The yeast prion [PSI⁺] is a self-propagating amyloidogenic isoform of the translation termination factor Sup35. Overproduction of the chaperone protein Hsp104 results in loss of [PSI⁺]. Here we demonstrate that this effect is decreased by deletion of either the gene coding for one of the major yeast ubiquitin-conjugating enzymes, Ubc4, or the gene coding for the ubiquitin-recycling enzyme, Ubp6. The effect of ubc4Δ on [PSI⁺] loss was increased by depletion of the Hsp70 chaperone Ssb but was not influenced by depletion of Ubp6. This indicates that Ubc4 affects [PSI⁺] loss via a pathway that is the same as the one affected by Ubp6 but not by Ssb. In the presence of Rnq1 protein, ubc4Δ also facilitates spontaneous de novo formation of [PSI⁺]. This stimulation is independent of [PIN⁺], the prion isoform of Rnq1. Numerous attempts failed to detect ubiquitinated Sup35 in the yeast extracts. While ubc4Δ and other alterations of ubiquitin system used in this work cause slight induction of some Hsps, these changes are insufficient to explain their effect on [PSI⁺]. However, ubc4Δ increases the proportion of the Hsp70 chaperone Ssa bound to Sup35, suggesting that misfolded Sup35 is either more abundant or more accessible to the chaperones in the absence of Ubc4. The proportion of [PSI⁺] cells containing large aggregated Sup35 structures is also increased by ubc4Δ. We propose that UPS alterations induce an adaptive response, resulting in accumulation of the large “aggregome”-like aggregates that promote de novo prion generation and prion recovery from the chaperone treatment.

Prions are infectious aggregated protein isoforms that cause fatal and incurable neurodegenerative diseases such as scrapie and “mad cow” diseases in mammals and Creutzfeldt-Jacob disease in humans (1). The pathology of prion diseases is reminiscent of the other noninfectious amyloidoses and protein assembly disorders associated with amyloid-like protein aggregation, such as Alzheimer, Huntington, and Parkinson diseases (2, 3).

Degradation of abnormal or damaged proteins occurs, at least in part, via the ubiquitin-proteasome system (UPS) (4). Targeting of a protein for degradation to the proteasome requires its conjugation to a 76-amino acid protein named ubiquitin (Ub) through the sequential action of several enzymes, including the Ub-conjugating enzymes (Ubc). This process can be reversed by the action of various deubiquitinating enzymes, including Ub-specific processing proteases (Ubp) that disassemble Ub conjugates and release free Ub (5). Mutations in different UPS components have been found to be associated with Alzheimer and Parkinson diseases (6). UPS failure can lead to aggregation of impaired proteins; in turn, aggregate formation is known to inhibit UPS function (7). Although proteasome inhibitors affect the turnover of mammalian prion proteins, and Ub is found in intracellular deposits of prion aggregates (8–10), the role of Ub-mediated proteolysis in prion formation is not defined.

Several yeast proteins possess prion properties (11–13). All known yeast prions contain QN-rich stretches, somewhat resembling poly(Q) stretches involved in aggregation disorders such as Huntington disease. Unlike the mammalian prion protein PrP, yeast prions do not kill cells, although some of them appear to be pathogenic in a longer run (14–16). Prion formation can antagonize the normal cellular function of a yeast protein, thus producing changes in phenotype that mimic conventional loss-of-function mutations. The yeast non-Mendelian element [PSI⁺] is a prion isoform of the yeast translation termination factor Sup35. [PSI⁺] cells are partially defective in termination of translation. [PIN⁺], a prion isoform of another protein of yet unknown function, Rnq1 (17), is usually needed for the de novo formation of [PSI⁺] but not for propagation of pre-existing [PSI⁺] (18–21).

The propagation of yeast prions is modulated by chaperone proteins of the Hsp100 and Hsp70 families (11, 12). The chaperone protein Hsp104, an ATPase known to promote solubilization of aggregated heat-damaged proteins in cooperation with other chaperones (22), is required for maintenance of all known yeast prions, possibly due to its ability to break prion aggregates into the oligomeric seeds, initiating new rounds of prion propagation. Transient overproduction of Hsp104 also

**Effects of Ubiquitin System Alterations on the Formation and Loss of a Yeast Prion**

Kim D. Allen†1,2, Tatiana A. Chernova91, E. Paula Tennant1, Keith D. Wilkinson5, and Yury O. Chernoff†3

From the 9School of Biology and Institute for Bioengineering and Bioscience, Georgia Institute of Technology, Atlanta, Georgia 30332-0230 and the 3Department of Biochemistry, Emory University School of Medicine, Atlanta, Georgia 30322

Received for publication, October 11, 2006, and in revised form, November 21, 2006 Published, JBC Papers in Press, December 1, 2006, DOI 10.1074/jbc.M609597200
Ubiquitin System Alterations Influence Yeast Prions

