

NF- κ B p105 Processing via the Ubiquitin-Proteasome Pathway*

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The p50 subunit of NF- κ B is generated by proteolytic processing of a 105-kDa precursor (p105) in yeast and mammalian cells. Here we show that yeast mutants in the ubiquitin-proteasome pathway inhibit or abolish p105 processing. Specifically, p105 processing is inhibited by a mutation in a 20 S proteasome subunit (*pre1-1*), by mutations in the ATPases located in the 19 S regulatory complexes of the proteasome (*ym1*, *ym2/sug1*, *ym5*, *cim5*), and by a mutation in a proteasome-associated isopeptidase (*doa4*). A ubiquitinated intermediate of the p105 processing reaction accumulates in some of these mutants, strongly suggesting that ubiquitination is required for processing. However, none of the ubiquitin conjugating enzyme mutants tested (*ubc1*, -2, -3, -4/5, -6/7, -8, -9, -10, -11) had an effect on p105 processing, suggesting that more than one of these enzymes is sufficient for p105 processing. Interestingly, a mutant “N-end rule” ligase does not adversely affect p105 processing, showing that the N-end rule pathway is not involved in degrading the C-terminal region of p105. Unexpectedly, we found that a glycine-rich region of p105 that is required for p105 processing in mammalian cells is not required for processing in yeast. Thus, p105 processing in both yeast and mammalian cells requires the ubiquitin-proteasome pathway, but the mechanisms of processing, while similar, are not identical.

The transcription factor NF- κ B can be activated by a large number of extracellular inducers, including interleukin-1 and tumor necrosis factor- α , and is a critical component in the stress response pathway (1–3). Activated NF- κ B has been implicated in the regulation of a large number of genes involved in the immune and inflammatory responses (4, 5). NF- κ B is a dimeric protein consisting of members of the Rel family of transcriptional activator proteins; it most often comprises a heterodimer of p50 (NF κ B1) and p65 (RelA). The p50 subunit is generated by proteolytic processing of a p105 precursor, whereas p65 is synthesized as a mature protein. In the absence of inducer, NF- κ B is sequestered in the cytoplasm either as a heterodimer of p105/p65 or of p50/p65 bound to a member of the I κ B family of inhibitor proteins. The C terminus of p105 bears a striking similarity to I κ B proteins and thus is likely to fulfill a similar function. In fact, in certain cell types, the NF κ B1 gene is alternatively transcribed (6) to generate an mRNA encoding the C-terminal region of p105 (7, 8). This protein, designated I κ B γ , can function as an inhibitor of NF- κ B.

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Inducers of NF- κ B lead to the site-specific phosphorylation of I κ B α (the best studied member of the I κ B family) (9, 10), followed by site-specific ubiquitination (11–14), and degradation by the proteasome (15–18). The sites of phosphorylation of I κ B- α have been elucidated (19, 20), as have the sites of ubiquitination (12, 13). Furthermore, a kinase complex responsible for the phosphorylation of I κ B- α has been identified (21). By contrast, proteolytic processing of p105 results in the selective degradation of the C terminus of p105, thereby removing the inhibitory sequence (22). Thus, there are in principle two (related) mechanisms by which cytoplasmic forms of NF- κ B could be activated: by p105 processing or the degradation of the I κ B protein.

The understanding of p50 generation is based on studies of the constitutive processing observed in mammalian and yeast cells. The case for regulated processing of p105 is less clear. Analogous to the situation for I κ B- α , treatment of cells with inducers of NF- κ B results in the phosphorylation of p105 (23–26), and an increase in the level of p50 is observed (27–29). However, it is not clear that this change is due to an increase in the rate of p105 processing upon phosphorylation or to an increase in the level of precursor (30). Since the NF κ B1 gene is positively autoregulated by NF- κ B, the increase in p50 could result in the presence of more p105 (31).

Irrespective of the role of p105 processing in NF- κ B activation, proteolytic processing is a critical step in the generation of the p50 subunit of NF- κ B. The p105 precursor protein is processed *in vivo* and *in vitro*, and the latter reaction was shown to require ATP (22). Biochemical studies in mammalian cell extracts demonstrated that p105 processing requires ubiquitination and the 26 S proteasome, and proteasome inhibitors were shown to inhibit p105 processing *in vivo* and *in vitro* (15). Subsequently, the reconstitution of a rabbit reticulocyte cell-free system that is able to support ubiquitination of p105 was reported (32), and a system allowing ubiquitin-dependent processing of p105 was characterized from HeLa cells.¹ Remarkably, the human p105 protein is also processed to p50 in wild-type yeast but not in strains carrying a proteasome mutation (15). Crude and fractionated yeast extracts can also support p105 ubiquitination. When these extracts were fractionated, the activity could be reconstituted by adding purified components that were shown to be involved in p105 processing in HeLa cells.² Recent work has elucidated a region of p105 (the “glycine-rich region”) that is sufficient to target the protein for processing (33).

Proteins not degraded through the lysosomal pathway are, with few exceptions, targeted to the proteasome (see below) via the covalent addition of branched polyubiquitin chains to the ϵ -amino group of one or more surface lysines (34–36). Ubiquitin is a heat-stable 76-amino acid protein that is found virtually unchanged from yeast to mammals; it is considered to be the

¹ O. Coux and A. Goldberg, manuscript in preparation.

² C. Sears, O. Coux, and A. Goldberg, unpublished observations.

most highly conserved protein known. The amide linkage of ubiquitin to a substrate protein is carried out by three classes of accessory enzymes in a cascade reaction. Ubiquitin activating enzymes (E1)³ activate ubiquitin by forming a high energy thiol ester intermediate (37). Ubiquitin conjugating enzymes (Ubc or E2) effect the second step in the covalent addition of ubiquitin to proteins, serving as carriers of the activated thiol ester form of ubiquitin (38). Individual E2s are believed to be responsible for ubiquitinating different groups of proteins, possibly with the help of equally diverse ubiquitin ligases (E3). These two classes of enzymes are responsible for catalyzing the formation of an isopeptide bond between the C terminus of ubiquitin and the ϵ -amino group of a lysine on the target protein. These or other E2-E3 complexes then form branched polyubiquitin chains on the mono-ubiquitinated substrate. There are currently 13 known E2s in the yeast *Saccharomyces cerevisiae*, and there may be overlapping specificities in their actions (39). In a reconstituted system, all three categories of affinity purified enzymes (E1, E2, and E3) are required for the breakdown of ¹²⁵I-albumin to acid-soluble material in the presence of ubiquitin and ATP (40). Additionally, mutants of these enzymes are able to inhibit ubiquitination and degradation of a variety of substrates (36, 38, 41). This pathway recognizes specific structural characteristics of the target proteins (42); in fact, it is possible to synthetically add a degradation signal to a heterologous protein (33, 43).

The 26 S proteasome is a large (\approx 2 MDa) multienzyme complex that is able to degrade ubiquitinated proteins (44, 45). The complex consists of the following three components: a 20 S, 700-kDa multicatalytic core and two 19 S terminal caps that are responsible for the regulatory functions of the proteasome (46–48). The proteolytic core particle of the mammalian proteasome contains 14 distinct subunits, each present twice within a given complex. These subunits form four seven-membered rings, which are stacked upon each other to form a cylindrical structure. The interior of the cylinder contains the proteolytic active sites of the particle. The substrate is thought to pass into this luminal space through a narrow channel located in the face of the cylinder. This channel opens into the 19 S particle. One 19 S particle binds to each face of the proteolytic core particle to form the 26 S proteasome. Among the components of the 19 S particle is a family of six putative ATPases. The importance of these ATPases for proteasome function is suggested by the strict ATP dependence of protein breakdown by the proteasome. In addition, point mutations in the ATP-binding domains of these proteins have dramatic effects on protein turnover by the proteasome.⁴ The exact roles of ATP in proteasome function are not known, but it has been postulated that they act as unfoldases, in part because the channel leading into the proteolytic lumen of the proteasome is too narrow (\approx 13 Å) for folded proteins to traverse. This measurement is based on the crystal structure of the *Thermoplasma acidophilum* 20 S proteasome (49). However, in the recently elucidated *S. cerevisiae* structure no terminal channel is visible at all (50), suggesting that the opening of this pore may be regulated.

