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Multiubiquitin Chain Binding and Protein Degradation Are Mediated by Distinct Domains within the 26 S Proteasome Subunit Mcb1

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The 26 S proteasome is a multisubunit proteolytic complex responsible for degrading eukaryotic proteins targeted by ubiquitin modification. Substrate recognition by the complex is presumed to be mediated by one or more common receptor(s) with affinity for multiubiquitin chains, especially those internally linked through lysine 48. We have identified previously a candidate for one such receptor from diverse species, designated here as Mcb1 for Multiubiquitin chain-binding protein, based on its ability to bind Lys48-linked mult ubiquitin chains and its location within the 26 S proteasome complex. Even though Mcb1 is likely not the only receptor in yeast, it is necessary for conferring resistance to amino acid analogs and for degrading a subset of ubiquitin pathway substrates such as ubiquitin-Pro-β-galactosidase (Ub-Pro-β-gal) (van Nocker, S., Sadis, S., Rubin, D. M., Glickman, M., Fu, H., Coux, O., Wefes, I., Finley, D., and Vierstra, R. D. (1996) Mol. Cell. Biol. 16, 6020–28). To further define the role of Mcb1 in substrate recognition by the 26 S proteasome, a structure/function analysis of various deletion and site-directed mutants of yeast and Arabidopsis Mcb1 was performed. From these studies, we identified a single stretch of conserved hydrophobic amino acids (LAM/LALRL/N (ScMcb1 228–234 and At-Mcb1 226–232)) within the C-terminal half of each polypeptide that is necessary for interaction with Lys48-linked multiubiquitin chains. Unexpectedly, this domain was not essential for either Ub-Pro-β-gal degradation or conferring resistance to amino acid analogs. The domain responsible for these two activities was mapped to a conserved region near the N terminus. Yeast and Arabidopsis Mcb1 derivatives containing an intact multiubiquitin-binding site but missing the N-terminal region failed to promote Ub-Pro-β-gal degradation and even accentuated the sensitivity of the yeast Δmcb1 strain to amino acid analogs. This hypersensitivity was not caused by a gross defect in 26 S proteasome assembly as mutants missing either the N-terminal domain or the multiubiquitin chain-binding site could still associate with 26 S proteasome and generate a complex indistinguishable in size from that present in wild-type yeast. Together, these data indicate that residues near the N terminus, and not the multiubiquitin chain-binding site, are most critical for Mcb1 function in vivo.

The ubiquitin/26 S proteasome pathway is a major route for the selective degradation of eukaryotic proteins. Through the removal of key regulatory components, the pathway helps control many aspects of cell homeostasis, growth, and development (1–4). Examples include cell cycle progression, maintenance of chromatin structure, DNA repair, enzymatic regulation, transcription, signal transduction, and programmed cell death. In addition, the ubiquitin pathway participates in cellular housekeeping and the stress response by removing abnormal and denatured proteins.

In the ubiquitin pathway, proteins are first enzymatically tagged for breakdown by the covalent attachment of one or more chains of ubiquitin monomers. This process is catalyzed by an enzymatic cascade, involving ubiquitin-activating enzymes (E1s), ubiquitin-conjugating enzymes (E2s), and ubiquitin-protein ligases (E3s), that couples ATP hydrolysis to ubiquitin ligation (1–3). Attachment is via an isopeptide bond between the C-terminal glycine of ubiquitin and free lysines either in the target or in the preceding ubiquitin in the chain. Within the multiubiquitin chain, Lys48 appears to be the preferred intermolecular linkage site (5, 6) but genetic evidence has implicated several other lysines as well (7). Unique among the ubiquitin pathway enzymes is the 26 S proteasome, a structure composed of multiple subunits and responsible for degrading proteins that are tagged for degradation by the ubiquitin pathway. The 26 S proteasome is composed of two 19 S regulatory complexes, each containing approximately 14 subunits, and a 20 S catalytic core. The 19 S regulatory complexes function to deliver ubiquitinated proteins to the 20 S core and to function as a ubiquitin-specific protease (7). The 20 S core is a cylindrical structure, 200 Å in diameter, composed of 28 subunits, 14 of which are organized into two rings of 14 subunits each. The 20 S core is specific for mult ubiquitin chains, especially those internally linked through lysine 48. We have identified previously a candidate for one such receptor from diverse species, designated here as Mcb1 for Multiubiquitin chain-binding protein, based on its ability to bind Lys48-linked mult ubiquitin chains and its location within the 26 S proteasome complex. Even though Mcb1 is likely not the only receptor in yeast, it is necessary for conferring resistance to amino acid analogs and for degrading a subset of ubiquitin pathway substrates such as ubiquitin-Pro-β-galactosidase (Ub-Pro-β-gal) (van Nocker, S., Sadis, S., Rubin, D. M., Glickman, M., Fu, H., Coux, O., Wefes, I., Finley, D., and Vierstra, R. D. (1996) Mol. Cell. Biol. 16, 6020–28). To further define the role of Mcb1 in substrate recognition by the 26 S proteasome, a structure/function analysis of various deletion and site-directed mutants of yeast and Arabidopsis Mcb1 was performed. From these studies, we identified a single stretch of conserved hydrophobic amino acids (LAM/LALRL/N (ScMcb1 228–234 and At-Mcb1 226–232)) within the C-terminal half of each polypeptide that is necessary for interaction with Lys48-linked multiubiquitin chains. Unexpectedly, this domain was not essential for either Ub-Pro-β-gal degradation or conferring resistance to amino acid analogs. The domain responsible for these two activities was mapped to a conserved region near the N terminus. Yeast and Arabidopsis Mcb1 derivatives containing an intact multiubiquitin-binding site but missing the N-terminal region failed to promote Ub-Pro-β-gal degradation and even accentuated the sensitivity of the yeast Δmcb1 strain to amino acid analogs. This hypersensitivity was not caused by a gross defect in 26 S proteasome assembly as mutants missing either the N-terminal domain or the multiubiquitin chain-binding site could still associate with 26 S proteasome and generate a complex indistinguishable in size from that present in wild-type yeast. Together, these data indicate that residues near the N terminus, and not the multiubiquitin chain-binding site, are most critical for Mcb1 function in vivo.

