Mechanism of Ubiquitin Recognition by the CUE Domain of Vps9p

Gali Prag, Saurav Misra, Eudora A. Jones, Rodolfo Ghirlando, Brian A. Davies, Bruce F. Horazdovsky, and James H. Hurley

Summary

Coupling of ubiquitin conjugation to ER degradation (CUE) domains are ~50 amino acid monoubiquitin binding motifs found in proteins of trafficking and ubiquitination pathways. The 2.3 Å structure of the Vps9p-CUE domain is a dimeric domain-swapped variant of the ubiquitin binding UBA domain. The 1.7 Å structure of the CUE:ubiquitin complex shows that one CUE dimer binds one ubiquitin molecule. The bound CUE dimer is kinked relative to the unbound CUE dimer and wraps around ubiquitin. The CUE monomer contains two ubiquitin binding surfaces on opposite faces of the molecule that cannot bind simultaneously to a single ubiquitin molecule. Dimerization of the CUE domain allows both surfaces to contact a single ubiquitin molecule, providing a mechanism for high-affinity binding to monoubiquitin.

Introduction

The covalent addition of ubiquitin and ubiquitin-like proteins is one of the most widespread regulatory post-translational modifications of proteins (Hershko and Ciechanover, 1998; Hochstrasser, 2000; Pickart, 2001). Ubiquitin is a 76 amino acid protein named for its extraordinary distribution from yeast to man. The C terminus of ubiquitin is conjugated to Lys residues of target proteins by the action of three enzymes: an ubiquitin-activating enzyme (E1), an ubiquitin-conjugating enzyme (E2), and an ubiquitin protein ligase (E3).

Ubiquitin is conjugated to proteins via an isopeptide bond between the C terminus of ubiquitin and a specific Lys residue in the ubiquitinated protein. Ubiquitin may be attached to proteins as a monomer or as a polyubiquitin chain. Ubiquitin polymers are formed when additional ubiquitin molecules are attached to Lys residues on a previously attached ubiquitin, usually Lys-48. Historically, interest in ubiquitination has centered on the role of polyubiquitin chains in targeting proteins for degradation by the 26S proteasome. It is now clear that ubiquitination regulates a much wider array of cell processes, including cell-cycle control, stress response, DNA repair, signaling, transcription, and gene silencing. Recently, intense interest has centered on the role of monoubiquitination as a sorting signal and regulator of endocytosis and endocytic trafficking, spurred in part by the discovery of new monoubiquitin recognition motifs (Shih et al., 2000; Katzmann et al., 2001; Hicke, 2001; Pickart, 2001; Bonifacino and Traub, 2003).

Many ubiquitinated proteins are recognized by domains that specifically bind to mono- and/or polyubiquitin. These include the UEV, UBA, UIM, and CUE domains or motifs (reviewed in Buchberger, 2002). The UEV domain is a counterpart of a ubiquitin-conjugating enzyme, as illustrated by the structure of the enzymatically inactive TSG101 UEV domain (Pornillos et al., 2002). The other three prominent ubiquitin binding domains, UBA, UIM, and CUE, are all small (20–50 amino acids) and are known or predicted to be α-helical. The ubiquitin binding UBA domain is found in DNA damage-inducible and other proteins (Hofmann and Bucher, 1996). UBA domains are three-helix bundles (Dieckmann et al., 1998; Withers-Ward et al., 2000; Mueller and Feigon, 2002) that can bind polyubiquitin with high affinity, and their physiological functioning is in at least some cases mediated by polyubiquitin binding (Wilkinson et al., 2001; Funakoshi et al., 2002). UBA domains also bind mono-ubiquitin (Vadlamudi et al., 1996; Bertolaet al., 2001b; Chen et al., 2001), bind to other proteins (Dieckmann et al., 1998; Withers-Ward et al., 2000), and form homodimers with each other (Bertola et al., 2001a). The UIM (ubiquitin interaction motif; Hofmann and Falquet, 2001) is a ubiquitin binding motif discovered in the proteasome subunit S5a. The S5a UIM binds polyubiquitin, but not monoubiquitin (Deveraux et al., 1994). In contrast, UIMs of many endocytic proteins bind monoubiquitinated proteins. The endocytic proteins eps15, Vps27p/Hrs, and Hse1p contain UIMs that bind monoubiquitin (Bilodeau et al., 2002; Klapiz et al., 2002; Oldham et al., 2002; Polo et al., 2002; Raiborg et al., 2002; Shih et al., 2002; Shekhtman and Cowburn, 2002). The UIMs of eps15 are essential for the ubiquitination stdep15 itself, representing a second function for eps15. Furthermore, interest in ubiquitination has centered on the role of polyubiquitin chains in targeting proteins for degradation by the 26S proteasome. It is now clear that ubiquitination regulates a much wider array of cell processes, including cell-cycle control, stress response, DNA repair, signaling, transcription, and gene silencing. Recently, intense interest has centered on the role of monoubiquitination as a sorting signal and regulator of endocytosis and endocytic trafficking, spurred in part by the discovery of new monoubiquitin recognition motifs (Shih et al., 2000; Katzmann et al., 2001; Hicke, 2001; Pickart, 2001; Bonifacino and Traub, 2003).
Little is known about mechanisms of ubiquitin recognition by the downstream effectors of ubiquitin signaling, and no structures of ubiquitin complexed to ubiquitin binding domains have been reported. The mechanism of discrimination between mono- and polyubiquitin is unknown. In order to shed light on this mechanism, we have obtained the crystal structure of the CUE domain of Vps9p alone and in complex with ubiquitin. On the basis of these structures and their functional analysis, we propose a mechanism for the differential recognition of monoubiquitin and polyubiquitin.

