Molecular and Cellular Biology, Nov. 1996, p. 6020–6028 Vol. 16, No. 11
0270-7306/96/$04.00+0
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The Multiubiquitin-Chain-Binding Protein Mcb1 Is a Component of the 26S Proteasome in *Saccharomyces cerevisiae* and Plays a Nonessential, Substrate-Specific Role in Protein Turnover

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Received 1 April 1996/Returned for modification 22 May 1996/Accepted 5 August 1996

The 26S proteasome is an essential proteolytic complex that is responsible for degrading proteins conjugated with ubiquitin. It has been proposed that the recognition of substrates by the 26S proteasome is mediated by a multiubiquitin-chain-binding protein that has previously been characterized in both plants and animals. In this study, we identified a *Saccharomyces cerevisiae* homolog of this protein, designated Mcb1. Mcb1 copurified with the 26S proteasome in both conventional and nickel chelate chromatography. In addition, a significant fraction of Mcb1 in cell extracts was present in a low-molecular-mass form free of the 26S complex. Recombinant Mcb1 protein bound multiubiquitin chains in vitro and, like its plant and animal counterparts, exhibited a binding preference for longer chains. Surprisingly, Δ*mcb1* deletion mutants were viable, grew at near-wild-type rates, degraded the bulk of short-lived proteins normally, and were not sensitive to UV radiation or heat stress. These data indicate that Mcb1 is not an essential component of the ubiquitin-proteasome pathway in *S. cerevisiae*. However, the Δ*mcb1* mutant exhibited a modest sensitivity to amino acid analogs and had increased steady-state levels of ubiquitin-protein conjugates. Whereas the N-end rule substrate, Arg–β-galactosidase, was degraded at the wild-type rate in the Δ*mcb1* strain, the ubiquitin fusion degradation pathway substrate, ubiquitin–Pro–β-galactosidase, was markedly stabilized. Collectively, these data suggest that Mcb1 is not the sole factor involved in ubiquitin recognition by the 26S proteasome and that Mcb1 may interact with only a subset of ubiquitinated substrates.

The ubiquitin-proteasome pathway is responsible for the selective elimination of many nuclear and cytosolic proteins in eukaryotes. In this pathway, chains of the 76-amino-acid protein ubiquitin become covalently attached to proteins destined for degradation. These multiubiquitin chains presumably serve as recognition signals for the ATP-dependent breakdown of the conjugated proteins by the 26S proteasome, a ~2-MDa proteolytic complex specific for ubiquitinated intermediates (23, 39, 49). The pathway is responsible for controlling the levels of many short-lived regulatory proteins, including the plant photoreceptor phytochrome A, cyclins, the yeast MATα2 repressor and Gα protein Gpa1, cJun, Mos, Gcn4, components of the NF-κB transcriptional complex, and p53 when associated with the E6 protein from "high-risk" papillomaviruses (19, 25, 27, 32–35, 44, 50). In addition, the ubiquitin-proteasome pathway functions to eliminate abnormal and denatured proteins (45).

Target specificity within the ubiquitin pathway is determined by two recognition steps. The first selects appropriate substrates for ubiquitination. This step involves two protein classes, the ubiquitin-conjugating enzymes (E2s) and ubiquitin-protein ligases (E3s), both of which exist as a diverse enzyme family with specificity toward distinct substrates (7, 28). The coordinated action of these factors generates a ubiquitin-protein adduct in which one or more multiubiquitin chains, linked internally via an isopeptide bond between the carboxyl-terminal Gly-76 of one ubiquitin and a lysine residue in an adjacent ubiquitin, are attached via their carboxyl termini to free lysyl ε-amino groups within the target. Lys-48 appears to be the most common residue involved in linking individual ubiquitins within the chain. In fact, free chains linked through this residue are among the most abundant ubiquitin conjugates in cell extracts from a variety of species (48, 52). Through interactions among neighboring ubiquitins, Lys-48-linked chains can assemble into compact polymers with twofold symmetry (8). In addition to Lys-48, genetic analyses have implicated two other surface-exposed lysines, Lys-29 and Lys-63, in chain assembly (1, 30, 48). The latter linkage may play a special role in error-prone DNA repair (48).

The second step in defining the specificity of ubiquitin-dependent proteolysis involves the recognition of multiubiquitinated proteins by the 26S proteasome. The 26S proteasome contains two subcomplexes of 20S and 19S (39). The 20S particle is a hollow cylinder composed of four stacked rings, each of which contains seven polypeptides. The catalytic sites of the protease are in the interior of the cylinder. The 19S particle associates with one or both ends of the 20S particle and imparts both the ATP dependence and specificity toward ubiquitinated substrates. This regulatory complex contains 15 or more subunits, ranging in size from 35 to 110 kDa; at least six of these subunits are members of a novel ATPase family that

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may assist in protein unfolding prior to polypeptide cleavage (9, 42).

Substrate selection by the 26S proteasome is presumably mediated by the interaction of specific components of the 19S regulatory complex with multiubiquitinated proteins. Although little is known of this essential step, a human 19S regulatory complex subunit, designated S5a, that could bind ubiquitin-lysozyme conjugates in vitro was recently identified (10, 51). The corresponding gene was subsequently identified in *Arabidopsis thaliana* through a protein interaction screen of a cDNA expression library with free, Lys-48-linked multiubiquitin chains as a probe (51). Sequence analyses showed that homologs to this gene, designated *AtMBP1* for *Arabidopsis thaliana* multiubiquitin-binding protein, are present in a wide variety of other eukaryotes as well, including *Saccharomyces cerevisiae*, *Drosophila melanogaster*, *Caenorhaditis elegans*, rice, and castor bean (51). Like S5a, biochemical analyses indicated that the *Drosophila* homolog, designated μ54p, is associated with the 19S regulatory complex (20).

Although both human S5a and *Arabidopsis At*Mbp1 proteins bind ubiquitin monomers, they exhibit a preference for multiubiquitin chains, especially those containing four or more ubiquitin moieties (10, 51). This preference for chains is in accord with the critical role of multiubiquitination, as opposed to monoubiquitination, in targeting proteins for degradation. Both their association with the 19S regulatory complex and their specificity for binding multiubiquitin chains suggested that members of this protein family are important ubiquitin recognition components of the 26S proteasome. However, this role remains to be directly confirmed. It also remains to be determined whether other 26S proteasome components are required for multiubiquitin chain recognition and, if so, whether the different components are restricted to recognizing specific ubiquitinated substrates or specific types of multiubiquitin chains (e.g., Lys-29, Lys-48, and Lys-63 linked). To help answer these questions, we have initiated a molecular genetic analysis of the Mbp1 homolog in *S. cerevisiae*, designated Mcb1 (for multiubiquitin-chain-binding protein). In the present study, we show that Mcb1 is present in cell extracts both in a free form and as a component of the yeast 26S proteasome. Unexpectedly, Δ*mcb1* deletion mutants are viable, grow at near wild-type rates, and degrade the bulk of short-lived protein normally. However, these mutant strains do exhibit a modest sensitivity to amino acid analogs and stabilize a subset of ubiquitin pathway targets. Thus, while Mcb1 appears to be involved in multiubiquitin chain recognition by the 26S proteasome, it is unlikely to be the sole recognition factor.

