Degradation of the yeast MATα2 transcriptional regulator is mediated by the proteasome

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Abstract Rapid degradation of specific regulatory proteins plays a role in a wide range of cellular phenomena, including cell cycle progression and the regulation of cell growth and differentiation. A major mechanism of selective protein turnover in vivo involves a large multi-subunit protease known as the proteasome or multi-catalytic proteinase. At the same time, the degradation of many cellular proteins requires their covalent ligation to the polypeptide ubiquitin. Here we show that the yeast S. cerevisiae MATα2 repressor, which is known to be ubiquitinylated in vivo, requires the proteasome for its rapid intracellular proteolysis.

Key words: Proteolysis; Proteasome; Ubiquitin; MATα2 repressor; Saccharomyces cerevisiae

1. Introduction

Protein degradation is an essential component of many cellular regulatory mechanisms. In eukaryotes, much of the nonspecific turnover of normally long-lived cellular proteins occurs in the lysosome (vacuole); various stress conditions, e.g. starvation, lead to a marked increase in lysosomal/vacuolar proteolytic rates [1-4]. On the other hand, a large fraction of normally short-lived cellular proteins are degraded by the proteasome, an essential, high molecular weight, multi-subunit protease located in both the cytoplasm and nucleus [5,8]. Studies with the yeast Saccharomyces cerevisiae have led to the discovery of the first in vivo substrates of the proteasome. These substrates include both unassembled and abnormal proteins [9] as well as naturally short-lived proteins whose turnover is regulated either by changes in growth conditions [10,11] or by progression through the cell cycle [12-15].

It has been shown that for many short-lived proteins, conjugation to ubiquitin is an obligatory step in their degradation [16-18]. Ubiquitin is an evolutionarily conserved 76-residue polypeptide, the carboxyl-terminus of which forms isopeptide linkages with the %-amino groups of lysine residues in substrate proteins. The degradation of at least some ubiquitinylated proteins has been shown to depend on the proteasome in vivo [6-8,19,20]; in these cases, the proteasome is thought to function in the context of a still larger protease complex, the so-called 26S protease [16-17]. Recently, Ishida et al. [21] have shown that the Mos protein kinase, which is known to be ubiquitinylated in vivo, can be degraded by the 26S enzyme in vitro.

Cell identity in S. cerevisiae is governed by the mating type, or MAT, locus. We have found that the MATα2 transcriptional repressor, which is encoded by the MATα locus and is required for repression of a cell-specific genes in a cells, has an in vivo half-life of ~4 min at 30°C [22]. The α2 protein is multiply ubiquitinylated in vivo, and attachment of a multi-ubiquitin chain to α2 is important for its rapid degradation [23,24]. At least four different ubiquitin-conjugating enzymes, Ubc4, Ubc5, Ubc6, and Ubc7, participate in the targeting of α2 for ubiquitin-dependent turnover [24]. The α2 repressor has been, until recently at least, the only endogenous protein known to be ubiquitinylated in S. cerevisiae cells, although the list of such substrates is likely to expand considerably over the next few years. Here we show that the proteasome is necessary for the rapid degradation of the α2 repressor in vivo. The α2 protein is the first naturally short-lived ubiquitinylated protein to be shown to also be an in vivo substrate of the proteasome.

2. Materials and methods

2.1. Chemicals

The proteinase inhibitors pepstatin A, chymostatin, antipain, leupeptin and aprotinin were obtained from the Peptide Institute (Osaka, Japan) or Sigma (St. Louis, MO). [*S]methionine was obtained from Amersham (Braunschweig, Germany or Arlington Heights, IL). Yeast nitrogen base without amino acids was purchased from Difco (Detroit, USA). Antibodies against carboxypeptidase Y (CPY) were described in [25]. The antibodies to the MATα2 repressor were described in [22]. Other chemicals were obtained from Pharmacia (Freiburg, Germany), Roth (Karlsruhe, Germany), Merck (Darmstadt, Germany) and Serva (Heidelberg, Germany).