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a large family of E2 and E3 isozymes working alone or in combination to recognize key degradation signals within the targets (1–3, 11). The second regulatory mechanism involves control of the steady-state level of ubiquitin-protein conjugates prior to breakdown. Conjugate levels are affected not only by the rate of ubiquitination but also by the rate of deubiquitination; a reaction catalyzed by a diverse family of ubiquitin-specific proteases that cleaves the junction between ubiquitin and the protein moiety (2). Through such deubiquitination, proteins can be rescued from degradation (e.g., see Refs. 12–14).

The third mechanism to achieve specificity is the association and breakdown of ubiquitin-protein conjugates by the 26 S proteasome. The 26 S proteasome is composed of two subcomplexes, designated the 19 S regulatory complex and the 20 S proteasome (4, 10, 15, 16). The 20 S proteasome contains the catalytic core of the protease; it exists as a hollow cylinder, created by the assembly of four stacked polypeptide rings, and confines the protease active sites within the lumen (17, 18). The 19 S regulatory complex binds to one or both ends of the 20 S particle. It contains ~15 subunits and confers both ATP and ubiquitin dependence to the holoenzyme complex. The function(s) of most of these subunits are unknown; six belong to the AAA family of ATPases (19, 20). Presumably, the 19 S complex recognizes appropriate targets through the multiubiquitin chain, unfolds the target moiety, and directs the unfolded polypeptide into the lumen of the 20 S complex for breakdown (4, 10). During or after this process, the multiubiquitin chain is disassembled by ubiquitin-specific proteases.

Recognition of ubiquitinated substrates by the 26 S proteasome is proposed to be mediated by one or a few common multiubiquitin chain receptors located in the 19 S particle. We recently identified a candidate for one such receptor from diverse species, designated here as Mch1 for Multiubiquitin chain-binding protein, based on its ability to bind Lys48-linked multiubiquitin chains and its presence in the 26 S proteasome (21, 22). (Additional names include Mbp1 (21), ASF-1 and S5a (23, 24), Sun1 (25), and p54 (26) for the multiubiquitin chain receptors located in the 19 S particle. We considered them as Mcb1 for Multiubiquitin chain-binding protein Mcb1 derivatives (see Fig. 2) were made in the Escherichia coli expression vector pET28a (Novagen, Madison, WI) by PCR strategies. The derived genes were verified as correct by DNA sequence analysis. The 5’-amplification oligonucleotides were designed to add an NdeI site at the native start codon or, for N-terminal-deleted constructions, at the degradation site to create a new start codon. Introduction of the 5’-terminal-deleted constructions, the 3’-amplification oligonucleotides were designed to add an internal stop codon. Introduced restriction sites and stop codons in the various oligonucleotides listed below are underlined and italicized, respectively. Positions of new start and stop codons for the terminal-deleted constructions are indicated in Fig. 2. The PCR products were cloned either directly or through intermediate vectors into pET28a using the NdeI site which was created by the design of the 3’-amplification oligonucleotide (EcoRI, underlined) or derived from intermediate cloning vectors (EcoRI, HindIII, SalI, or NdeI).

For wild-type yeast MCB1 (ScMCB1), the coding region was PCR-amplified from genomic DNA (isolated from strain S288C) using oligonucleotides GCAGTAAACGCCCATATGTTGTTGGAAGTACAG (primer 1) and CTAATTTAGAGGAAAGATTCAACTACCTCCGG (position 75–103 downstream of stop codon) (31). The PCR fragment was cloned through intermediate vectors pGEMT (Promega, Madison, WI) and pET29 (using NdeI/NcoI sites; Novagen) into pET28a using the NdeI site at the 5’ end, which was created by the design of the 5’-amplification oligonucleotide (EcoRI, underlined) or derived from intermediate cloning vectors (EcoRI, HindIII, SalI, or NdeI).

