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A ubiquitin-binding motif required for intramolecular monoubiquitylation, the CUE domain

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Monoubiquitylation is a regulatory signal, like phosphorylation, that can alter the activity, location or structure of a protein. Monoubiquitin signals are likely to be recognized by ubiquitin-binding proteins that transmit the regulatory information conferred by monoubiquitylation. To identify monoubiquitin-binding proteins, we used a mutant ubiquitin that lacks the primary site of polyubiquitin chain formation as bait in a two-hybrid screen. The C-terminus of Vps9, a protein required in the yeast endocytic pathway, interacted specifically with monoubiquitin. The region required for monoubiquitin binding mapped to the Vps9 CUE domain, a sequence previously identified by database searches as similar to parts of the yeast Cue1 and mammalian Tollip proteins. We demonstrate that CUE domains bind directly to monoubiquitin and we have defined crucial interaction surfaces on both binding partners. The Vps9 CUE domain is required to promote monoubiquitylation of Vps9 by the Rsp5 *hect* domain ubiquitin ligase. Thus, we conclude that the CUE motif is an evolutionarily conserved monoubiquitin-binding domain that mediates intramolecular monoubiquitylation.

Keywords: CUE domain/monoubiquitylation/Rsp5/ubiquitin-binding protein/Vps9

Introduction

Post-translational modifications of cellular proteins by a chemical group or a small polypeptide serve as regulatory signals that alter the activity or location of a protein. These modifications may alter the conformation of a protein, sterically hinder protein–protein interactions or create a new landscape to attract binding partners. Ubiquitin is a 76 amino acid polypeptide that is highly conserved in evolution. It is the best characterized member of a class of small protein modifiers, ubiquitin and ubiquitin-like proteins, that regulates the activities of a diverse array of cellular proteins (reviewed in Hershko and Ciechanover, 1998; Hochstrasser, 2000; Jentsch and Pyrowolakis, 2000).

Ubiquitin is most often linked to proteins through the ε-amino group of lysine residues. Because ubiquitin itself carries surface lysines, polyubiquitin chains can form. Polyubiquitin chains linked through ubiquitin Lys48 mediate the well-characterized role of ubiquitin in targeting proteins for degradation by the 26S proteasome (reviewed in Hershko and Ciechanover, 1998). Monoubiquitylation, the addition of a single ubiquitin unit to a protein, is also an important cellular regulatory signal (Hicke, 2001). The characterized roles of monoubiquitin include acting as a sorting signal to direct protein traffic in the endocytic pathway, regulating vesicle budding machinery, modifying histones, regulating transcriptional machinery and controlling intranuclear localization. More regulatory roles for monoubiquitin are likely to exist.

One way in which cells may interpret and transmit the information conferred by monoubiquitin signals is through monoubiquitin-binding proteins. Two sequence motifs, originally identified through database searches, are known to bind directly to monoubiquitin. UBA (ubiquitin-associated) motifs are ~40 amino acid domains that were recognized because they are found in many proteins that function in ubiquitylation or deubiquitylation (Hofmann and Bucher, 1996). In assays with purified proteins, UBA domains bind monoubiquitin, but have a greater affinity for polyubiquitin chains (Bertolaet *et al*., 2001; Chen *et al*., 2001; Wilkinson *et al*., 2001; Funakoshi *et al*., 2002). It is not known whether monoubiquitylated or polyubiquitylated proteins are binding partners for specific UBA domains *in vivo*. The structure of the UBA domain as determined by nuclear magnetic resonance (NMR) is a bundle of three α-helices, and this bundle contains a distinct hydrophobic surface region that is predicted to be the site of interaction with ubiquitin (Dieckmann *et al*., 1998; Mueller and Feigon, 2002).

A second ubiquitin-binding domain is the UIM (ubiquitin-interacting motif). This is a 20 amino acid sequence motif that was identified using iterative database searches with the sequences from the S5a subunit of the proteasome that interact directly with polyubiquitin chains (Hofmann and Falquet, 2001). UIMs bind to monoubiquitin directly, albeit with low affinity (Polo *et al*., 2002; Raiborg *et al*., 2002; Shih *et al*., 2002), and are present as tandem pairs or triplets in many proteins. UIMs are found in a number of proteins important in the endocytic pathway (epsins, Eps15 and Hrs), where they are critical for function, and are likely to bind monoubiquitylated partners in the cell (Raiborg *et al*., 2002; Shih *et al*., 2002). Endocytic UIM proteins are themselves monoubiquitylated, and this ubiquitylation event requires the protein's UIM domains (Klapisz *et al*., 2002; Oldham *et al*., 2002; Polo *et al*., 2002).

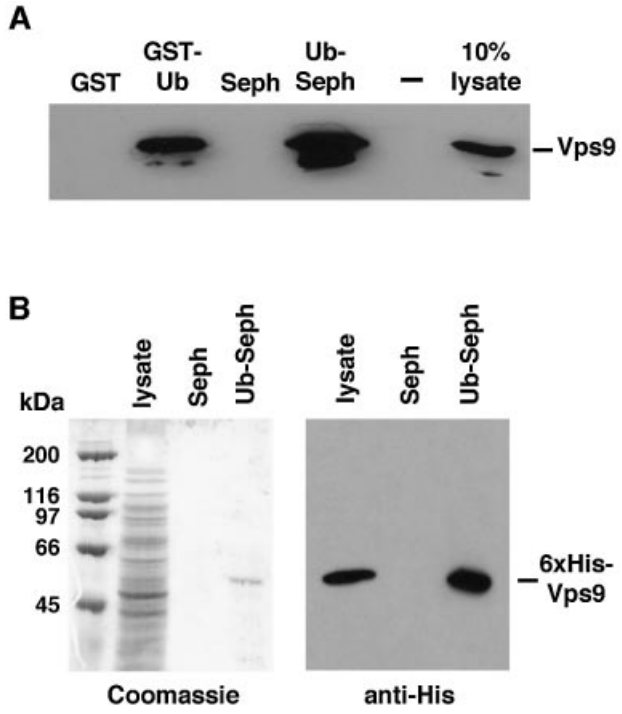

Fig. 1. Vps9 binds to monoubiquitin. (**A**) Lysate prepared from cells expressing Vps9-HA was incubated with Sepharose beads bound to GST, GST–Ub, monoubiquitin (Ub–Seph) or no protein (Seph). Total lysate (10% volume) and proteins eluted from each type of bead were analyzed by SDS–PAGE, followed by an anti-HA immunoblot. (**B**) Lysate from *E.coli* expressing His_6-Vps9 was incubated with Sepharose or Ub–Sepharose beads. Total lysate and eluted proteins were separated on a 16.5% Tris-tricine gel and analyzed by Coomassie Blue staining or by immunoblotting with anti-histidine antiserum.