Isopeptidases, enzymes that cleave covalently attached ubiquitin from its substrate, have been described (51, 52). These enzymes act to replenish the intracellular pool of free ubiquitin by cleaving them from proteolyzed fragments, or by cleaving ubiquitin fusions post-translationally. Some isopeptidases are also thought to be involved in proteasomal degradation, per-

haps even as components of the proteasome (53, 54). This is reasonable since removal of the polyubiquitin chains is probably necessary for the degradation of target proteins.

Previous studies have demonstrated that the proteasome is required for p105 processing in yeast but did not directly demonstrate a role for other steps in the ubiquitin-proteasome pathway. Therefore, the possibility formally existed that the proteasome was required for some step upstream of the p105 processing event. This possibility was accentuated by the fact that the proteasome is not known to “process” any other protein but rather to degrade its targets entirely to oligopeptides. If the ubiquitin-proteasome pathway is involved in p105 processing, it should be possible to inhibit processing by interfering at multiple points in the pathway. If the pathway is directly involved, it should also be possible to isolate, *in vivo*, the ubiquitinated intermediate in the processing reaction. In this paper we show that other components of the ubiquitin-proteasome pathway are essential for processing of p105 in yeast. Specifically, mutations in several proteasome-associated ATPase subunits are able to inhibit p105 processing. A proteasome-associated ubiquitin peptidase/isopeptidase is also able to inhibit processing. Interestingly, this mutant accumulates ubiquitinated p105. We also show that degradation associated with the N-end rule, which also relies on the ubiquitin-proteasome pathway, is not necessary for processing nor are mutants in this pathway able to stabilize the C-terminal fragment of p105. In addition, we demonstrate that a panel of ubiquitin-conjugating enzyme mutants are still able to process p105 at nearly wild-type levels. This suggests either overlapping specificities of the enzymes or the presence of another E2 that is responsible, or at least competent, for processing p105 in yeast. Finally we demonstrate that a glycine-rich region that is required for p105 processing in mammals is not required for processing in yeast.

EXPERIMENTAL PROCEDURES

Transient Transfections—Cos-1 cells (55) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Life Technologies, Inc.) and were transfected with 20 μ g of p105 expression plasmid in 100-mm culture dishes using the calcium phosphate method (56). The cells were harvested 48 h after transfection.

Yeast Transformations—Yeast strains used in all experiments are listed in Table I. Plasmids used in all experiments are listed in Table II. All yeast strains were grown to saturation in YPDA until saturated. Saturated cells (1 ml) were spun down and resuspended in 100 μ l of lithium acetate solution (0.1 M lithium acetate, 40% polyethylene glycol in TE pH 7.5). Plasmid (3 μ g) was added, and the mixture was incubated at 30 °C for 30 min. Cells were transferred to 42 °C for 15 min, then spun down, resuspended in TE, and plated on SD agar dropout plates lacking leucine, tryptophan, or uracil (depending on the plasmid used for transformation). Single colonies were picked and grown overnight in liquid SD dropout medium lacking the same amino acids.

Cell Extract Preparation—Transformed yeast cells were grown to an A_{600} of 1.1–1.4 in SD dropout liquid medium lacking the amino acid that was coded for by the plasmid's auxotrophic marker. Approximately 2.5×10^7 cells (5 ml) were spun at 3000 rpm for 15 min at 4 °C to precipitate the cells. The pellet was resuspended in 100 μ l of 20 mM Tris-Cl, pH 7.9, 10 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml leupeptin, 10 μ g/ml pepstatin, 0.01 units/ml aprotinin, 5% glycerol (v/v). An equal volume of 0.45–0.55-mm acid-washed glass beads was added, and the cell suspension was vortexed 5 times at maximum speed for 30–60 s at 4 °C, with 2 min on ice between each vortexing. Cell breakage was determined by visual inspection under a microscope. The suspension was centrifuged for 60 min at maximum speed in a microcentrifuge at 4 °C. The crude extract was collected and used immediately or quick-frozen on dry ice and stored at –80 °C.

Cos-1 cells were lysed by three cycles of freezing on dry ice followed by thawing at 37 °C. Cell debris was precipitated by centrifuging at 15,000 rpm for 20 s, and protein content was determined by the Bradford assay.

³ The abbreviations used are: E1, ubiquitin activating enzyme; E2, ubiquitin carrier protein; E3, ubiquitin ligases; GRR, glycine-rich region; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

⁴ D. Rubin and D. Finley, manuscript in preparation.

TABLE I
Mutant yeast strains

Name	Genotype	Category	Ref.
cim5	MATa ura3-52 leu2-dI his3-d200 ade2-101 lys2-801 trp1-dI cim5-1	ATPase cim5	69
SY147	Sub62 background with both SUG1 and YTA2 deleted and yta2R on a trp marked CEN plasmid and SUG1 on a LEU marked cen plasmid MATa lys2-801 leu2-3,112 ura3-52 his3- Δ 200 trp1-1 yta2::HIS3 sug1::HIS3 (Dp75)(Sp5)	ATPase yta2, sug1	Footnote 4
DY65	Sub62 background with YTA1 deleted and yta1S on a cen plasmid: MATa lys2-801 leu2-3,112 ura3-52 his3- Δ 200 trp1-1 yta 1::HIS3 (Dp43)	ATPase ytal	Footnote 4
DY62	Sub62 background with YTA5 deleted and yta5RF on a CEN plasmid MATa lys2-801 leu2-3,112 ura3-52 his3- Δ 200 trp1-1 yta5::HIS3 (Dp42)	ATPase yta5	Footnote 4
doa4	MATalpha ura3-52 lys2-801 his3-d200 trp1-1(am) leu2-3.2-112 DOA4::LEU2	Isopeptidase	53
pre1-1	MATa his3-11,15 leu2-3,112 ura3 pre1-1	Proteasome β -type subunit	59
pre2-1	MATa his3-11,15 leu2-3,112 ura3 pre2-1	Proteasome β -type subunit	60
pre2-2	MATa his3-11,15 leu2-3,112 ura3 pre2-2	Proteasome β -type subunit	60
bby47	MATa ubr1- Δ 1::LEU2 trp1-1 ura3-52 his3- Δ 200 leu2-3,112 lys2- 801 gal	Ubr1 ubiquitin ligase	74
ky203	MATa ura3-52 leu2-d2 bas1-2 bas2-2 gen4-dl adc8-GCN4 cdc34- 2	Ubiquitin-conjugating enzyme Ubc3 mutant	96
ky206	MATa ura3-52 leu2-d2 bas1-2 bas2-2 gen4-dl adc8-GCN4 rad6-dl	Ubiquitin-conjugating enzyme Ubc2 mutant	97
pas2	MATa ura3-53 trp1 leu2-3 PAS2::LEU	Ubiquitin-conjugating enzyme Ubc10 mutant	98
ubc11	MATa lys2-801 leu2-3.2-112 ura3-52 his3 Δ 200 trp1-1(am) ubc11 Δ 1	Ubiquitin-conjugating enzyme Ubc11 mutant	Footnote 5
ubc6,7	MATalpha lys2-801 leu2-3,2-112 ura3-52 his3 Δ 200 trp1-1(am) UBC6::LEU2 UBC7::HIS3	Ubiquitin-conjugating enzyme Ubc6,7 mutant	100, 101
ubc8	MATalpha adc2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100 UBC8:URA3	Ubiquitin-conjugating enzyme Ubc8 mutant	102
ubc9	MATalpha lys2-801 leu2-3,2-112 ura3-52 his3 Δ 200 trp1-1(am) ubc9-1(ts)	Ubiquitin-conjugating enzyme Ubc9 mutant	103
yw022	MATa his3-d200 leu2-3,2-112 lys2-801 trp1-1(am) ura3-52 UBC4::HIS3, UBC5::LEU2	Ubiquitin-conjugating enzyme Ubc4/Ubc5	104
yw06	MATa his3-d200 leu2-3,2-112 lys2-801 trp1-1(am) ura3-52 UBC1::HIS3	Ubiquitin-conjugating enzyme Ubc1 mutant	105