MATERIALS AND METHODS

Yeast strains, media, genetic techniques, and growth conditions. All genetic manipulations were performed in strain DF5 (*MAT***a**/*MAT*α *lys2-801*/*lys2-801 leu2-3,112*/*leu2-3,112 ura3-52*/*ura3-52 his3-Δ200*/*his3-Δ200 trp1-1*/*trp1-1*) (15) except when otherwise noted. Strain S288C was used as the wild-type DNA source for the PCR-based amplification of the *MCB1* gene. Strains DY85 (wild-type) and SUB459 were used for purification of 26S proteasome. SUB459 carried a plasmid directing the expression of the Pre1 protein tagged at the carboxyl terminus with hexahistidine and otherwise is isogeneic to the *pre1* strain YW071 (41). Plasmids directing the expression of ubiquitin–Arg–β-galactosidase (ub–Arg–β-gal) and ubiquitin–Pro–β-galactosidase (ub–Pro–β-gal) were as previously described (3). Phenotypic analyses were carried out with *MAT***a** derivatives. Transformation was carried out as described by Gietz et al. (18). Standard techniques were used for tetrad dissection (40). Synthetic medium consisted of 0.7% yeast nitrogen base (Difco) supplemented with amino acids, uracil, adenine, and 2% glucose. Specific amino acids were omitted from the medium when necessary to maintain selection. Arginine and phenylalanine were omitted from medium supplemented with canavanine and *p*-fluorophenylalanine, respectively, and methionine was omitted from the medium for pulse-labeling experiments. Cultures were grown at 30°C except when indicated. For analyses of plating efficiency in response to UV irradiation, cells were plated and irradiated at 254 nm with a Stratalinker UV cross-linker (Stratagene) and the plates were incubated in the dark.

Cloning of *MCB1*. The *MCB1* gene was amplified by PCR in a 50-μl reaction mixture containing 100 ng of genomic DNA, 10 mM Tris-HCl (pH 9.0; 25°C), 50 mM KCl, 0.1% Triton X-100, 2.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates, 1.25 U of *Taq* DNA polymerase (Promega), and 240 nM oligonucleotide primers (5′-TGCATCATTGCGAATACCGAG-3′ and 5′-CTATTTAGAGGA AGAGATCTCAAACCTGG-3′). The reaction mixture was incubated at 94°C for 4 min and then subjected to 20 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min. The final, 72°C incubation was extended by 10 min. The product was subcloned, and the DNA sequence was determined in its entirety from both strands. Nucleotide and amino acid sequence analyses were performed with programs from the University of Wisconsin Genetics Computer Group software package (12).

Production and purification of recombinant Mcb1. By using PCR with a mutagenic primer (5′-GCAGTAACCGCCATATGGTATTGGAAGCTACAG-3′), an *Nde*I restriction site was introduced into the *MCB1* gene at the presumed start codon. This allowed introduction of the gene into the *Escherichia coli* expression vectors pET29 and pET28 (Novagen) for production of full-length Mcb1 or Mcb1 containing an amino-terminal hexahistidine tag, respectively. *E. coli* BL21(DE3) cells carrying pET29*MCB1* or pET28*MCB1* were induced in log phase by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to the medium and lysed 3 h later by sonication. Recombinant full-length Mcb1 protein was purified from the soluble fraction by a combination of anion-exchange chromatography and gel filtration. The cells were lysed in 50 mM Tris-HCl (pH 7.5; 22°C)–2 mM tetrasodium EDTA–0.5 mM dithiothreitol. Clarified cell lysate was applied to a DE-52 column (Whatman; bed volume, 10 ml) equilibrated in the same buffer. Protein eluting between 50 and 100 mM NaCl was applied to a 1.9- by 95-cm Sephadex G-75 column (flow rate, 18 cm/h) equilibrated in 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES, pH 8.0; 4°C)–100 mM NaCl–0.5 mM dithiothreitol. Immunopositive fractions were pooled and concentrated by ultrafiltration. Recombinant Mcb1 was analyzed for multiubiquitin-chain-binding activity as previously described for *Arabidopsis At*Mbp1, using ^{125}I-labeled, Lys-48-linked multiubiquitin chains prepared with *Zm*Ubc7 enzyme (51).

Purification of the 26S proteasome. A clarified yeast extract was fractionated by sucrose density centrifugation (38). An equal volume of each fraction was assayed for the ability to hydrolyze the substrate Suc-LLVY-AMC in the presence of ATP (41). Purification of the yeast 26S proteasome either by conventional chromatography or by nickel-chelate affinity chromatography was described previously (41). Briefly, for purification by conventional chromatography, the eluate from the DEAE–Affi-Gel Blue column was further fractionated by anion-exchange and gel filtration chromatography (41). For nickel chelate affinity chromatography, eluates from the DEAE–Affi-Gel Blue column were loaded directly onto Ni-nitrilotriacetic acid agarose columns (Qiagen, Inc., Chatsworth, Calif.). Nonspecifically bound proteins were eluted with 100 mM NaCl and 15 mM imidazole. 26S proteasome was eluted with 100 mM NaCl and 100 mM imidazole (41).

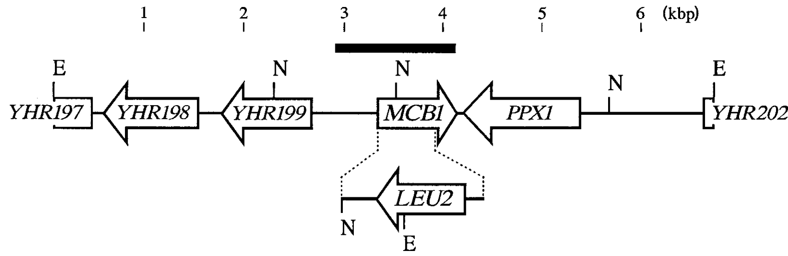
DNA and RNA gel blot analyses. Yeast genomic DNA and total RNA were isolated on Qiagen nucleic acid purification columns by using protocols supplied by the manufacturer. DNA (2 μg) was digested with appropriate restriction endonucleases and fractionated by electrophoresis on a 1% agarose gel. RNA (5 μg) was fractionated on 1% agarose–formaldehyde gels. Nucleic acids were transferred to Zeta-probe membranes (Bio-Rad, Melville, N.Y.) by the method described by Sambrook et al. (43). The probe was labeled with [^{32}P]dCTP via random priming (43) and hybridized to membrane-bound nucleic acids at 65°C for 8 to 16 h in 0.25 M Na_2HPO_4 (pH 7.2; 22°C)–1 mM tetrasodium EDTA–7% sodium dodecyl sulfate (SDS).

Strain construction. The Δ*mcb1*::*LEU2* allele was produced as follows. A 1,460-bp *Ssp*I fragment from pRS415 (Stratagene) containing the *LEU2* gene was first ligated into the *Eco*RV site of pBluescript II KS (Stratagene) to create the plasmid pKS*LEU2*. A 342-bp *Bam*HI fragment from pET29*MCB1* containing 224 bp of *MCB1* coding region and 82 bp of 3′ noncoding region was ligated into the *Bam*HI site of pKS*LEU2* to create plasmid pKS*LEU2MCB1*T. The oligonucleotide primers 5′-TGCATCATTGCGAATACCGAG-3′ and 5′-CTGTAGCT TCCAATACCATATGGCGGTTACTGC-3′ were used in PCR to amplify the *MCB1* promoter region from yeast genomic DNA (for the conditions used, see above). The PCR product was cloned into pGEM-T (Promega) to create p*MCB1*P. An *Apa*I-*Sal*I fragment from p*MCB1*P containing the entire PCR product was ligated into the *Apa*I-*Sal*I sites of pKS*LEU2MCB1*T. An *Apa*I-*Xba*I fragment from the resulting plasmid containing the *LEU2* gene flanked by *MCB1* sequence was used for transformation.