Here we report the identification and characterization of a new ubiquitin-binding domain, the CUE domain. CUE motifs are 42–43 amino acid sequences that were identified in a database search because they are similar to a region of the yeast Cue1 protein (Ponting, 2000). Because Cue1 acts to recruit the Ubc7 ubiquitin-conjugating enzyme to the endoplasmic reticulum (ER) membrane (Biederer *et al*., 1997), CUE domains were proposed to be a scaffold for interaction with ubiquitin-conjugating enzymes (Ponting, 2000). We demonstrate that yeast and human CUE domains present in proteins involved in diverse cellular functions interact directly with monoubiquitin. Some CUE domains, such as the Cue1 CUE domain, bind ubiquitin weakly. Others, including the Vps9 CUE domain, bind monoubiquitin efficiently due to the presence of a MF sequence adjacent to the invariant proline characteristic of the CUE domain. CUE domains, although unrelated in sequence to UIMs, also promote the monoubiquitylation of proteins within which they are found.

Results

The C-terminus of Vps9 binds to monoubiquitin

To identify cellular monoubiquitin-binding proteins in yeast that function to interpret and transmit information from a monoubiquitin signal, we performed a two-hybrid screen with a modified form of ubiquitin in which a principal site of polyubiquitin chain formation was removed by the mutation of Lys48 to arginine (Ub^{K48R}). Three independent clones encoding the C-terminus of the Vps9 (vacuolar protein sorting) protein were identified in this screen. Vps9 is a guanine nucleotide exchange factor that regulates a Rab-like GTPase, Vps21, and is required for the transport of proteins from both the biosynthetic and endocytic pathways into the lysosome-like vacuole (Burd *et al*., 1996; Hama *et al*., 1999).

To confirm that Vps9 is a ubiquitin-binding protein, lysates were prepared from yeast cells expressing Vps9 tagged with a hemagglutinin (HA) epitope and were incubated with monoubiquitin affinity matrices. Vps9-HA bound to ubiquitin–Sepharose and to immobilized GST–ubiquitin, but not to control beads (Figure 1A). Recombinant His_6-tagged Vps9 (His_6-Vps9) expressed in *Escherichia coli* also specifically bound to ubiquitin–Sepharose (Figure 1B). These data demonstrate that Vps9 binds to monoubiquitin directly.

The clones identified in our two-hybrid screen suggested that the ubiquitin-binding region of Vps9 was contained within the C-terminus, amino acids 351–451. Binding of ubiquitin to full-length and truncated fragments of Vps9 was analyzed by two-hybrid experiments and by incubation of recombinant Vps9 fragments with ubiquitin–Sepharose. The ubiquitin-binding region mapped within amino acids 408–451 (Figure 2), corresponding to a CUE domain (Ponting, 2000; http://smart.embl-heidelberg.de). CUE domains are amino acid sequences similar to regions of the yeast Cue1 and mouse Tollip proteins, and have been proposed to be a scaffolding domain to recruit ubiquitin-conjugating enzymes (Ponting, 2000). Truncation into the Vps9 CUE domain from either end abolished ubiquitin interaction (Figure 2A; data not shown). Together, these experiments indicate that the Vps9 CUE domain was sufficient for direct interaction with ubiquitin.

CUE motifs are monoubiquitin-binding domains

The consensus CUE domain sequence consists of 42–43 amino acids that contain an invariant proline and a conserved di-leucine-like motif (Ponting, 2000; see Figure 5A). We individually mutated these conserved residues in the Vps9 CUE domain (Pro421, Leu446 and Leu447) to alanine and found that each mutation reduced the ability of the recombinant CUE domain to bind to GST–Ub (Figure 3). Binding of the mutant CUE domains to ubiquitin–Sepharose was reduced to an even greater extent (our unpublished data). These findings indicate that the conserved residues of the CUE domain are important for direct interaction with ubiquitin and suggest that the CUE domain structure is responsible for binding to ubiquitin.

Eight yeast proteins carry CUE domains (Ponting, 2000, 2002; see Figure 5A). Seven proteins (Vps9, Cue1, Def1, Don1, Ygl110, Yml101 and Yor042) carry one CUE domain, and one protein (Ykl090) has two CUE domains. Four of these proteins have previously assigned names and functions. We will refer to the remaining four unidentified open reading frames (ORFs) as Cue2 (Ykl090), Cue3 (Ygl110), Cue4 (Yml101) and Cue5 (Yor042) (*Saccharomyces* Genome Database designations). To test whether the CUE domain is a general

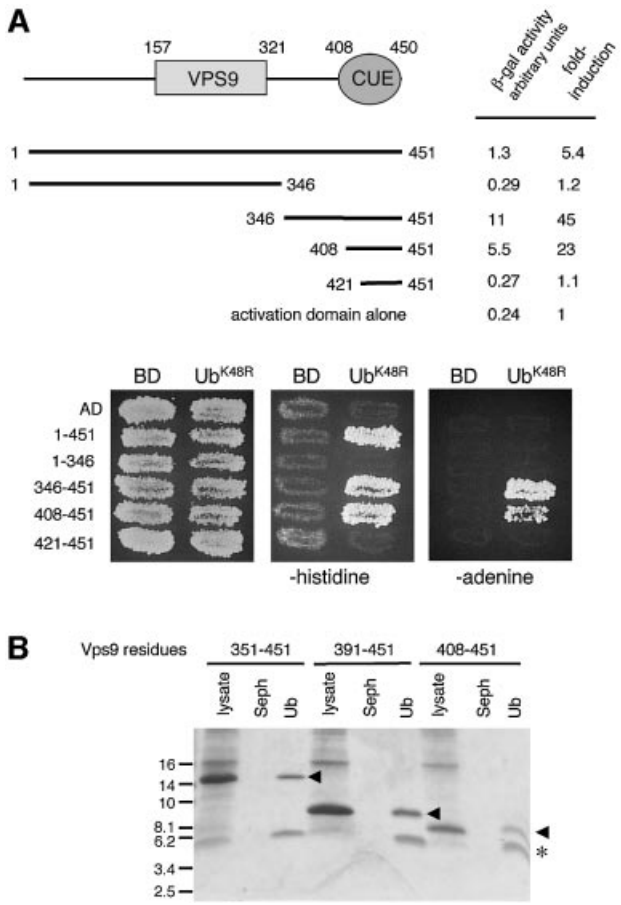

Fig. 2. The CUE domain of Vps9 interacts with ubiquitin directly. (**A**) Schematic of Vps9 indicating defined domains (http://smart.embl-heidelberg.de). Fragments of Vps9 fused to the Gal4 activation domain (AD) were assayed for interaction with Ub^{K48R} fused to the Gal4-binding domain (BD) by the yeast two-hybrid method. Growth on medium lacking histidine or adenine indicated a positive interaction. Growth on medium lacking adenine indicated a stronger interaction than growth on medium lacking histidine. The interaction between Ub^{K48R} and individual domains was quantified by assaying β-galactosidase activity in cell lysates. The background resulting from a strain co-expressing BD alone and AD alone was normalized to 1. (**B**) Bacterial lysates from cells expressing C-terminal fragments of Vps9 were incubated with Sepharose or Ub–Sepharose. Total lysates and eluted proteins were analyzed by Coomassie Blue staining. The arrowheads indicate the mobilities of Vps9 fragments. An endogenous bacterial polypeptide (*) also bound to Ub–Sepharose.

