The Yeast Ubr1 Ubiquitin Ligase Participates in a Prominent Pathway That Targets Cytosolic Thermosensitive Mutants for Degradation

Farzin Khosrow-Khavar, Nancy N. Fang, Alex H. M. Ng, Jason M. Winget, Sophie A. Comyn, and Thibault Mayor

Department of Biochemistry and Molecular Biology, Center for High-Throughput Biology, University of British Columbia, Vancouver, BC V6T 1Z4, Canada

ABSTRACT Mutations causing protein misfolding and proteolysis are associated with many genetic diseases. The degradation of these aberrant proteins typically is mediated by protein-quality control pathways that recognize misfolded domains. Several E3 ubiquitin ligases have been shown to target cytosolic misfolded proteins to the proteasome. In this study, we characterized a panel of more than 20 cytosolic thermosensitive mutants from six essential genes in Saccharomyces cerevisiae. These wild-type proteins are stable at restrictive temperature. In contrast, we found that a large portion of the mutants is degraded at nonpermissive temperature in a proteasome-dependent manner. Approximately one-third of the assessed unstable mutants are targeted by the Ubr1 ubiquitin ligase. In two cases, efficient degradation of the thermosensitive mutants is abrogated in the absence of Ubr1 alone, whereas in a third case it is reliant on the dual deletion of Ubr1 and the nuclear E3 ligase San1. We found that the impairment of the degradation of these quality control substrates at the restrictive temperature is associated with the suppression of thermosensitive phenotype. This study confirms that Ubr1 plays an important role in the degradation of cytosolic misfolded proteins and indicates that degradation mediated by protein quality control is a major cause for the conditional lethality of mutated essential genes.

KEYWORDS protein quality control misfolding ubiquitin proteasome system E3 ligase Ubr1 San1 Saccharomyces cerevisiae

The eukaryotic cell has developed critical degradative protein quality control (QC) pathways to eliminate misfolded and/or damaged polypeptides (Buchberger et al. 2010). Failure to remove these aberrant proteins can lead to the formation of oligomers or aggregates that are associated with human proteopathies such as Parkinson and Huntington disease (Hol and Scheper 2008; Roth et al. 2008). On the other hand, degradation of misfolded proteins as the result of mutations can also be detrimental and lead to loss of function diseases, such as cystic fibrosis and phenylketonuria (Cheng et al. 1990; Lichter-Konecki et al. 1988). In this case, the degradative QC depletes from the cell proteins that are partially misfolded (as a result of mutations) but otherwise functional.

The ubiquitin proteasome system plays a major role in degradative QC, in which misfolded proteins are first conjugated to poly-ubiquitin chains and then recognized and degraded by the proteasome (Finley 2009). Protein ubiquitylation is an ATP-driven process catalyzed by a cascade of three enzymes: E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme), and E3 (ubiquitin ligases). The conjugation of ubiquitin normally occurs on substrate lysine residues and substrate recognition is typically facilitated by E3 ligases (Pickart and Eddins 2004).

Distinct QC pathways have been identified in different cellular compartments. Perhaps the best characterized of the degradative QC pathways is the endoplasmic reticulum-associated degradation, in which newly synthesized misfolded proteins are recognized by chaperone proteins in the ER, retrotranslocated to the cytoplasm, poly-ubiquitylated, and degraded by the proteasome (Anelli and Sitia 2008; Hirsch et al. 2009). In the yeast nucleus, San1 ligase is involved in the poly-ubiquitylation and degradation of nuclear misfolded proteins.
In this report, we have analyzed a panel of ts alleles corresponding to different cytosolic proteins and observed a variable stability profile for the different mutants. We further characterized the unstable alleles and found that the Ubr1 pathway plays a major role in the degradation of these misfolded proteins.