To create the corresponding complementing plasmid pCHG1*MCB1*, a *Nde*I-*Sac*I fragment from pET29*MCB1* containing the entire *MCB1* coding sequence and 82 bp of *MCB1* 3′ sequence was ligated into the *Nde*I-*Sac*I sites of p*MCB1*P. An *Apa*I-*Sac*I fragment from the resulting plasmid was ligated into the *Apa*I-*Sac*I sites of pCHG1, a pRS313-derived plasmid (47) (a gift of P. James, University of Wisconsin—Madison). The *MCB1* gene in pCHG1*MCB1* contained an *Nde*I site introduced at the presumed start codon but was otherwise identical to the wild-type *MCB1* gene.

Immunological techniques. For protein extraction, yeast cultures were grown in liquid medium to an optical density at 600 nm of 0.8. Cells were collected by

A



B

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Yeast   MVLEATVLVIDNSEYSRNGDFPRTRFEAQIDSVEFIFQAKRNSNPENTVGLISGAGANPR   60
Arabi   MVLEATMICIDNSEWMRNGDYSPSRLQAQTEAVNLLCGAKTQSNPENTVGILTMAGKGVR
Human   MVLESTMVCVDNSEYMRNGDFLPTRLQAQQDAVNIVCHSKTRSNPENNVGLITLAN.DCE
Droso   MVLESTMISFDNSDFQRNGDYFPTRLIVQRDGINLVCLTKLRSNPENNVGLMTLSN.TVE

Yeast   VLSTFTAEFGKILAGLHDTQIEGKLHMATALQIAQLTLKHRQNKVQHQRIVAFVCSPISD  120
Arabi   VLTTPTSDLGKILACMHGLDVGGEINLTAAIQIAQLALKHRQNKNQRQRIIVFAGSPIKY
Human   VLTTLTPDTGRILSKLHTVQPKGKITFCTGIRVAHLALKHRQGKNHKMRIIAFVGSPVED
Droso   VLATLTSDAGRIFSKMHLVQPKGEINLLTGIRIAHLVLKHRQGKNHKMRIVVFVGSPINH

Yeast   SRDELIRLAKTLKKNNVAVDIINFGE..IEQNTELLDEFIAAVNNPQEETSHLLTVTPGP  178
Arabi   EKKALEIVGKRLKKNSVSLDIVNFGEDDDEEKPQKLEALLTAVNN..NDGSHIVHVPSGA
Human   NEKDLVKLAKRLKKEKVNVDIINFGEE..EVNTEKLTAFVNTLNGKDGTGSHLVTVPPGP
Droso   EEGDLVKQAKRLKKEKVNVDIVSFGDH..GNNNEILTAFINALNGKDGTGSHLVSVPRGS

Yeast   RLLYENIASSPIILEEGSSGMGAFGGSGGDSDANGTFMDFGVDPSMDPELAMALRLSMEE  238
Arabi   NALSDVLLSTPVF..TGDEGASGYVSAAAAAAAAGGDFDFGVDPNIDPELALALRVSMEE
Human   .SLADALISSPILAGEGG.AMLGLGASD.........FEFGVDPSADPELALALRVSMEE
Droso   .VLSDALLSSPIIQGEDGMGGAGLGGNV.........FEFGVDPNEDPELALALRVSMEE

Yeast   EQQRQE......................................................  244
Arabi   ERARQEAAAKKAADEAGQKDKDGDTASAS.........................QETVAR
Human   QRQRQEEERRRAAAASAAEAGIATTGTE...................DSDDALLKMTIS.
Droso   QRQRQESEQRRANPDGAPPTGGDAGGGGVSGSGPGNEESAGAENEANTEEAMLQRALAL

Yeast   ............................................................  244
Arabi   TTDKNAEPMDEDSALLDQAIAMSVGDVNMSEAADEDQDLALALQMSMSGEESSEATGAGN
Human   QQEFGRTGLPDLSSMTEEEQIAYAMQMSLQGAEFGQAESADIDASSAMDTSEPAKEEDDY
Droso   STETPEDNLPDFANMTEEEQIAFAMQMSMQDAPDDSVTQQAKRPKTDEANAPMDVDEDYS

Yeast   ................................RLRQQQQQDQPEQSEQPEQHQDK*   268
Arabi   NLLGNQAFISSVLSSLPGVDPNDPAVKELLASLPDESKRTEEEESSSKKGEDEKK*
Human   DVMQDPEFLQSVLENLPGVDPNNEAIRNAMGSLASQATKDGKKDKKEEDKK*
Droso   EVIGDPAFLQSVLENLPGVDPQSEAVRDAVGSLNKD..KDKKSDGKDSQKK*
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C

	Yeast MCB1	*Arabidopsis* MBP1	*Drosophila* μ54p
Human S5a	69/50	71/49	75/60
Drosophila μ54p	66/42	69/45	
Arabidopsis MBP1	68/45		

centrifugation and lysed by vortexing with glass beads in lysis buffer (50 mM Tris-HCl [pH 8.0; 4°C], 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 14 mM 2-mercaptoethanol, 2 mM tetrasodium EDTA), with the addition of 0.5 mM phenylmethylsulfonyl fluoride (100 mM in isopropanol) and 100 nM pepstatin A just before use. Proteins were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and electroeluted onto polyvinylidene difluoride membranes (Immobilon-P; Millipore Corp., Bedford, Mass.) or nitrocellulose membranes (HAHY; Millipore). Anti-Mcb1 serum was produced in a New Zealand White rabbit with highly purified, recombinant Mcb1 as the antigen (see above). Immunoblot analyses were performed with either these immunoglobulins or affinity-purified anti-oat-ubiquitin immunoglobulins (27). Antibodies to Cim5 and Sug1 were a gift from Carl Mann (Centre d'Etudes de Saclay, Gif-sur-Yvette Cedex, France). The presence of the primary antibodies was visualized with alkaline phosphatase-labeled goat anti-rabbit immunoglobulins (Kirkegaard and Perry Laboratories, Gaithersburg, Md.) and the substrates nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

Degradation assays. To measure bulk protein turnover, wild-type and Δ*mcb1* cells were grown to exponential phase (optical density at 600 nm, 0.4 to 1.0) at 30°C in synthetic glucose medium supplemented with amino acids. Each culture was divided into 1-ml aliquots and incubated for 1 h at either 25 or 37°C. Half of the cultures at each temperature were supplemented with canavanine (500 μg/ml) during the last 15 min. Cell cultures were pulse-labeled for 2 min with 50 to 100 μCi of ^{35}S-labeled protein labeling mix (New England Nuclear, Boston, Mass.). The cells were washed twice with 0.5 ml of prewarmed chase medium (labeling medium containing 0.5 mg of cycloheximide per ml and 1 mg of methionine per ml), resuspended in 0.6 ml of chase medium, and returned to the appropriate temperature to initiate the chase. At 10-min intervals, duplicate 5-μl aliquots were spotted onto Whatman 3MM filters presoaked in 50% trichloroacetic acid (TCA). Dried filters were incubated in 10% TCA for 10 min, brought to a boil, washed sequentially in 10% TCA and 95% ethanol, dried again, and counted in a scintillation counter. The amount of TCA-precipitable radioactivity at each time point was normalized to the zero time point. The results of three independent experiments were averaged.