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through intermediate vector pGEM into pET28a using NdeI/SalI and NdeI/NdeI sites, respectively. For Arabidopsis N12, N22, N32, and N44, C4AAGGACATGTTGATTCACTCCTACTGTC, ATTTCG-GGAGGAGATCATATAAGAACGGCTGAA (primer 5), TGGT-GTGAAGGTCGCTGCTACCTCTCTGTC, or GGGCGGCGAGGAGCCTATAGGACGAGG, respectively, was used as the 5'-oligonucleotide and the KS primer was used as the 3'-oligonucleotide. Corresponding PCR products were cloned separately into pET28a using the 5' NdeI site and a 3' HindIII site derived from the cDNA vector. For Arabidopsis N1, N12, N22, and N32, primer 5 and primer 4 were used for PCR amplification. The PCR product was cloned through intermediate vector and amino acid sequence MGSSHHHHHHSSGLVPRGSH, was encoded by Arabidopsis I2 and I3, primer 5 or GAGGGTCAAGTGGCATATGATCGTGCGGCCTGTGCTGTC. An example of the om- oligonucleotide and 1% Nonidet P-40, 0.5% deoxycholate, was used as the 5'-oligonucleotide and AACTGAGGCTTGTTAAGGCGGAAAGAATGTC, respectively, was used as the 3'-oligonucleotide. The PCR products were cloned directly into pET28a using NdeI/EcoRI sites.

For all the site-directed mutants (i.e. yeast N5 and G and Arabidopsis D5/G, D5, N5/G, N5, D/G, D, and G) (see Fig. 2), the QuickChange™ mutagenesis strategy (Stratagene) was employed using either ScMCB1 or AtMCB1 present in pET28a. The oligonucleotides used for the mutagenesis were designed according to the manufacturer's guidelines to have the noncomplementary nucleotides bracketed by 10-22 complementary nucleotides.

Purification of the Yeast 26 S Proteasome—The 26 S proteasome was partially purified from wild-type and mcb1 yeast strains by conventional chromatography as described previously (34). The peak of hydrolytic activity (as measured using the substrate Suc-LLVY) from the DEAE-Aff-Gel blue column (Bio-Rad) was loaded onto a MonoQ column (Pharmacia Biotech Inc.). Protein was eluted using a linear gradient from 0 to 0.5 M NaCl. Fractions were assayed for peptide activity and the presence of Mcb1 and Sugu1/Cim3 (23). The peak of peptidase activity eluted at around 350 mM NaCl.

26 S proteasome preparations were further refined by non-denaturing PAGE using a modification of the protocol of Hoffman et al. (35). PAGE employed a single gel layer consisting of 0.18 M Tris borate (pH 8.3), 5 mM MgCl2, 1 mM ATP, 1 mM dithiothreitol, and 4% acrylamide gel (for 26 S proteasome preparations) or 5% acrylamide gel (for partially purified 26 S proteasomes) with 0.1% ammonium persulfate. The running buffer was the same as above without acrylamide. Xylene cyanol was added to the samples, and the samples were electrophoresed at 100-150 mM until the xylene cyanol migrated through the gel. The position of the 26 S proteasome was visualized by UV light following an overlay with Suc-LLVY-AMC. The fluorescent gels were transilluminated with a UV light and photographed with a Polaroid camera. Sugu1/Cim3 and Mcb1 were detected by immunoblot analysis as described above. Anti-Sugu1/Cim3 serum was generously provided by Dr. Carl Mann (Center d’Etudes de Saclay, Gif-sur-Yvette Cedex, France).

RESULTS

Mutational Analysis of Yeast and Arabidopsis Mcb1—To identify domains in Mcb1 necessary for multiquitin chain recognition, association with the 26 S proteasome, and in vivo functions, a parallel structure/function analysis of yeast and Arabidopsis Mcb1 was initiated by constructing various deletion and amino acid substitution mutants. Amino acid sequence comparisons of Mcb1 homologs from Arabidopsis (21), Drosophila (26), humans (24), and moss3 revealed four highly conserved regions that may function in these capacities (designated domains I-IV, Fig. 1, A and B). Whereas the Drosophila, human, and moss sequences average 47% identity to Arabidopsis Mcb1 over their entire length, the four conserved domains average 72% identity. Yeast Mcb1 also contains the first three conserved domains but is lacking the C-terminal region that includes domain IV (22). Among all five proteins, domain III exhibits the greatest conservation (82% identity). It contains a short stretch of conserved hydrophobic residues (LAL/26 S proteasome H V Q) surrounded by charged amino acids and is preceded by an invariant sequence GYDV (Fig. 1C). Beal et al. (36) previously proposed, from binding studies of mutant multiquitin chains with the 26 S proteasome, that repeated hydrophobic patches formed within assembled multiquitin chains are important for binding to the complex. Based on their data, we have suggested that this conserved hydrophobic patch

3 H. Fu, P. Girod, and R. D. Vierstra, unpublished data.
within Mcb1 could participate in this association (21). Moreover, because Arabidopsis Mcb1 also contains a second LALAL patch near the C terminus of the protein (residues 310–314), it was possible that mult ubiquitin chain binding was strengthened by the cooperative action of these motifs.