MATERIALS AND METHODS

Yeast strains and plasmids

Media preparation and molecular biology techniques were performed using standard procedures. Yeast strains used in this study are outlined in supporting information, Table S1. The ts alleles used in this study were generously provided by Phil Hieter and were previously obtained by random polymerase chain reaction mutagenesis of essential genes that were then integrated in their endogenous loci after selection for conditional ts lethality (Ben-Aroya et al. 2008, 2010). In this study, the yeast temperature-sensitive strains were tagged C-terminally by homologous recombination using 13MYC-KanMX6 module (Longtine et al. 1998). The deletions were generated by directly knocking out the specific gene by homologous recombination (Rothstein 1991) or by crossing with cells carrying single gene deletion (can1::STE2pr-sphHIS5, his3Δ1, leu2Δ0, ura3Δ0, met1Δ0, geneX::NatMX4) (Baryshnikova et al. 2010; Tong and Boone 2006) followed by tetrad dissection.

Immunocytochemistry

Protein degradation was examined by cycloheximide chase assays. Cells were grown in YPD to exponential phase (OD600 = 1) at permissive temperature (25°C) before the addition of cycloheximide (100 μg/mL). The cells were then either incubated at the permissive temperature or restrictive temperature (37°C). For proteasome inhibition experiments, cells were grown to exponential phase in synthetic complete medium (0.17% yeast nitrogen base without ammonium sulfate) supplemented with 0.1% proline and 2% glucose as the carbon source (Liu et al. 2007). The overnight grown culture was diluted with fresh media supplemented with 0.003% SDS at OD600 0.2. The cells were treated with 20 μM MG132 or control dimethyl sulfoxide (DMSO) for 30 min and then with 100 μg/mL cycloheximide. Samples were collected at indicated time points and lysed with a modified Laemmli lysis buffer (50 mM Tris-HCl pH 6.8, 2% sodium dodecyl sulfate [SDS], 10% glycerol) with glass beads. Protein concentration was determined by DC-Protein assay (Bio-Rad). Equal amounts of protein were resolved by SDS polyacrylamide gel electrophoresis (PAGE). To assess solubility, cells were lysed with glass beads in a native lysis buffer [0.5% NP-40, 20 mM HEPES, 200 mM NaCl, 1 mM ethylene diamine tetraacetic acid, protease inhibitors (Roche Applied Science), 1 mM phenanthroline]. The lysates were first precleared by centrifugation in a microfuge (2000 g, 5 min at 4°C), then further fractionated to soluble and insoluble fractions by centrifugation (16,000 g, 10 min, 4°C). Immunoblots were performed with 9E10 monoclonal mouse antibody (1:7000 dilution) against MYC-tagged proteins, and anti-Pgkl monoclonal rabbit antibody as loading control (1:10,000 dilution; gift from J. Thorner, University of California). Antimouse and antirabbit secondary antibodies, coupled to Cy3 and Cy5, respectively (Mandel Scientific, 1:10,000), were used. Protein bands were quantified using Li-Cor Odyssey Fluorescent detection system.

Structure prediction

MODELER (Sali and Blundell 1993; Shen and Sali 2006) was used to generate models of Pro3 using homologs from several different organisms as templates (PDB codes 1YQG, 2AHR, 2IZZ, 2RCY). First a structurally influenced alignment of the templates was generated.
using the salign function, and then the Pro3 sequence was appended and aligned. A total of 100 models were generated on the basis of this alignment and scored using the DOPE statistical potential. The best-scoring model was used to fit mutated residues. To generate a model of the dimer, two copies of the best-scoring Pro3 model were structurally aligned (using PyMol) to chains A & E of PDB structure 2RCY, the most closely related homolog. Models of Pro3-1 and Pro3-2 were generated in the same way, by replacing the relevant amino acids in the alignment.

**RESULTS**

A large portion of ts mutants is degraded at restrictive temperature

We reasoned that a large fraction of ts mutants might be unstable at restrictive temperature. We selected from a mutant library generated by polymerase chain reaction mutagenesis (Ben-Aroya et al. 2008) a panel of 22 ts alleles of six essential genes encoding for primarily cytosolic proteins: Pro3, Gus1, Guk1, Gln1, Ugp1, and Gsr1. Delta 1-pyrroline carboxylate reductase (Pro3) is a multimeric enzyme involved in the conversion of delta-1-pyrroline carboxylate reductase to proline in the cytosol (Brandriss and Falvey 1992). Glutamyl tRNA synthetase (Gus1) and Glycyl tRNA synthetase (Gsr1) are cytosolic enzymes that ligate amino acids to cognate tRNA (Delarue 1995; Galani et al. 2001). Glutamine synthetase (Gln1) is a metabolic enzyme in the cytosol that catalyzes amination of glutamate to form glutamine (Benjamin et al. 1989). Guanylate kinase (Guk1), localized both in the cytosol and nucleus, converts GMP to GDP and is required for mannose chain elongation in the eukaryotic cell wall (Shimma et al. 1997). UDP-glucose pyrophosphorylase (Ugp1) is a cytosolic enzyme involved in the formation of UDP-glucose from glucose-1-phosphate and UTP (Lai and Elias 2000). All these proteins were estimated to be present in high abundance in the cell (>10,000 copies/cell) and relatively stable (t1/2 > 40–300 min) (Belle et al. 2006).