Results and Discussion

The Vps9p CUE Domain Is a Helical Dimer

The structure of the G440E mutant of the CUE domain of Vps9p (residues 394–451) was determined by multi-wavelength anomalous dispersion (MAD) using the signal from the two SeMet residues and refined at 2.3 Å resolution (Figure 1A and Table 1). The G440E mutation was obtained as an artifact of the PCR reaction used to subclone the CUE domain into the protein expression system for crystallization.
Table 1. Crystallographic Data, Phasing, and Refinement Statistics

Data Collection and Phasing

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<td>c = 61.38</td>
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<td>35.6 (6.2)</td>
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<td>8.6 (40.8)</td>
<td>7.2 (28.5)</td>
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<td>FOM - RESOLVE</td>
<td>0.57 (0.17)</td>
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Refinement

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<td>Residues in core φ–γ region</td>
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1 Statistics are shown for the peak wavelength of the Selenomethionine MAD datasets. Statistics for the inflection wavelength (0.97924 Å for apo and 0.97931 for complex) and remote wavelength (0.95645 Å for apo and complex) were very similar.

The structure consists of three α helices and two connecting loops (Figure 1B). Helices α2 and α3 are antiparallel to the longer helix α1. The Vps9p CUE domain forms extensive dimer contacts across a crystallographic two-

Table 2. Mutational Analysis of CUE Function

<table>
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<tr>
<th>Sample</th>
<th>Interface¹</th>
<th>Apparent Kd (μM)²</th>
<th>Ubiquitination³</th>
<th>Puncta per Cell⁴</th>
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<tr>
<td>Vps9-CUE wild-type</td>
<td></td>
<td>20 ± 1</td>
<td>100</td>
<td>3.0 ± 1.7</td>
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<tr>
<td>M419D</td>
<td>mono/dimer</td>
<td>NB</td>
<td>12</td>
<td>7.9 ± 2.5</td>
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<td>F420D</td>
<td>mono/dimer</td>
<td>NB</td>
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<td>8.0 ± 2.8</td>
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<tr>
<td>L427D</td>
<td>dimer</td>
<td>171 ± 14</td>
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<td>8.9 ± 2.6</td>
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<tr>
<td>D430A</td>
<td>dimer</td>
<td>71 ± 8</td>
<td>64</td>
<td>4.8 ± 2.5</td>
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<tr>
<td>K435A/K436A</td>
<td>dimer</td>
<td>60 ± 6</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>G440E</td>
<td>dimer</td>
<td>26 ± 2</td>
<td>ND</td>
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<td>C442T</td>
<td>none</td>
<td>34 ± 1</td>
<td>ND</td>
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<td>V443D</td>
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<td>9</td>
<td>8.3 ± 2.8</td>
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<tr>
<td>D444A</td>
<td>mono/dimer</td>
<td>61 ± 6</td>
<td>65</td>
<td>6.6 ± 2.4</td>
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<td>L447E</td>
<td>mono/dimer</td>
<td>133 ± 8</td>
<td>29</td>
<td>8.1 ± 2.5</td>
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¹ Mono/dimer indicates mutants that are in the α1/α3 interface common to the monomer and dimer; dimer indicates mutants that are in the α2 interface unique to the dimer; none, C442T is a control mutant for disulfide formation and not directly part of an interface.

² ITC measurements. NB, no detectable binding within a limit of ~500 μM; ND, not determined.

³ Percent wild-type in vivo ubiquitination levels, quantified with a UVP bioimaging system.

⁴ >50 cells scored per data point.
fold axis (Figure 1B). Helix $\alpha 3$ of one monomer nests between $\alpha 1$ and $\alpha 2$ of its symmetry-related mate. The interface buries 780 Å² of solvent-accessible surface area from each monomer. The dimer in the crystal is bridged by disulfide bonds between Cys-432 and Cys-442.

Sedimentation equilibrium centrifugation was used to determine whether Vps9p-CUE is a dimer in solution. An excellent fit was obtained to a reversible monomer-dimer equilibrium (Figure 1E) with a $K_d$ (dimerization) of 1 mM. The mutation C442T was engineered to prevent intermolecular disulfide bond formation and has near wild-type affinity for ubiquitin (Table 2). The sedimentation behavior of the C442T mutant was identical to that of wild-type (data not shown), indicating that the dimer is not an artifact of oxidation.