The turnover of ub–Pro–β-gal and Arg–β-gal in wild-type and Δ*mcb1* cells carrying plasmids expressing either of these two reporter proteins was measured (3). Cells were grown to exponential phase at 30°C in synthetic medium supplemented with 2% raffinose, 2% galactose, and amino acids. The methods for pulse-chase and immunoprecipitation were essentially the same as those described by Finley et al. (16), except that trichodermin was omitted from the chase and the methionine concentration in the chase medium was increased to 1 mg/ml. Immunoprecipitated samples were separated by SDS-PAGE, and the dried gels were exposed to Phosphorimager cassettes for 3 to 7 days.

RESULTS

Identification and cloning of *MCB1*. In a previous study, we identified in the EMBL/GenBank database an *S. cerevisiae* DNA sequence highly similar to the *Arabidopsis AtMBP1* cDNA (57.1% nucleotide sequence identity over 763 bp). This gene, designated *YHR200* by Johnston et al. (31) and here designated *MCB1*, is located on chromosome VIII between *YHR199* (a gene of unknown function) and the exopolyphosphatase gene *PPX1* (53) (Fig. 1A). The *MCB1* gene was amplified by PCR from genomic DNA, sequenced in its entirety, and confirmed as being identical to that reported by Johnston et al. (31). No other sequence present in the complete yeast genome database displayed significant homology to *MCB1*. Furthermore, DNA gel blot analysis of chromosomal DNA with an *MCB1* probe detected only fragments derived from the *MCB1* gene, even at low stringency and with extended exposures of the autoradiograph (data not shown). These observations indicate that *S. cerevisiae* does not contain a closely related homolog of *MCB1.*

MCB1 encodes a 268-residue protein with a predicted mass of 29,747 Da and a predicted pI of 4.56 (Fig. 1B). The Mcb1 protein exhibits significant amino acid sequence homology to *Arabidopsis At*Mbp1 (68% similarity and 45% identity) and its human (S5a [69% similarity and 50% identity]) and *Drosophila* (μ54p [66% similarity and 42% identity]) homologs (Fig. 1B and C). These homologs exhibit a comparable degree of homology with each other (Fig. 1C). However, Mcb1 is ~120 amino acids shorter than the others in the group. The carboxyl terminus of Mcb1 contains a glutamine-rich tract (14 of the last 29 residues) adjacent to a domain highly conserved in all other Mcb1 homologs (residues 217 to 239 in Fig. 1B). This domain comprises one of the repeated elements proposed to be involved in multiubiquitin chain recognition by the *At*Mbp1 protein (51). A search of sequences listed in the *PROSITE Dictionary of Protein Sites and Patterns* failed to identify any significant, previously characterized sequence motifs within the Mcb1 protein.

When the *MCB1* gene was expressed in *E. coli*, it directed the synthesis of a protein with an apparent molecular mass of 40 kDa, substantially larger than the molecular mass of ~30 kDa predicted from its sequence. A similar disparity between the expected and observed molecular mass was previously seen with recombinant *At*Mbp1, S5a, and μ54p (14, 20, 51). Antiserum generated against recombinant Mcb1 recognized a 40-kDa protein present in wild-type yeast extracts that was absent in extracts from the Δ*mcb1* deletion mutant (see below), indicating that this protein was the product of the *MCB1* gene. During SDS-PAGE, this protein comigrated with recombinant Mcb1, suggesting that Mcb1 produced in *E. coli* was not structurally modified. Upon extended development of the immunoblot, a 28-kDa protein was detected as well. This species was also present in extracts from Δ*mcb1* cells, indicating that it was not a degradation product or modified form of Mcb1 (data not shown). Like *Arabidopsis At*Mbp1 and human S5a, recombinant yeast Mcb1 bound Lys-48-linked multiubiquitin chains following SDS-PAGE and immobilization on nitrocellulose membranes (Fig. 2A and B). Also like *At*Mbp1 and S5a, this binding was selective for longer chains (Fig. 2C). Quantitation of the bands in Fig. 2C by Phosphorimager analysis indicated that in vitro, Mcb1 preferentially bound chains longer than four ubiquitin units (data not shown).

The *MCB1* gene encodes a component of the 26S proteasome. Immunoblot analysis of total cell lysates subjected to sucrose gradient fractionation detected a bimodal distribution of Mcb1. One peak included the most slowly sedimentating fractions, and the other peak coincided with rapidly sedimentating fractions that contained 26S proteasome enzymatic activity (Fig. 3A and B). The presence of the 26S proteasome in the latter fraction was confirmed by using antibodies specific for Cim5, a likely ATPase previously shown to be a component of the 26S complex (17, 41). Unlike Mcb1, Cim5 was detected only in the rapidly sedimentating fraction, suggesting that the appearance of Mcb1 in an apparent free form did not result from posthomogenization dissociation of the 26S complex (Fig. 3A and B).

To verify that Mcb1 was associated with the 26S proteasome, the complex was purified by two independent methods and analyzed immunologically for Mcb1. One purification method used multiple conventional chromatography steps, whereas the

FIG. 1. Structure and genomic organization of yeast *MCB1*. (A) Organization of the region of chromosome VIII containing the *MCB1* gene, based on the results of Johnston et al. (31). Boxes define the open reading frames; arrowheads indicate the direction of transcription. The solid box depicts the position of a molecular probe used in DNA and RNA gel blot analyses. The region of the *MCB1* gene replaced with the *LEU2* marker is shown. Restriction sites used in DNA gel blot analysis are indicated: N, *Nde*I; E, *Eco*RV. (B) Amino acid sequence alignment of *S. cerevisiae* (Mcb1), *Arabidopsis* (AtMbp1 [51]), human (S5a [14]), and *Drosophila* (μ54p [20]) Mcb proteins. Residues that are identical or similar between these sequences are displayed in black and grey, respectively. Numbers correspond to the amino acid sequence positions of yeast Mcb1. This figure was generated with the computer program BoxShade 2.7 (26). (C) Amino acid sequence relationships between the Mcb1 homologs characterized to date. Numbers reflect the homology (percent similarity/percent identity) between the corresponding sequences. Sequences are as in Fig. 1B.

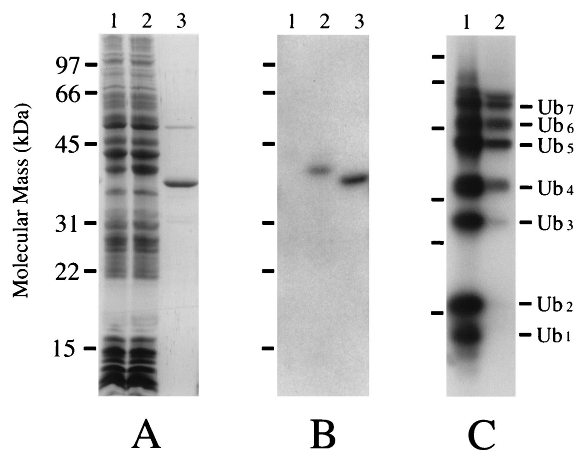

FIG. 2. Expression and multiubiquitin-chain-binding activity of recombinant Mcb1 protein. (A) Expression and purification of Mcb1. Cell lysate from *E. coli* carrying pET28 (lane 1) or pET28*MCB1* (lane 2) or 0.125 μg of purified, recombinant, full-length Mcb1 (lane 3) was fractionated by SDS-PAGE and stained for total protein. (B) Protein as in panel A was transferred to nitrocellulose membranes and probed with ^{125}I-labeled, Lys-48-linked multiubiquitin chains. The Mcb1 protein in lane 2 carries an amino-terminal, hexahistidine tag and migrates more slowly than the nontagged Mcb1 protein in lane 3. (C) ^{125}I-labeled multiubiquitin chains before binding (lane 1) and after elution from Mcb1 (lane 2). The majority of bound radioactivity was eluted from the immobilized Mcb1 protein by heating in an SDS-containing buffer. The migration positions of molecular mass standards and free multiubiquitin chains are indicated on the left and right, respectively.