ubiquitin-binding motif, we expressed in bacteria His_6-tagged CUE domains from six of the yeast CUE domain proteins and an unrelated SH3 domain, which is similar in size and was not expected to bind ubiquitin. Each of the tagged CUE domains was immobilized on metal affinity beads and incubated with lysate from *E.coli* cells expressing GST or GST–Ub. The CUE domains differed in their ability to bind to GST–Ub, suggesting that different CUE domains have different ubiquitin-binding affinities. The Vps9 CUE domain bound GST–Ub to the greatest extent, followed by the two CUE domains from Cue2 and the Cue3 CUE domain (Figure 4A). The Cue5 CUE domain consistently, but weakly, bound GST–Ub above background (Figure 4A; data not shown). In this experiment, the CUE domains from Cue1 and Cue4 did not bind GST–Ub reproducibly above the background binding of

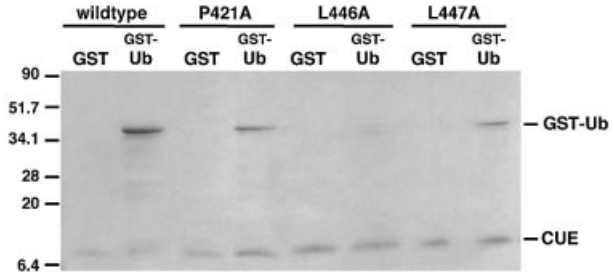

Fig. 3. Conserved CUE domain residues are important for monoubiquitin binding. Equal amounts of His_6-tagged Vps9 CUE domain (408–451) and the indicated mutant variants were immobilized on metal affinity beads and incubated with bacterial lysates expressing GST or GST–Ub. After extensive washing, the beads were boiled. Lysates and eluted proteins were separated on a 15% SDS–polyacrylamide gel and analyzed by Coomassie Blue staining.

GST alone (Figure 4A). However, weak but specific binding of the Cue1 and Cue4 CUE domains to GST–Ub was detected with a sensitive immunoblot (see Figure 5C; our unpublished data).

To confirm that the Cue1 CUE domain binds monoubiquitin, and to measure the difference in ubiquitin-binding affinity between the Vps9 and Cue1 CUE domains, we performed isothermal titration calorimetry (ITC) with purified CUE polypeptides (Figure 4B). The dissociation constants and enthalpies of Vps9 and Cue1 CUE domain binding to ubiquitin were $K_d = 20.3 \pm 1.4$ μM and $\Delta H_{apparent} = -10.81$ kcal/mol for Vps9-CUE, and $K_d = 160 \pm 20$ μM and $\Delta H_{apparent} = -2.6$ kcal/mol for Cue1-CUE, thus there is a 8-fold difference in affinities between the strongest and weakest ubiquitin-binding CUE domains.

Mammalian proteins that carry a CUE domain include two proteins involved in signaling from the interleukin-1 receptor, Tollip and TAB2 (Ponting, 2000). Among all the defined CUE domains, the Vps9 CUE domain is most similar to that of Tollip. To test whether the Tollip CUE domain binds to ubiquitin, we expressed this CUE domain as a His_6-tagged protein in *E.coli.* His_6-Tollip CUE was immobilized on metal affinity beads and incubated with bacterial lysates expressing GST or GST–Ub. The Tollip CUE domain bound specifically to GST–Ub (Figure 4C). Bacterially expressed full-length Tollip also bound to GST–Ub in a similar experiment, although to a lesser extent (data not shown).

The Vps9 CUE domain bound efficiently to K48-linked oligo- and polyubiquitin chains (Ub_2 and larger), whereas the Cue1 CUE domain did not (Figure 4D). This observation indicates that the Cue1-CUE, which interacts weakly with monoubiquitin, also does not bind polyubiquitin chains efficiently.

CUE domain and ubiquitin residues important for interaction

To determine how CUE domains that bind monoubiquitin efficiently differ from those that do not bind effectively, we compared the amino acid sequences of the CUE domains we had analyzed (Figure 5A). CUE domains that bound significantly to GST–Ub in our assay carry a phenylalanine in the position before the invariant proline. This observation suggested that a Phe–Pro (FP) sequence

in a CUE domain is important for ubiquitin binding. To test this idea, we mutated Phe420 to alanine in the Vps9 CUE domain. This mutation inhibited ubiquitin binding (Figure 5B). In the CUE domains that bound most effectively to monoubiquitin, FP is preceded by a methionine or leucine. To determine whether a large hydrophobic amino acid in this position facilitates ubiquitin binding, we mutated Met419 to alanine, a small hydrophobic amino acid. M419A inhibited binding to ubiquitin (Figure 5B), consistent with the observed weak binding of the Cue5 CUE domain to ubiquitin, which carries an AFP sequence. These data, together with the analysis of P421A, L446A and L447A mutations described earlier (Figure 3), suggest that an FP sequence and a conserved di-leucine-like motif are part of the CUE ubiquitin-binding surface. To test further the idea that an FP motif in the CUE domain is crucial for efficient ubiquitin binding, we mutated the LAP sequence in Cue1-CUE to MFP. This mutation significantly increased the ability of Cue1-CUE to bind monoubiquitin (Figure 5C). Mutating the Cue1 di-leucine-like motif (YL→LL) had no effect.

On ubiquitin, two functional surfaces have been defined (Beal *et al*., 1996; Shih *et al*., 2000; Sloper-Mould *et al*., 2001). One surface includes residues required for ubiquitylation and deubiquitylation and encompasses a hydrophobic patch (Leu8, Ile44 and Val70) that participates in binding to the proteasome, UIMs and UBA domains (Beal *et al*., 1996; Shih, 2002; Shih *et al*., 2002). The other surface is required specifically for non-proteasome-dependent functions, including endocytosis (Sloper-Mould *et al*., 2001). The key reside on this second surface is Phe4. To test which of these regions might be involved in CUE domain binding, we incubated immobilized Vps9 CUE domain with *E.coli* lysates containing GST, GST–Ub, GST–Ub^{I44A} or GST–Ub^{F4A}. The interaction of the Vps9 CUE domain requires ubiquitin Ile44, but not Phe4 (Figure 6).