TABLE 1
S. cerevisiae strains used

| Strain | Genotype or description | Reference |
|--------|-------------------------|-----------|
| GT81-1C | MATa ade1–14 (UGA) his3Δ200 leu2–3,112 lys2–801 | 14 |
| GT81-1D | MATa strain isogenic to GT81-1C | 14 |
| GT159 | [psi− PIN+] derivative of GT81-1C | 26 |
| GT409 | [psi− PIN+] derivative of GT81-1C | This study |
| GT234 | [psi− pin+] derivative of GT81-1D | This study |
| GT349 | abc4Δ::HIS3MX6 disruptant of GT81-1C | This study |
| GT386 | [psi− PIN+] derivative of GT349 | This study |
| GT387 | [psi− pin+] derivative of GT349 | This study |
| GT340-12C | abc5Δ::HIS3MX6 disruptant of GT81-1D | This study |
| GT347 | [psi− PIN+] derivative of GT340-12C | This study |
| GT385-1A | [psi− pin+] derivative of GT340-12C | This study |
| GT564 | rnp1Δ::HIS3MX6 disruptant of GT409 | This study |
| GT820 | rnp1Δ::HIS3MX6 disruptant of GT81-1C | This study |
| WTY105 | ubp6Δ::HIS3MX6 disruptant of GT81-1C | This study |
| GT832-7B | ubp6Δ::HIS3MX6 ubc4Δ::HIS3MX6 isogenic to GT654 | This study |
| GT147 | ubp6Δ::HIS3MX6 ubc4Δ::HIS3MX6 isogenic to GT81-1C | 26 |
| GT389-1A | ubp6Δ::HIS3MX6 ubc4Δ::HIS3MX6 isogenic to GT81-1C | 26 |
| GT389-1A | ubc4Δ::HIS3MX6 disruptant of GT81-1D | This study |
| WTY191 | ssa1Δ::HIS3MX6 disruptant of GT81-1D | 32 |
| GT156 | erg6Δ::HIS3MX6 disruptant of GT81-1C | This study |
| MYH501 | MATA his3Δ200 leu2–3,112 lys2–801 | 59 |
| WTY271 | pdr1Δ::kanMX6 disruptant of MYH501 | This study |

Cures yeast cells of [PSI+], although not of the other prions. Ssa proteins of the Hsp70 family also play an important role in prion propagation. Ssa overproduction antagonizes [PSI+] curing by excess Hsp104 and promotes de novo [PSI+] formation in the [PIN+] strains (23, 24), while Ssa mutations destabilize [PSI+] (25). Another chaperone of the Hsp70 family, Ssb, consistently manifests itself as a [PSI+] antagonist (26). Hsp40 chaperones that act as co-chaperones of Hsp70s were shown to control propagation of [PIN+] (27) and modulate aggregation of heterologous poly(Q) proteins in yeast (28, 29). Mechanisms of the chaperone effects on prions are not yet completely understood. As some of the chaperones of Hsp70 and Hsp40 families were implicated in targeting misfolded proteins for Ub-dependent degradation (30, 31), it is possible that some of the effects of chaperones on prions could be mediated by UPS.

We employed [PSI+] as a model to analyze the possible involvement of the UPS in modulation of prion formation, propagation, and clearance. Previously, we have shown that depletion of cellular Ub levels (by deleting the deubiquitinating enzyme Ubp6) decreases both the phenotypic manifestation of [PSI+] and de novo induction of [PSI+] by overproduced Sup35 protein in the [PIN+] background (32). Here, we demonstrate that both ubp6Δ and deletion of a gene coding for the major yeast Ub-conjugating enzyme, Ubc4, antagonize [PSI+] curing by excess Hsp104. Moreover, ubc4Δ also significantly increased spontaneous formation of [PSI+]. Our data implicate the UPS as one of the major modulators of prion formation and clearance in the yeast cells.

EXPERIMENTAL PROCEDURES

Yeast Strains—The Saccharomyces cerevisiae strains used in this study are listed in Table 1. GT81-1C and GT81-1D, isogenic haploid strains that derived from self-homologous diploid GT81 (14), contain the [PSI+]-suppressible marker ade1–14 (UGA) (33). The [psi− PIN+] and [psi− pin−] derivatives resulted from curing the isogenic [PSI+] strains by overproduction of Hsp104 or guanidine hydrochloride (GuHCl) treatment, respectively (33). All of the single deletion strains used were constructed for this study by one step PCR-mediated direct gene replacement with the pFA6a-His3MX6 module (34). Primers sequences are available by request. Double and triple knock-out strains were obtained by mating the appropriate deletion strains and then sporulating and dissecting the resulting diploid strain. Presence of double and multiple disruptions in the spore clones was confirmed by PCR.