Immunoblot—Protein content was determined by the Bradford assay. Crude extract (20–50 μ g) was mixed 2:1 with 3 \times sample loading buffer (6% SDS, 15% 2-mercaptoethanol, 30% glycerol, and 0.3 mg/ml bromphenol blue in 188 mM Tris-Cl, pH 6.8), heated at 90 $^{\circ}$ C for 10 min, and separated by 10% Tris-Tricine SDS-polyacrylamide gel electrophoresis. Separated proteins were electrophoretically transferred onto nitrocellulose membrane at 100 mA for 120 min (30). The blotted membrane was blocked with 5% skim milk in Tris-buffered saline containing 0.05% Tween 20 (TTBS buffer) for 15–30 min. Anti-ubiquitin, anti-Gal4 DNA-binding, or anti-p105 antibody (0.1 μ g/ml), diluted in TTBS containing 1.5% skim milk, was added and incubated for 1.5 h at 4 $^{\circ}$ C. The bound antibodies were detected by alkaline phosphatase-conjugated anti-rabbit IgG or horseradish peroxidase-conjugated anti-rabbit IgG secondary antibodies (Bio-Rad) followed by AP substrate (Bio-Rad) or enhanced chemiluminescence detection (Amersham Corp.) according to the manufacturer's instructions.

Bradford Assay—Protein extract (5 μ l) was added to 95 μ l of 0.15 M NaCl. Coomassie Brilliant Blue solution (1 ml of 0.1% Coomassie Brilliant Blue G-250, 5% ethanol, 8.5% phosphoric acid) (57) was added to the tube, and the mixture was vortexed and allowed to stand for 2 min at room temperature. The A_{595} was compared with a bovine serum albumin standard curve to determine protein content.

Immunoprecipitations—A volume of supernatant corresponding to approximately 200 μ g of crude extract was raised to 1 ml in IP buffer (1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 50 mM Tris, pH 7.5, 1 mM phenylmethylsulfonyl fluoride, 2 μ g/ml aprotinin, 1 μ g/ml leupeptin, 1 μ g/ml pepstatin) and incubated overnight at 4 $^{\circ}$ C with a 1:50 dilution of anti-ubiquitin antibody. Antigen-antibody complexes were collected on Protein A-Sepharose beads (Pharmacia Biotech Inc.) and washed four times in IP buffer and twice in wash buffer B (150 mM NaCl, 5 mM EDTA, 50 mM Tris, pH 7.5, 1 mM phenylmethylsulfonyl fluoride, 2 μ g/ml aprotinin, 1 μ g/ml leupeptin, 1 μ g/ml pepstatin). Proteins were released from the beads by addition of 3 \times sample loading buffer, boiled for 10 min, subjected to SDS-polyacrylamide gel electrophoresis, and immunoblotted with p105 antibody.

Immunoblot Quantitations—Immunoblots were scanned by a densitometer at 600 dpi² and 65,536 densities. Band density was quantitated

by volume/area integration analysis using Molecular Dynamics' Image-Quant software.

Antisera—All antisera used are described previously. They include antisera raised against the p105 nuclear localization signal (antibody 1157) and against the C terminus (I κ B- γ ; 1140), both gifts of Nancy Rice (National Cancer Institute, Fredrick, MD) (58). Antiserum against the Gal4 DNA-binding domain was purchased from Santa Cruz Biotechnology, Inc. (SC-577). Rabbit polyclonal antiserum was purchased from Sigma (R-9133). Anti-ubiquitin antiserum was a gift from Vito Palmella (Proscript, Cambridge, MA).

RESULTS

Effects of Proteasome Subunit Mutations on the Processing of p105—We examined p105 processing, via Western analysis, in yeast strains mutant for proteasome subunits. As shown previously, p105 is processed to p50 in yeast (Fig. 1, lane 1), but a mutation in the β -type subunit, *pre1-1* (59), that decreases the chymotrypsin-like peptide hydrolysis activity of the proteasome blocks the formation of the mature p50 product (Fig. 1, lane 2; Fig. 6). Interestingly, other mutations (*pre2-1* and *pre2-2*) that affect the same peptide specificity of the proteasome (60) did not prevent the production of p50 (Fig. 1, lanes 4 and 5). *Pre2*, like *pre1* mutants, are defective in the chymotrypsin-like activity of the proteasome; however, processing was not abolished in *pre2-1* and *pre2-2* mutants. Furthermore, in these mutants the steady-state ratio of p105 to p50 was comparable with that of wild-type (Fig. 6). There is, however, an accumulation of lower mobility forms of p105 in the *pre2* alleles (Fig. 1, lanes 4 and 5), suggestive of ubiquitin conjugation. When the Western analyses were overexposed via enhanced chemiluminescence, these conjugates became conspicuous,

⁵ D. Finley, unpublished observations.

TABLE II
Plasmids

Name	Description	Ref.
Dp42	Yoplac111 with the CIM5 promoter and the coding region of YTA5 with K229R and S241F amino acid substitutions	Footnote 4
Dp43	Yoplac111 with the CIM5 promoter and the coding region of TYA1 with a K228S amino acid substitution	Footnote 4
Dp75	Yoplac111 with the CIM5 promoter and the coding region of YTA2 with a K219R amino acid substitution	Footnote 4
ADNSp105myc BXG1	Yeast 2-m, LEU2 based p105 expression construct pECE72 based mammalian Gal4 expression construct	15 Gift from Thomas Scholl (Myriad Genetics Laboratories, Salt Lake City, UT)
cDNAP105myc	pcDNA1 (Invitrogen) based mammalian p105 expression construct	22
CSM3-1	Mammalian Gal4::CIITA chimera—a CIITA fragment (amino acids 1–161) was cloned between the <i>EcoRI</i> and <i>PstI</i> sites in pBXG1	This work
CSM3-5	Mammalian Gal4::p105(372–503)::CIITA chimera—p105 fragment (amino acids 371–503) was cloned into pBXG1 derived Gal4::CIITA construct via <i>EcoRI</i> and <i>SalI</i> sites	This work
CSM3-7	Mammalian Gal4::p105(372–544)::CIITA chimera—a p105 fragment (amino acids 371–503) was cloned into pBXG1 derived Gal4::CIITA construct via <i>EcoRI</i> and <i>SalI</i> sites	This work
CSM1-1	Mammalian Δ GRR construct—amino acids 376–397 were excised via polymerase chain reaction mutagenesis from pcDNAP105myc	This work
CSY3-1	Yeast Gal4::CIITA chimera—a CIITA fragment (amino acids 1–161) was cloned between the <i>EcoRI</i> and <i>PstI</i> sites in pGBT9	This work
pCSY3-5	Yeast Gal4::p105(372–503)::CIITA chimera—a p105 fragment (amino acids 372–503) was cloned into pGBT9 derived Gal4::CIITA construct via <i>EcoRI</i> and <i>SalI</i> sites	This work
CSY3-7	Yeast Gal4::p105(372–544)::CIITA chimera—a p105 fragment (amino acids 372–503) was cloned into pGBT9 derived Gal4::CIITA construct via <i>EcoRI</i> and <i>SalI</i> sites	This work
CSY1-1	Yeast Δ GRR construct—amino acids 376–397 were excised via polymerase chain reaction mutagenesis from pADNSp105myc	This work
G1p105	Yeast pG1 (106) based 2- μ m, TRP1 based p105 expression construct	Gift from Vito Palombella (Proscript, Cambridge, MA)
GBT9	Yeast 2- μ m, TRP1 based Gal4 expression construct (99)	Gift from Thomas Scholl (Myriad Genetics Laboratories, Salt Lake City, UT)
YES2p105	pYES2 (Invitrogen) derived 2- μ m, URA3 based p105 expression construct	This work
Sp5	YCplac22 with the CIM5 promoter and the coding region of SUG1 with a K195R amino acid substitution	Footnote 4

smearing to the gel origin, but were never visible in the wild-type strain (data not shown). The accumulation of ubiquitinated p105 is consistent with the behavior of these *pre2* mutants on other endogenous cellular proteins (60). There is no such accumulation of p105 conjugates evident in the *pre1-1* mutant, even when the immunoblot is overexposed. *pre1-1,2-1* double mutants do abolish p105 processing, indicating that the *pre1-1* effect is dominant to *pre2-1* (data not shown). Therefore all proteasome mutants examined had some effect on p105 processing, either by inhibiting processing or by leading to the accumulation of high molecular weight conjugates.