The CUE domain of Vps9 is required for intramolecular ubiquitylation by Rsp5

While testing the binding of Vps9 to ubiquitin, we observed a species of Vps9-HA in yeast lysates that migrated at 77 kDa, 12 kDa larger than the major doublet form of the protein (Figure 7A, lane 1). To test whether the 77 kDa band was an ubiquitylated form of Vps9, we transformed cells with plasmids overexpressing either wild-type ubiquitin or a c-myc-tagged version. Overexpression of ubiquitin increased the amount of the 77 kDa Vps9 form (Figure 7A, lane 2). In addition, expression of

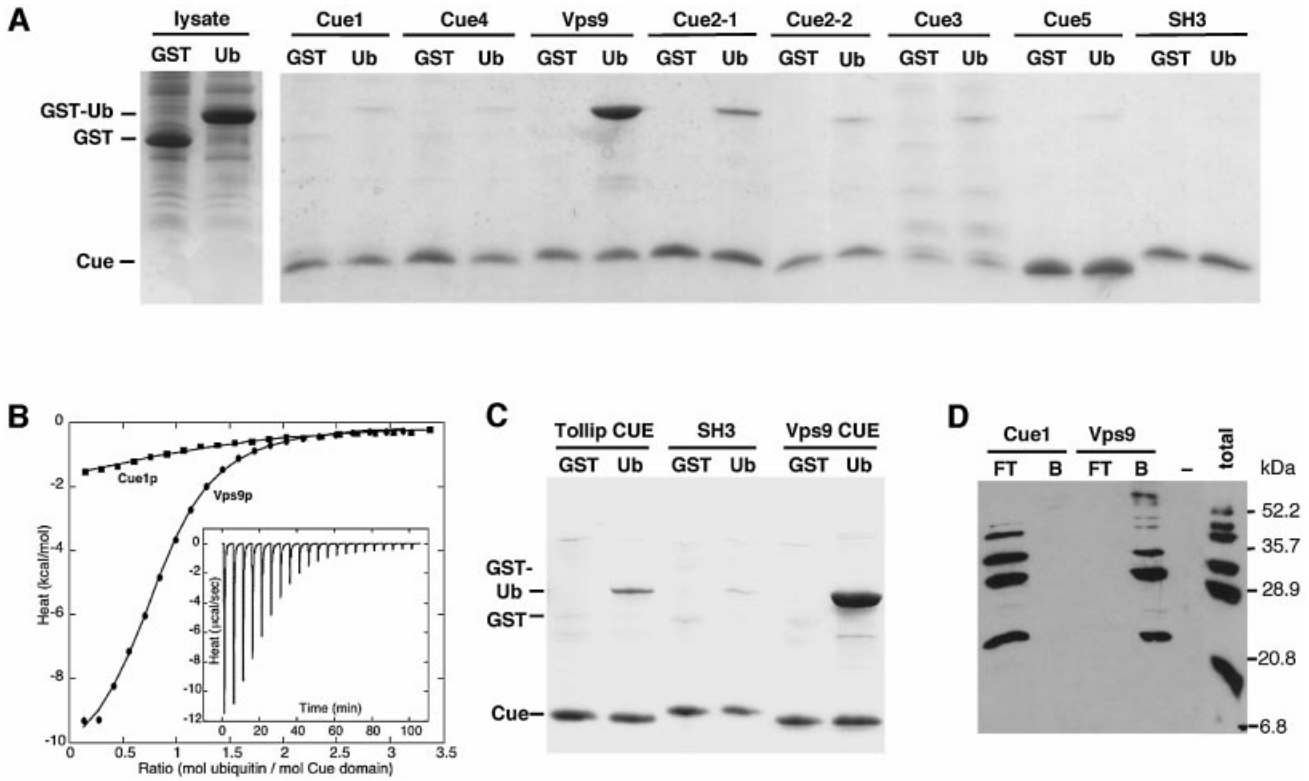

Fig. 4. The CUE motif is a general ubiquitin-binding domain. (**A**) CUE domains from yeast CUE proteins and a control SH3 domain from the yeast Rvs167 protein were expressed as His_6-tagged polypeptides in *E.coli*. Binding to GST–Ub was performed as described in the legend to Figure 3, except GST/GST–Ub total lysates and eluted proteins were separated on a 16.5% Tris-tricine gel. Vps9 (amino acids 408–451), Cue1 (amino acids 65–106), Cue2-1 (amino acids 8–50), Cue2-2 (amino acids 55–97), Cue3 (amino acids 316–358), Cue4 (amino acids 74–115), Cue5 (amino acids 97–139) and SH3 (Rvs167 amino acids 428–482). (**B**) ITC analysis of Vps9-CUE and Cue1-CUE binding to ubiquitin. Titration curves are shown for the Vps9 and Cue1 CUE domains. Inset: a representative experimental ITC trace of Vps9-CUE. The differential heat signals from injections of 4 mM ubiquitin into 200 µM Vps9 or Cue1 CUE domains are shown (after subtraction of blank data as described in Materials and methods). (**C**) CUE domains from yeast and human CUE proteins were analyzed as in (A). Tollip CUE (amino acids 229–271). (**D**) Binding of ubiquitin chains to Vps9 and Cue1 CUE domains. Oligo- and polyubiquitin chains (Ub_2 and greater) were incubated with Vps9 and Cue1 CUE domains immobilized on metal affinity beads. Total ubiquitin chains (total) and ubiquitin in the flow-through (FT) and bound (B) fractions were fractionated by SDS–PAGE and detected on an immunoblot with ubiquitin antiserum.

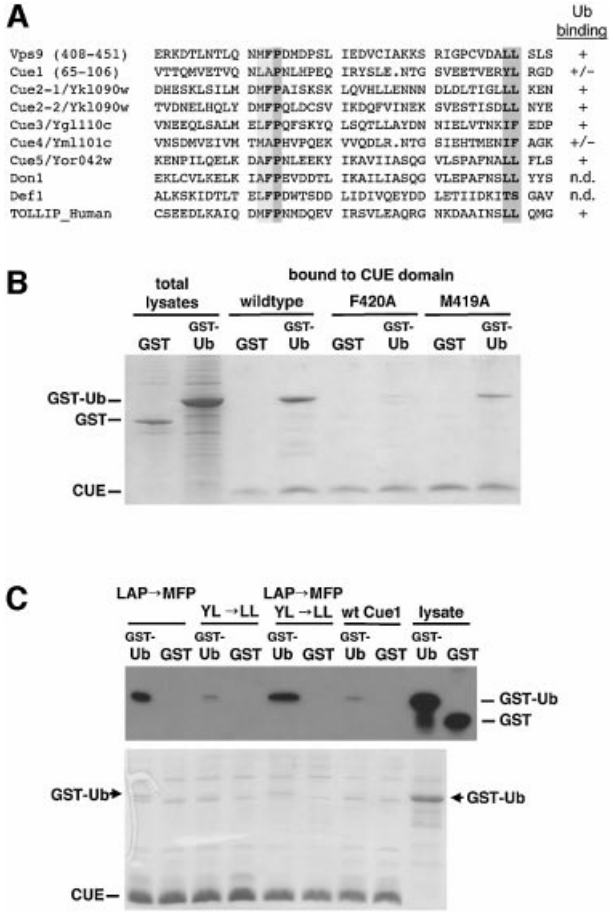

Fig. 5. A CUE domain FP motif is important for binding to monoubiquitin. (**A**) Alignment of yeast CUE domains identified by database searches for sequences similar to regions of Cue1 and Tollip (Ponting, 2000). The CUE domain invariant proline and highly conserved di-leucine motif are highlighted in dark gray. X-Phe residues that precede the invariant proline are highlighted light gray. (**B**) Equal amounts of His_6-tagged Vps9 CUE domain (408–451) and the indicated mutant variants were immobilized on metal affinity beads. Binding to GST and GST–Ub was performed as described in the legend to Figure 3. (**C**) Equal amounts of His_6-tagged Cue1 CUE domain (amino acids 65–106) and the indicated mutant variants were immobilized on metal affinity beads. Binding to GST and GST–Ub was performed as described in the legend to Figure 3, except that total lysates and eluted proteins were analyzed by an anti-GST immunoblot and by Coomassie Blue staining.