**A UBA-like Fold for the CUE Domain**

Despite negligible sequence identity (17% with Rad23 UBA[1]), the CUE domain structure revealed significant structural homology with the UBA domain. The first two helices of the apo CUE domain can be superimposed on the corresponding helices of the UBA domains of Rad23 (Figure 1C). The rmsd for 22 Cα positions (excluding $\alpha 3$) is 2.1 Å for the CUE domain with Rad23-UBA[1] and 1.8 Å for Rad23-UBA[2]. The UBA domains have a hydrophobic motif $\Phi$GAR (where $\Phi$ is hydrophobic and AR is aromatic) at the end of $\alpha 1$ in the UBA structures (Figure 1C) that coincides with the critical MFP motif of the CUE domain (Figure 1D). This hydrophobic site corresponds to the Vpr binding site in Rad23 UBA domains, as shown by NMR chemical shift perturbations (Dieckmann et al., 1998; Withers-Ward et al., 2000; Mueller and Feigon, 2002), and the ubiquitin binding site of the Rad23-UBA domain(s) as judged by mutational analysis (Bertolaet et al., 2001b). Taking the structural and functional similarities together, CUE and UBA can be grouped into a superfamily of three-helical ubiquitin binding domains.

**The Vps9p CUE Domain Dimerizes by Domain Swapping**

The $\alpha 3$ helix of one CUE monomer interacts with $\alpha 1$ and $\alpha 2$ of its partner using interactions equivalent to those of $\alpha 3$ of the UBA monomer with $\alpha 1$ and $\alpha 2$. The CUE domain undergoes a monomer:dimer equilibrium in solution. We assume that in solution, the unliganded CUE monomer is in a closed, globular conformation similar to the UBA domain. We have modeled the structure of...
this closed monomer, assuming that the observed dimer is the product of domain swapping (Liu and Eisenberg, 2002), in which the two monomers have exchanged their α3 helices. The model was constructed using the program Swiss-Pdb Viewer (Guex and Peitsch, 1997). The loop connecting helices 2 and 3 of one monomer was deleted and rebuilt to connect helix 2 of one monomer and helix 3 of the other. In this model, all three helices of the CUE and UBA domains are superimposable (Figure 1C). The hydrophobic core packing interactions in the closed CUE monomer and UBA cores are equivalent (Figure 1D). Domain-swapped oligomers such as CUE that do not have a known closed monomer structure but do have a homolog with a closed monomer structure are classified as quasi-domain swapped (Liu and Eisenberg, 2001).

Structure of the CUE Dimer:Ubiquitin Complex

The structure of the K435A/K436A mutant of the Vps9p-CUE domain in complex with ubiquitin was determined by MAD phasing from SeMet CUE protein crystallized in complex with native bovine ubiquitin and refined to a resolution of 1.7 Å (Figure 2A, Table 1). The K435A/K436A mutant binds ubiquitin (Table 2) and was engineered using the strategy of Derewenda and coworkers (Longenecker et al., 2001) to facilitate crystallization of the Vps9p-CUE complex after crystallization of the wild-type complex failed.

The crystallized complex contains two ubiquitin molecules and two CUE domains per asymmetric unit. The two ubiquitin molecules are well ordered, with the exception of two and three mobile residues in their respective C termini. The two molecules are superimposable on the structure of ubiquitin crystallized alone with rmsd values of 1.1 and 0.9 Å for all Cα positions (Vijay-Kumar et al., 1987; pdb entry 1ubq). One of the CUE domain monomers is ordered from residues 408–437 and partially ordered in residues 440–444 (Figure 2B). The second CUE monomer is ordered from 416–451, where the prime (′) indicates the second CUE chain. There are residual positive difference density features in Fo-Fc syntheses that are uninterpretable as an atomic model but probably represent partially disordered regions. These features may explain the relatively high free R factor (Table 1). The Vps9p CUE domain in the crystallized complex is a domain-swapped dimer, but the ubiquitin-bound CUE dimer is not disulfide bonded.

The bound CUE dimer undergoes a dramatic conformational change (Figure 3) relative to the apo structure. Residues 398–431 and 437–451 belong to a rigid core that shifts by only 1.2 Å rmsd (Cα positions). With these residues used for reference, α3 moves 28 Å (Cα of 444), and the N terminus of α2 moves 21 Å (Cα of 424). Residues 434–437 form an extended coil that pivots to allow a large movement between α2 and α3. The entire α1/α2′ unit rotates by roughly 180° relative to its orientation in the apo structure. The conformational changes in the CUE dimer bend it 122° into the rod-like apo structure, pushing it into the shape of a basket with an opening 36 Å across, 16 Å wide, and 8 Å deep.

The CUE dimer wraps itself partway around one of the two ubiquitin molecules in the asymmetric unit (Figures 2B and 2C). The interface buries 654 Å² of solvent-accessible surface area each from the ubiquitin and the CUE dimer. The CUE domain binds to a hydrophobic patch on ubiquitin defined by Leu-8, Ile-44, and Val-70 identified as a binding site for the proteasome, UIMs, and UBA domains (Beal et al., 1996; Shih et al., 2000; Siöger-Mould et al., 2001; Shih et al., 2002). Ile-44 was shown by mutational analysis to bind to the Vps9p CUE domain (Shih et al., 2003), consistent with our structural observations.