To assess the stability of the selected proteins, wild-type and ts alleles were C-terminally tagged with 13 MYC epitopes. We observed that the tested wild-type alleles are fully stable after the addition of the translation inhibitor cycloheximide to cells growing at 37°C for 3 hr (Figure 1A). We then assessed the viability of the C-terminally tagged mutant alleles on synthetic complete media at permissive (25°C) and restrictive temperatures (37°C). We found that 19 of the 22 strains maintained a complete loss of viability phenotype at restrictive temperature, two strains displayed a subdued phenotype (pro3-2 and ugp1-1), and one strain was fully viable (guk1-5; Figure 1B). We confirmed that the untagged guk1-5 mutant remained ts at restrictive temperature in our growth conditions (data not shown). We reasoned that the addition of the C-terminal tag rescues the ts phenotype of guk1-5 (which was not further characterized) while not affecting the temperature sensitivity of most tagged alleles.

We next assessed the stability of the tagged ts mutant proteins at restrictive temperature. We found that 10 of the 21 assessed proteins displayed 50% or greater reduction in protein levels after the addition of cycloheximide to cells growing at restrictive temperature for 3 hr (Figure 1C). Distinct stability was observed for different alleles of the same protein, as for the guanylate kinase (Guk1). Two alleles (Guk1-6 and Guk1-9) were almost completely degraded, two other alleles displayed substantial reduction (Guk1-7, Guk1-8), whereas another ts allele was stable (Guk1-10). Overall, we observed that approximately one-half of the ts mutants displayed greater protein turnover at the restrictive temperature. These results suggest that a large portion of yeast ts alleles encodes for unstable proteins.

The ts mutant Pro3-1 is preferentially degraded at restrictive temperature

We next sought to examine the turnover rate of the Pro3-1 mutant in more detail because of its pronounced degradation in the first assay. We confirmed that Pro3-1 is efficiently degraded over a 3-hr cycloheximide incubation at the nonpermissive temperature, whereas it is fully stable at the permissive temperature (Figure 2A). In contrast, wild-type Pro3 is completely stable at both the permissive and restrictive temperatures during the same 3-hr chase period. Similarly, we found that the second mutant, Pro3-2, was also fully stable at both the permissive and nonpermissive temperatures (Figure 2A). Interestingly, this mutant displayed a subdued ts phenotype, which might be explained by the greater stability of the protein at 37°C as compared with Pro3-1.

Upon sequencing the mutant alleles, we found that both pro3-1 and pro3-2 mutant alleles each have four missense mutations and one silent mutation in their open-reading frames (Figure 2B). We next mapped the mutations of Pro3-1 on a homology structure model. It has been shown that Pro3 is a multimer in higher eukaryotes (Meng et al. 2006). Two of the mutated residues (H236, K261) are found near the binding interface involved in Pro3 homo-dimerization and the two other sites (A27, V133) are located within the catalytic domain of Pro3 (Figure 2C). Interestingly, the lysine (K261) residue of Pro3 is closely located to its counterpart in the second homo-dimer unit (inset in Figure 2C). In addition, the histidine (H236) is located near a negatively charged glutamic acid (E199) of the opposing subunit. These data indicate that both residues may be important for the formation of the Pro3 homo-dimer. One possibility is that these two mutated residues may affect dimerization and stability of Pro3-1. In agreement with this notion, the DOPE (i.e., discrete optimized protein energy) (Shen and Sali 2006) scores of the homology model structures generated for Pro3-1 were significantly greater compared with wild-type Pro3 and Pro3-2 (Figure 2D), further indicating that the conformation of this mutant may be less stable. To test this idea, we examined the solubility of the three alleles of Pro3 after centrifugation at the nonpermissive temperature. We found that after a 30-min incubation period at 37°C, a significant portion of Pro3-1 (>30%) is deposited in the insoluble fraction (Figure 2E). In contrast, only minute portions of Pro3-2 and of the wild-type Pro3 are insoluble in these conditions. These results indicate that, upon shifting the cells to the restrictive temperature, a large portion of Pro3-1 likely misfolds and aggregates.