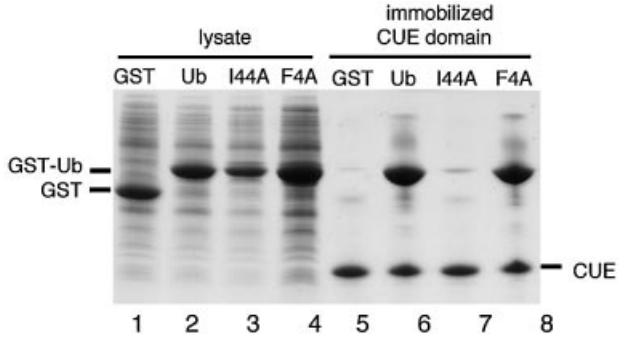

Fig. 6. CUE domain binding requires Ile44 of ubiquitin. The Vps9 CUE domain (residues 408–451) was immobilized on metal affinity beads and incubated with bacterial lysates expressing GST, GST–Ub, GST–Ub^{I44A} or GST–Ub^{F4A}. The lysates and eluted proteins were analyzed by Coomassie Blue staining. The mobilities of the CUE domain, GST and GST–Ub are indicated.

c-myc-ubiquitin caused a 1–2 kDa decrease in mobility of this species (Figure 7A, lane 3), a size shift consistent with the addition of a myc epitope tag.

To support the idea that Vps9 is monoubiquitylated, we tested whether the presence of the 77 kDa Vps9 band was dependent on components of the cellular ubiquitylation machinery. Specifically, we prepared lysates from cells carrying mutations in ubiquitin ligases whose function is implicated in the endocytic pathway. The Vps9 77 kDa form was present at normal levels in cells lacking the Vps8, Pib1 and Tul1 proteins (our unpublished data), which are confirmed or putative RING finger ubiquitin ligases that act in the late endocytic and biosynthetic pathways. In contrast, a temperature-sensitive mutant defective in the gene encoding the Rsp5 *hect* domain ubiquitin ligase, *mdp1-1* (*rsp5*) (Zoladek *et al*., 1997; Fisk and Yaffe, 1999), had a significantly reduced level of 77 kDa Vps9 (Figure 7B). Non-ubiquitylated Vps9 was present at the same level in both wild-type and *rsp5* cells. These observations demonstrate that the 77 kDa form of Vps9 is ubiquitylated, and suggest that Rsp5 is the ubiquitin ligase that modifies Vps9.

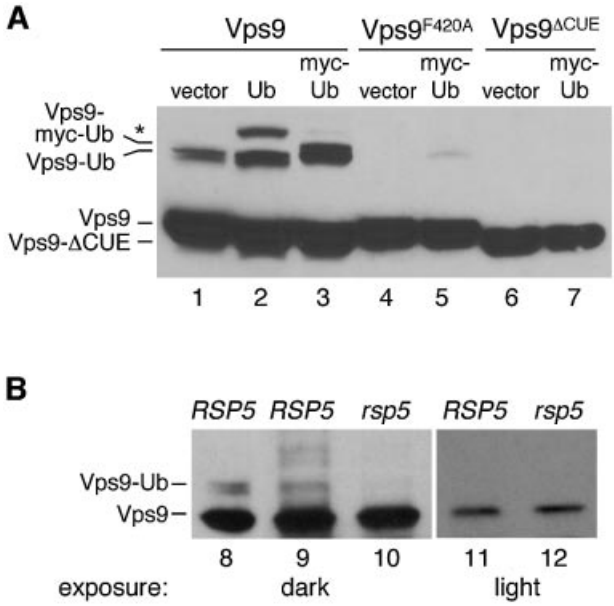

Fig. 7. CUE-dependent monoubiquitylation of Vps9 by Rsp5. (**A**) Plasmids encoding His_6-tagged Vps9 and mutant variants were co-transformed into yeast cells with an empty vector, or with plasmids encoding wild-type (Ub) or c-myc-tagged ubiquitin (myc-Ub). Ubiquitin overexpression was induced in cells prior to preparing the yeast lysates. Cell lysates were prepared and analyzed by anti-His immunoblot. In cells that did not overexpress ubiquitin, a high molecular weight form of Vps9 that migrated at 77 kDa was observed (lane 1). The overexpression of wild-type ubiquitin yielded an increase in 77 kDa Vps9, as well as inducing the appearance of an uncharacterized Vps9 species (*). The overexpression of c-myc-ubiquitin resulted in increased mobility of the 77 kDa Vps9 species (compare lane 3 with lane 2). Deletion of the Vps9 CUE domain or introduction of the F420A mutation severely inhibited Vps9 ubiquitylation (lanes 5 and 7). (**B**) A centromere-based plasmid encoding HA-Vps9 was transformed into *mdp1-1/rsp5* and isogenic wild-type cells (*RSP5*). Lysate from the multicopy *VPS9* wild-type strain analyzed in (A) lane 1 was used to indicate the mobility of the Vps9 77 kDa species (*RSP5*, lane 8). Higher ubiquitylated forms of Vps9 are observed in one strain background (lane 9) in addition to monoubiquitylated Vps9. A lighter exposure in which the 77 kDa species is not visible in the wild-type strain lysate is shown to indicate that each strain contains equivalents amounts of non-ubiquitylated Vps9.