2.2. Media

Complete minimal (CM) dropout medium was prepared according to Ausubel et al. [26], containing 0.67% yeast nitrogen base without amino acids and 2% glucose, supplemented with adenine, uracil and amino acids.

2.3. Strains

Yeast strains used were: WCG4α (MATα his3-11,15 ura3 leu2-3,112 can6 GAL); WCG4-1α (MATα pre1-1 his3-11,15 ura3 leu2-3,112 can6 GAL); WCG4-11α (MATα pre2-2 his3-11,15 ura3 leu2-3,112 can6 GAL); WCG4-122α (MATα pre1-1 pre2-2 his3-11,15 ura3 leu2-3,112 can6 GAL); WCG4-1122α (MATα pre1-1 pre2-2 his3-11,15 ura3 leu2-3,112 can6 GAL); YBR-41α (MATα pre1-1 his3-11,15 ura3 leu2-3,112 can6 GAL); YS18 (MATα ura3Δ his3-11,15 ura3 leu2-3,112) and 954-5Δ (MATα pre1-1 ura3Δ his3-11,15 ura3 leu2-3,112).

2.4. Molecular biological techniques and plasmids

Isolation, purification and analysis of DNA were carried out according to standard procedures [27]. For transformation, either a standard protocol [26] or a modified protocol [6] was followed. All yeast strains except YS18 and 954-5Δ were transformed with a high copy YEp13-based MATα plasmid [28] that expressed the MATα2 protein.

2.5 Immunoprecipitation and pulse-chase assays

Labeling of cells, preparation of cell extracts, immunoprecipitation
with antisera specific for MATα2 and for carboxypeptidase yscY, SDS-PAGE and fluorography were done as described by Hochstrasser and Varshavsky [22]. Prior to labeling, cells were grown 24 h at 30°C in CM medium. After centrifugation cells were diluted into fresh, prewarmed CM medium yielding an OD600 of 0.5 and incubated for 3-5 h at temperatures as outlined in the figure legends. Degradation rates of MATα2 were measured as follows: the 35S content of the MATα2 repressor protein was determined by a gel scanner from dried, scintillant-impregnated gels containing the immunoprecipitated MATα2 protein. Radioactivity was plotted on a semilogarithmic graph. The radioactivity at time point zero (r = 0 min) was set to 100%.

For the experiments examining degradation of α2 expressed only from the chromosomallyMATα2 locus (Fig. 1), overnight 30°C cultures in CM medium were diluted to an initial OD600 of 0.2, grown for 3 h at 30°C, and then grown for another 3 h at 38°C. Pulse-chase analysis was done at 38°C. Degradation rates were determined using excised α2-containing gel fragments to measure 35S content by scintillation counting, as described previously [24].

3. Results and discussion

Because the yeast MATα2 repressor is extremely short-lived in vivo and must be ubiquitinated prior to degradation [22-24] and because the proteasome has been implicated in ubiquitinylated protein turnover (see section 2), we examined the possibility that the proteasome is involved in the degradation of the α2 protein. We conducted pulse-chase experiments to follow the degradation of α2 in both wild-type and mutant cells defective for particular peptidase activities of the multi-catalytic proteasome. Yeast mutants bearing mutations in different subunits of the proteasome were previously shown to have substantially lost their ability to cleave specific chromogenic and fluorogenic peptide substrates. The pref-1 and pref-2 mutants show defects in the proteasome's chymotrypsin-like activity, whereas pref-4-1 mutants are defective for the peptidyl-glutamyl-peptide cleaving activity of the protease [6,19,20].