Based on these sequence comparisons, a series of N- and C-terminal deletion mutants were generated from both yeast and Arabidopsis Mcb1 with a special emphasis on the four conserved domains (Fig. 2). Domain III was further analyzed by a collection of site-directed mutants designed to alter the hydrophobicity of the LAL/MALRLV motif. The mutant proteins were expressed in E. coli and tested for their ability to bind Lys48-linked mult ubiquitin chains in vitro. Various Arabidopsis and yeast Mcb1 mutants were also expressed in yeast and examined for their ability to complement the phenotypic defects of Δmcb1 (22) and for their ability to integrate into the yeast 26 S proteasome.

The Conserved Hydrophobic Sequence in Mcb1 Is Critical for Binding Mult ubiquitin Chains—Evaluating the chain binding activity of the various yeast and Arabidopsis Mcb1 derivatives was facilitated by the discovery that each derivative accumulated to high levels in a soluble form when expressed in E. coli (Fig. 3A). Similar to the parental molecules (21, 22), the apparent molecular mass (as measured by SDS-PAGE) of the various deletions was substantially greater than their presumed mass. This discrepancy was especially strong for the N-terminal deletions, suggesting that the C-terminal portion has an unusual structure in the presence of SDS. The site-directed mutants that replaced all five of the LAL/MAL residues in domain III with either aspartic acid or asparagine (D5/G, N5/G, D5, and N5) (see Fig. 3) also migrated noticeably slower than the parental polypeptides.

Chain binding was assayed using total protein from induced cultures that had been subjected to SDS-PAGE and electrophoretically transferred onto nitrocellulose membranes (28). The transfer efficiency of the various Mcb1 derivatives differed substantially. This was especially true for the smaller deletion mutants (i.e. yeast N53 and Arabidopsis N53, N4, I1, I2, and I3), which required much lower currents and shorter electrophoretic transfer times. To ensure that equal amounts of protein were transferred onto the membranes, the electrophoresis conditions were varied accordingly and the amount of each Mcb1 protein transferred was verified subsequently by protein staining of the membranes (data not shown). Duplicate membranes were then probed with Lys48-linked mult ubiquitin chains prepared in vitro and radiolabeled with 125I. It should be emphasized that this assay is a qualitative measure of chain binding and may not precisely reflect the relative affinities in solution.

Binding analysis of the N- and/or C-terminal deletions revealed that a region encompassing domains III of yeast and Arabidopsis Mcb1 (amino acids 204–268 and 202–264, respectively) contains a site required for mult ubiquitin chain binding. As shown in Fig. 3B, every derivative containing an intact domain III showed binding activity (e.g. Arabidopsis and yeast N53 and N52, and Arabidopsis C51 and C52); even polypeptides containing just domain III surrounded by a few additional residues were competent (i.e. yeast N53 and Arabidopsis I1, I2, and I3). In contrast, every deletion that removed all or part of domain III failed to bind chains (e.g. yeast C51 and Arabidopsis C53, N53, and N54). Domains I and II were not essential as demonstrated by the strong binding activity of yeast and Arabidopsis N51 and N52. Loss of the first 60 amino acids of yeast Mcb1 actually improved the association of chains with the membrane-bound protein by as much as 5-fold (Fig. 3B). Of interest is the absence of chain binding activity of the Arabidopsis N-terminal deletion N54, in which domain IV and the second LALAL patch are intact (residues 310–314) (21), and the presence of chain binding activity for the Arabidopsis C52, which is missing both domains but containing an intact domain III. These data are inconsistent with a major role for both domain IV and the second LALAL patch in chain recognition.

An essential role for domain III in mult ubiquitin chain recognition was shown more conclusively through the analysis of various site-directed mutants (Figs. 2 and 3B). Substituting the hydrophobic LALAL (226–230) sequence in AtMcb1 with five aspartic acids abolished chain binding, implicating the
FIG. 2. Schematic diagrams of yeast and Arabidopsis Mcb1 mutants. Various yeast (ScMcb1) and Arabidopsis (AtMcb1) Mcb1 derivatives were constructed using PCR strategies (see “Experimental Procedures”). Names of various mutants are designated to the left. The numbering indicates the position of the initiator methionine, the position of the C-terminal residue, or the site of amino acid substitutions. Gray boxes identify the conserved regions (domains I–IV) defined in Fig. 1. The black boxes show the hydrophobic patch, the sequences of which are described above the boxes. The hatched boxes indicate the second LALAL box in AtMcb1. Amino acid sequence alterations in the hydrophobic patch are shown above the box in the corresponding mutants. The ability of various Mcb1 derivatives to bind multiubiquitin chains (see Fig. 3), to confer amino acid analog resistance to the yeast \( \text{Δmcb1} \) mutant (see Fig. 5), and to degrade Ub-Pro-\( \beta \)-gal (see Fig. 8) are summarized to the right. Assay for Ub-Pro-\( \beta \)-gal degradation was performed only with the yeast Mcb1 proteins. ND, not determined.