The first highly conserved motif is the sequence MFP and Phe-420 directly bind to ubiquitin residues Ile-44, Ala-46, Gly-47, His-68, Leu-69, and Val-70 (Figures 2D and 2E). Pro-421 serves as a helix-breaker at the end of α1, and contacts Ala-46 and Gly-47 of ubiquitin. The C-terminal conserved motif in the CUE domain consists of φxx/I/V/L, where φ is a large hydrophobic residue. In Vps9p, the first position φ corresponds to Val-443, and the latter two positions correspond to Leu-446′ and Leu-447′. Val-443′ interacts with ubiquitin Arg-42, Ile-44, and Val-70. Leu-447′ interacts with ubiquitin Arg-42, Gly-47, Lys-48, and Gin-49. Leu-446′ is buried in the center of the hydrophobic core and is required for stability, rather than binding. A salt bridge is formed between Glu-444′ and ubiquitin Arg-42, and there is a hydrogen bond between Asn-418 and His-68.

The MFP and C-terminal conserved motifs bind the same surface of ubiquitin, surrounding Ile-44, but the third conserved motif interacts with the opposite side of the interface, around Leu-8, Ile-36, and Leu-73 (Figures 2F and 2G). In the CUE domains, the central portion of the α2 helix contains the conserved sequence (I/L/V)xxL. The first conserved position in this sequence corresponds to Ile-428 in Vps9p. Ile-428 is a key residue in the α1/α2 packing interface, making contact with Leu-413 and Leu-416 on α1. The second conserved position, which is a Leu in nearly all other CUE domains, is occupied by Cys-432 in Vps9p. In the CUE dimer complex, ubiquitin interacts with α2′. The two strongest hydrophobic interactions in this site are made by Leu-427′ and Val-431′. Leu-427′ interacts with ubiquitin Glu-34, Gly-35, and Ile-36, while Val-431′ interacts with Leu-71. Additional hydrophobic contacts in this site are made between Ile-433′ and ubiquitin Leu-8, and between Ala-434′ and Leu-71. The Cβ of Ala-435′, which replaces Lys-435 in wild-type, makes contact with Leu-73. The aliphatic moiety of the Lys side chain in wild-type is predicted to make more extensive interactions. The loss of these interactions would explain the reduced affinity of K435A/K436A for ubiquitin (Table 2, Figure 5A). Asp-430′ interacts with Thr-9. The α2′ contact residues are less conserved than the MFP and LL motifs, so this contact may be a feature of the high-affinity monoubiquitin binding subclass of CUE domains, rather than all CUE domains.

The last contact region is formed by part of the linker between the domain-swapped monomers (residues 438′–440′). The side chain of Arg-438′ approaches the C terminus of the ordered part of ubiquitin at Leu-73. Ile-439′ makes a hydrophobic contact with Val-70, and Gly-440′ contacts Arg-42 and Val-70. The domain-swapped conformation allows this linker region to wrap halfway around the C terminus of ubiquitin.
The CUE Dimer Interface Is Required for High-Affinity Ubiquitin Binding

To determine the relative contributions of the CUE monomer and dimer to ubiquitin binding, we modeled the interaction of ubiquitin with a closed CUE monomer (Figure 3A). The CUE monomer can interact with ubiquitin through α1 and α3, which contain the MFP motif (Met-419 and Phe-420) and the LL motif (Leu-447). The monomer interface also includes Val-443 and Asp-444. The surface of α2, which includes Leu-427, Asp-430, and Val-431, is on the side of the CUE monomer that faces away from ubiquitin and has no interactions with it. Several ubiquitin hydrophobic residues (e.g., Leu-8 and Leu-73) that are functionally important in ubiquitin-dependent internalization (Sloper-Mould et al., 2001) make no contact with the monomer. These residues are only buried in the dimer interface. The monomer buries 419 Å² of solvent-accessible surface area from each partner.

Mutations were constructed to test which of the two faces of the CUE domain were involved. Several mutants were made in which surface hydrophobic residues were replaced with acidic residues. The mutant proteins M419D, F420D, L427D, D430A, D444A, and L447E were stable enough to be expressed and purified at wild-type levels. The interactions between all of these mutant proteins and ubiquitin were measured by isothermal titration calorimetry (ITC). The α1/α3' interface mutants M419D and F420D had undetectable binding by ITC (Table 2, Figure 5A). The two other mutants in α1/α3' interface residues, D444A and L447E, reduced, but did not abolish, binding. Mutants in the α2' interface, L427D and D430A, both reduced binding. The hydrophobic mutant L427D produced the strongest effect, a nearly 10-fold reduction in affinity.

The mutational analysis shows that both interaction surfaces of the CUE domain are required for strong binding to ubiquitin. The most disruptive mutations in the α1/α3' interface abolish binding completely. This is consistent with the conservation of these residues and previous mutational analysis. This finding rules out the binding of a CUE monomer to ubiquitin via α2 of the monomer, since in this model, the MFP and LL motifs do not contact ubiquitin. Disruption of the α2' interface reduces binding by roughly 10-fold. This finding leads us to conclude that a CUE monomer is capable of binding to ubiquitin via an interface formed by its α1 and α3 helices. It also sets an upper limit of ~170 μM to the affinity of the monomer for ubiquitin. The low affinity of the monomer for ubiquitin is consistent with the small amount of solvent-accessible surface area buried in the interface. Because disruption of either interface greatly impairs or abolishes ubiquitin binding, we conclude that both interfaces are required for high-affinity binding. The surface area buried in the combined interface is consistent with the high affinity of CUE for ubiquitin determined from sedimentation analysis (see below).
Both interfaces can be presented to a single ubiquitin molecule in the dimer, but not in the monomer. The only reasonable explanation for the mutational data is that the CUE monomer is capable of binding ubiquitin with low affinity, but the dimer is the only form of the CUE domain capable of binding with high affinity.