Plasmids—The plasmids used in this study were as follows: 1) centromeric (low copy) URA3 plasmids: pYS104 (35) from S. Lindquist, expressing Hsp104 from its endogenous (PHSP104) promoter; pYS-GAL104 (36) from S. Lindquist, expressing Hsp104 under the control of galactose-inducible (Pgal) promoter; pmCUP1-GFP and pmCUP1-NM-GFP from S. Lindquist (37), producing, respectively, green fluorescent protein (GFP), and the N-terminal and middle domains of Sup35 fused to GFP (Sup35NM-GFP) from copper-inducible (Pcup) promoter; pmCUP1-SUP35-HA (23) expressing HA-tagged Sup35 from the copper-inducible (Pcup) promoter; and control plasmids pRS316GAL (38) and pmCUP1 (37); 2) a 2-μm URA3 plasmid pUBC4 from M. Hochstrasser (39), expressing Ubc4 from its endogenous promoter, and a control plasmid pFL44 from F. Lacroute (40); 3) a centromeric TRP1 plasmid pFL39-GAL-HSP104 (41), expressing Hsp104 from the Pgal promoter, and a control plasmid pFL39 from F. Lacroute (40); 4) the 2-μm TRP1 plasmids YEp112 and YEp105 from M. Ellison (39), expressing, respectively, Ubi-HA and Ubi-myc from the (Pcup) promoter; 5) a 2-μm plasmid pHL105 (26), provided by S. Lindquist that produces Hsp104 from the highly expressed glyceraldehyde phosphate dehydrogenase (Pgal) promoter, and a control plasmid YEp13 (42).

Media and Growth Conditions—Standard yeast media, cultivation conditions, procedures for yeast growth, transformation, sporulation, and tetrad analysis were used (43). Yeast cultures were grown at 30°C unless otherwise specified. Gal medium contained 2% galactose instead of glucose.
Ubiquitin System Alterations Influence Yeast Prions

medium contained 2% galactose and 2% raffinose instead of glucose. To induce expression of genes under the \( P_{\text{CUP1}} \) promoter, CuSO\( \text{4} \) (50 or 100 \( \mu \text{M} \)) was added to the media. For drug sensitivity assay, strains were grown on YPD medium to an \( A_{600} \) of 1.0, and 2.5 \( \mu \text{l} \) of serial 10-fold dilutions were plated on YPD (1% yeast extract, 2% peptone, 2% glucose) medium with anisomycin (20 \( \mu \text{g/ml} \)). Growth on the plates was recorded after 2 and 5 days. The presence of \([\text{PSI}^+]\) was monitored by its ability to suppress the reporter allele \( ade1–14 \) (UIGA) as described previously (33). In the absence of \([\text{PSI}^+]\), these cultures develop light pink or white color on YPD and are able to grow on \(-\text{Ade} \) medium. In the presence of \([\text{PSI}^+]\), \( ade1–14 \) cultures develop red color on YPD and are unable to grow on \(-\text{Ade} \) medium.

**Assays for \([\text{PSI}^+]\) Curing**—Assays for \([\text{PSI}^+]\) curing by GuHCl were performed as described previously (33). Briefly, to measure \([\text{PSI}^+]\) curing by overproduced Hsp104, yeast cultures were transformed with the Hsp104 expression vectors or empty control vectors and analyzed as outlined below. For plate assays with the plasmids expressing Hsp104 from the endogenous \( P_{\text{HSP104}} \) or constitutively active \( P_{\text{GPD}} \) promoters, plasmid-containing cultures were grown on the medium selective for the plasmid and then velveteen replica plated onto the medium not selective for the plasmid and lacking adenine or onto YPD medium. For plate assays with the galactose-inducible \( P_{\text{GAL}} \) promoter, cultures were grown on selective medium containing Gal and then velveteen replica-plate onto selective media containing glucose but lacking adenine or onto YPD medium. For all plasmids, the efficiency of \([\text{PSI}^+]\) curing was assessed by decreased growth on \(-\text{Ade}\) medium and increased intensity of red color on YPD medium compared with the control cultures. For quantitative assays, yeast cultures containing the plasmids bearing \( HSP104 \) under \( P_{\text{GAL}} \) promoter were pregrown in selective medium containing Gal and then inoculated into the same medium and growth on \(-\text{Ade} \) medium. All deletion strains remained cytologically detectable aggregates in these conditions (data not shown), confirming that aggregation is specific to the Sup35-GFP protein.

**RESULTS**

**Alterations of the UPS Decrease \([\text{PSI}^+]\) Curing by Overproduced Hsp104**—Transient overproduction of the wild-type chaperone Hsp104 eliminates the yeast prion \([\text{PSI}^+]\) (46). To check whether or not alterations of the UPS affect \([\text{PSI}^+]\) curing by excess Hsp104, we have constructed an isogenic series of yeast strains with deletions of the \( UBC4, \ UBC5, \) or \( UBP6 \) gene in the background of \([\text{PSI}^+]\) strain GTB1-1C as described under “Experimental Procedures.” All deletion strains remained \([\text{PSI}^+]\), and neither deletion affected mitotic stability of \([\text{PSI}^+]\) under normal conditions (data not shown).