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hydrophobic patch in particular (mutant D5, Fig. 3B). A more subtle alteration, conversion to five asparagines in both yeast and Arabidopsis Mcb1 (mutant N5, Fig. 3B), also abolished binding activity as did a point mutation in AtMcb1, which replaced Leu\( ^{228} \) with aspartic acid (mutant D); this single substitution within the patch was sufficient to reduce binding over
10-fold. In addition to the five contiguous hydrophobic residues, an adjacent hydrophobic amino acid one residue C-terminal from the patch (Leu234 or Val232 in yeast and Arabidopsis Mcb1, respectively) (Fig. 1) was also critical. Conversion of this amino acid to glycine was sufficient to dramatically impair chain binding (mutant G, Fig. 3B). As expected, Arabidopsis mutants combining the G substitution with mutations D5, N5, and D (D5/G, N5/G, and D/G, Fig. 3B) also failed to bind chains. Although the hydrophobic patch in domain III was clearly essential for multikinuin chain binding, sequence flanking this patch also appeared to influence binding strength. Arabidopsis NA3, which contained the intact hydrophobic stretch but was missing 10 residues within the N-terminal portion of domain III (encompassing the conserved GVDP motif), showed no binding activity (Fig. 3B). Likewise, Arabidopsis I3, which was missing residues more N-terminal to the patch (residues 151–201), showed reduced binding activity as compared with I2 (Fig. 3B).

As reported previously, the Mcb1 family of proteins prefers binding Lys48-linked multiubiquitin chains over ubiquitin monomers, especially those chains containing three or more ubiquitins (21, 22, 24, 28, 37). To test whether domain III alone is sufficient for this selectivity, the profile of multiubiquitin chains bound to full-length yeast Mcb1 was compared with those of the mutants NA2 and NA3. 125I-Labeled chains were incubated with the nitrocellulose-bound proteins, and chains that associated were released subsequently by heating the corresponding regions of the membrane in an SDS-containing buffer. Radioactive chains eluted from equivalent surface areas of the nitrocellulose were subjected to SDS-PAGE and autoradiography. As shown in Fig. 4, the profile of chains bound to NA2 and NA3 was similar to that bound to wild-type yeast Mcb1; chains lengths ≥3 ubiquitins were preferentially enriched as compared with free ubiquitin. It should be noted that the stronger signal obtained with chains released from NA2 reflected the greater amount of radiolabeled chains initially bound to the immobilized protein (see Fig. 3B). Similar results were obtained when full-length Arabidopsis Mcb1 was compared with its mutants, Cα2, NA2, and I3 (data not shown).

The N-terminal Region of Mcb1 Is Required for Amino Acid Analog Resistance—Although MCB1 is not essential in yeast, deletion of this gene results in several phenotypes (22). The most notable is an enhanced growth sensitivity to amino acid analogs, presumably due to the accumulation of abnormal proteins that are normally removed by the ubiquitin pathway (38). To identify region(s) of Mcb1 required for this function, we expressed a number of the yeast and Arabidopsis Mcb1 derivatives (see Fig. 2) in the yeast Δmcb1 strain and examined their ability to complement the growth defect on analog-containing medium.

The wild-type and mutant proteins were expressed from a high copy 2μ plasmid (pRS424) under the control of the yeast MCB1 promoter. All but one of the yeast and all of the Arabidopsis Mcb1 derivatives could be expressed to levels easily detected by immunoblot analysis with anti-Arabidopsis Mcb1 sera (Fig. 5A). Protein levels, estimated from the immunoblots, ranged from approximately 0.1 (e.g. Arabidopsis CA1) to 10 times (e.g. yeast Mcb1 and G) the level of ScMcb1 found normally in wild-type yeast (WT). The notable exception was yeast NA2, which appeared to be expressed at extremely low levels as it could be detected only following extensive development of the immunoblots (data not shown). Although most polypeptides appeared to be stable in vivo, breakdown products of several were observed, especially those containing N-terminal deletions (e.g. yeast NA1 and Arabidopsis NA1 and NA2) and the Arabidopsis site-directed mutants D5/G, D5, and N5 (Fig. 5A).

The growth of yeast Δmcb1 strains expressing the various Mcb1 derivatives was examined on medium containing canavanine (CAN) and p-fluorophenylalanine (pFP), analogs of arginine and phenylalanine, respectively. As can be seen in Fig. 5B, the levels of CAN and pFP used were sufficient to reduce the growth of Δmcb1 by over 100-fold as compared with wild-type yeast. Analog resistance could be completely restored by reintroducing ScMcb1 and restored to at least 50% of the wild-type levels by ectopic expression of AtMcb1. When the mutant proteins were tested, we found that the hydrophobic patch in domain III, necessary for the multikinuin chain binding, was not required for amino acid analog resistance. C-terminal deletions removing most of domain III (yeast CA1 and Arabidopsis CA3) as well as site-directed mutants affecting residues within the hydrophobic patch (yeast N5 and G, and Arabidopsis D5/G, D5, N5/G, N5/D, D, and G) could rescue the growth defect even though none could bind chains by the solid-phase assay used in Fig. 3. The level of resistance provided by these mutants was comparable to that of wild-type yeast and those Δmcb1 strains expressing full-length yeast and Arabidopsis Mcb1. In contrast, the N-terminal region encompassing domain I was essential for analog resistance. Expression of the various N-terminal mutations missing domain I (yeast NA1 and NA2 and Arabidopsis NA1, NA2, NA3, and NA4) not only failed to restore resistance, it actually accentuated the analog sensitivity of Δmcb1 (Fig. 5B). This was especially true for the strains expressing yeast NA1 or NA2, which exhibited a severe growth defect on CAN/pFP-containing medium.