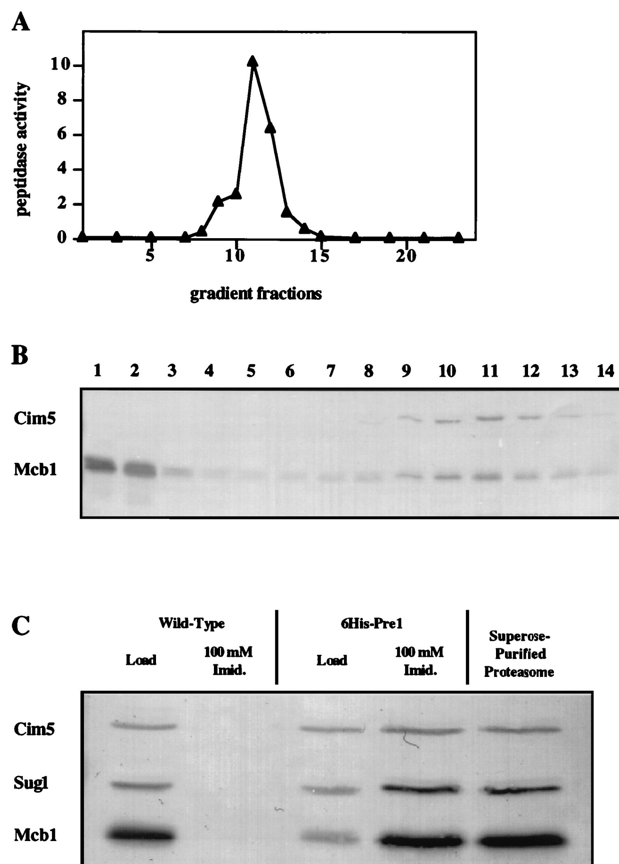

FIG. 3. Association of Mcb1 with the 26S proteasome. (A) Analysis of sucrose density centrifugation fractions from total yeast cell lysate. Triangles represent peptidase activity against the substrate Suc-LLVY-AMC (measured in arbitrary fluorescence units). (B) Fractions from the sucrose gradient were analyzed by SDS-PAGE and immunoblotting with antibodies specific for Cim5 or Mcb1. The 26S proteasome coelutes with the major peak of peptidase activity, as indicated by the presence of Cim5. A small shoulder peak of peptidase activity (fraction 9) is most probably due to free 20S complex. (C) The 26S proteasome was purified by two methods. The Pre1 subunit of the 26S proteasome was tagged with a hexahistidine (6His) epitope. Extracts from 6His-Pre1-expressing and wild-type control strains were fractionated on Ni affinity columns. The epitope-tagged complex elutes at 100 mM imidazole (Imid.), as indicated by either peptidase activity or ubiquitin-conjugate degrading activity (41). Equal proportions of wild-type and 6His-Pre1 imidazole eluates were run on SDS-PAGE gels (12% polyacrylamide) and immunoblotted. The blot was sequentially treated with Cim5, Sug1 (Cim3), and Mcb1 antibodies. Sug1, Cim5, and Mcb1 were detected in the 100 mM imidazole eluate of extract from the 6His-Pre1 strain but not in the wild-type strain. Sug1 (Cim3), Cim5, and Mcb1 proteins are also detected in the Superose-purified 26S proteasome preparation. This preparation was identical to that used by Rubin et al. (41).

other exploited nickel-chelate affinity chromatography to isolate the 26S complex bearing a hexahistidine-tagged, 20S-particle subunit, Pre1 (Fig. 3C). The latter method provided a rapid and efficient means of partially purifying the 26S complex (41). In both of these procedures, Mcb1 copurified with the 26S proteasome as demonstrated by its coelution with two known components of the 19S regulatory complex, Cim5 and Sug1 (Fig. 3C) (41).

Mcb1 has a nonessential function in the ubiquitin proteolytic pathway. To help define a functional role for *MCB1*, a deletion strain in which most of the *MCB1* coding region was replaced with the *LEU2* gene was engineered. The positions of the *LEU2* insertion and corresponding *MCB1* deletion are depicted in Fig. 1A. DNA isolated from Leu$^+$ transformants was subjected to DNA gel blot analysis with a fragment of the *MCB1* gene as a probe (Fig. 1A and 4A). Transformants exhibiting the restriction pattern expected for proper disruption of the *MCB1* gene were sporulated and subjected to tetrad analysis. In each case, all four products of meiosis were viable, and leucine auxotrophy segregated in a 2:2 pattern (data not shown). The viability of the Δ*mcb1* strain was surprising, given the proposed role for Mcb1 as a ubiquitin recognition component of the 26S proteasome.

We verified that *MCB1* expression was eliminated in the Leu$^+$ segregants by RNA gel blot analysis and by immunoblot analysis. Both the ~950-nucleotide *MCB1* RNA and the 40-kDa Mcb1 protein present in the wild-type segregant were undetectable in the Leu$^+$ segregant (Fig. 4B and C, respectively). Because of the proximity of the *PPX1* gene, a significant portion of the *MCB1* coding region was left unaltered to preserve any *cis* elements important for transcription of *PPX1*. In no case was an RNA species or immunoreactive protein observed that would be consistent with expression of the remaining *MCB1* sequence. Microscopic examination of these mutants revealed neither gross physical abnormalities nor an obvious defect in sporulation of the corresponding Δ*mcb1*/Δ*mcb1* diploid strain (data not shown). In fact, Δ*mcb1* mutants exhibited only a very minor growth defect under standard growth conditions (Fig. 5 and data not shown). In addition, Δ*mcb1* mutants exhibited a wild-type response to stress conditions. The plating efficiency of the Δ*mcb1* strain was almost identical to that of the wild type after exposure to elevated temperatures (38.5°C) for extended periods or irradiation with graded fluences of UV (254-nm) light (Fig. 6).