Deletion strains and wild-type control strain were transformed with the plasmids producing Hsp104 from either the galactose-inducible \( P_{\text{GAL}} \) or a strong constitutive \( P_{\text{GPD}} \) promoter, as well as by empty control vectors. In the case of \( \text{ubc4}A \), we also moderately overexpressed \( HSP104 \) by providing a single plasmid-borne copy under the control of its endogenous promoter. Levels of overexpressed Hsp104 were similar in wild-type and UPS mutants as confirmed by Western blot analysis (Fig. 1A and data not shown). The efficiency of \([\text{PSI}^+]\) curing in the presence of either \( P_{\text{GAL}}-HSP104 \) (Fig. 1A) or \( P_{\text{GPD}}-HSP104 \) (data not shown) constructs was detected by color on YPD medium and growth on \(-\text{Ade} \) medium. Efficiency of curing was decreased in \( \text{ubc4}A \) and \( \text{ubp6}A \) single deletion strains, compared with the wild-type strain. \([\text{PSI}^+]\) loss in the presence of an
Ubiquitin System Alterations Influence Yeast Prions

Extra copy of \( P_{\text{HSP104}}-\text{HSP104} \) was also decreased in \( ubc4 \Delta \) (data not shown). Quantitative assays employing the \( P_{\text{GAL}}-\text{HSP104} \) construct demonstrated that the effect of double \( ubc4 \Delta \) \( ubp6 \Delta \) deletion on \([\text{PSI}^+]\) curing by excess Hsp104 is the same as the effect of single \( ubc4 \Delta \) deletion (Fig. 1B). This result suggests that both proteins influence \([\text{PSI}^+]\) curing via one and the same pathway and that Ubc4 acts before Ubp6. Such a conclusion is in agreement with the known roles of both enzymes in the Ub-proteasome degradation pathway. Interestingly, the efficiency of \([\text{PSI}^+]\) curing by excess Hsp104 was not altered in the \( ubc5 \Delta \) strain (data not shown), despite the fact that \( ubc5 \) is known to have a role that is very similar to and partly redundant with that of Ubc4. A double \( ubc4 \Delta \) \( ubc5 \Delta \) deletion strain was not viable in the genetic background of our strains.

The only other known mutation decreasing \([\text{PSI}^+]\) curing by excess Hsp104 is the double deletion of the genes \( SSB1 \) and \( SS2 \), coding for the Ssp protein of the Hsp70 family (26). This deletion is designated below as \( ssp1/2 \Delta \). To check if \( ssp1/2 \Delta \) and deficiencies in the UPS components influence \([\text{PSI}^+]\) loss through one and the same pathway, we compared \([\text{PSI}^+]\) curing by excess of Hsp104 in several strains: the single \( ubc4 \Delta \) deletion strain, the double \( ssp1/2 \Delta \) strain, and the triple \( ubc4 \Delta \) \( ssp1/2 \Delta \) deletion strain. The triple \( ubc4 \Delta \) \( ssp1/2 \Delta \) deletion decreased the efficiency of \([\text{PSI}^+]\) curing in comparison to both single \( ubc4 \Delta \) or double \( ssp1/2 \Delta \) deletions (Fig. 1C). The additive effect of \( ubc4 \Delta \) and \( ssp1/2 \Delta \) indicates that these mutations influence \([\text{PSI}^+]\) curing by excess Hsp104 via different pathways.

Spontaneous Formation of \([\text{PSI}^+]\) Is Increased in the \( ubc4 \Delta \) Background—Next, we checked whether spontaneous \([\text{PSI}^+]\) formation is influenced by the UPS alterations. In the \([\text{psi}^+]\) strain bearing the \( ade1-14 \) reporter allele, spontaneous \([\text{PSI}^+]\) formation can be detected as the emergence of small \( Ade^+ \) revertants (papillae) on a patched colony growing on \(-Ade\) medium. Papillae are usually observed only in the presence of \([\text{PIN}^+]\), the prion form of the Rnq1 protein (18–20). The \([\text{psi}^-\text{pin}^-]\) strains containing non-prion form of Rnq1 rarely papillate on \(-Ade\) and, if they do, the resulting \( Ade^+ \) papillae usually contain a chromosomal mutation rather than the \([\text{PSI}^+]\) prion. Surprisingly, we observed that both \([\text{psi}^-\text{pin}^-\text{PIN}^-]\) (Fig. 2A) and \([\text{psi}^-\text{pin}^-]\) (Fig. 2B) strains bearing the \( ubc4 \Delta \) deletion exhibited significantly increased frequency of the spontaneous \( Ade^+ \) papillae (compared with the isogenic \([\text{psi}^-\text{PIN}^-]\) and \([\text{psi}^-\text{pin}^-\text{PIN}^-]\) \( UBC4^+ \) strains, respectively). In contrast, neither \( ubc5 \Delta \) nor \( ubp6 \Delta \) exhibited an increase in papillation, in either \([\text{psi}^-\text{PIN}^-]\) or \([\text{psi}^-\text{pin}^-]\) backgrounds. The \( ubc5 \Delta \) \([\text{psi}^-\text{pin}^-]\) strain is shown as an example in Fig. 2B. One should note that the negative result for \( ubp6 \Delta \) is somewhat ambiguous, as \( ubp6 \Delta \) is known to weaken growth of the \([\text{PSI}^+]\) strains on \(-Ade\) medium (32), so that some newly arisen \( Ade^+ \) derivatives might remain unnoticed in this strain.