When we used a more quantitative assay involving plating...
efficiency as a measure of analog resistance, similar results were obtained (Fig. 6). At the levels of CAN/pFP used here, only 
−10% of the yeast Δmcb1 cells harboring the 2μ vector alone formed colonies. Plating efficiency was restored to near wild-
type levels by expression of any of the site-directed mutations altering the hydrophobic patch in domain III or almost any of 
the C-terminal deletions. The only exception was Arabidopsis C31, but its slightly lower efficiency was likely caused by the 
poor expression of this mutant protein in yeast (see Fig. 5B). In 
contrast, the strain expressing yeast NΔ1 was >100 times more sensitive to the analogs than Δmcb1, whereas the strains ex-
pressing yeast NΔ2 or Arabidopsis NΔ1, NΔ2, NΔ3, or NΔ4 were −3−7 times more sensitive (Fig. 6). The hypersensitivity 
induced by yeast NΔ1 and NΔ2 was particularly surprising given the low levels of protein present (especially for NΔ2; see 
Fig. 5A) and suggested that the deletions were behaving in a 
dominant negative fashion. This negative effect was only seen 
when Δmcb1 was exposed to the amino acid analogs. When 
Δmcb1 strains expressing the N-terminal deletions were grown 
on complete medium without analogs, plating efficiency was 
indistinguishable from that of Δmcb1 and wild-type yeast 
(data not shown).

It was previously shown that purified Arabidopsis Mcb1 is a 
potent inhibitor of ubiquitin-dependent proteolysis in vitro, 
preumably by binding ubiquitin conjugates in a free form and 
blocking their subsequent interaction with Mcb1 and other 
receptors associated with the 26 S proteasome (39). Based on 
this inhibitory action, it was possible that the growth defect 
observed for the N-terminal deletions was caused by their 
ability to bind multiubiquitin chains through domain III even 
though another function(s) was now impaired (e.g. assembly 
into the 26 S proteasome, interaction with other proteasome 
subunits; see below). To test this possibility, we generated 
 yeast and Arabidopsis double mutants combining the NΔ1 de-
letions with the site-directed L234G or V232G mutations (mu-
tation G) that abolished multiubiquitin chain binding in vitro 
(see Fig. 3B). When yeast NΔ1/G was expressed in Δmcb1, the 
severe growth defect of NΔ1 was suppressed by over 10-fold 
(Fig. 6B). However, the NΔ1/G-expressing strain was still 7 
times more sensitive to CAN/pFP than Δmcb1. For Arabidopsis 
NΔ1/G, no decrease in analog sensitivity was seen as compared 
with that of Arabidopsis NΔ1 (Fig. 6B). These data suggest that 
the negative effect observed for NΔ1 does not require a func-
tional multiubiquitin chain-binding site.

If the N-terminal deletions could behave in a dominant neg-
ative fashion, they could be useful tools to poison selected 
parts(s) of the ubiquitin pathway in vivo (e.g. UFD pathway) (9). 
To test this possibility, we expressed yeast Mcb1 and NΔ1 from 
a high copy 2μ plasmid in wild-type yeast and tested for growth 
inhibition on CAN/pFP plates. Whereas NΔ1 was expressed to
levels similar to that of endogenous ScMcb1, ectopic expression of ScMcb1 resulted in a -10-fold increase in ScMcb1 protein (Fig. 7A). Both the ScMcb1- and NΔ1-expressing wild-type strains showed the same growth resistance to the analogs as wild-type yeast expressing the plasmid alone (Fig. 7B). This lack of dominance suggests that the N-terminal mutants cannot interfere with the function of endogenous ScMcb1. Moreover, it showed that ScMcb1 does not hinder yeast cell survival when expressed above normal levels, thus diminishing its potential value as a pathway inhibitor in vivo.