Effects of Mutations in Proteasomal ATPase Subunits on the Processing of p105—19 S multi-subunit regulatory complexes confer ubiquitin and ATP dependence to the proteasome (39, 61, 62). A gene family (found from yeast to higher eukaryotes) encoding six highly conserved ATPases is localized to the 19 S regulatory caps (63–68)⁴. All six ATPases, Yta1, Yta2, Yta5, Cim5, Sug1, and Sug2, are essential in yeast (69–71). We have engineered mutant strains expressing the ATPases with conservative and non-conservative substitutions of an invariant lysine in the Walker type A sequence motif of the ATPase domains (72). General characterization of these mutants is reported elsewhere.⁴ Although many of the mutants were not viable, three were characterized by a slow growth phenotype and the accumulation of ubiquitinated cellular proteins. p105 processing was severely inhibited in all three mutants (Fig. 2,

lanes 2–4; Fig. 6). The *sug1R/yta2R* and *yta5RF* mutants were able to produce some level of p50, as observed on overexposed blots (data not shown). Single mutants of *sug1R* and *yta2R* also inhibited processing of p105 but to a lesser extent than the double mutant (data not shown).

Ghislain *et al.* (69) isolated *cim5-1*, a temperature-sensitive mutant shown to stabilize ubiquitin conjugated proteins that are normally rapidly degraded by the 26 S proteasome (69). The *cim5-1* strain also inhibited processing of p105 to p50 at the non-permissive temperature (Fig. 2, lane 5). The ratio of p105/p50 is approximately 9-fold greater than in the wild-type strain (Fig. 6) 120 min after the shift to non-permissive temperature. Therefore, all of the ATPase mutants severely altered the ratios of p105 to p50, suggesting that Yta5, Yta1, Yta2, Sug1, and Cim5 all play a role in the ability of the complex to process p105. These mutations do not prevent complex assembly,⁴ suggesting they may play a direct role in the mechanism of p105 processing.

Effects of Mutations in a Proteasome-associated Isopeptidase on the Processing of p105—Papa and Hochstrasser (53) identified Doa4, a proteasome-associated protease that is able to cleave peptide- and isopeptide-linked ubiquitin. The *doa4* mutant was also shown to stabilize substrates for the ubiquitin-mediated proteolytic pathway. This mutant was assayed for inhibition of p105 processing via Western analysis. Although the mutant was able to produce p50 at 30 °C, there was a clear

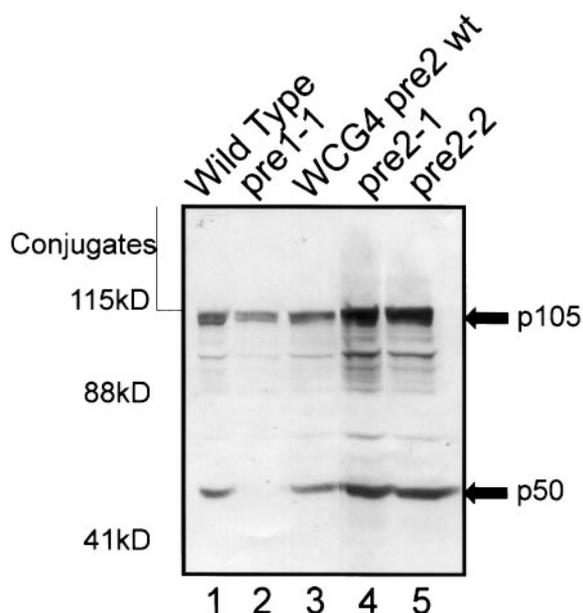


FIG. 1. Effect of proteasome mutants on p105 processing. Yeast proteasome mutants and wild-type (*wt*) strains were transformed with plasmids expressing human p105. Extracts (20 μ g) were subjected to 10% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane, and probed with anti-NF- κ B p50 antibody 1157. The membrane was incubated with alkaline-phosphatase-conjugated goat anti-rabbit IgG. The blot was developed with the AP substrate as per the manufacturer's instructions. Molecular mass markers are indicated on the left, and the strains are indicated at the top, and the locations of p105 and p50 are indicated on the right.

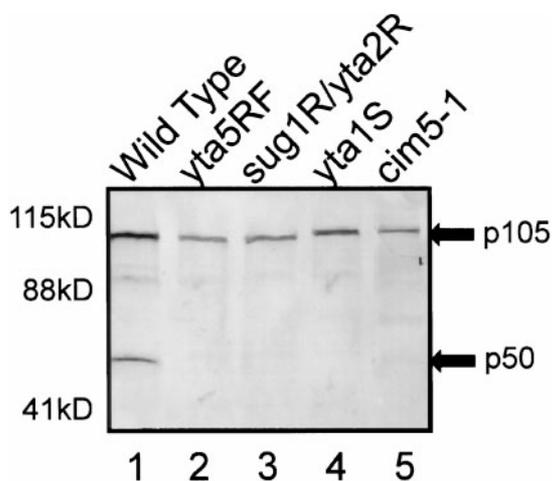


FIG. 2. Effect of proteasome-associated ATPase mutants on p105 processing. Yeast ATPase mutants and wild-type strains were transformed with plasmids expressing human p105. ATPase mutants: *yta5RF*, expressing *YTA5* with a conservative Lys-229 to Arg substitution and a Ser-241 to Phe substitution 12 residues downstream (the second site Ser-241 to Phe mutation suppresses the lethality of the first mutation); *sug1R/yta2R*, expressing *SUG1* with a conservative Lys-195 to Arg substitution and *YTA2* with a Lys-219 to Arg substitution; and *yta1S*, expressing *YTA1* with a non-conservative Lys-228 to Ser substitution. *cim5-1* has been described previously (69). Western was performed, and the figure was formatted as in Fig. 1.

build-up of higher molecular weight derivatives of p105 (Fig. 3A, lane 2). At elevated temperature (38.5 $^{\circ}$ C), p105 processing was blocked, and high molecular weight conjugates of p105 accumulated (Fig. 3A, lane 3; Fig. 6). After 2 h at 38.5 $^{\circ}$ C the conjugates were abundant, and p50 could no longer be observed. No conjugates are observed in wild-type cells at 38.5 $^{\circ}$ C. If the *doa4* strain is incubated at the elevated temperature for 2 h, then returned to the permissive temperature for 1 h, a

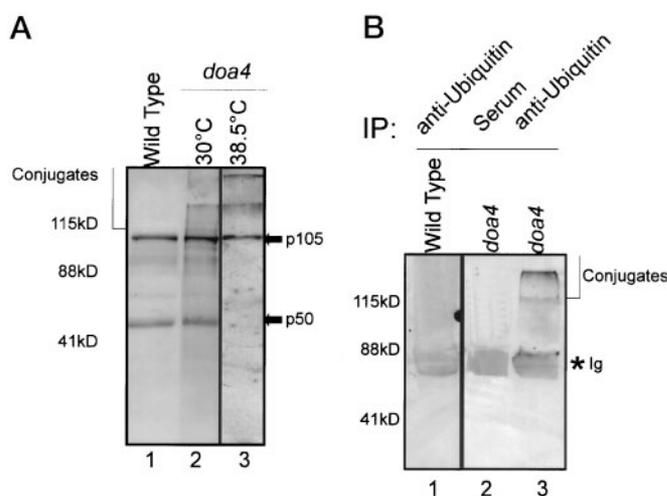


FIG. 3. Effect of a *doa4* isopeptidase mutant on p105 processing. A, the yeast *doa4* mutant and wild-type strains were transformed with plasmids expressing human p105. Extracts were made 120 min after transfer of parallel cultures to 30 and 38.5 $^{\circ}$ C. Extracts (50 μ g) were subjected to Western analysis, and the figure was formatted as in Fig. 1. B, yeast proteasome mutants and wild-type strains were transformed with plasmids expressing human p105. Extracts were immunoprecipitated (IP) with anti-ubiquitin antibodies or rabbit preimmune sera (R-9133). Western was performed as in Fig. 1. Molecular mass markers are indicated on the left; the strains and immunoprecipitating antibody are indicated at the top, and the locations of p105 and p50 are indicated on the right. The asterisk (*) indicates the presence of the immunoglobulin from the immunoprecipitation.

weak band corresponding to p50 is observed (data not shown).