The Vps9p CUE Domain Functions as a Dimer In Vivo

In order to assess the role of the CUE dimer interface in vivo, the CUE interface mutants were incorporated into intact Vps9p and assessed in vivo in yeast (Figures 5B and 5C). One function of the Vps9p CUE is to promote ubiquitination of Vps9p itself (Davies et al., 2003; Shih et al., 2003). Mutations in the interface common to both the monomer and dimer either blocked (M419D, F420D, V443D) or reduced (D444A, L447E) ubiquitination. Mutations in the α2 interface, which is only formed in the dimer, either completely blocked (L427D) or sharply reduced (D430A) ubiquitination. The rank order of the effects of the mutants on ubiquitination in vivo and direct binding in vitro are nearly identical (Table 2). The Vps9p-CUE is required for the efficient endocytosis of the mating factor receptor Ste3p. Analysis of a Ste3-GFP reporter indicated a partial defect in Ste3p trafficking with the increased appearance of perivacuolar puncta in yeast expressing Vps9p alleles bearing the CUE domain mutations described above (Table 2; Figure 5C). Mutations at either the monomer/dimer common ubiquitin binding interface or the unique ubiquitin binding interface of the dimer interfere with Vps9p function in vivo to similar degrees. Thus the entire ubiquitin binding interface of the CUE domain dimer is required for in vivo function, not just the portion of the interface present in the CUE monomer.

We tested whether Vps9p-CUE was capable of dimerizing in vivo and whether interactions between other portions of Vps9p could affect the stability of the dimer in vivo using the yeast two-hybrid system (Figure 5D). A collection of bait and prey fusions were constructed that expressed various portions of Vps9p, and interaction was scored using a β-galactosidase reporter system (see Experimental Procedures). Prey fusions that encoded full-length Vps9p (residues 1–451) interacted into intact Vps9p and assessed in vivo in yeast (Figures 5B and 5C). One function of the Vps9p CUE is to promote ubiquitination of Vps9p itself (Davies et al., 2003; Shih et al., 2003). Mutations in the interface common to both only the Vps9 CUE domain (residues 408–451). Additionally, the bait fusion containing only the CUE domain also interacted with a prey fusion that contained only the CUE domain, indicating that the CUE domain robustly interacts with itself in vivo. These results support the conclusion that the Vps9p CUE domain dimerizes in vivo.

Quantitation of CUE Monomer and Dimer Complexes with Ubiquitin in Solution

We sought to determine whether ubiquitin complexes with monomers and dimers could be detected in solution using sedimentation equilibrium centrifugation. To test the role of the dimer interface in complex formation, studies were executed in parallel on wild-type and on the α2 interface mutant L427D. The L427D mutant was predicted to destabilize the ubiquitin complex with the CUE dimer, but not with the CUE monomer. In order to
Figure 5. Functional Analysis of the CUE Dimer

(A) ITC titration curves for: •, wild-type; 1, M419D; ○, F420D; △, L427D; ◊, D430A; □, K435A, K436A; ▲, G440E; ○, C442T; ▼, D444A; and ▼, L447E. Inset: representative experimental ITC trace. The differential heat signal from injection of 4.0 mM ubiquitin into 200 μM wild-type Vps9p CUE domain is shown (after subtraction of data from injection of ubiquitin into a buffer blank).

(B) Lysates were generated from Δvps9 (lane 1) and Δvps9 yeast strains expressing wild-type and mutant alleles encoded on a plasmid. Western analysis with Vps9p antiserum was performed, and the sizes of the unmodified and ubiquitinated forms of Vps9p are indicated.

(C) Microscopic analysis of Ste3-GFP (green) in ΔVps9 (ΔVps9) and ΔVps9 yeast strains expressing wild-type (WT), L427D, or M419D alleles. The vacuolar limiting membrane is labeled with FM4-64 (red), and the perimeter of the cell was visualized with blue light.

(D) L40 yeast that were cotransformed with the indicated bait and prey plasmids were grown on selective media, transferred to a nitrocellulose, lysed, and the presence of β-galactosidase was determined using a colorimetric filter assay.