Increased spontaneous papillation of the \( ubc4 \Delta \) strain on \(-Ade\) media was eliminated by reintroduction of the wild-type

same way as in part B, with the only difference that \(-Trp\) media were used for the incubation and plating. Despite some variations in the efficiencies of \([\text{PSI}^+]\) curing between B and C, which are apparently due to different \( P_{\text{GAL}}-\text{HSP104} \) plasmids used, differences between the wild-type and \( ubc4 \Delta \) strains are maintained in these two variants of experiment.
Ubiquitin System Alterations Influence Yeast Prions

**A**

[psi PIN+]  
\[
\text{UBC4- ubc4Δ}  
\]

- Ade

**B**

[psi pin]  
\[
\text{UBC4- ubc4Δ}  
\]

- Ade

**C**

ubc4Δ  
\[
\text{ubc4Δ + pUBC4}  
\]

- UrA - Ade

**D**

His-pin  
\[
(\text{UBC4}) (\text{ubc4Δ})  
\]

- Ade

**FIGURE 2. Ubc4Δ increases spontaneous [PIN+] formation.** A and B, Ubc4Δ but not ubc5Δ increases the frequency of spontaneous [PIN+] formation in both [PIN+] (A) and [pin-] (B) backgrounds but not in the strain lacking Rq1 protein (mq1Δ). C, experiments were performed in the [psi PIN+] strains GT159 (UBC4Δ), GT386 (ubc4Δ), and GT548 (ubc5Δ; data not shown), the [psi pin+] strains GT409 (UBC4Δ), GT387 (ubc4Δ), and GT385-1A (ubc5Δ), and the [psi pin+] mq1Δ strains GT564 (UBC4Δ mq1Δ) and GT820 (ubc4Δ mq1Δ). All strains were isogenic except for the markers indicated. Independent colonies of each strain were patched on YPD medium and replica plated onto -Ade medium. The -Ade plates were photographed after 14 days of incubation. A sample of independent Ade derivatives was confirmed to contain [PIN+] by using an assay based on the loss of Ade phenotype following GuHCl treatment (see “Results”). C, increased spontaneous formation of A pepilae in the ubc4Δ strain is compensated by the plasmid pUBC4 containing the wild-type UBC4 gene. The [psi pin+] ubc4Δ strain GT385-14C was transformed with either control vector pFL44 (ubc4Δ) or pUBC4 (ubc4Δ + pUBC4). Transformants were patched on -Ura medium and replica plated onto -Ura-Ade medium. Papillation was scored after 14 days. One typical transformant is shown in each case. D, formation of Ade pepilae co-segregates with ubc4Δ in tetrad analysis. The [psi PIN+] ubc4Δ strain GT386 was crossed to the isogenic wild-type strain GT234. The resulting diploid strain was sporulated and dissected. One typical tetrad is shown. His+ corresponds to ubc4Δ (see Table 2 for numbers).

UBC4+ gene on a plasmid (Fig. 2C). When the ubc4Δ [psi PIN+] strain was mated to the isogenic [psi pin+] UBC4 strain of the opposite mating type, and the resulting diploid was sporulated and dissected, the increased papillation on -Ade co-segregated with the ubc4Δ marker (HIS3) in tetrad analysis (Fig. 4D and Table 2). These data confirm that increased papillation is due to lack of UBC4 gene rather than any independent alteration in the yeast genome.

A number of Ade colonies that had independently arisen from UBC4+ and ubc4Δ strains were tested for the curability of Ade+ phenotype by growth in the presence of GuHCl, an agent known to eliminate yeast prions (33, 47). While the majority of colonies obtained in the [PIN+] strains were curable by GuHCl, about 77% Ade+ colonies obtained in the UBC4+ [psi pin+] strain were incurable, indicating that they originated from mechanisms other than prion formation, such as reversion of the ade1-14 mutation or suppressor mutation. In contrast, 94% of Ade+ colonies obtained in the ubc4Δ [psi pin+] strain were curable by GuHCl. All GuHCl-curable Ade+ colonies isolated from this strain were also curable by the PPGPD-HSP104 plasmid, known to eliminate [PIN+] as described above. This confirms that majority of spontaneous Ade+ colonies detected in the ubc4Δ [psi pin+] strain result from [PIN+] formation rather than from suppressor or reverse mutation. Quantitative assay confirmed that the differences in rates of spontaneous [PIN+] formation between the ubc4Δ and wild-type strains in both [psi PIN+] (data not shown) and [psi pin+] (Table 3) backgrounds are statistically significant.

One possible explanation for the spontaneous [PIN+] generation in the [psi pin+] strain is that the prion form of Rq1 ([PIN+]) accumulates first and subsequently facilitates the formation of the prion form of Sup35 ([PSI+]). To test this possibility, we have checked whether the [PIN+] prion is present in the [PSI+] derivatives obtained from the [pin-] ubc4Δ strain. For this purpose, 37 independently obtained Ade- derivatives of this strain were cured of [PIN+] by overproduction of Hsp104 from the PPGPD promoter. Excess Hsp104 eliminates [PIN+] but not [PIN+] (18). Resulting [psi-] derivatives were cured of the PPGPD-HSP104 plasmid and tested for the presence of [PIN+] by mating them to the [psi- pin+] strain of opposite mating type bearing the multicopy SUP35 plasmid. No [PSI+] formation was detected in the resulting diploids, indicating that all original [PSI+] derivatives remained [pin-]. Therefore, increased spontaneous formation of [PSI+] in the [pin-] ubc4Δ strain is not due to generation of [PIN+].