It was possible that the high molecular weight species of p105 was not ubiquitin-associated. With an anti-ubiquitin Western analysis, it is not possible to demonstrate that the conjugates are, in fact, p105. This is because other ubiquitin-conjugated proteins are stabilized in this mutant, and this results in a high background on a Western analysis (data not shown). To clearly demonstrate that the high molecular weight bands in Fig. 3 are ubiquitinated p105, the extracts were immunoprecipitated with anti-ubiquitin antibodies and then immunoblotted with anti-p105/50 antibodies (Fig. 3B). The immunoprecipitated extracts from *doa4*, but not the wild-type strain, contained the same high molecular weight conjugates corresponding to ubiquitinated p105. Preimmune serum did not precipitate any anti-p105/50 reactive bands from *doa4* cells. If *doa4* cells are cotransformed with his-tagged ubiquitin, the same banding pattern is observed with anti-poly-His antibodies (data not shown). This demonstrates that p105 is conjugated to ubiquitin and not to a ubiquitin cross-reactive protein. Despite several attempts, it was not possible to immunoprecipitate with anti-p105 antibodies then immunoblot with anti-ubiquitin antibodies; no bands or smear were visible (unconjugated p105 is not recognized by anti-ubiquitin antibodies). This may be due to the interference of conjugated ubiquitin with recognition by the antibody under native conditions. These data demonstrate the accumulation of ubiquitin-conjugated p105 in a proteasome-associated isopeptidase mutant, *doa4*. Additionally, if ubiquitinated p105 is compelled to accumulate in these cells at elevated temperature, there is a concurrent reduction in p50 production, strongly suggesting a precursor-product relationship.

Effects of Mutations in Ubiquitin Conjugating Enzymes on the Processing of p105—As previously mentioned, ubiquitin conjugating enzymes are essential constituents of ubiquitin-proteasome-mediated proteolysis. We therefore speculated that mutants in this class of enzyme might abolish p105 processing.

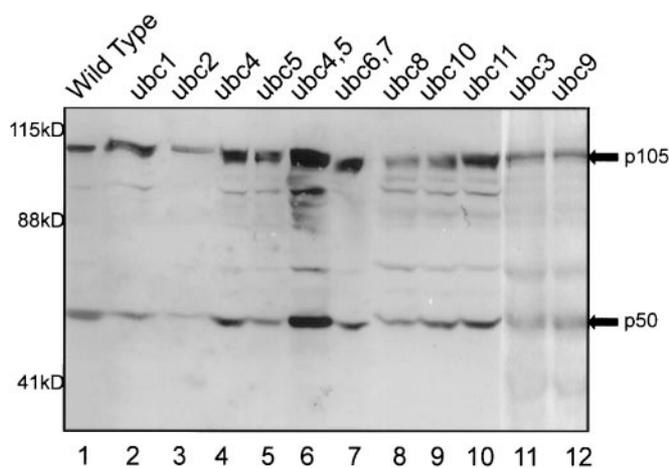


FIG. 4. Effect of ubiquitin conjugating mutants on p105 processing. Yeast ubiquitin conjugating enzyme mutants and wild-type strains were transformed with plasmids expressing human p105. Western was performed, and the figure was formatted as in Fig. 1.

Null or temperature-sensitive mutants in the known yeast E2s were examined, via Western analysis, for defective p105 processing (Fig. 4; Fig. 6). All of the individual mutants retained the ability to process p105. Furthermore, double mutants in *ubc4/ubc5* and *ubc6/ubc7* were also able to process p105. Not only was processing not abolished, but the steady-state ratios of p105 to p50 were roughly comparable for the entire panel of mutants (see below). These mutants were tested at both logarithmic and stationary phases of growth, and the immunoblots were overexposed, but there was no evidence of inhibition of processing or of high molecular weight conjugates. These data suggest that the Ubc responsible for p105 processing is not one of the 11 tested or that the enzymes that act on p105 are redundant.

Effects of Mutations in the N-end Rule Pathway on the Processing of p105—One signal that targets proteins to the ubiquitin-dependent proteolytic pathway comprises the residue at the N terminus of a mature protein (73), hence the name N-end rule. Bartel *et al.* (74) have cloned and characterized the E3 (Ubr1) that recognizes these destabilizing N-terminal residues in yeast. In the absence of this gene, N-end rule proteolysis is suppressed.

The C-terminal fragment of p105 cannot be recovered during p105 processing in yeast or mammalian cells (15). Pulse-chase labeling data demonstrate that radioactive p105 is converted into p50 with no obvious intermediates (data not shown). The N-end rule may be responsible for the processing of the C-terminal fragment of p105 if it is cleaved in an endoproteolytic event. The cleaved fragment of p105 would possess a new N-terminal residue that is very unlikely to be methionine. The N-end rule machinery may then be responsible for degrading the fragment. If this were true, the *ubr1* mutation should stabilize the C-terminal fragment. To examine this possibility, extracts from this mutant were probed with anti-I κ B- γ antibodies (Fig. 5, lane 3) that do not recognize p50. I κ B- γ is an alternate transcript of NF κ B1 and consists of the \approx 70-kDa C terminus of p105. These extracts contain the expected size band corresponding to p105 but do not contain a new band corresponding to the C-terminal fragment. Furthermore, we reasoned that any of the mutants assayed in this work could be responsible for degrading the C-terminal fragment. To examine this possibility, protein blots of all the mutants were probed with anti-I κ B- γ . There was no evidence of a stabilized band corresponding to the C terminus of p105 in any of the mutants tested (data not shown). These data demonstrate that none of the mutants tested are responsible for degrading the C-termi-

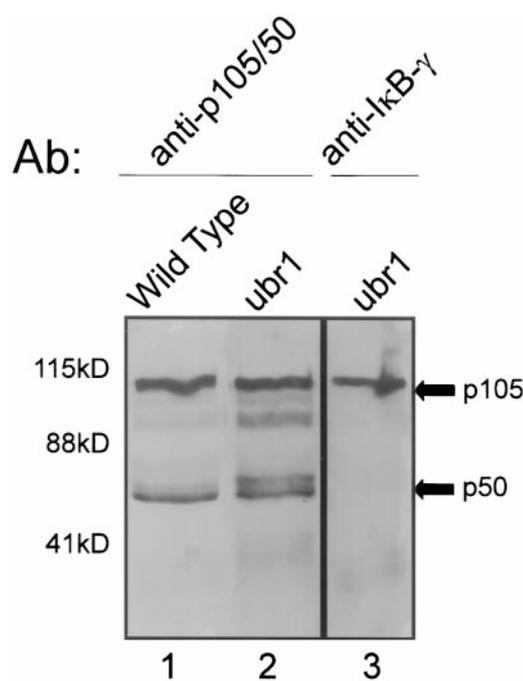


FIG. 5. Effect of an N-end rule mutant on p105 processing. A yeast *ubr1* mutant and wild-type strain were transformed with plasmids expressing human p105. Extracts (20 μ g) were subjected to 10% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane, and probed with anti-NF- κ B p50 antibody (Ab) (antibody 1157), or anti-I κ B- γ antibody (1140). Western was performed, and the figure was formatted as in Fig. 1.

nal fragment of p105 after a hypothetical endoproteolytic cleavage.