(E–G) Sedimentation equilibrium profiles at 280 nm and 20.0°C. (E) A 1:1 mixture of CUE and ubiquitin fit to a noncooperative interaction (equation [2]), illustrating that wild-type CUE is not properly fit by this model. (F) A 2:1 mixture of CUE and ubiquitin fit to a cooperative interaction (equation [1]). (G) A 1:1 mixture of L427D CUE and ubiquitin fit to a noncooperative interaction (equation [2]). Symbols correspond to data collected at 24,000 (circles, shifted by +0.2 A280), 26,000 (triangles, shifted by +0.2 A280), and 28,000 (squares, shifted by +0.2 A280) rpm.

model the data without overfitting, two simplified models were constructed. In one model, the interactions in the dimer are cooperative and in the other, they are noncooperative. Both models contain only one adjustable parameter, $K_0$, which represents the association constant for the formation of a 1:1 CUE:ubiquitin complex.
plex (CU). In the cooperative model, the CUE dimer interacts with ubiquitin in only one manner such that the free energy of this 2:1 complex formation is twice that for 1:1 complex formation. In the noncooperative model, the free energies of formation of the C\textsubscript{2}U and U\textsubscript{2}C complexes from the CUE dimer and free ubiquitin are identical to that for 1:1 complex formation. Data for the wild-type CUE did not fit the noncooperative model (Figure 5E), but excellent fits to the cooperative model were obtained (Figure 5F). The fit to the data yields a wild-type CUE:ubiquitin \( K_d \) value of 1.2 \( \mu \text{M} \) (within error, 0.5–3.0 \( \mu \text{M} \)) and a wild-type monomer:ubiquitin \( K_d \) value of 1.1 \( \mu \text{M} \) (within error, 0.7–1.9 \( \mu \text{M} \)). Complexes with the L427D CUE:ubiquitin mixture gave an excellent fit to the noncooperative model (Figure 5G), with a CUE:ubiquitin \( K_d \) value of 3 \( \mu \text{M} \) (within error, 2–4 \( \mu \text{M} \)); the \( K_d \) values for the L427D mutant monomer and dimer are identical given the noncooperative nature of the model. The low \( \approx 1 \) \( \mu \text{M} \) affinity of the CUE monomer agrees well with the limit of \( \approx 170 \) \( \mu \text{M} \) derived from ITC of mutants. The high \( \approx 1 \) \( \mu \text{M} \) affinity of the Vps9p CUE dimer for a single ubiquitin monomer would be consistent with a physiological function for the dimer in monoubiquitin recognition.

Lattice Contacts Explain Multiple Binding Modes

The presence of two ubiquitin monomers per CUE dimer in the crystal was surprising, since the CUE dimer wraps around only one of the two ubiquitin molecules. Lattice contacts with the other ubiquitin molecule show that the same residues that make specific contacts with the primary ubiquitin are used by the second ubiquitin molecule to hold the crystal lattice together. The hydrophobic surface surrounding Leu-8, Ile-44, and Val-70 of the second ubiquitin molecule forms lattice contacts with the convex “back” sides of two different CUE dimers in the crystal (Figure 6). Each CUE dimer has two “left-over” hydrophobic contact sites that do not interact with the primary ubiquitin. Because the CUE dimer is bent to wrap around the primary ubiquitin molecule in a concave manner, the side of the CUE dimer opposite to the primary ubiquitin contact is convex in shape. The convex shape makes it impossible for the back side of the dimer to wrap around a second ubiquitin. The Ile-44 region of the second ubiquitin contacts the dimer-related \( \alpha_2 \) surface of one CUE dimer, burying 608 \( \AA^2 \) of solvent-accessible surface area. The Ile-36/Leu-73 region of the same ubiquitin molecule contacts the dimer-related \( \alpha_1 / \alpha_3 \) interface of a different CUE dimer in the crystal lattice, burying 263 \( \AA^2 \) of solvent-accessible surface area. Taken together, the two separate surfaces on the back side of two separate lattice-related CUE dimers form a hydrophobic basket. The basket is a remarkable mimic of the MFP LL surface and the dimer-related \( \alpha_2 \) surface are exposed and can interact with additional ubiquitin molecules with low affinity. These interactions give rise to the 1:1 CUE:ubiquitin stoichiometry in the crystal, the apparent 1:1 binding of CUE to ubiquitin by ITC, and the presence of 2:2 complexes in solution as judged by the sedimentation equilibrium analysis. The presence of multiple complexes in solution explains why the CUE (monomer + dimer):ubiquitin \( K_d \) value of 20 \( \mu \text{M} \) obtained by ITC is higher than the CUE dimer:ubiquitin \( K_d \) obtained by fitting the sedimentation data. The heat released in the ITC experiment represents all binding modes, while the sedimentation analysis treats binding of the CUE monomer and dimer as separate events.

Mechanism for Recognition of Monoubiquitin and Polyubiquitin

It is widely believed that monoubiquitin recognition is a physiological function of the Vps9p-CUE domain. Never-
theless, Vps9p-CUE also binds polyubiquitin in vitro (Shih et al., 2003). Since the terminal ubiquitin in a polyubiquitin chain is equivalent to a monoubiquitin modification, monoubiquitin binding domains must bind polyubiquitin. The converse does not apply, since a ubiquitin monomer offers fewer potential interaction surfaces than a polymer. The challenge in monoubiquitin recognition is for a targeting domain to achieve an affinity high enough to bind a monoubiquitinated protein at physiological concentrations.

The structural analysis shows how different members of a family of related domains could interact with either mono- or polyubiquitin, using the same underlying molecular interfaces in different oligomerization states. Differentiation of the CUE domain allows both interaction surfaces of the CUE domain to be presented simultaneously to a single ubiquitin monomer (Figure 6G). This leads to an extensive contact surface between the ubiquitin monomer and the CUE dimer that would not be possible if both molecules were monomers. This suggests an elegant hypothesis for mono- versus polyubiquitin recognition in which the molecular interactions are identical, and specificity is controlled by the dimerization states of the binding domain. Given the structural similarities of CUE to UBA and the functional similarity of CUE to UIM, it will be interesting to see if such a principle applies to these domains as well.