N-Terminal Domain Is Required for Degradation of Ub-Pro-β-Gal—Prior phenotypic analysis of Δmcb1 (22) showed that ScMcb1 is essential for the breakdown of Ub-Pro-β-gal, a synthetic ubiquitin fusion degraded by the ubiquitin pathway. Unlike ubiquitin fusion substrates bearing other amino acids besides proline at the junction, the ubiquitin moiety in Ub-Pro-β-gal is not cleaved following synthesis (33). This ubiquitin then serves as an acceptor site for further ubiquitination by the UFD subpathway involving the E2/E3 pair encoded by UBC4/5 and UFD4 (9). To identify the domains within Mcb1 required for this breakdown, the stability of Ub-Pro-β-gal was examined by pulse-labeling in Δmcb1 strains expressing various yeast Mcb1 derivatives. Whereas Ub-Pro-β-gal was extremely stable in the Δmcb1 strain, it was rapidly degraded when the ScMCB1 gene was reintroduced (t1/2 = 12 min (Fig. 8)). This instability was similar to that obtained with wild-type yeast and was independent of whether a high copy 2μ vector (pRS424) or a low copy CEN plasmid (pRS314) was used for expression (data not shown and Fig. 8). Each of the modifications tested that deleted amino acid analog sensitivity of wild-type yeast. Yeast Mcb1 (ScMcb1) or the N-terminal deletion NΔ1 were expressed in wild-type (WT) yeast from the high copy 2μ plasmid. A, expression of the ScMcb1 and NΔ1 proteins in yeast. Crude extracts (15 μg) from the Δmcb1 strain, wild-type yeast, and wild-type yeast ectopically expressing ScMcb1 or NΔ1 proteins were subjected to SDS-PAGE and immunoblotted with an Arabidopsis Mcb1 antiserum. The migration positions of the ScMcb1 and NΔ1 proteins are indicated by arrows. B, colonies formed by the various yeast strains when plated on medium containing the amino acid analogs, canavanine and p-fluorophenylalanine (1.5 and 25 μg/ml, respectively). Cultures were grown initially in analog-free medium, resuspended in analog-containing medium to an A600 = 1.0, and then spotted in 10-fold serial dilutions (left to right) onto solidified medium supplemented with the analogs. Colony growth was observed after 6 days.
both species were similar in wild-type and various ScMcb1 derivatives. Various ScMcb1 derivatives were introduced into a Δmcb1 yeast strain expressing the Ub-Pro-β-gal reporter protein. Descriptions of the various Mcb1 mutants are shown in Fig. 2. The metabolic stability of the reporter protein was determined by pulse-chase analysis as described previously (22). Ub-Pro-β-gal was immunoprecipitated from cell lysates and subjected to SDS-PAGE. The level of Ub-Pro-β-gal was quantitated by PhosphorImager analysis and expressed as a percentage of that present at t = 0. In the data shown here, ScMcb1 and mutant derivatives were expressed from a high copy 2μ vector (pRS424) (31). For ScMcb1 and the site-directed mutant N5, similar results were obtained (data not shown) with a low copy CEN vector (pRS314) (43).

The metabolic stability of the reporter protein was determined by pulse-chase analysis as described previously (22). Ub-Pro-β-gal in yeast strain expressing the Ub-Pro-β-gal in yeast mcb1 strains containing various ScMcb1 derivatives. Various ScMcb1 derivatives were introduced into a Δmcb1 yeast strain expressing the Ub-Pro-β-gal reporter protein. Descriptions of the various Mcb1 mutants are shown in Fig. 2. The metabolic stability of the reporter protein was determined by pulse-chase analysis as described previously (22). Ub-Pro-β-gal was immunoprecipitated from cell lysates and subjected to SDS-PAGE. The level of Ub-Pro-β-gal was quantitated by PhosphorImager analysis and expressed as a percentage of that present at t = 0. In the data shown here, ScMcb1 and mutant derivatives were expressed from a high copy 2μ vector (pRS424) (31). For ScMcb1 and the site-directed mutant N5, similar results were obtained (data not shown) with a low copy CEN vector (pRS314) (43).

Analysis of both deletion and site-directed mutants localized a region essential for multiubiquitin chain binding to a hydrophobic patch (LALAL) within domain III, a highly conserved domain present in all Mcb1 proteins (Fig. 1) (22). While it is possible that other regions also participate, data presented here suggest that this motif provides the major interaction site. (i) Mutants missing sequences either C- or N-terminal to the patch (e.g. Arabidopsis CΔ1 and CΔ2 and yeast NΔ1 and NΔ2) show no significant reduction in chain binding. (ii) Deletion mutants containing just domain III flanked by short sequences (e.g. yeast NΔ3 and Arabidopsis I3 containing only 65 and 63 amino acids, respectively) were still capable of binding multiubiquitin chains, albeit at lower levels. (iii) Single amino acid substitutions in the hydrophobic patch, (changing residue Leu102 (yeast) or Val102 (Arabidopsis) to glycine) were sufficient to dramatically impair binding. (iv) The smallest yeast and Arabidopsis Mcb1 mutants containing only domain III had a binding preference for multiubiquitin chains comparable to their full-length counterparts. For Arabidopsis Mcb1 in particular, our data eliminate a significant role for the second LALAL patch near the C terminus of the protein (residues 310–314). A deletion containing just this domain (NΔ4) failed to bind chains whereas a deletion missing this domain (CΔ2) had full binding activity by the solid-phase assay used here.

Our results are consistent with those recently published by Haracska and Udvardy (27) who found that domain III in Drosophila Mcb1 (p54) contains a major chain-binding site. In contrast to our work, they also detected a second chain-binding site within the conserved domain IV, but possibly with weaker affinity. Our mutational analysis does not support a major role for this second site. Domain IV is not present in yeast Mcb1. Although present in Arabidopsis Mcb1, the ability of a single amino acid substitution in domain III (e.g. Val322 → Gly) to completely block chain binding indicates that a second site, if it is present, cannot work autonomously. However, we cannot rule out the possibility that a second site exists but was not
detected by the different assay conditions used here (higher temperature incubations and higher salt washes).