A *ubr1* mutant was assayed, via Western analysis, for its ability to process p105 (Fig. 5, lane 2). The production of p50 was not prevented in this mutant, establishing that the N-end rule is not required for p105 processing in yeast. As with the E2s, overexposure of the blot did not reveal inhibition of processing or high molecular weight conjugates. Also, the ratio of p105 to p50 was comparable to wild-type in this mutant (Fig. 6). Note that the ubiquitin conjugating enzyme (Ubc2) that is required for the activity of this E3 was tested (see above) and was also found to be unnecessary for processing. These data demonstrate that the N-end rule machinery is not involved in the processing of p105 to p50.

Steady-state Ratios of p105 to p50—Immunoblots were analyzed with a scanning densitometer to determine the relative concentrations of NF- κ B p105 to p50. Fig. 6 depicts the ratio of p105 to p50 in each of the mutants tested. The ratios in all of the wild-type strains were approximately 1.5, and the ratios of most of the mutants fluctuated around that number. However, it is clear that *pre1*, all of the examined ATPase mutants, and *doa4* (at 38.5 $^{\circ}$ C) demonstrated greatly elevated levels of p105 compared with p50. As is evident in the blots, these mutants did not produce more p105 than the wild-type strains; thus, the entire effect on the ratio was due to the absence of p50 production.

Processing of GRR Deletion Mutants—Recent work has elucidated a region of NF- κ B p105 (the glycine-rich region; GRR) that is required for processing in mammalian cells (33, 75). To determine whether the processing of p105 is dependent on the glycine-rich region in yeast (as it is in mammals), 22 amino acids corresponding to the GRR (amino acids 376–397) were excised from constructs expressing wild-type human p105 (Fig. 7A). As expected, the GRR deletion abolished processing of p105 in Cos-1 cells (Fig. 8, lane 4). However, the same deletion

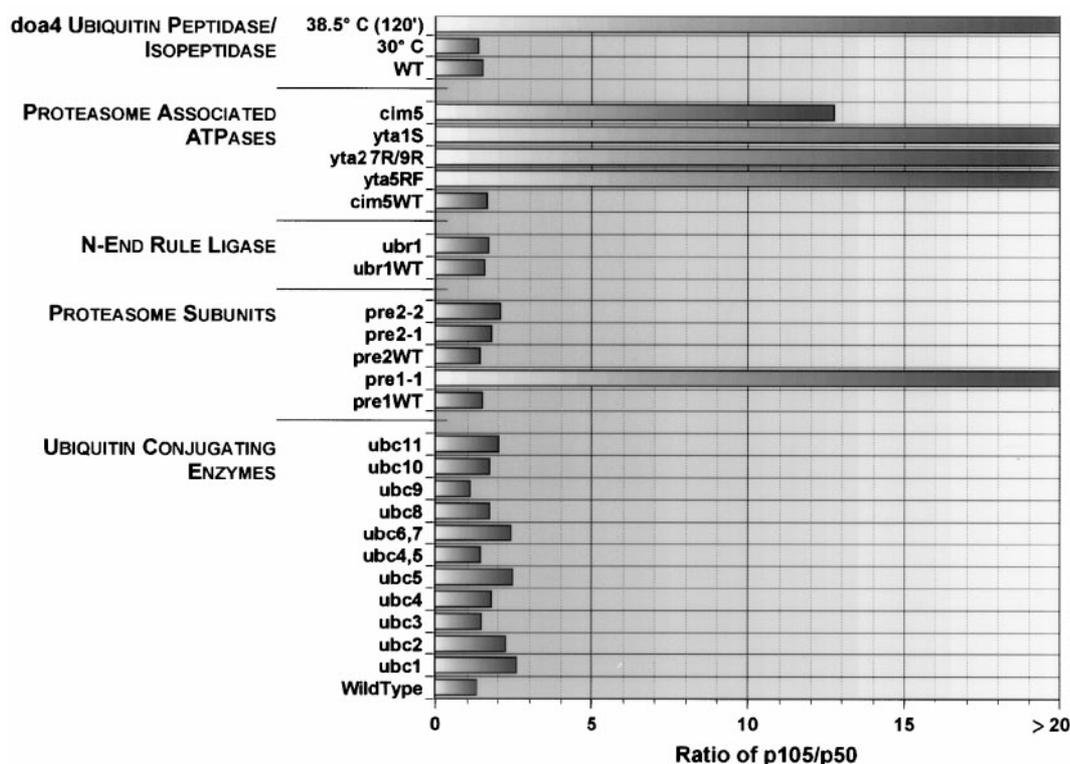


FIG. 6. Ratio of p105 to p50 in the ubiquitin-proteasome pathway mutants. Densitometrically scanned immunoblots from all of the mutants were subjected to volume/area integration analysis with Molecular Dynamic's ImageQuant. The ratio of the integrated peaks for p105 compared with p50 are plotted. Ratios greater than 20 are cut off at the >20 mark on the value axis. Numbers above that are not meaningful since the p50 band is too close to background for accurate quantitation.

did not abolish the p50 band in a wild-type yeast strain (Fig. 8, lane 2). These data demonstrate that p105 processing in yeast cells differs from mammalian cells; it does not require the glycine-rich region.

Processing of Heterologous GRR Constructs in Yeast—In mammalian cells, the glycine-rich region of p105 is sufficient to confer proteolytic processing in heterologous proteins (33). To examine whether the GRR functions in this manner in yeast, several constructs were developed that fused the GRR sequence with a chimeric protein (Fig. 7B). Fig. 9 demonstrates the result of introducing these constructs into yeast and mammalian cells. Gal4 DNA-binding domain alone or fused to the activation domain of a class II transcriptional activator (CIITA) was produced in both organisms and was not processed (Fig. 9, lanes 1, 2, 5, and 6). When fragments containing the GRR region of p105 were fused between the DNA-binding and activation domains, the protein was processed in Cos-1 cells (Fig. 9, lanes 7 and 8), consistent with the results of Lin and Ghosh (33). However, the same constructs, when introduced into wild-type yeast, were not processed to any detectable level (Fig. 9, lanes 3 and 4). Fusions involving only the minimal GRR fused between Gal4 and CIITA behaved identically to the larger constructs (data not shown). Even when the blots were overexposed, no band corresponding to a processed product could be observed in yeast. These data demonstrate that, in contrast to the mammalian system, the GRR region of p105 is not sufficient to confer processing to a heterologous construct in yeast.

DISCUSSION

We have demonstrated that NF- κ B p105 is processed to the mature p50 protein by the ubiquitin-proteasome pathway in yeast. The processing mechanism occurs through a ubiquitinated p105 intermediate, and blocking the pathway at a post-conjugative stage inhibits the formation of p50. No single ubiquitin conjugating enzyme mutant is able to arrest p105

processing. However, several mutants do alter the steady-state ratio of p105 to p50 and the absolute level of p105 as observed by quantitative immunoblot analysis. On the other hand, mutations in the isopeptidase and ATPase activities associated with the proteasome result in profound inhibition of p105 processing. Proteasome mutants are also shown to have an effect on p105 processing by the ubiquitin-proteasome pathway. It is surprising that p105 is processed at all in *S. cerevisiae*, since there is no Rel family member in yeast. This suggests that a proteolytic processing mechanism is conserved in evolution (older even than the Rel family) and may therefore have some function in yeast.

The mechanism of ubiquitin conjugate formation, disassembly, and conjugate degradation by an ATP-dependent mechanism is conserved from yeast to higher eukaryotes. Ubiquitin itself, as previously mentioned, is the most highly conserved protein encountered. Despite three amino acid differences between yeast and human ubiquitin, both proteins behave identically in every assay tested so far (76). Furthermore, it is possible to clone human ubiquitin pathway genes by functional complementation of yeast mutants (77), and purified mammalian proteins are able to reconstitute ubiquitination and p105 processing in yeast extracts.² These data suggest an early common evolutionary origin of the ubiquitin-proteasome pathway.