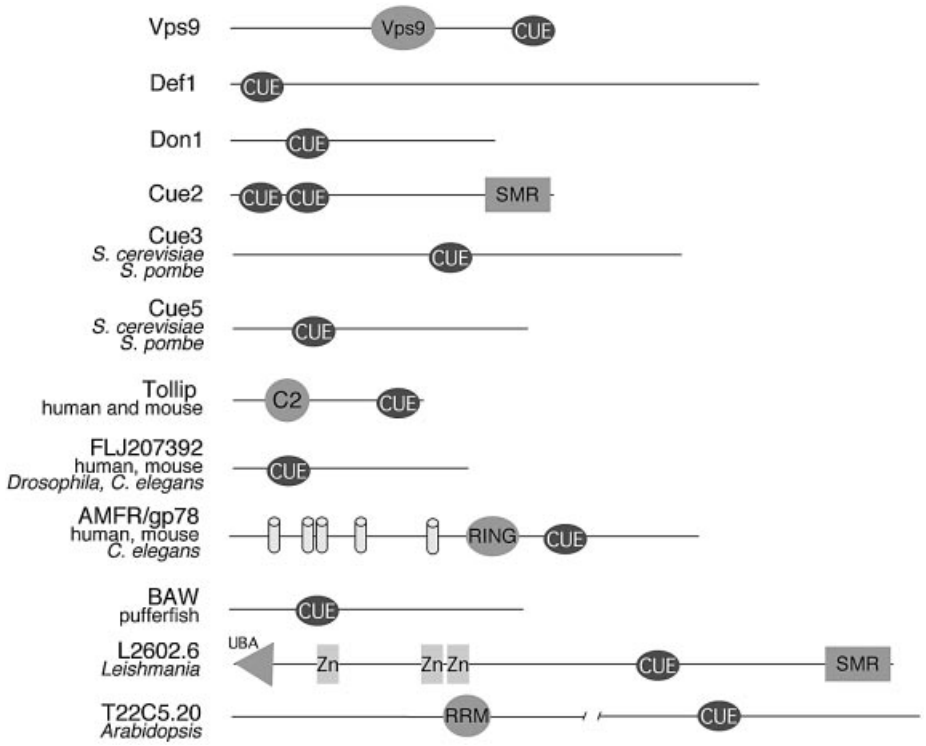

Fig. 8. Ubiquitin-binding CUE proteins. Proteins that carry a CUE domain characterized by an FP and a di-leucine-like sequence, and therefore are likely to bind ubiquitin efficiently, are shown, and defined domains are indicated (http://smart.embl-heidelberg.de). Cylinders indicate the predicted transmembrane domain.

The 12 kDa difference in mass between the ubiquitylated species and the major 65 kDa form of the protein is consistent with monoubiquitylation, although higher molecular weight oligo-ubiquitylated species are detected in some strain backgrounds (e.g. Figure 7B, lane 9). The ubiquitylated form of Vps9 is likely to be physiologically significant because ubiquitylated Vps9 was detected in cells that express normal levels of ubiquitin and Vps9 (Figure 7B, lane 9; our unpublished data).

Several CUE domain proteins have been shown to interact with ubiquitin-conjugating enzymes and are required for specific ubiquitylation reactions (Biederer *et al*., 1997; Fang *et al*., 2001). One of these, gp78/AMFR, carries a CUE domain (Ponting, 2000), suggesting that CUE domains may generally be involved in ubiquitylation reactions. We examined whether Vps9 monoubiquitylation was dependent on the CUE domain by expressing mutant forms of Vps9 in cells overexpressing c-myc-ubiquitin. Vps9 that carried a precise truncation of the CUE domain, $Vps9^{\Delta CUE}$, was not ubiquitylated (Figure 7A, lanes 6 and 7). Vps9 that carried a point mutation in the CUE domain that inhibited monoubiquitin binding, $Vps9^{F420A}$, was ubiquitylated very poorly (Figure 7A, lanes 4 and 5). These experiments demonstrate that the CUE domain of Vps9 is required for Vps9 monoubiquitylation.

Discussion

CUE domains are monoubiquitin-binding motifs

CUE domains directly bind monoubiquitin with varying affinities. The affinity of ubiquitin binding to the six yeast CUE domains we analyzed spanned an 8-fold range. The affinity for ubiquitin depends primarily on the three amino acids that precede and include the invariant proline in the conserved CUE motif. In the Vps9 CUE domain, this sequence is MFP, and the phenylalanine is most critical for ubiquitin binding. This was demonstrated by mutating Phe420 in Vps9-CUE, which severely inhibited ubiquitin binding, and by replacing LAP in Cue1-CUE with MFP, which significantly increased Cue1-CUE ubiquitin binding. Binding to ubiquitin also requires a highly conserved di-hydrophobic sequence found near the end of the CUE motif. The possibility remains that sequences outside the defined CUE domains may be necessary for efficient ubiquitin binding in some cases. However, a large fragment containing both CUE domains of Cue2 did not bind significantly more GST–Ub than each Cue2 CUE domain alone (data not shown). Other CUE residues must also influence the affinity for ubiquitin because different CUE domains that carry both MFP and LL (Vps9, Cue2-1, Cue2-2 and Tollip) bind to ubiquitin to different extents in our assay (Figure 4). We also demonstrated that the CUE domains that do not bind monoubiquitin efficiently are also not likely to interact well with polyubiquitin, because the Cue1 CUE domain did not bind K48-linked polyubiquitin chains under conditions in which the Vps9 CUE domain did.

A recent iterative database search conducted by Kay Hofmann (MEMOREC, Germany) identified a subset of CUE domains that are distantly related to UBA domains (K.Hoffman, personal communication). These domains included the Vps9, Cue2, Cue3 and Cue5 CUE motifs. In this analysis, Cue1 and Cue4 CUE domains sorted into a

Table I. Plasmids

Name	Description	Reference
pAS2-1	GAL4-binding domain (BD)	Clontech
pACT2	GAL4 activation domain (AD)	Clontech
pEMBL-VPS9	His_6-VPS9 (2μ)	Hama *et al.* (1999)
pES7	CUP1-c-myc-UBIQUITIN (2μ)	Michael Ellison, University of Alberta
pGT9-2	GAL4 AD-VPS9	Hama *et al.* (1999)
pGT9HA-2	VPS9-HA (2μ)	Hama *et al.* (1999)
pQEVPS9	His_6-VPS9	Hama *et al.* (1999)
YEplac195	*URA3*-marked 2μ vector	Gietz and Sugino (1998)
LHP537	GAL4 BD-UBI^{K48R}	This study
LHP585	CUP1-*UBI* (2μ)	This study
LHP1150	GAL4 AD-$VPS9^{1–346}$	This study
LHP1211	GAL4 AD-$VPS9^{345–451}$	This study
LHP1232	GAL4 AD-$VPS9^{408–451}$	This study
LHP1233	GAL4 AD-$VPS9^{421–451}$	This study
LHP1263	HA-VPS9 (*CEN*)	This study
LHP1317	pET30-$VPS9^{408–451}$	This study
LHP1559	pET30-$CUE1^{65–106}$	This study
LHP1560	pET30-$CUE4^{74–117}$	This study
LHP1561	pET30-$CUE2^{8–50}$	This study
LHP1562	pET30-$CUE2^{55–97}$	This study
LHP1563	pET30-$CUE3^{316–358}$	This study
LHP1564	pET30-$CUE5^{97–139}$	This study
LHP1583	pET30-$VPS9^{408–451,L447A}$	This study
LHP1595	pET30-$VPS9^{408–451,M419A}$	This study
LHP1598	pET30-$VPS9^{408–451,F420A}$	This study
LHP1599	pET30-$VPS9^{408–451,P421A}$	This study
LHP1600	pET30-$VPS9^{408–451,L446A}$	This study
LHP1653	pET30-Tollip	This study
LHP1654	pET30-$Tollip^{229–271}$	This study
LHP1680	His_6-$VPS9^{F420A}$ (2μ)	This study
LHP1685	His_6-$VPS9\Delta^{CUE}$ (2μ)	This study
LHP1789	pET30-$CUE1^{65–106,Y102L}$	This study
LHP1790	pET30-$CUE1^{65–106,L76M,A77F}$	This study
LHP1791	pET30-$CUE1^{65–106,L76M,A77F,Y102L}$	This study

different CUE subfamily. Thus, an independent bioinformatics analysis supports our empirical ranking of CUE domains according to their ubiquitin-binding ability.