Strains bearing mutations in PRE1 and/or PRE2 were previously found to be defective in the turnover of artificial protein substrates known to be multi-ubiquitinylated in yeast cells [7,8], but the extent to which bulk high molecular mass ubiquitinylated proteins accumulated in the different mutants varied considerably [19,20]. None of the mutants showed any obvious accumulation of ubiquitinylated proteins at a growth temperature of 30°C. While the single pref-1 mutant exhibited a moderate increase in the steady state levels of bulk multi-ubiquitinylated proteins under stress conditions (either growth in the presence of the amino acid analog canavanine or at high temperature [37°C]), the pref-2-2 mutant showed very little accumulation of such proteins. However, introduction of the pref-2 mutation into a pref-1 strain lead to a pronounced increase in the levels of high molecular mass ubiquitinylated proteins under these conditions [19]. Interestingly, pref-4-1 cells, despite being completely defective in the peptidyl-glutamyl-peptide splitting activity of the proteasome, as measured with the chromogenic peptide Cbz-Leu-Leu-Glu-D-naphthylamide, did not accumulate high molecular mass ubiquitinylated proteins [20]. However, when introduced into a pref 1 mutant strain, the pref-1 mutation increased the levels of these apparent proteolytic intermediates relative to those seen in the single pref-1 mutant [20].

When yeast cells were grown and labeled at 30°C with [35S]methionine and thereafter chased with nonradioactive methionine at the same temperature, only a weak stabilization of the α2 repressor could be found in pref-1 cells relative to wild-type cells (not shown). However, incubation of cells at 38°C prior to labeling and pulse-chase analysis at the same temperature (using an antibody affinity-purified against α2 protein for immunoprecipitation [22]) revealed a stable stabilization (~10-fold) of α2 in the pref-1 mutant relative to the parental wild-type cells (Fig. 1). The accentuation of the proteolytic defect at 38°C is consistent with the exacerbation of phenotypic abnormalities previously noted in the pref-1 mutant at high temperature [6].

We repeated these experiments in isogenic strains carrying different combinations of proteasomal mutations. To increase the α2 signal, we overexpressed the α2 protein from a multicopy MATα2 plasmid. As an internal control, we precipitated the vacuolar enzyme carboxypeptidase yscY along with α2 (Fig. 2). The protein bands visible on the gel in the molecular weight range between carboxypeptidase yscY and MATα2 are due to unspecific protein material that is precipitated by the carboxypeptidase yscY antibody (not shown). Whereas in wild-

Fig. 1. Inhibition of MATα2 degradation in a pref-1 proteasomal mutant. Following 3 h of growth at 38°C, pulse-chase analysis was done as described in section 2. Cells were labeled for 5 min with [35S]Translabel and chased for the indicated periods of time (min) at 38°C. MATα2 protein was immunoprecipitated with an affinity-purified anti-α2 antibody [22]. (Ubiquitinylated α2 protein is generally difficult to detect using the experimental protocol described in section 2 — see [23].) The calculated half-life of α2 was 6.7 min in wild-type (YS18) and 60 min in pref-1 (954-5D) cells under these conditions.
Preliminary experiments with strains WCG4a (wild-type), YBR-4la (pre4-1), radioactive a2 protein remaining at each timepoint in pulse-chase experiments were separated by SDS-PAGE and visualized by fluorography. Levels of stabilization of a2 in wild-type and mutant strains. Immunoprecipitated proteins were quantified by densitometry. The introduction of the pre2 mutation into the prel mutant strain caused a further stabilization of the a2 protein (Fig. 2C), a result that correlates with the increased accumulation of ubiquitinylated proteins in the prel pre2 mutant relative to that seen in prel cells [19]. The degree of a2 stabilization in the double mutant relative to wild-type cells was ~5-fold (Figs. 2 and 3). Consistent with the finding that high molecular mass ubiquitinylated proteins do not accumulate in prel pre2 cells, the a2 repressor was degraded at wild-type rates in these same mutant cells (Fig. 3).

Previous in vitro and in vivo data had lead to the view that multi-ubiquitinylated proteins are proteolyzed by a proteasome-containing protease complex [16-18]. The a2 protein was known to be multiply ubiquitinylated in vivo, and its degradation is strongly ubiquitin-dependent [22-24]. Together with the data from the present paper, these results indicate that naturally short-lived substrates of the ubiquitin system such as a2 are targeted to a proteasome-dependent proteolytic pathway in vivo. This study uncovers the proteasome as to be a mediator of transcriptional regulation. Together with the findings of its involvement in the regulation of metabolic enzymes [10,11] and the cell cycle [12-14] the proteasome emerges as to be a central tool in eukaryotic cell regulation.

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References