Experimental Procedures

Crystallization of the Vps9p-CUE Domain

The G440E mutant of the CUE domain of S. cerevisiae Vps9p was expressed and purified (Shih et al., 2003). SeMet G440 Vps9p CUE domain was expressed in E. coli strain B834 (DE3) and purified. The Vps9p CUE domain was concentrated to 30 mg/mL; dialyzed into 50 mM NaCl, 20 mM Tris (pH 7.7), and 10 mM DTT; and crystallized in 1.9–2.1 M ammonium sulfate and 100 mM Tris-HCl (pH 8.2–8.8). Crystals were cryoprotected in mother liquor supplemented with 25% ethylene glycol and frozen in liquid propane.

Crystallization of the Vps9p-CUE:Ubiquitin Complex

The Vps9p-CUE K435A/K436A mutant was mixed with bovine ubiquitin (Sigma) at a 1:1 molar ratio. The complex was isolated from unbound material on a Superdex 75/560 gel filtration column (Pharmacia). Native protein was concentrated to 43 mg/ml and crystallized in the presence of 17% polyethylene glycol 3350 and 200 mM MgCl2. SeMet CUE:ubiquitin crystals were obtained in similar conditions. Crystals of the complex were cryoprotected in mother liquor supplemented with 18% glycerol and frozen in liquid propane or in the cryostream.

Structure Determination

MAD data sets were collected from apo and ubiquitin bound CUE crystals at beamlines 19ID and 22ID, respectively, at the Advanced Photon Source, Argonne National Laboratory. MAD data were collected at three wavelengths, at 95 K, and in 1° oscillation frames, and reduced using DENZO and Scalepack (Otwinowski and Minor, 1997). Se atoms were located and phases were calculated with SOLVE (Terwilliger and Berendzen, 1999). Density modification of the initial maps was performed using RESOLVE (Terwilliger, 2000). The resulting maps were used to build atomic models in O (Jones et al., 1991). The models were initially refined using CNS (Brünger et al., 1998; Table 1). The complex model was subsequently refined at 1.7 Å using Refmac5 of the CCP4 suite programs (CCP4, 1994).

Site-Directed Mutagenesis

Site-directed mutagenesis of Vps9p CUE domain mutants were constructed using the GeneTailor mutagenesis kit (Invitrogen) and confirmed by DNA sequencing.

Isothermal Titration Calorimetry

CUE domains and bovine ubiquitin (Sigma) were dialyzed against 100 mM NaCl, 50 mM Na/K phosphate buffer (pH 7.5), and 1 mM DTT. Protein concentrations were adjusted to 200 μM and 4.0 mM for CUE and ubiquitin, respectively. Measurements were performed on a MicroCal VP-ITC instrument at 30°C. Ubiquitin was injected into 1.4 ml of buffer containing CUE domain in 21 injections of 10 μl each. Traces were corrected by subtracting blank measurements and analyzed using Origin 5.0 (MicroCal). Binding constants were calculated by fitting the integrated titration data to a one-site binding model (Table 2).

Sedimentation Equilibrium

CUE, CUE-L427D, and ubiquitin in 150 mM NaCl, 50 mM Tris-HCl (pH 7.5), and 1 mM Tris (2-carboxyethyl) phosphine hydrochloride (TCEP) or 2-mercaptoethanol were loaded at 0.5–0.9 A280. Mixtures containing 1:1 and 2:1 molar ratios of CUE:ubiquitin and CUE-L427D:ubiquitin were loaded at 0.85 A280. Experiments were conducted at 20.0°C and 280 nm on a Beckman Optima XL-A analytical ultracentrifuge at rotor speeds of 24,000, 26,000, and 28,000 rpm. Data for ubiquitin were analyzed as a single ideal solute to obtain the buoyant molecular mass, M, of the CUE:ubiquitin and CUE-L427D:ubiquitin complexes. Values of M were calculated using densities obtained from standard tables and calculated values of n (Perkins, 1986). Sedimentation equilibrium data for CUE and CUE-L427D were analyzed as reversible monomer-dimer equilibrium to obtain a dimerization equilibrium constant, Kd (Jenkins et al., 1996).

For the 1:1 and 2:1 mixtures of CUE:ubiquitin and CUE-L427D:ubiquitin were analyzed in terms of the following equilibria:

\[
\begin{align*}
K_{\text{eq}}: & \quad C + U \rightleftharpoons C_2U \\
K_S: & \quad C + U \rightleftharpoons CU \\
K_C: & \quad C_2U + U \rightleftharpoons C_2U_2
\end{align*}
\]

where C and U are CUE and ubiquitin. Two models were considered. In the cooperative model, the cooperativity results in the exclusive formation of C2U with ΔG = 2ΔG0 or K = K0. In both models, it is assumed that K0 = K01. The model equations, values for the constants, and their error limits are described in the online Supplemental Data, Web supplement C. C, cells were labeled with [35S]methionine for 2 hr, and cyclohexamide was added (3 μM) during the last 45 min of the chase period. Labeled cells were visualized on an Olympus IX70 inverted microscope with a Rhodamine filter. Ste3-GFP was visualized with a FITC filter. Images were collected with a Photometric digital camera and deconvolved using DeltaVision (Applied Precision).