Protein degradation in Δ*mcb1* mutants. The growth defect of the Δ*mcb1* deletion strain was substantially enhanced by addition to the medium of the arginine and phenylalanine analogs canavanine and *p*-fluorophenylalanine, respectively (Fig. 5 and data not shown). Previous studies have shown that uptake of these analogs increases the production of abnormal

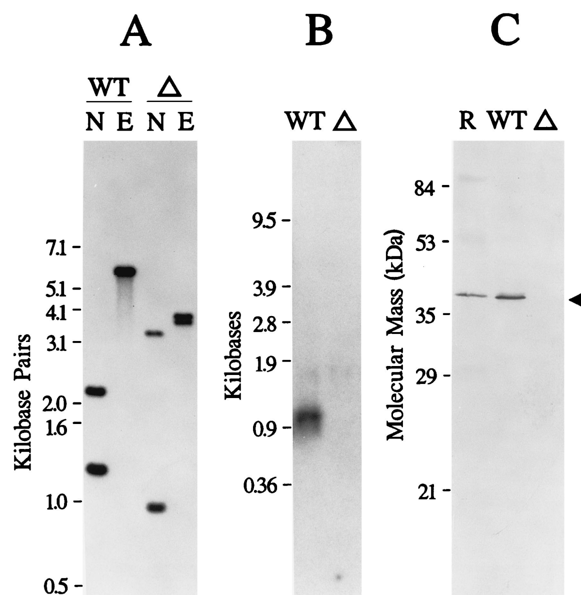

FIG. 4. Molecular characterization of *MCB1* in the wild-type and Δ*mcb1* haploid strains. (A) DNA gel blot analysis. Yeast genomic DNA from the wild-type (WT) or Δ*mcb1* strain (Δ) was digested with the restriction endonuclease indicated (N, *Nde*I; E, *Eco*RV) and subjected to DNA gel blot analysis with a fragment of the *MCB1* gene as a probe (Fig. 1A). DNA size standards are indicated. (B) RNA gel blot obtained with the *MCB1* probe. Approximately 5 μg of total RNA was run in each lane. RNA size standards are indicated. (C) Immunoblot analysis. Total yeast cell lysate was separated on an SDS-PAGE gel (12% polyacrylamide) and immunoblotted with Mcb1 antibodies. Lane R contains 5 ng of purified, recombinant Mcb1. The migration positions of molecular mass standards (in kilodaltons) are marked. The arrowhead indicates the migration position of Mcb1.

proteins, which are in turn degraded by a ubiquitin-dependent mechanism (45). The sensitivity of the Δ*mcb1* strain to these analogs was directly related to the loss of the *MCB1* gene, as demonstrated by the rescue of this growth defect by expression of the *MCB1* gene on an extrachromosomal plasmid (Fig. 5). To see if this phenotype resulted from a failure to degrade ubiquitin-protein conjugates, we measured the degradation rates of short-lived and canavanine-containing proteins in the

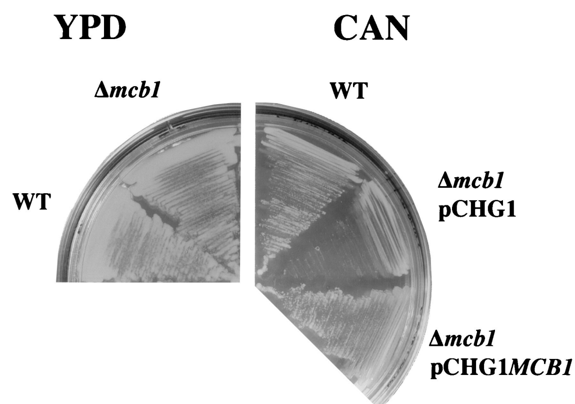

FIG. 5. Growth defect conferred by the Δ*mcb1* mutation. Growth of the Δ*mcb1* deletion strain was similar to that of the wild-type strain (WT) on rich medium (YPD). However, on minimal medium supplemented with 6 μg of canavanine per ml (CAN), the Δ*mcb1* strain (carrying the centromeric cloning vector pCHG1) failed to form colonies after 6 days. This defect was rescued by expression of the *MCB1* gene on plasmid pCHG1*MCB1*.

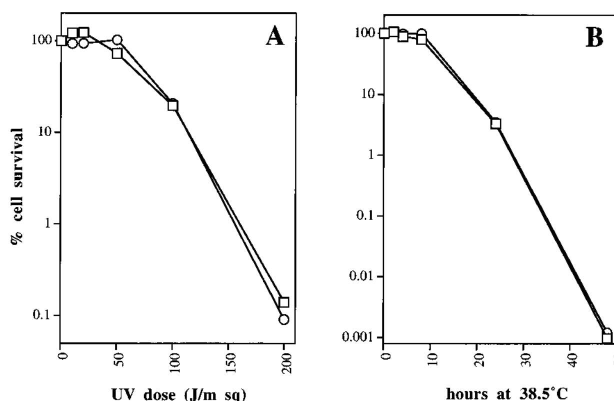

FIG. 6. Response of Δ*mcb1* mutants to UV radiation or heat stress. The plating efficiency of wild-type (squares) and Δ*mcb1* mutant (circles) strains was examined following UV irradiation or heat stress. Cultures growing exponentially in YPD medium were spread onto YPD plates in serial dilutions. Plates were exposed for various times to either 254-nm UV light (A) or 38.5°C (B) and subsequently incubated for 3 days at 23°C before colonies were counted.

wild-type and Δ*mcb1* strains. The bulk of short-lived proteins was found to be degraded at a normal rate of Δ*mcb1* mutants (Fig. 7A). In addition, wild-type and Δ*mcb1* strains exhibited an indistinguishable rate of bulk protein turnover in the presence of canavanine or at elevated temperature (Fig. 7A). Although it was unexpected that the Δ*mcb1* strain would be sensitive to canavanine in the absence of any observable defect in the degradation of canavanine-containing proteins, this phenomenon has been observed with other ubiquitin pathway mutants (48).

Immunoblot analysis of the mutant strain with anti-ubiquitin immunoglobulins showed an approximately twofold enhanced level of ubiquitin-protein conjugates in the mutant compared with the wild-type strain when grown under normal physiological conditions (Fig. 7B). The accumulation of these conjugates was enhanced during growth on medium containing canavanine or *p*-fluorophenylalanine (Fig. 7B). Two of the more intense immunoreactive species which showed preferential accumulation in the deletion mutant probably represented free multiubiquitin chains linked through Lys-48, on the basis of their comigration with synthetic yeast multiubiquitin chain standards (52).

We next explored the effect of the Δ*mcb1* mutation on the degradation of ub–Arg–β-gal and ub–Pro–β-gal, known substrates of ubiquitin-dependent proteolysis that are targeted for breakdown by two distinct mechanisms. ub–Arg–β-gal becomes a substrate of the N-end rule pathway (3) following its processing by ubiquitin carboxyl-terminal hydrolases to produce free ubiquitin and Arg–β-gal. Degradation of this substrate is dependent in part on the E2 enzyme Rad6 (Ubc2) and the E3 enzyme Ubr1 (4, 13). When expressed in wild-type cells, Arg–β-gal was degraded with a half-life of 5 to 10 min, similar to published reports for this substrate (Fig. 8) (3). A similar degradation rate was observed in the Δ*mcb1* cells, indicating that Mcb1 is not essential for proteolysis of this substrate by the N-end rule pathway.

In contrast to ub–Arg–β-gal, an amino-terminal fusion of ubiquitin to Pro–β-gal cannot be efficiently processed by ubiquitin carboxyl-terminal hydrolases and is degraded instead by the ubiquitin fusion degradation pathway, which is dependent in part on the E2 enzymes Ubc4/5 and a likely E3 enzyme, Ufd4 (30). Pulse-chase analyses demonstrated that ub–Pro–β-gal was degraded with a half-life of 5 to 10 min in wild-type