Ubiquitination is a regulated process, under spatial and temporal control. Cyclins are ubiquitinated and degraded only at the onset of anaphase (78). Likewise, only soluble and not mitochondrial bound hexokinase is recognized by the conjugating machinery and degraded (79). Exact control is also in accord with the need to regulate rapidly the activity of a transcriptional activator. Correspondingly, the cellular level of the yeast transcription factor Gcn4 is found to be regulated by the ubiquitin-proteasome proteolytic pathway (80). Recent work

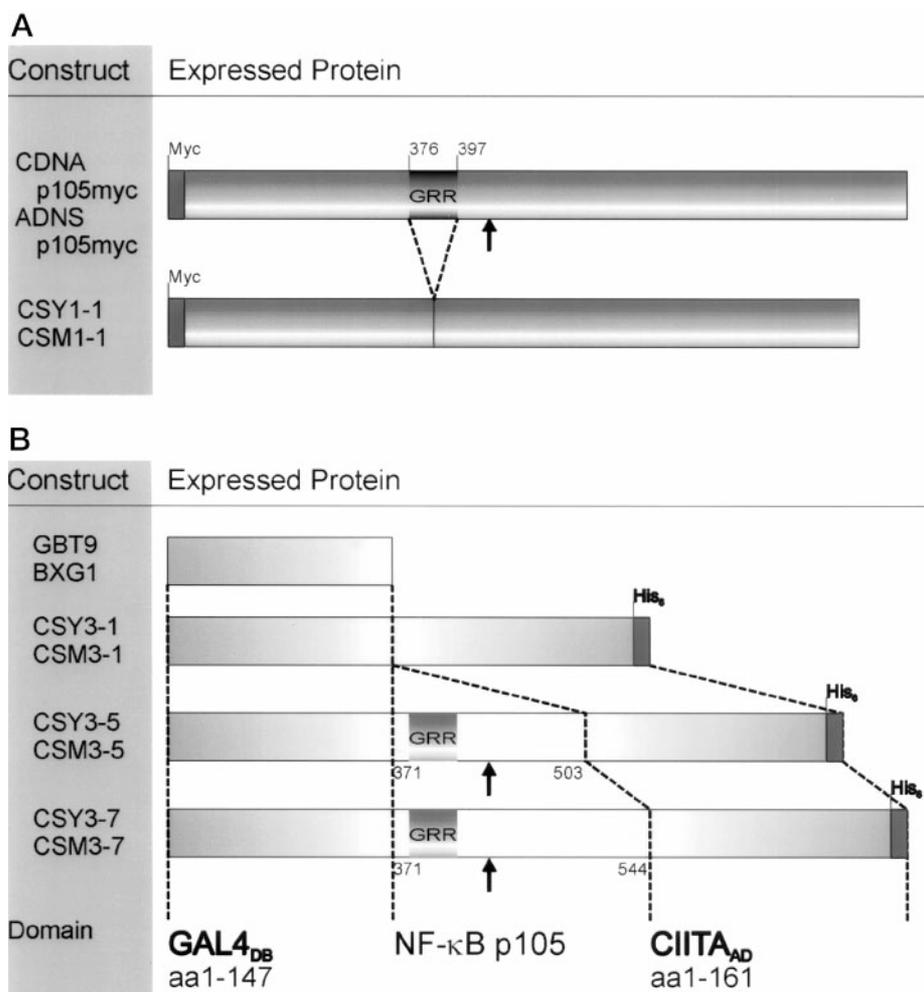


FIG. 7. Schematic diagrams of the glycine-rich region (GRR) constructs. *A*, yeast and mammalian constructs expressing a myc-tagged full-length NF- κ B p105 were used as the basis for excising the glycine-rich region (Table II). *Arrow* indicates the location of the processing site in the full-length protein. *B*, four constructs were designed for expression in both yeast and mammalian cells (Table II). Every construct contains the Gal4 DNA-binding domain. The next three constructs contain the transcriptional activation domain from the class II transcriptional activator (*CIITA*) with a C-terminal poly-His tag. The two largest constructs contain the GRR and various sizes of the surrounding p105 protein fused between the two domains. The regions of p105 are indicated *below* the two constructs. The names of the constructs are listed at the *left*, and the yeast construct is named first in each case. *Arrows* indicate the predicted cleavage sites of the chimeras.

has also demonstrated that a member of the Ubc4/5 family is indirectly involved in I κ B- α degradation in mammalian cells (21). It was therefore conceivable that one could discover the yeast Ubc(s) responsible for the ubiquitination of p105. Our inability to inhibit processing indicates that either no E2 is uniquely responsible for p105 processing or (one of) the E2(s) responsible for p105 processing in yeast is not one of the 11 examined.

Our data demonstrate that the ubiquitin-proteasome pathway is directly involved in processing p105 through a ubiquitinated intermediate. Despite that, it is not entirely surprising that we were unable to find the responsible ubiquitin conjugating enzyme(s) in this mutant analysis. Although some ubiquitination events are specific to a single enzyme (81), most are catalyzed by a family of conjugating enzymes. For example the Mat- α 2 repressor is the target of four enzymes that comprise members of two families of Ubcs that respond to two distinct signals in the protein (82). Similarly, catabolite inactivation of fructose-1,6-bisphosphatase is subject to the activity of three Ubcs (83). Ubiquitination of the previously mentioned bZip transcriptional regulator, Gcn4, is regulated by two Ubcs (80). Even short artificial nonapeptide sequences that are shown to target β -galactosidase to the proteasome were found to be recognized by up to three Ubcs (43). It is therefore possible (and

even likely) that p105 processing is the result of a similar combinatorial ubiquitination mechanism. Processing would therefore not be inhibited except in a strain lacking all of the required enzymes. Additional support for this view is found in recent work that shows two reticulocyte derived E2s are capable of ubiquitinating p105 *in vitro* (32). One of the enzymes is E2-F1, for which it is unclear whether a functional homologue exists in yeast; the other is Ubch5 (a member of the Ubc4/5 family). Furthermore, a HeLa cell-free system has been established in which more than one Ubc is again found to be involved in p105 processing.¹

Proteolysis via the N-end rule relies on the function of the proteasome for degradation (84). A cleaved p105 would result in fragments that would clearly have new N termini. These new N-terminal residues might be highly susceptible to the N-end rule machinery. This led us to speculate that the lack of any detectable C-terminal fragment was due to degradation via a strongly destabilizing N-terminal residue. However, null mutations in both *Ubr1* and *Ubc2* (both of which are essential for the operation of the N-end rule) did not stabilize the C-terminal fragment nor did they have any visible effect on processing. Furthermore, even the most destabilizing residues of the N-end rule would not be expected to destroy a protein so rapidly that it could never be detected, as is the case for the p105 C-terminal

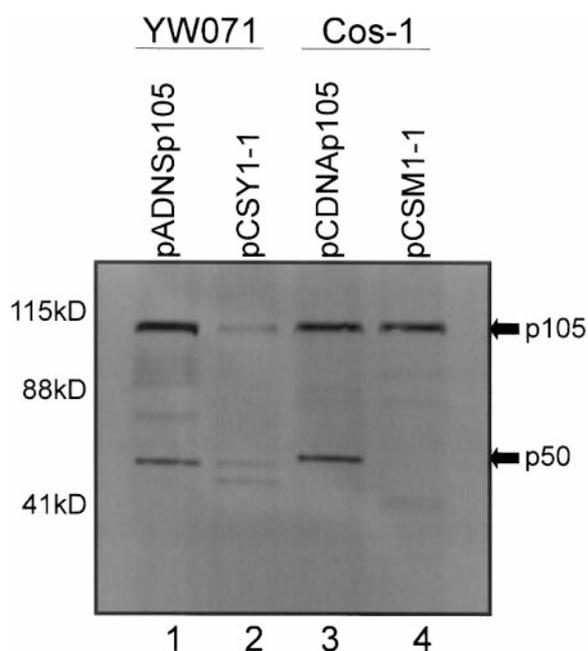


FIG. 8. **Processing of p105 constructs lacking the GRR.** Yeast wild-type strain YW071 and Cos-1 cells were transformed with plasmids expressing human p105 and a p105 deletion mutant lacking the glycine-rich region (amino acids 376–397). Extracts (20 μ g) were subjected to 10% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane, and probed with anti-NF- κ B p50 antibody 1157. The membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG. The blot was developed with the enhanced chemiluminescence (horseradish peroxidase) substrate as per the manufacturer's instructions. The figure is formatted as in Fig. 1.

