and UBA. Actually, our results from NMR chemical shift perturbation support the viewpoint that the helix-1 and loop-1

of UBA form a predominately hydrophobic surface, which is required for Ub binding (Figs. 5, 6). This is further confirmed by the combination of mutagenesis and fluorescence titration studies that show that the Ala12-to-Glu mutation of UBA completely abolishes the UBA-Ub interaction. By comparing the Ub-interacting surface on UBA and its counterpart on cUBA, we find that the hydrophobic part on cUBA is interrupted by a negatively charged residue Glu12 (Fig. 6A).

The UBA domains of c-Cbl and Cbl-b can mediate their homo- and heterodimerization *in vivo* (Bartkiewicz et al. 1999; Liu et al. 2003). Very recently, Gehring's group reported the mechanism by crystallographic studies underlying the Cbl dimerization that is mediated by the UBA domains (Peschard et al. 2007). Both cUBA and UBA form homodimers in crystal and probably in solution, and UBA is able to bind with Ub relatively strongly whereas cUBA is not. The results from our experiments can exclude the possibility that dimerization influences their Ub binding properties, for mutation of

Figure 5. The ubiquitin-interacting surface on UBA. (A) Overlay of a representative region of $[^1\text{H}, ^{15}\text{N}]$ -HSQC spectra of ^{15}N -labeled cUBA alone (red) and at a molar ratio of 1:2 with unlabeled ubiquitin (blue). (B) Overlay of the $[^1\text{H}, ^{15}\text{N}]$ -HSQC spectra of ^{15}N -labeled UBAb alone (red) and with ubiquitin at a molar ratio of 1:0.5 (blue). (C,D) Ribbon and side chain presentation (C) and surface display (D) of the surface of UBA domain that interacts with ubiquitin. The broadened resonances are displayed in green (N6, D8, A9, K10, A12, G16, and A31), and the shifted ones are colored in yellow (L14, M15, and E22).

the key dimerization residues (I30E/I41E) in cUBA, and UBAb has no significant impact on the Ub binding abilities of them (Fig. 7C). In addition, cUBA has an ordered C terminus; there is another possibility that this part prevents the binding of cUBA to Ub through steric hindrance effect. Again, the C-terminal deletion of cUBA does not improve Ub binding (Table 2). So far, there is still a possibility that, besides helix-1, other parts of UBA, like helix-2, may considerably contribute to the specific interaction. Nevertheless, we propose that Ub binding of the UBA domains is independent on their domain dimerization, and there are two distinct surfaces on UBAs, one for specific Ub binding predominantly in helix-1 and loop-1 regions, and the other for homo- or heterodimerization in the C-terminal region. Although cUBA does not bind Ub, it still has the possibility to bind other UbL-containing proteins, for the UBA domain of HOIP binds directly to the UbL domain of HOIL-1L but not to Ub (Kirisako et al. 2006). Further studies will be needed to identify the proteins that can interact with the cUBA domain.

The previous work suggested that helix-1 in UBA domains contributes to the Ub binding (Davies et al.

2004), and the present study provides a further insight into the differential Ub binding properties of the UBA domains. Combining the solution structural and biochemical information, we conclude that the alanine residue in the center of the hydrophobic patch of these UBA domains from Cbl proteins plays very important roles in the Ub binding specificity, but the domain dimerization, as ever unexpected, does not appear to have relevance to the binding property.

Materials and Methods

Sample preparation

The genes encoding cUBA (residues 851–906 of c-Cbl, numbered 1–56) and UBAb (residues 926–982 of Cbl-b, numbered 1–57) were chemically synthesized and cloned into pET-32M vector and expressed in *Escherichia coli* strain BL21(DE3) (Novagen). Site-directed mutagenesis was carried out on this vector via the regular PCR method. Cells were allowed to grow overnight in 5 mL of LB media at 37°C and subsequently transferred into 0.5 L of M9 minimal media containing $^{15}\text{NH}_4\text{Cl}$ and/or $^{13}\text{C}_6$ -glucose (Cambridge Isotope Laboratories, Inc.) for

Figure 6. Comparison of the electrostatic surfaces of cUBA (A) and UBAb (B). The orientation of both cUBA and UBAb is identical to that of panels C and D in Figure 4. The positive charges are shown in blue, and the negative charges are colored in red. The surface presentation shows that the Ub recognition surface on UBAb is predominately hydrophobic with positively charged edges while the corresponding surface on cUBA is something negatively charged in the center. The Ub recognition surface on UBAb and its counterpart on cUBA are highlighted by green parallelograms and labeled with amino acid residues. The dimeric interfaces (indicated by green circles) of cUBA (C) and UBAb (D) are rotated by $\sim 180^\circ$ relative to those in A and B, respectively. Residues I30 and I41 that are involved in homodimerization are labeled. The PDB codes for the structures are 2JUI for cUBA and 2JNH for UBAb. (E) Glutamic acid; (A) alanine; (I) isoleucine.

preparation of $^{13}\text{C}/^{15}\text{N}$ -labeled or ^{15}N -labeled proteins, respectively. The thioredoxin-His₆ tag fusion proteins were purified by Ni-NTA affinity chromatography (Qiagen), and the tags were removed by on-column cleavage with thrombin, leaving Gly-Ser residues at their N termini. The cleaved proteins were further purified by gel filtration (Superdex-75) using the AKTA FPLC system (Amersham Biosciences). The protein purities were $>95\%$ as judged by SDS-PAGE.