The loss of stability of the ts mutants at restrictive temperature is dependent on the proteasome

We postulated that the degradation of the Pro3-1 mutant is mediated by a proteasome-dependent QC pathway. Incubation of pro3-1 temperature-sensitive cells with the proteasome inhibitor MG132 impaired the rapid turnover of the mutant protein after shifting the cells at 37°C (Figure 3A). This finding indicates that degradation of Pro3-1 is, at least partially, dependent on the proteasome.

We next examined whether the other nine unstable ts mutants are also targeted to the proteasome. A large subset of the unstable mutant proteins is completely stabilized by proteasome inhibition (Figure 3B), whereas two others are only partially stabilized (gln1-1 and guk1-9) and one ts allele degradation is not dependent on the proteasome (guk1-3). This finding suggests that the proteolysis of most unstable ts mutants is mediated by the proteasome, whereas a few other mutants are degraded by a different pathway in the cell (e.g. macro-autophagy).
A large fraction of ts mutant proteins are degraded at restrictive temperature. (A) The stability of wild-type cytosolic proteins was assessed at 37°C after the addition of cycloheximide to cells grown at 25°C. Cells were collected at the indicated time (min), and equal amount of proteins were analyzed by Western blotting using anti-MYC 9E10 antibody and anti-Pgk1 antibodies. (B) Yeast strains carrying the indicated C-terminally tagged ts alleles, and wild-type BY4741 cells were streaked on synthetic complete media, and viability was determined after three days of growth at the indicated temperatures. (C) The stability of mutant proteins was determined after a 3-hr cycloheximide treatment at 37°C as in (A). Asterisks denote alleles that displayed more than a 50% protein level decrease.
Double deletion of UBR1 and SAN1 significantly stabilizes Pro3-1 and suppresses lethality of pro3-1

We next sought to determine which ubiquitin ligase involved in a QC pathway may be required for targeting Pro3-1 for degradation by the proteasome. We directly assessed the stability of Pro3-1 in cells containing single deletion of SAN1, UBR1, UBR2, HRD1, DOA10, RKR1, and HUL5, which are the major yeast ubiquitin ligases involved in the degradation of misfolded proteins. We found that none of the tested single deletions had a significant effect on the stability of Pro3-1 when the cells were shifted to the restrictive temperature along with addition of cycloheximide (Figure 4A). In contrast, we found that there was a significant reduction of the turnover of Pro3-1 in the absence of both Ubr1 and San1 E3 ligases in comparison with control cells (Figure 4B). This finding indicates that Ubr1 and San1 together play a major role in targeting of Pro3-1 for degradation.

We then determined whether pro3-1 lethality could be rescued by a reduced turnover of the mutant protein. We assessed the viability of different pro3-1 strains with single and double deletions of UBR1 and SAN1. We found that the single deletions of UBR1 and SAN1 did not suppress the ts phenotype, whereas the double ligase deletion restored the growth at the nonpermissive temperature (Figure 4C). This finding suggests that the conditional lethality of pro3-1 is most likely...
attributable to the QC degradation of the misfolded polypeptide, leading to depletion of the essential protein at restrictive temperature.

**Ubr1 is an important constituent of the cytosolic QC machinery**

We then sought to determine whether the other ts alleles degraded in a proteasome-dependent manner (partially or fully) are affected by the absence of Ubr1. We found that lethality of both *ugp1-3* and *gln1-2* is restored by *UBR1* deletion (Figure 5A). Because single deletion of *UBR1* rescues the loss of function, we performed add-back experiments with either wild-type or inactive RING mutant (C1220S) Ubr1 ubiquitin ligase. Lethality was restored in presence of the wild-type but not of the inactive ligase, further indicating that the phenotype was dependent on the ubiquitin ligase activity of Ubr1 (Figure 5B). Double deletion of both *UBR1* and *SAN1* did not suppress lethality of the other alleles, nor did it further augment the recovery of *ugp1-3* and *gln1-2* (data not shown). These data suggest that, in some cases, targeting of QC substrates for degradation is mainly mediated by the Ubr1 E3 ligase.