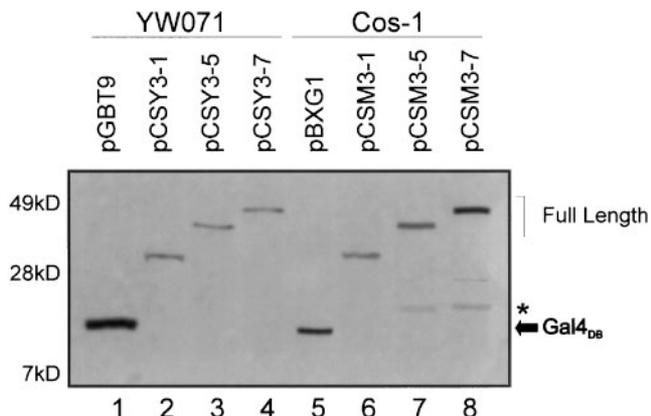


FIG. 9. **Processing of heterologous constructs containing the GRR.** Yeast wild-type strain YW071 (59) and Cos-1 cells were transformed with plasmids expressing chimeric proteins. The chimera is of the yeast Gal4 DNA-binding domain (amino acids 1–147) combined with the activation domain of the mammalian class II transcriptional activator (amino acids 1–161). Fragments of NF- κ B p105 were inserted between these domains (amino acids 371–503 or 371–544 of NF- κ B p105). Western was performed as in Fig. 8. Molecular mass markers are indicated on the left, and the strains and transfected constructs are indicated at the top, and the locations of Gal4 and the full-length chimeras are indicated on the right. The asterisk indicates the presence of processed products from the chimeric proteins.

fragment (data not shown). These data suggest that there may not be any C-terminal fragment, but rather that processing via the proteasome may be processive and terminate at p50.

The proteasome and its ancillary machinery are ubiquitous in the cell. The complex can be found associated with any cellular fraction including the endoplasmic reticulum, nuclear, and plasma membranes (85). We have examined the effect of several mutants that alter the chymotrypsin-like activity of the

proteasome. This peptidase function has been shown to be involved in ubiquitin-mediated degradation (59). Aclacinomycin A, which selectively disrupts the chymotrypsin-like activity of the proteasome, inhibits the degradation of ubiquitinated proteins (86). In our assay, the *pre2-1* and *pre2-2* mutants (also defective in the chymotrypsin activity) did not inhibit processing of p105, whereas the *pre1-1* allele drastically reduced processing. However, the *pre2* alleles are not null mutants, and they do present a much milder phenotype (compared with (*pre1-1*) with regard to defects in proteolysis (60). It is important to remember that all of the proteasome mutants are “leaky” alleles since null mutations are lethal. It may be that the residual proteolytic activity in the *pre2* mutants is sufficient to allow processing.

The *pre2* mutant does, however, accumulate high molecular weight p105-reactive species, similar to those observed in *doa4* strains (see below). The appearance of these conjugates is consistent with the presence of multi-ubiquitinated p105. It is possible that the *pre2* mutations indirectly interfere with the isopeptidase activity associated with the proteasome, and the *pre1* mutant does not. Conversely, *pre1* mutants may somehow interfere with an aspect of p105 recognition by the conjugation mechanism (*i.e.* block some earlier step), whereas the *pre2* mutants do not.

The regulatory component of the 26 S proteasome (the 19 S particle) is responsible for the ATPase activity and the ubiquitin dependence of the proteasome. Proteasomal ATPases are postulated to be involved in unfolding of the proteolytic substrate (62). Six of the ≈ 20 subunits of the mammalian PA700 proteasome regulator contain nucleotide binding consensus domains (66) and belong to a large family of proteins called the AAA-type ATPases. At least 12 members of this family exist in budding yeast, and several of them are also known to be components of the 26 S proteasome (70). Proteasomal AAA-type ATPases are highly homologous to one another and are strongly conserved in evolution. Members of this family are also known components of the proteasome in species as diverse as fission yeast (87), invertebrates (88), and mammals (89). Point mutations that impair the ATPase activity in *S. cerevisiae* proteasome-associated ATPases are shown here to inhibit processing of p105. This result is surprising since one would assume that, even in these ATPase mutant backgrounds, the 26 S proteasome particle would still possess wild-type subunits (since there are still five wild-type genes present in these mutants). If their functions were redundant, the proteasome should still function. These data suggest that inhibition of one of these subunits is able to impair the function of the entire proteasome, at least with regard to p105 processing. This model is supported by other research that has also demonstrated that 26 S ATPases are non-redundant in *Schizosaccharomyces pombe* (87).

The *doa4* mutant profoundly inhibited p105 processing. In addition, at elevated temperatures this mutant accumulated ubiquitinated p105 and halted any detectable p50 production. This strongly suggests a precursor-product relationship between ubiquitinated p105 and p50. In other words, p105 must be ubiquitinated and dealt with by the proteasome before p50 is detectable. The ubiquitinated product cannot be p50 because these conjugates are observed when probing with I κ B- γ antibodies that do not recognize p50 (data not shown). This effect cannot be due simply to the inhibition of general de-ubiquitination since many other ubiquitin hydrolases are present in these cells. There is considerable evidence for specificity in the function of isopeptidases. Cell fate determination in the *Drosophila* eye is controlled by a ubiquitin hydrolase, *fat facets* (90), whereas another isopeptidase, *uch-D*, is specifically impli-

cated in *Drosophila* oogenesis (91).

It is not likely that a protein with a covalently attached chain of ubiquitin could be injected into the small aperture of the proteasome; this opening is only large enough to accept something approximately as large as an α -helix. There is therefore some controversy regarding the stage of proteasome-mediated proteolysis at which de-ubiquitination occurs. Wilkinson *et al.* (92) suggest that in mammalian cells, polyubiquitin chains are probably removed late in the proteolytic process (*i.e.* not before the ubiquitinated protein is degraded), at which time the released chain is disassembled by isopeptidase T (92). However, other evidence suggests that de-ubiquitination may occur earlier, perhaps while bound to the proteasome (53). Our data support the latter view.

The mechanism involved in regulating p105 processing in mammals is not well understood. There is evidence that p105 is constitutively phosphorylated in unstimulated cells (23, 93) and also inducibly phosphorylated, possibly at different sites, upon activation (25, 29, 93–95). This inducible phosphorylation may lead to enhanced processing and thus greater levels of p50 after activation. However, there is some evidence that the increase in p50 is due to enhanced transcription of p105 and rapid processing of this newly formed precursor (30). The NF κ B1 promoter does indeed have binding sites for a p50/p65 heterodimer (31).

The genetic analysis presented here strongly supports the conclusion that p105 processing in yeast directly requires the ubiquitin-proteasome pathway. Similarly, biochemical studies in mammalian cell extracts and the use of specific proteasome inhibitors, *in vitro* and *in vivo*, unequivocally demonstrate that this pathway is also required for p105 processing in mammalian cells (15, 16). Thus, by these criteria it appeared that p105 was processed in yeast and mammalian cells by the same mechanism. It is therefore surprising to find that the GRR region of p105, which is essential for p105 processing in mammalian cells (33), is not required for processing in yeast. One interpretation of the GRR requirement in mammalian cells is that p105 is processed by a two-step mechanism that involves a GRR-dependent endoproteolytic cleavage of p105, followed by rapid degradation of the C terminus. This interpretation is based on the observation that, at least in one case, the processing of a heterologous protein containing the GRR leads to the generation of stable N-terminal and C-terminal processing products (33). The absence of a GRR requirement for p105 processing in yeast suggests that a two-step mechanism may not be used. Rather, there may be a ubiquitin-proteasome-dependent processive degradation from the C terminus, which stops at the C-terminal processing site of p50. In any case, our experiments demonstrate a fundamental difference in the mechanism of p105 processing in yeast and in mammalian cells. Remarkably, however, both mechanisms require the ubiquitin-proteasome pathway and both result in the generation of p50. Comparative studies of the yeast and mammalian systems may help us to understand the evolution and underlying mechanisms involved in ubiquitin-proteasome-dependent regulation.

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