The construct pET-3a-Ub for expressing Ub was transformed into *E. coli* strain BL21(DE3). Cells containing the plasmid were grown at 37°C in M9 minimal media using $^{15}\text{NH}_4\text{Cl}$ as the sole nitrogen source. The expression of Ub was induced by adding IPTG to a final concentration of 0.2 mM when an OD_{600} reached ~ 0.6 . The protein was purified by acetic acid denaturation, ion exchange chromatography (SP XL16/10), and gel

filtration (Superdex-75). Fractions containing ^{15}N -labeled Ub were pooled together, dialyzed against water, lyophilized, and then stored at -20°C . The unlabeled Ub and its mutant F45W (Khorasanizadeh et al. 1993) were prepared by using LB media, following the similar purification procedure as that of the ^{15}N -labeled one.

NMR spectroscopy

The NMR experiments were carried out at 25°C on a Varian Unity INOVA 600-MHz spectrometer equipped with four RF channels and a triple-resonance pulsed-field gradient probe. The samples were prepared with 1 mM protein dissolved in 90% $\text{H}_2\text{O}/10\%$ D_2O buffer containing 20 mM sodium phosphate (pH 6.5), 50 mM NaCl, and 1 mM DTT. All the NMR spectra were processed using NMRPipe (Delaglio et al. 1995) and analyzed using Sparky (T.D. Goddard and D.G. Kneller). Two-dimensional ^{15}N -edited HSQC and three-dimensional CBCA(CO)NH, HNCACB, HNCO, HNHA, (H)CC(CO)NH-TOCSY, and HCCH-TOCSY experiments were performed to obtain the chemical shift assignments of the backbone and side-chain atoms. The three-dimensional ^{15}N - and ^{13}C -edited NOESY-HSQC spectra were collected to confirm the chemical shift assignments and generate distance restraints for structure calculations.

Structure calculation

The structure calculations were performed using the program CNS (Brunger et al. 1998) with the ARIA2 module (Rieping et al. 2007). Distance restraints were derived from inter-proton NOEs. Dihedral angles (ϕ and ψ) were calculated from backbone chemical shifts using the program TALOS (Cornilescu et al. 1999). Hydrogen bond restraints were obtained using the secondary structural information from the program CSI (Wishart and Sykes 1994) and confirmed by intermediate-range NOEs. For both of these domains, 200 structures were calculated in the last iteration and the 15 lowest-energy structures were selected for refinement in water, respectively. The final structures were analyzed using the program packages MOLMOL (Koradi et al. 1996) and PROCHECK (Laskowski et al. 1996).

NMR titration experiments

The UBA-ubiquitin titration experiments were monitored by a series of two-dimensional ^{15}N -edited HSQC experiments. The buffering systems were the same as for recording three-dimensional spectra. During the titration, the molar ratios of UBA/Ub and Ub/UBA were gradually increased from 0.5:1 to 3:1. The spectra were also recorded on a 600-MHz spectrometer at 25°C .

Fluorescence titration experiments

For preparing samples for fluorescence titration experiments, the two UBA domains and their mutants were expressed in

Figure 7. Affinities of the UBA domains and their mutants for Ub. (A) Titration curves for the binding of wild-type cUBA and UBAb to Ub F45W. The relative fluorescence intensity change (Δ RFI) of the F45W mutant of Ub is plotted against the concentration of the UBA domains. (B) Titration curves for the binding of UBAb mutants to Ub F45W. (C) Titration curves for the binding of I30E/I41E mutant of UBAb and E12A/I30E/I41E of cUBA to Ub F45W. (D) Analytical gel filtration of UBAb and its I30E/I41E mutant. The I30E/I41E mutant of UBAb gives an elution volume larger than that of the wild type.

LB media and purified using the same method as above. Fluorescence measurements were performed on a HITACHI F-4010 fluorescence spectrometer at room temperature. The concentration of F45W ubiquitin was 15 μ M in a square 3-mL quartz cuvette. The excitation wavelength was 285 nm (5-nm band pass), and the emission was observed at 353 nm (10-nm band pass). The molar ratios of UBA/F45W were gradually increased from 0.2:1 to 8:1. The dissociation constants for UBA-Ub complexes were calculated by fitting the data sets with the single binding site model (Hu et al. 2004).

Analytical gel filtration

Analytical gel filtration was performed on a Superose-12 HR 10/30 column (Pharmacia Biotech) with a flow rate of 0.5 mL/min and a sample injection of 0.1 mL. The elution buffer for the analysis was 20 mM sodium phosphate (pH 6.5), 50 mM NaCl, and 1 mM DTT; the protein concentrations of the samples were 6–10 mg/mL. The resolution volume of the elution profile is <0.1 mL.

Protein Data Bank accession codes

Structural coordinates have been deposited in the RCSB Protein Data Bank and are available under the accession code 2JUU

Table 2. Dissociation constants (K_D) for the binding of cUBA, UBAb and their mutants to Ub

UBA	K_D (μ M)
UBAb: WT	53.1 $\pm$ 3.1
K10E	39.3 $\pm$ 3.7
A9S/K10E	69.7 $\pm$ 5.2
A12E	>10,000
G16S	79.0 $\pm$ 12.4
A12E/G16S	>10,000
A9S/K10E/A12E/G16S	>10,000
I30E/I41E	85.8 $\pm$ 12.9
cUBA: WT	>10,000
E12A	>10,000
E12A(1–47)	>10,000
E12A/I30E/I41E	>10,000

Each value is the average of three independent experiments. Data are presented as mean $\pm$ MD.

for c-Cbl UBA (cUBA) and 2JNH for Cbl-b UBA (UBAb), respectively.

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