Thirty-two proteins encoded in the genome databases carry CUE domains (http://smart.embl-heidelberg.de). Of these, 20 carry FP and di-leucine-related sequences and are likely to bind to monoubiquitin (Figure 8). Several higher eukaryotic CUE domain proteins carry LP, rather than FP, and di-leucine-like sequences. Mutation of Phe420 to leucine in the Vps9 CUE domain inhibited ubiquitin binding almost as much as Phe420 to alanine (data not shown), suggesting that LP CUE domains do not bind monoubiquitin efficiently.

A previous study demonstrated that Cue1 interacts with ubiquitin, but only in the presence of the ubiquitin-conjugating enzyme, Ubc7 (Biederer *et al*., 1997). The biological function of Cue1 is to recruit ubiquitin-conjugating enzymes to the ER membrane to ubiquitylate specific ER-associated degradation substrates. This observation, together with our demonstration of a role for the Vps9 CUE domain in intramolecular monoubiquitylation, suggests that all CUE domains may interact with the ubiquitylation machinery. A subset of CUE domain proteins, such as Cue1, may only interact with ubiquitin efficiently in the presence of conjugating enzymes, whereas other CUE proteins, such as Vps9 and Cue2, may bind to ubiquitin signals directly and serve as ubiquitin receptors, in addition to participating in intra- or extramolecular ubiquitylation reactions. The presence of a CUE domain within a protein strongly suggests that it regulates or is regulated by a ubiquitylation reaction.

In the case of Vps9, deletion of the CUE domain has no effect on protein transport through the biosynthetic or endocytic pathways of the marker proteins we have assayed, including Ste2, carboxypeptidase Y, carboxypeptidase S and the fluid-phase endocytic marker, lucifer yellow (data not shown). We hypothesize that the CUE domain in this protein negatively regulates Vps9 activity, because the Vps9 CUE domain binds to the Vps9 N-terminus (our unpublished data), in addition to binding monoubiquitin.

CUE domains, like UIM and UBA domains, require ubiquitin Ile44 for interaction, but not ubiquitin Phe4. This finding suggests that all three domains may interact with ubiquitin in a similar way. In fact, both UBA and CUE domains consist of three known or predicted α-helices. The UBA domains of human Rad23 are three helix barrels with an exposed hydrophobic patch that could interact with the Leu8, Ile44, Val70 patch of ubiquitin (Dieckmann *et al*., 1998; Mueller and Feigon, 2002). Like the CUE domain, the UBA domain carries a highly conserved FP sequence that resides near its predicted hydrophobic interaction surface.

Monoubiquitin binding and intramolecular ubiquitylation

A similarity between UIM and CUE domains is that both domains are required to ubiquitylate the proteins within

which they are found. We refer to this as intramolecular ubiquitylation. Polo *et al*. (2002) proposed that UIM domains may promote intramolecular ubiquitylation by directly binding to the ubiquitin of a thioester-linked ubiquitin-E3 (ubiquitin ligase) catalytic intermediate. This idea is supported by the observation that both UIM and CUE domains mediate intramolecular ubiquitylation through *hect* domain ligases, which form a thiolester intermediate. Another possibility is that monoubiquitin-binding domains bind to ubiquitylation enzymes independently of binding to ubiquitin. This model is consistent with the function of Cue1 in recruiting ubiquitin-conjugating enzymes and with the presence of the gp78 CUE domain in a RING finger ligase, although it is not known if the CUE domains in these proteins are important for their ubiquitylation functions.

For at least two monoubiquitin-binding domains (UIM and CUE), ubiquitin binding and intramolecular monoubiquitylation are coupled. This coupling suggests that the monoubiquitin moiety conjugated to the protein may interact with the monoubiquitin-binding site to regulate its activity. Monoubiquitin binding by the UIM and CUE domains in the cell must be regulated to avoid occupation of the domains by the free intracellular pool of monoubiquitin. In many cases, the attached monoubiquitin might occupy the monoubiquitin-binding domain to restrict access of these domains to their ubiquitylated binding partners. Cleavage of monoubiquitin from the UIM/CUE protein would expose the ubiquitin-binding domain to bind a monoubiquitylated partner in a temporally and spatially appropriate manner.

Materials and methods

Plasmids, strains and reagents

Plasmids used for this study are listed in Table I. To insert an HA epitope at the N-terminus of Vps9, a *Not*I site was introduced after the first methionine codon of *VPS9* in pPS91 (Hama *et al*., 1999). *Not*I-*VPS9* was then subcloned into the centromere-based vector YCplac111 and a fragment encoding an HA_3 tag flanked by *Not*I sites was inserted, resulting in LHP1263. Plasmids encoding His_6-CUE domains were generated by ligation of DNA encoding the appropriate polypeptides into pET-30, using the LIC cloning according to the manufacturer's protocol (Novagen, Madison, WI). DNA encoding the yeast CUE domains (Vps9, amino acids 408–450, 351–451 or 391–451; Cue1, amino acids 65–106; Cue2-1, amino acids 8–50; Cue2-2, amino acids 55–97; Cue3, amino acids 316–358; Cue4, amino acids 74–115; and Cue5, amino acids 97–139) were amplified from yeast genomic DNA. DNA encoding the Tollip CUE domain (amino acids 229–271) and full-length Tollip protein were amplified from the human expressed sequence tag (EST) IMAGE clone 3452585 (ResGen, Carlsbad, CA). Expression and purification of GST and GST–Ub from *E.coli* have been described previously (Shih *et al*., 2002). All mutations were introduced into plasmids by Quikchange™ mutagenesis (Stratagene, La Jolla, CA) and confirmed by automated sequencing. Mutagenic oligonucleotide sequences and the details of plasmid construction are available upon request.

Yeast strains were propagated in synthetic media with dextrose, and supplemented with the appropriate amino acids for plasmid maintenance (Sherman, 1991). *mdp1-1* (*rsp5*, LHY1118) and isogenic wild-type (LHY1117) strains were a gift of Michael Yaffe, University of California, San Diego, CA. All recombinant proteins were expressed in BL21-Codon Plus bacterial strains (Stratagene, La Jolla, CA) except His_6-Vps9, which was expressed in M15 cells (Qiagen, Valencia, CA). All bacterial strains were propagated in standard (LB) medium supplemented with ampicillin, kanamycin and/or chloramphenicol for plasmid maintenance. Anti-His and anti-GST antiserum were purchased from Amersham-Pharmacia (Piscataway, NJ).