To check whether Rq1 protein in its non-prion form influences formation of the [PSI+] prion in the ubc4Δ background, we deleted the Rq1 gene in both wild-type and ubc4Δ [psi-] strains and found that rqn1Δ dramatically reduced frequency of the spontaneous [PSI+] papilae in the ubc4Δ strain, making it indistinguishable from the wild-type strain (Fig. 2B). These data show that the presence of the Rq1 protein is required for the increased de novo prion formation observed in the ubc4Δ cells. To our knowledge, this is the first evidence demonstrating a

| Phenotype | No. of spores |
|-----------|--------------|
| His+ Pin- | 21           |
| His+ Pin+ | 1            |
| His- Pin- | 1            |
| His- Pin+ | 22           |
| Total     | 45           |

**TABLE 2 Meiotic co-segregation of the Pap phenotype with ubc4Δ:HIS3**

The ubc4Δ:HIS3 [psi- Pin+] strain GT386 was crossed to the isogenic UBC4 [psi- pin+] strain GT234. Resulting diploids were sporulated and dissected. Each clone was assayed for the frequency of spontaneous [PIN+] formation. High frequency is designated as high papillation (Pap+) phenotype, while low frequency is designated as low papillation (Pap-) phenotype. In majority of the spores, ubc4Δ (His-) and UBC4+ (His+) co-segregate with Pap+ and Pap- respectively. Rare exceptional spores (His+ Pap- and His+ Pap+) occur at the frequency similar to the expected frequency of meiotic conversion of the HIS3 marker.
role for the non-prion form of Rnq1 in the formation of a heterologous prion. In contrast, an antagonistic effect of ubc4Δ on [PSI+] curing by excess Hsp104 was detected in the [PSI+] rna1Δ strain as clearly as in the [PSI+] RNQ1+ background (data not shown). This agrees with the previous observation that Rnq1 prion is involved in de novo [PSI+] formation but not in the maintenance of a pre-existing [PSI+] prion (19, 20).

Effects of UPS Alterations and Ssb Deficiency on Ub Levels and Antibiotic Sensitivity—We and others (32, 48) have previously observed that the ubp6Δ deletion results in depletion of free Ub and increased sensitivity to the translation inhibitor anisomyacin and that most of the detectable phenotypes of ubp6Δ are due to Ub depletion. The proposed (30) involvement of Ssb in UPS-dependent protein degradation makes it tempting to speculate that some effects of ssb1/2Δ on [PSI+] could be through alteration of UPS. To examine potential mechanisms by which defects of UPS influence prion formation, we first characterized the levels of Ub and sensitivity to anisomyacin in the strains with ubc4Δ, ubp6Δ, and ssb1/2Δ deletions. As expected, ubp6Δ reduced levels of free Ub in comparison with the isogenic wild-type strain (Fig. 3A). Interestingly, levels of free Ub were also reduced in the double ssb1/2Δ mutant (Fig. 3A). The ubc4Δ strain exhibited a slight increase in the levels of free Ub (Fig. 3A) apparently due to impaired conjugation. Deleting UBC4 in the ubp6Δ or ssb1/2Δ deletion strains (Fig. 3A) partially restored free Ub levels suggesting that the effects of UPS alterations on [PSI+] curing are not simply due to decreased Ub levels.

As shown previously for ubp6Δ (32), decreased Ub levels correlate with increased sensitivity to anisomyacin. We found that like ubp6Δ, both ubc4Δ and ssb1/2Δ mutants were more sensitive to anisomyacin than the wild type strain. The ubp6Δ strain exhibited the highest level of sensitivity, the ubc4Δ strain exhibited an intermediate sensitivity, and ssb1/2Δ was the least sensitive. The double ubc4Δ ubp6Δ deletion strain was more sensitive to anisomyacin than either single mutant, while the sensitivity of the triple ubc4Δ ssb1/2Δ deletion strain was closer to that of the ubc4Δ than to the ssb1/2Δ strain (Fig. 3B). These data indicate that decreased Ub levels and decreased Ub conjugation affect resistance to anisomyacin in a partially independent fashion. Interactions between Ubc4, Ubp6, and Ssb affect both Ub levels and anisomyacin sensitivity differently than interactions between the same players affect the [PSI+] curing assays. This suggests that the effects of UPS alterations on [PSI+] cannot be explained as a simple consequence of the alterations in Ub levels.