We next assessed the turnover of *ugp1-3* and *gln1-2* mutant proteins at restrictive temperature in the absence of the Ubr1 ubiquitin ligase. In both instances, deletion of *UBR1* caused stabilization of the mutant proteins after shifting the cells at the nonpermissive temperature in the presence of cycloheximide (Figure 5C). Overall, these results support the idea that the Ubr1 E3 ligase plays a crucial role in the degradation of misfolded proteins.
indicate that UBR1 is involved in the proteasomal degradation of a substantial number (3/9) of unstable ts alleles of cytosolic proteins.

To gain further understanding of the targeting of the mutant proteins by Ubr1, we tested whether the deletion of SSE1, encoding for the Hsp70 nucleotide exchange factor, could also rescue the lethality of the ts allele. It was previously shown that degradation of the VHL model substrate in yeast requires SSE1 (McClellan et al. 2005). Similarly, the degradation of three engineered Ubr1 substrates required SSE1 (Heck et al. 2010; Prasad et al. 2010). In addition, in vitro ubiquitylation of tGdn1-GFP (truncation fragment of Gdn1 fused to GFP) was dramatically reduced when using extracts derived from sse1Δ cells (Heck et al. 2010), indicating that Sse1 may mediate or enhance the recognition of the misfolded substrate by Ubr1. We therefore tested whether deletion of SSE1 could rescue the lethality of pro3-1, ugp1-3, and gln1-2. We found that lethality of gln1-2 was partially rescued in sse1Δ cells (Figure 5D), whereas no effect was observed for pro3-1 and ugp1-3 at different nonpermissive temperatures (35, 36, or 37°C; data not shown). These data indicate that SSE1 may only be involved in the degradation of a subset of Ubr1-misfolded substrates.

DISCUSSION

Degradative QC pathways have been classically elucidated using model protein substrates. In this study, we show that ts mutants of cytosolic proteins display key features of QC substrates that can then be used to characterize the cytosolic QC machinery. Using this panel of model substrates, we found that the ubiquitin ligase Ubr1 plays a major role in targeting cytosolic ts substrates for degradation.

In most cases, ts mutants arise by mutations in essential genes that lead to a loss of viability under restrictive conditions. These mutations may impair protein function by altering the catalytic activity, the propensity to bind to physiological partners, as well as by affecting steady-state levels (by modifying protein expression or stability). We hypothesized that missense mutations that lead to conditional lethality may affect the steady-state levels of a large portion of essential proteins by inducing misfolding and degradation. We observed an increased protein turnover for approximately half the tested alleles under restrictive conditions (10/21), suggesting that reduced protein half-life may be a major cause of the ts phenotype. Importantly, each time the degradation of a ts mutant was reduced by genetic manipulations, the viability of the cell at nonpermissive temperature was restored.
In most cases, the turnover of these unstable \( ts \) mutant is dependent (fully or partially) on the proteasome activity, indicating that the ubiquitin proteasome system plays a key role in targeting these mutant proteins. Interestingly, degradation of these normally long-lived proteins occurs presumably after folding to their native state at permissive temperature. In contrast to the turnover of newly synthesized proteins after translation (typically degraded within a few minutes), the half-life of these mutants at nonpermissive temperature was relatively long (>30 min). Hence, the \( ts \) phenotype of these alleles may arise from a relatively slow decay of the essential proteins.