Yeast two-hybrid analysis

The yeast strain pJ69-4a (*MAT***a**, *trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ LYS2::GAL1 HIS3, GAL2-ADE2, met2::GAL7-lacZ*) (James *et al*., 1996) was used for all yeast two-hybrid analyses. Plasmids encoding full-length Vps9 (pGT9-1; Hama *et al*., 1999) or Vps9 fragments (amino acids 1–346, 346–451, 408–451 or 421–451) fused in-frame to the Gal4 activation domain in pACT2 (Clontech, Palo Alto, CA) were co-transformed with a plasmid encoding the Gal4-binding domain (pAS2-1, Clontech) or the Gal4-binding domain fused to Ub^{K48R} (LHP537) into pJ69-4a. Two-hybrid interactions were tested by plating transformants on medium lacking histidine or adenine and observing growth after 72 h at 30°C. The strength of the interaction was quantified using the liquid β-galactosidase assay with *o*-nitrophenyl-β-D-thiogalactopyranoside (ONPG) as a substrate according to the Yeast Protocol Handbook (Clontech, Palo Alto, CA).

Ubiquitin-binding experiments

Yeast strains expressing Vps9-HA or His_6-Vps9 were constructed by transforming pGT9HA-2 or pEMBL-VPS9 (kindly provided by Bruce Horazdovsky, Mayo Clinic, Rochester, MN) into LHY2427 (*MAT***a** *vps9Δ::kanMX4 his3Δ leu2Δ met15Δ ura3)*. The preparation of yeast lysates for ubiquitin-binding experiments has been described previously (Shih *et al*., 2002). Binding of proteins expressed in *E.coli* and yeast to ubiquitin was assayed as described previously (Shih *et al*., 2002).

Recombinant proteins and CUE domains were expressed by inducing an *E.coli* culture in logarithmic growth phase with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 24 or 37°C for 3–4 h. The cells were harvested, suspended in 1× phosphate-buffered saline (PBS), 1% Triton X-100, 10% glycerol (~2 ml of lysis buffer per 1 g of wet-weight bacteria) and lysed by sonication (three times for 20 s each). Cell debris was removed by centrifugation at 31 000 *g* for 15 min at 4°C, and this cleared lysate was used for binding experiments.

To immobilize His_6-tagged polypeptides onto TALON resin (Clontech, Palo Alto, CA), the lysate was prepared by sonication as described above, except that the buffer used was 50 mM Na_2HPO_4, 300 mM NaCl at pH 7.0. Binding of His_6-polypeptides to TALON beads and subsequent binding experiments with *E.coli* lysates containing GST fusion proteins have been described (Shih *et al*., 2002).

To measure binding of polyubiquitin chains to immobilized His_6-CUE domains, purified K48-linked ubiquitin chains (Affiniti Research Products Ltd, Exeter, UK) were dissolved at 5 μg/μl in 50 mM Tris–HCl pH 7.6. Ubiquitin chains (5 μg) were incubated with 6 μg of immobilized CUE domain in 200 μl of 1× PBS, 1% Triton X-100 overnight at 4°C. Beads were washed four times with 1× PBS, 1% Triton X-100, and bound ubiquitin was eluted by boiling in Laemmli sample buffer. The presence of ubiquitin chains in bound and flow-through samples was analyzed by fractionation of the samples by 15% SDS–PAGE followed by transfer to nitrocellulose membranes. Proteins on the membranes were denatured further by incubation in 1× Tris-buffered saline (TBS), 0.37% formaldehyde. Ubiquitin was detected with P4G7 (Covance, Berkeley, CA), a monoclonal antibody that recognizes monoubiquitin and polyubiquitin chains (Kahana and Gottschling, 1999).

Isothermal titration calorimetry

The *Saccharomyces cerevisiae VPS9* and *CUE1* genes were cloned from yeast genomic DNA (Invitrogen) using PCR. The CUE domains of yeast Cue1 (residues 44–117) and Vps9 (residues 394–451) were subcloned into the pHis-parallel2 vector (Sheffield *et al*., 1999). The CUE domain proteins were expressed in *E.coli* strain BL21(DE3)-IRL (Novagen) and purified using nickel-NTA resin. The His_6 tags were cleaved during overnight incubation with TEV (tobacco etch virus) protease. The cleaved tags, TEV protease and uncleaved proteins were removed by a second pass over the nickel-NTA column. Lyophilized bovine erythrocyte ubiquitin (Sigma) was dissolved in ITC buffer [100 mM NaCl, 50 mM Na/K phosphate buffer pH 7.5 and 1 mM dithiothreitol (DTT)]. All proteins were dialyzed extensively in ITC buffer. ITC measurements of Cue1 and Vps9 CUE domains were performed on MicroCal MCS-ITC and VP-ITC instruments, respectively, at 30°C. Ubiquitin (final concentration 4 mM) was injected into 1.4 ml of buffer containing CUE domains (200 μM) in 21 injections of 10 μl each. Blank measurements were obtained by a second set of ubiquitin injections into 1.4 ml of the saturated samples. Experimental traces were corrected by subtracting the blank measurements, and analyzed further using Origin 2.9 software (MicroCal). Binding constants were calculated by fitting the integrated titration data.

Detecting ubiquitylation of Vps9

To monitor Vps9 ubiquitylation, *vps9*Δ strains expressing His_6-Vps9, His_6-Vps9^{F420A} and His_6-Vps9Δ^{CUE} were transformed with an empty vector (YEplac195), or a plasmid encoding copper-inducible ubiquitin (LHP585) or c-myc-tagged ubiquitin (pES7). Ubiquitin overexpression was induced by adding 100 μM copper sulfate to a logarithmic phase yeast culture for 4 h. Six OD_{600} units of cells were harvested and lysed by the addition of 1 ml of lysis buffer (10 mM Tris–HCl pH 7.0, 1 mM EDTA, 0.2 N NaOH, 0.5% β-mercaptoethanol, 5 mM *N*-ethylmaleimide). After incubation on ice for 10 min, trichloroacetic acid (TCA) was added to 10% final concentration followed by another 10 min incubation on ice. The precipitates were collected by centrifugation and washed twice with 100% cold acetone. Laemmli sample buffer (2% SDS, 2% β-mercaptoethanol, 10% glycerol, 62.5 mM Tris–HCl pH 6.8, 0.01% bromophenol blue) was added to the precipitates and the samples were heated to 100°C for 5 min. The proteins were resolved by 9% SDS–PAGE and transferred to nitrocellulose membranes (Schleicher & Schuell, Moorestown, NJ). The membranes were incubated with 5% non-fat dried milk, 0.1% NP-40, 50 mM Tris–HCl at pH 7.5, 0.15 M NaCl, before incubation with monoclonal anti-His antiserum (Amersham-Pharmacia, Piscataway, NJ) and goat anti-mouse immunoglobulin conjugated with horseradish peroxidase (Sigma, St Louis, MO). The immunoblot was developed with SuperSignal reagent (Pierce, Rockford, IL).

For the analysis of Vps9 ubiquitylation in *rsp5/mdp1* mutant cells, a plasmid encoding HA-Vps9 (LHP1263) was transformed into LHY1117 (*RSP5*) and LHY1118 (*mdp1-1*). The transformants were grown at 24°C, shifted to 37°C for 1–2 h and lysates were prepared as described above. The lysates were fractionated and Vps9 was detected as described above.

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