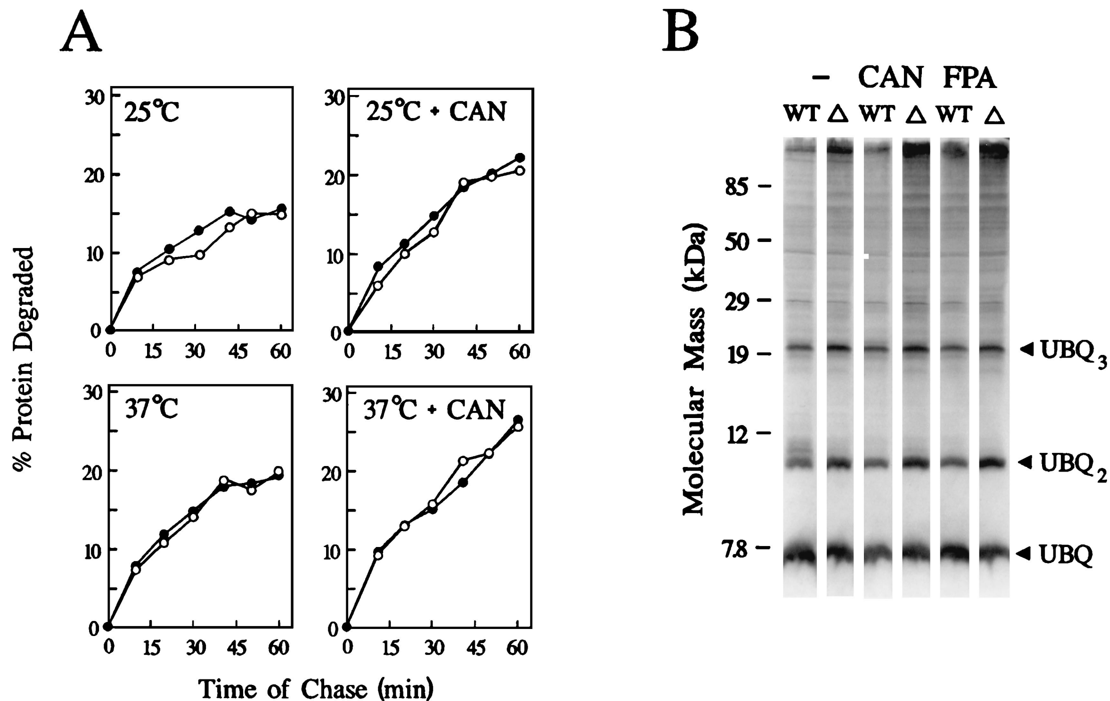

FIG. 7. Bulk protein degradation in wild-type and Δ*mcb1* strains. (A) The turnover of short-lived and abnormal proteins in wild-type (open circles) and Δ*mcb1* (solid circles) strains was determined by pulse-chase analysis as described in Materials and Methods. Top left, exponential cell cultures grown at 25°C; top right, exponential cell cultures grown at 25°C and adjusted to contain 500 μg of canavanine (CAN) per ml 15 min prior to the pulse-chase; bottom left, exponential cell cultures grown at 25°C and shifted to 37°C 1 h prior to the pulse-chase; bottom right, exponential cell cultures grown at 37°C adjusted to contain 500 μg of canavanine per ml 15 min prior to the pulse-chase. The results shown are means of three independent experiments. (B) Profile of ubiquitin conjugates in wild-type and Δ*mcb1* mutants. Total protein was extracted from log-phase cultures as described in Materials and Methods, except that the extraction buffer was supplemented with 5 mM *N*-ethylmaleimide. Canavanine (CAN) and *p*-fluorophenylalanine (FPA) were used at 3 and 25 μg/ml, respectively. Approximately 50 μg of protein was loaded on an SDS-PAGE gel (15% polyacrylamide) and immunoblotted with anti-ubiquitin immunoglobulins. The migration positions of free ubiquitin (UBQ) and multiubiquitin chains (UBQ_2 and UBQ_3) are marked, as well as the migration positions of molecular mass standards (in kilodaltons).

cells. However, in the Δ*mcb1* strain, this substrate was substantially stabilized, with a half-life of >2 h (Fig. 8). Similar results were obtained with a strain coexpressing ubiquitin to high levels (data not shown), indicating that the selective stabilization of ub–Pro–β-gal was not caused by depletion of free ubiquitin in the ub–Pro–β-gal-expressing strain (30). The inhibition of ub–Pro–β-gal proteolysis was also not related to a failure to ubiquitinate the substrate, because the electrophoretically retarded, ubiquitinated forms of ub–Pro–β-gal were observed in extracts from both wild-type and Δ*mcb1* cells (Fig. 8A).

DISCUSSION

The 26S proteasome functions as an essential component of the ubiquitin-dependent proteolytic pathway by degrading multiubiquitinated target proteins. This proteolytic complex is crucial for cellular function, as evidenced by the lethality associated with deletion of genes encoding various subunits of both the 20S proteolytic core (22, 46) and the associated, 19S regulatory complex (*CIM3* [*SUG1*] and *CIM5* [17]). Our results demonstrate that the yeast *MCB1* gene encodes a multiubiquitin-binding component of the 26S proteasome. Phenotypic analysis of the Δ*mcb1* mutant strain indicates that the encoded protein functions in substrate degradation by the 26S complex. However, the lack of an essential function for Mcb1 implies the existence of additional recognition factors. Their ubiquitin-binding properties could overlap with those of Mcb1 or be distinct with respect to various ubiquitin-ubiquitin linkages.

Interestingly, Mcb1 was detected not only in a particle-bound form but also in a low-molecular-mass form. Immunoblot analysis indicated that the abundance of 26S proteasome-bound Mcb1 relative to other 26S proteasome subunits was comparable throughout various purification steps (Fig. 3C and data not shown), suggesting that the protein is a tightly bound component of the complex and that presence of the apparent free form was not due to posthomogenization dissociation from the 26S complex. The *Drosophila* homolog of Mcb1 has also been detected in a low-molecular-mass form (20), suggesting that the presence of Mcb1 in both free and complexed states may be an evolutionarily conserved property. A free form of Mcb1 expands the functional possibilities for the protein. For example, Mcb1 could alternate between a free form and a particle-bound form to shuttle target proteins to the site of proteolysis and thus could function to control the degradation of specific ubiquitin pathway substrates. This idea is consistent with the inhibition of 26S proteasome-dependent proteolysis in vitro by high exogenous levels of *Arabidopsis* *At*Mbp1 (11). Alternatively, free Mcb1 may have a function unrelated to the 26S complex, perhaps in ubiquitin-dependent but 26S proteasome-independent lysosomal degradation (24). The Mcb1 homolog in humans, S5a, is apparently identical to the previously identified antisecretory factor (14, 29, 51). This protein was originally isolated by its ability to inhibit cholera-induced intestinal fluid secretion when administered intravenously. At present, it is unclear how this activity could be related to the intracellular function of Mcb1 in the ubiquitin-proteasome pathway. The antisecretory activity could result from the binding of Mcb1 to unidentified, ubiquitinated mol-