These \( ts \) alleles of cytosolic proteins constitute a new class of model substrates to study QC. So far, mainly ectopic artificial substrates, such as truncated carboxypeptidase Y or GFP, as well as general misfolding stresses, have been used to elucidate yeast protein QC pathways in the cytoplasm (Fang et al. 2011; Heck et al. 2010; Nillegoda et al. 2010; Prasad et al. 2010). The use of these approaches does not always fully mimic the physiological conditions or encapsulate the full spectrum of substrates targeted by protein QC. The panel of \( ts \) substrates used in this study are advantageous because they are expressed under the endogenous promoters. We found that as few as two missense

Figure 5 The Ubr1 ubiquitin ligase is required for the degradation of Ugp1-3 and Gln1-2. (A) Viability of \( ugp1-3 \) and \( gln1-2 \) haploid strains (\( UBR1 \) or \( ubr1 \Delta \)) was assessed on plates with yeast peptone dextrose at the indicated temperatures. (B) Viability of \( ugp1-3 \) and \( gln1-2 \) haploid strains (\( UBR1 \) or \( ubr1 \Delta \)) with an empty plasmid (pRS315) or expressing wild-type or inactive RING mutant Ubr1 were assessed on SD-LEU plates at the indicated temperatures after 1/5 dilutions of the cell cultures. (C) Levels of Ugp1-3 and Gln1-2 in \( UBR1 \) and \( ubr1 \Delta \) cells were assessed at 37\(^\circ\) after the addition of cycloheximide to cells grown at 25\(^\circ\) (OD\(_{600} \) ≈ 1). Levels of the MYC-tagged mutant proteins were determined by western blot analysis using 9E10 and anti-Pgk1 antibodies. The graph represents relative levels, normalized with Pgk1 signal, quantified at the indicated time points and averaged from three independent experiments (with standard deviations). (D) Viability of \( gln1-2 \) haploid strains (with \( UBR1 \) or \( SSE1 \) deletions) was assessed on plates at the indicated temperatures. Two different \( SSE1 \) strains were assessed.
mutations can lead to the decreased stability of the ts mutants guk1-6 and guk1-8 (data not shown). Misfolding and degradation can be induced by shifting cells to nonpermissive temperature without inducing a full stress response. Thus, interactions with these ts model substrates are likely pertinent to physiological conditions and potentially relevant to human autosomal recessive genetic diseases caused by protein misfolding.

We next sought to determine how prominent the role of Ubr1 is in cytosolic QC by using the panel of cytosolic ts mutants. To date, six of seven E3 ligases involved in protein QC have been either exclusively or partially implicated in cytosolic QC in Saccharomyces cerevisiae (Bengtson and Joazeiro 2010; Fang et al. 2011; Gardner et al. 2005; Heck et al. 2010; Lewis and Pelham 2009; Nillегода et al. 2010; Prasad et al. 2010; Ravid et al. 2006), and it is not clear which is (or are) the primary pathway(s) in the cell. We found that Ubr1 plays a substantial role in cytosolic QC, as the proteasome degradation of three of nine cytosolic mutant proteins was significantly reduced by UBR1 deletion. In two other cases (guk1-6 and guk1-8), we also observed a recovery in some—but not all—cells, which may be attributable to the reversion of the phenotype by additional mutations (data not shown). In one case (pro3-1), only deletion of both SAN1 and UBR1 leads to a significant stabilization of the mutant, indicating that both ubiquitin ligases are redundant. The same observation was made in previous detailed analyses of artificial model substrates (Heck et al. 2010; Prasad et al. 2010). Intriguingly, the authors of previous studies have shown that SSE1 was important for the degradation of Ubr1 substrates. In our hands, only gln1-2 was partially rescued by SSE1 deletion. One possibility is that by increasing the temperature, several chaperone proteins may be induced and compensate for the absence of Sae1. Interestingly, San1 was shown to interact with the truncated version of Pro3 by yeast two-hybrid assay (Rosenbaum et al. 2011). The targeting of cytosolic proteins by San1 ligase may depend on their propensity to bind to San1 and/or to shuttle to the nucleus. Note that as the degradation of Pro3-1 was only partially blocked by proteasome inhibition, it suggests that another proteasome-independent pathway is also involved in the turnover of that mutant.

It remains unclear which pathways target the other unstable mutants. Single deletions of Doa10 and Hul5, as well double deletion of San1 and Ubr1, do not rescue the lethality of the other ts alleles (data not shown). One possibility is that some of the unstable proteins are ubiquitylated by several redundant ubiquitin ligases (e.g., Ubr1 and Doa10); alternatively, these mutants could also be targeted by another, not yet identified, Q3 E3. Future studies will help to delineate these degradative QC pathways further.

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