How the binding site in domain III interacts with multiubiquitin chains is not understood. That Mcb1 can still bind multiubiquitin chains even after SDS denaturation and fixation onto nitrocellulose membrane indicates that the binding site in domain III is not essential for the phenotypic function of Mcb1, i.e. resistance to amino acid analogs or for the substrate Suc-LLVY-AMC. The identity of a single dominant binding site in Mcb1 is inconsistent with a model that binding involves multiple patches within Mcb1 interacting with complementary sites formed by the repeated ubiquitin monomers within the chains. It also suggests that the greater affinity of Mcb1 for longer mult ubiquitin chains does not result from increased cooperative interactions among complementary repeated binding sites in both Mcb1 and multiubiquitin chains. Instead, the greater affinity could arise from the enhanced probability that one of the ubiquitin moieties will interact with domain III or from the possibility that longer multiubiquitin chains stabilize a structural motif that is absent in the ubiquitin monomer. In support of the latter, structural differences between the ubiquitin dimer, trimer, and tetramer have been noted (6, 40).

Surprisingly, analysis of the various yeast and Arabidopsis Mcb1 mutants with alternatively linked chains and the structural solution of Mcb1/multiubiquitin complexes will be useful in this regard.

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the ability to degrade Ub-Pro-β-gal. Instead, we found that a distinct domain within the first 60 N-terminal residues (domain I) was critical. Whereas mutations affecting domain III could fully complement the ∆mcb1 deletion, mutants missing domain I failed to promote the degradation of Ub-Pro-β-gal and actually made the ∆mcb1 strain more sensitive to amino acid analogs.

Our ability to separate the phenotypic functions of Mcb1 from its ability to recognize multiubiquitin chains raises a question as to the role of Mcb1 in 26 S proteasome function. Because domain III mutants lack phenotypic consequences, it can be argued that the multiubiquitin chain binding activity seen in vitro is not relevant to the in vivo function of Mcb1. While definitive proof is not yet available, several observations point against this possibility. First, Mcb1 is the only subunit of purified rabbit and Drosophila 26 S proteasomes that displayed an affinity for multiubiquitin chains in vitro (27, 28). Second, the region identified here as involved in ubiquitin binding (domain III) represents the most conserved region of the molecule, implicating it as a critical domain for function. Third, both the hydrophobic nature of domain III and its preference for multimeric over monomeric and dimeric ubiquitin conforms well with predictions made concerning the specificity of a ubiquitin receptor within the 26 S proteasome (5, 28, 36). Fourth, this structural motif is highly specific for multiubiquitin binding because even single amino acid substitutions within domain III were sufficient to substantially impair binding (mutants D and G).

A more likely possibility is that Mcb1 has multiple functions. One function involves multiubiquitin chain recognition but is dispensable by virtue of the presence of other multiubiquitin receptors. The other function, which is more apparent phenotypically, is unclear but presumably requires the N-terminal region. Interestingly, a recent study has also indicated that the N-terminal domain of Mcb1 is important. It showed that human Mcb1 (or S5a) can associate both in vitro and in vivo with Id1, a helix-loop-helix protein that interacts with and blocks transcription of the immunoglobulin heavy chain by virtue of the presence of other multiubiquitin receptors. The other function, which is more apparent phenotypically, is unclear but presumably requires the N-terminal region. Interestingly, a recent study has also indicated that the N-terminal domain of Mcb1 is important. It showed that human Mcb1 (or S5a) can associate both in vitro and in vivo with Id1, a helix-loop-helix protein that interacts with and blocks transcription of the immunoglobulin heavy chain.

What is the in vivo function of the N-terminal region? One obvious possibility is that domain I is required for assembly of Mcb1 into the 26 S proteasome. The hypersensitivity of yeast Mcb1 mutant NΔ1 could be explained by its failure to integrate into the complex while still retaining its multiubiquitin chain binding partners including Nin1 (25) and Soi1, identified by genetic screens and yeast two-hybrid analysis, respectively, as 19 S subunits that interact with Mcb1. Expression of Mcb1 mutants missing the N-terminal domain could interfere with proper assembly of these and potentially other 19 S subunits into the 26 S complex. The location of key residues in domain I as well as the identification of proteins that interact with this region will be critical to clarify the function(s) of Mcb1.

If Mcb1 is not the only ubiquitin receptor in the 26 S proteasome, what are other candidates? Besides Mcb1, no other 26 S proteasome subunits have been shown to have an affinity for multiubiquitin chains using the solid-phase assay (21, 27, 28). It is possible that other receptors were not identified by this assay because they failed to survive SDS denaturation and fixation onto nitrocellulose membranes or because they exist as multisubunit complexes. Both Nin1 and Soi1 have some interaction with Mcb1, but neither have been shown yet to have any ubiquitin binding activity. It is also possible that other ubiquitin receptors are not tightly associated with the 26 S proteasome and dissociate upon purification of the 19 S complex. An attractive possibility is that ubiquitin receptors freely dissociate from the 19 S complex allowing them to shuffle multiubiquitinated proteins to the 26 S proteasome. The fact that a substantial portion of cellular Mcb1 can also be found in a free form, not associated with the 26 S proteasome, is consistent with this notion (21, 22, 26). One possible candidate of this type is p62, a human phosphotyrosine-independent SH2 domain ligand, that has an affinity for ubiquitin (42). Clearly, a combination of biochemical and genetic approaches will be required to identify these additional receptor elements.

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Multiubiquitin Chain Binding and Protein Degradation Are Mediated by Distinct Domains within the 26 S Proteasome Subunit Mcb1
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