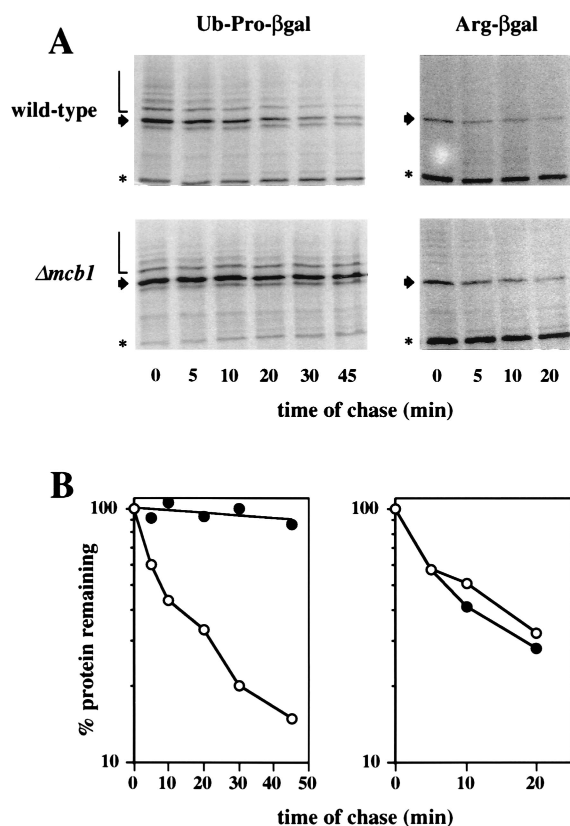

FIG. 8. Degradation of Arg–β-gal and ub–Pro–β-gal in wild-type and Δ*mcb1* strains. Wild-type and Δ*mcb1* strains were transformed with plasmids expressing ub–Pro–β-gal and ub–Arg–β-gal reporter proteins (3). The metabolic stability of the reporter proteins in the two strains was determined by pulse-chase analysis as described in Materials and Methods. (A) Phosphorimager analysis of the relevant SDS-PAGE gel sections. The arrow indicates the position of the β-galactosidase reporter protein, and the asterisk indicates a stable, 90-kDa breakdown product. The 90-kDa species is commonly observed during pulse-chase analysis of short-lived β-galactosidase reporter proteins (3). Brackets denote the ubiquitinated forms of the β-galactosidase reporter protein. (B) Graphical representation of the data. The intensity of the β-galactosidase reporter protein bands shown in panel A was determined by Phosphorimager analysis. The wild-type strain is depicted by open circles, and the Δ*mcb1* strain is depicted by solid circles. Left panel, ub–Pro–β-gal; right panel, Arg–β-gal.

ecules on the cell surface or to intracellular proteins following uptake of Mcb1 into the cell.

Like *Arabidopsis At*Mbp1 and human S5a, recombinant yeast Mcb1 has the ability to bind selectively Lys-48-linked multiubiquitin chains in vitro and exhibits a similar selectivity for longer chains. Beale et al. (5), using synthetic, Lys-48-linked multiubiquitin chains composed of mutant ubiquitins, have implicated hydrophobic patches formed by repeating ubiquitin units in the 26S proteasome-multiubiquitin chain interaction. A reiterated hydrophobic sequence in *At*Mbp1 (and its human and *Drosophila* homologs) may provide a complementary binding site (14, 51). Although the Mcb1 protein exhibits strong amino acid sequence conservation with its homologs at the amino-terminal hydrophobic repeat, the second, carboxyl-terminal repeat is conspicuously absent in Mcb1. On the basis of our data showing that yeast Mcb1 has a similar binding affinity to its plant and animal homologs, this second repeated sequence may not be crucial for binding multiubiquitin chains. Recent analysis of carboxyl-terminal deletions of *At*Mbp1 and Mcb1 has supported the possibility that only the first hydrophobic patch is required (16a).

In addition to its in vitro binding affinity, another line of evidence suggests that Mcb1 binds Lys-48-linked chains in vivo. Among the major ubiquitin conjugates exhibiting increased steady-state levels in the Δ*mcb1* mutant are species previously identified by van Nocker and Vierstra (52) as free multiubiquitin chains linked through Lys-48. However, Mcb1 cannot be the sole component of the 26S complex responsible for Lys-48-linked chain recognition. Because the Lys-48 linkage is apparently essential for viability (16), such a scenario would be inconsistent with the mild phenotype of the Δ*mcb1* mutant. In addition, Δ*mcb1* mutants exhibit no defect in degradation of the N-end rule substrate Arg–β-gal, which is modified with a homogeneous, Lys-48-linked chain, at least in vitro (6, 16).

The existence of two ubiquitin linkages in vivo in addition to Lys-48 (i.e., Lys-29 and Lys-63 [1, 30, 48]) raises the possibility that Mcb1 can recognize multiubiquitin chains linked through other lysine residues as well. The mammalian homolog of Mcb1, S5a, recognizes chains linked through Lys-6, Lys-11, and Lys-48, at least in vitro (2). If Mcb1 has the ability to recognize Lys-63-linked chains, it probably would not be the sole recognition factor for this linkage. Spence et al. (48) showed that expression of an Arg-63 mutant ubiquitin, which would fail to form Lys-63-linked chains, conferred sensitivity to UV radiation and other DNA-damaging agents. Likewise, we expect that a failure to recognize these chains should lead to a similar phenotype. Because these phenotypes were not seen with the Δ*mcb1* mutant, these data suggest that Mcb1 does not play a primary role in recognition of Lys-63-linked chains.

Consistent with the proposed function of Mcb1 in the ubiquitin-proteasome pathway, Δ*mcb1* mutants exhibit a mild sensitivity to amino acid analogs, a phenotype also associated with lesions in genes encoding polyubiquitin (15) and the ubiquitin-conjugating enzymes Ubc4/5 (45). Also consistent with a role of Mcb1 in ubiquitin-dependent protein degradation, the ubiquitin fusion degradation pathway substrate ub–Pro–β-gal is selectively stabilized in Δ*mcb1* mutants. Because ub–Pro–β-gal degradation appears to require the formation of Lys-29-linked chains (30), the strikingly distinct effects of *MCB1* deletion on the degradation of ub–Pro–β-gal versus ub–Arg–β-gal could reflect a role for Mcb1 in the recognition of Lys-29-linked chains. However, a unique role is unlikely on the basis of the severe growth defect associated with expression of an Arg-29 mutant ubiquitin (48). A different interpretation is suggested by the finding that mutations in genes encoding two other 19S complex components, Cim3 (Sug1) and Cim5, preferentially stabilize ub–Pro–β-gal, albeit far less dramatically than Δ*mcb1* (17). In contrast, mutations in the genes *PRE1* and *PRE2* encoding subunits of the 20S proteolytic core stabilize both ub–Pro–β-gal and Arg–β-gal (21, 46). Thus, it is conceivable that degradation of ub–Pro–β-gal is a more sensitive indicator of general defects in proteolysis.

A likely explanation of our results is that ubiquitin-protein conjugates are recognized, through their ubiquitin moieties, by still unidentified factors in addition to Mcb1. One candidate in this regard is the ubiquitin carboxyl-terminal hydrolase Doa4 (Ubp4), which may be associated with the 26S proteasome and probably functions in removing ubiquitin from substrates before or after their proteolysis (36, 37). On the basis of the restrictive nature of the screen employed to identify *AtMBP1* (51) and the existence of additional, uncharacterized polypeptides associated with the yeast 26S complex, the involvement of multiple factors in conjugate recognition is plausible. The identification of additional multiubiquitin-chain-binding factors will be instrumental in testing the hypothesis that multiubiquitin chains function to mediate the binding of proteolytic substrates to the 26S complex.

ACKNOWLEDGMENTS

We thank Carl Mann and Michel Ghislain for antibodies to Cim3 and Cim5, Bonnie Baxter and Phil James for reagents and advice, and Tanya Falbel and Bonnie Baxter for critical reading of the manuscript.

This work was supported by U.S. Department of Agriculture grant 94-37031-03347 and Department of Energy/National Science Foundation/U.S. Department of Agriculture collaborative program on research in plant biology grant BIR 92-20331 to R.V. and S.V.N., an NIH grant (GM43601) to D.F., an American Cancer Society Fellowship to S.S., an NIH Fellowship to D.M.R., a Human Frontiers Science Program Fellowship to O.C., and a Damon Runyon-Walter Winchell Cancer Research Foundation Fellowship to M.G.

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