Ubiquitin System Alterations Influence Yeast Prions

| Strain | Median frequency | Median rate for [PSI+] | 95% confidence limit |
|--------|------------------|------------------------|---------------------|
| [ps+ pin+] ubc4Δ | 4.1 × 10⁻⁶ | 7.1 × 10⁻⁷ | (5.9 – 8.3) × 10⁻⁷ |
| [ps+ pin+] ubc4Δ | 1.7 × 10⁻⁶ | 4.8 × 10⁻⁷ | (4.1 – 5.5) × 10⁻⁷ |

* Proportion of [PSI+] colonies was determined in the random sample of the independently arisen Ade+ colonies on the basis of GuHCl curability assay (see “Results” for details).

Despite numerous tries, none of the experiments produced any evidence for the existence of ubiquitinated Sup35. Neither were steady state levels of Sup35 influenced by the proteasome inhibitors (Fig. 3E). Likewise, a large scale analysis of the ubiquitinated proteins in yeast (50) failed to detect Sup35, although ubiquitinated forms of another prion protein (Rnq1) and some members of the Hsp70 family were detected. These negative results do not completely exclude the possibility that there is a small and short-lived ubiquitinated fraction of Sup35 that cannot be identified by the approaches used. However, at least in the case of the excess Hsp104-mediated [PSI+] curing, the ubiquitinated fraction of Sup35 should be sizeable for ubc4Δ to influence the outcome, and our results make such an explanation unlikely. Therefore, we propose that UPS alterations influence [PSI+] via a more complex mechanism than simply by altering Sup35 ubiquitination.
Ubiquitin System Alterations Influence Yeast Prions

Levels of Sup35 and Hsps in the Strains with UPS Alterations—

In the strains where it is viable, the \( ubc4\Delta \) \( ubc5\Delta \) double deletion increases background levels of some stress-inducible chaperones, specifically Hsp70s (51). As both Sup35 levels and Hsp levels control \([\text{PSI}^+]\) propagation (12), one possibility could be that UPS alterations influence \([\text{PSI}^+]\) via alterations of either Sup35 or Hsp levels. To address this possibility, we checked the effects of UPS alterations on the levels of Sup35 and levels of Hsps known to influence \([\text{PSI}^+]\).

Our experiments detected no differences in Sup35 levels between the wild-type strain and isogenic strains bearing the single \( ubc4\Delta \) and \( ubp6\Delta \) or double \( ubc4\Delta ubp6\Delta \) deletions. This observation was confirmed for both \([\text{PSI}^+]\) (Fig. 4A) and \([\text{psi}^-]\) (data not shown) backgrounds. There was also no detectable change in the levels of Ssb, Hsp40-Ydj, or Hsp40-Sis1 chaperones in any of these strains (Fig. 4A). Level of Hsp104 and total Ssa protein were slightly but reproducibly increased by \( ubc4\Delta \) UPS defects. For this purpose, we have employed the Sup35NM-GFP construct that bears the N-proximal portion of Sup35 (encompassing the prion-forming and middle domains) fused to the green fluorescent protein. Expression of this construct from the copper-inducible \( P_{\text{CUP1}} \) promoter results in accumulation of “clumped” or “dot”-like fluorescent aggregates in a fraction of the \([\text{PSI}^+]\) cells, while diffused fluorescence is detected in the \([\text{psi}^-]\) strain (Fig. 4). These data indicate that lack of Ubc4 increases either ability of Ssa to recognize Sup35 or proportion of the Sup35 protein that can be recognized by Ssa or both. The possible relevance of this observation to the \( ubc4\Delta \) effect on \([\text{PSI}^+]\) is discussed below (see “Discussion”).

Aggregation Status of the Sup35 Protein in Strains with UPS Mutations—Next, we checked whether the aggregation status of the Sup35 protein is altered in strains with \([\text{PSI}^+]\) or \([\text{psi}^-]\) (Fig. 4A and B) and \([\text{psi}^-]\) (data not shown) backgrounds. This increase in Ssa levels was primarily due to induction of the normally silent \( Ssa3 \) and \( Ssa4 \) genes, as confirmed by using an Ssa3/4-specific antibody (data not shown).

A slight but not statistically significant increase in Hsp104 levels was also detected in the \( ubp6\Delta \) strain. However, neither Hsp104 nor Ssa levels were increased in the double \( ubc4\Delta ubp6\Delta \) mutant. The defect of \([\text{PSI}^+]\) curing by excess Hsp104 detected in this strain (see above), cannot be explained by alterations of the Hsp104 or Ssa levels.

Previously, we have shown that Sup35 and Ssa proteins physically interact in vivo (23). Interestingly, co-immunoprecipitation experiments demonstrated that the fraction of Ssa bound to Sup35 is increased about 3-fold in the \( ubc4\Delta \) strain compared with the isogenic wild-type strain (Fig. 4, C and D), while total amount of Ssa in the cell is increased at only about 1.5-fold (Fig. 4B). These data indicate that lack of Ubc4 increases either ability of Ssa to recognize Sup35 or proportion of the Sup35 protein that can be recognized by Ssa or both. The possibility that this observation to the \( ubc4\Delta \) effect on \([\text{PSI}^+]\) is discussed below (see “Discussion”).