FM4-64 Labeling and Ste-GFP Localization Assay

Vacular morphology was analyzed by labeling with FM4-64 (Molecular Probes, Eugene, OR) as described (Vida and Emr, 1995), except that labeling was at a concentration of 16 μM at 30°C, cells were chased 1 hr, and cyclohexamide was added (3 μM) during the last 45 min of the chase period. Labeled cells were visualized on an Olympus IX70 inverted microscope with a Rhodamine filter. Ste3-GFP was visualized with a FITC filter. Images were collected with a Photometric digital camera and deconvolved using DeltaVision (Applied Precision).

Two-Hybrid Analysis

Yeast strain L40 (Vojtek et al., 1993) was transformed with bait plasmid alone (pJUL11) (JulienFlores, et al., 1995) or with bait plasmids that also encoded the indicated portions of Vps9p. These plasmids were cotransformed with the prey plasmid alone (pGAD-GH), or prey plasmids encoding full-length or the indicated portions of Vps9p, or prey plasmid expressing ubiquitin. Cotransformants were selected on minimal media, and interaction was scored using a β-galactosidase filter assay (Vojtek et al., 1993).
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Accession Numbers

The coordinates have been deposited in the PDB under identifiers 1MN3 and 1P3Q.
Table S1. Equilibrium Concentrations of CUE and Ubiquitin Complexes

Data are based on an initial loading concentration of 0.5 mM CUE and 0.5 mM ubiquitin. Equilibrium concentrations were obtained by fitting the sedimentation equilibrium data to the following mathematical models.

Cooperative model:

\[ C_r = C_{o,1} \exp[HM_{b1}(r^2 - r_o^2)] + C_{o,2} \exp[HM_{b2}(r^2 - r_o^2)] + C_{o,1}^2 \exp[\ln(K_{dim}) + 2HM_{b1}(r^2 - r_o^2)] + C_{o,1}C_{o,2} \exp[\ln(K_0) + H(2M_{b1} + M_{b2})(r^2 - r_o^2)] + C_{o,1}^2C_{o,2}^2 \exp[\ln(K_{dim}) + 2\ln(K_0) + H(2M_{b1} + 2M_{b2})(r^2 - r_o^2)] + E, [1] \]

Noncooperative model:

\[ C_r = C_{o,1} \exp[HM_{b1}(r^2 - r_o^2)] + C_{o,2} \exp[HM_{b2}(r^2 - r_o^2)] + C_{o,1}^2 \exp[\ln(K_{dim}) + 2HM_{b1}(r^2 - r_o^2)] + C_{o,1}C_{o,2} \exp[\ln(K_0) + H(2M_{b1} + M_{b2})(r^2 - r_o^2)] + C_{o,1}^2C_{o,2}^2 \exp[\ln(K_{dim}) + 2\ln(K_0) + H(2M_{b1} + 2M_{b2})(r^2 - r_o^2)] + E, [2] \]

where \( C_{o,1} \) is the concentration of Cue at a reference point \( r_o \), \( C_{o,2} \) is the reference concentration of ubiquitin, \( H \) represents \( \omega^2/2RT \), \( \omega \) is the angular speed in rads\(^{-1} \), \( R \) is the gas constant, \( T \) is the absolute temperature, \( E \) is a small baseline correction, and \( M_{b1} \) and \( M_{b2} \) represent the buoyant molecular masses of Cue and ubiquitin, respectively. Each data set collected at different rotor speeds were analyzed simultaneously on an absorbance based scale (SigmaPlot 8.02 [SPSS, Inc.]) using experimentally determined values for \( \ln(K_{dim}) \), to yield the global parameter \( \ln(K_0) \). This parameter was used to determine values for \( K_0 \), \( K_1 \), and \( K_2 \). Within the experimental precision of the method, identical values of \( \ln(K_0) = 6.8 \pm 0.5 \) were obtained. This results in values of 900 M\(^{-1} \), \( 8.1 \times 10^5 \) M\(^{-1} \), and 900 M\(^{-1} \) for \( K_0 \), \( K_1 \), and \( K_2 \), respectively. Based on the error, limits for \( K_0 \) and \( K_2 \) are 1500 to 540 M\(^{-1} \), whereas limits for \( K_1 \) are 22 to 3.0 \( \times 10^5 \) M\(^{-1} \). In the case of the L427D CUE, 1:1 and 2:1 mixtures were studied in TCEP containing buffers. Data were analyzed in terms of a noncooperative model (equation 2) to yield an average \( \ln(K_0) \) of 5.9 \( \pm 0.3 \) (Figure 5E–5G) and values of \( K_0 = K_2 = 350 \) M\(^{-1} \) and \( K_1 = 700 \) M\(^{-1} \). Limits for \( K_0 \) and \( K_2 \) are 450 to 270 M\(^{-1} \), whereas limits for \( K_1 \) are twice these values.