**DISCUSSION**

Our data show that mutational alterations of the UPS, specifically deletions of the genes \( UBC4 \), coding for one of the
Ubiquitin System Alterations Influence Yeast Prions

In addition to increasing \([PSI^+]\) resistance to curing by excess Hsp104, \(ubc4\Delta\) also increased spontaneous \([PSI^+]\) formation. This is the first mutation shown to have such a “protein mutator” effect in the absence of the other pre-existing prion, \([PIN^+]\). In principle, both effects of \(ubc4\Delta\) on \([PSI^+]\) could be explained by a model, suggesting that misfolded Sup35 protein, occasionally generated spontaneously in the cells lacking prion or produced in resus of disaggregating activity of Hsp104 on prion aggregates, is normally targeted by Ubc4-dependent ubiquitination for degradation via the proteasome. In the absence of Ubc4, this misfolded protein is not eliminated, increasing the probability of turning it into a prion. This model predicts that a fraction of Sup35 is ubiquitinated. Moreover, in order to explain the anti-curing effects of \(ubc4\Delta\), the ubiquitinated fraction of Sup35 should be sizeable, at least in the prion-containing cells that overproduce Hsp104. However, neither direct approaches employed in our work (see above, Fig. 3, C and D) nor large scale analysis performed by another group (50) detected Sup35 among ubiquitinated proteins. Moreover, the prion domain of Sup35 contains a QN-rich stretch resembling poly(Q) proteins, and it is known that proteasomal degradation of proteins with poly(Q) stretches is inefficient (53). Although we cannot completely exclude a possibility that the small short-lived fraction of misfolded ubiquitinated Sup35 may exist in the yeast cells, it seems unlikely that elimination of such a fraction could explain all the observed effects of \(ubc4\Delta\) on \([PSI^+]\).
Ubiquitin System Alterations Influence Yeast Prions

Some UPS alterations are known to have broad pleiotropic effects, for example by inducing stress response. As some stress-induced proteins are shown to influence prions, we have checked whether levels of these proteins are altered in the cells with UPS alterations. Indeed, we have detected slightly increased levels of Hsp104 and Ssa in ubc4Δ and slightly increased level of Hsp104 in ubp6Δ. However, neither of these chaperones was induced in the double ubc4Δ ubp6Δ mutant. Moreover, neither overproduction of Hsp104 nor overproduction of Ssa leads to a detectable increase in spontaneous [PSI+] formation in the [psi− pin−] strain (data not shown). Therefore, it is highly unlikely that effects of UPS alterations on [PSI+] are explained by slight alterations in the levels of these chaperones. Remarkably, additive effects of ubc4Δ and ssb1/2Δ on [PSI+] curing by excess Hsp104 indicate that Hsp70-Ssb also influences prion resistance to excess Hsp104 via a pathway that does not involve Ubc4 and vice versa.

Interestingly, we have observed that while total levels of Ssa protein are only slightly increased in the ubc4Δ strain, the amount of Ssa bound to Sup35 is increased significantly. This could occur by one of the following two mechanisms: either 1) an increase in the affinity of Ssa to Sup35 or 2) an increase in the fraction of Sup35 that can be bound by Ssa. It is worth noting that these mechanisms are not mutually exclusive. The first mechanism is supported by the observation that Ssa was detected among ubiquitinated proteins (50). Thus, it cannot be excluded that its activity is somehow influenced by ubiquitination, which is defective in ubc4Δ strains. On the other hand, known patterns of Ssa interactions with other proteins speak in favor of the second mechanism. Indeed, Ssa with the help of the Hsp40 co-chaperones is known to bind partially unfolded proteins; therefore it is quite likely that it binds a partially misfolded fraction of Sup35. Increased Ssa binding to Sup35 in the absence of Ubc4 may therefore reflect either increased proportion of misfolded Sup35 in these conditions or its increased accessibility to Ssa.

Why, then, is misfolded Sup35 increased in abundance in the ubc4Δ cells if not due to lack of its Ub-promoted degradation? One possible explanation could be drawn from our observation that the fraction of cells containing large Sup35 aggregates is increased in the [PSI+] ubc4Δ culture (Fig. 4E). We have previously indicated (45) that large cytologically detectable aggregates of Sup35 share some (but not all) features of mammalian aggresomes, large cytoskeleton-associated structures generated by misfolded aggregation-prone proteins in the mammalian cells (54). It has previously been shown that aggresome formation is induced by UPS inhibition (55). It is therefore possible that defects of UPS, such as ubc4Δ, induce an adaptive response in the yeast cells, which results in increased formation of the large aggresome-like structures. This response could be triggered by accumulation of the misfolded proteins due to defective Ub-conjugation but not necessarily by accumulation of misfolded Sup35 per se. In case of prion proteins, such aggresome-like structures promote segregation of the prion state from the action of chaperones and therefore contribute to increased resistance of prion state to the prion curing agents. In the non-prion cells, occasional “aggresome” formation by Sup35 initiates de novo prion generation.

Ssa protein has been implicated in disassembly of the large Sup35 aggregates into the smaller prion-propagating units (56), and our preliminary data suggest that Ssa can bind at least some cytologically detectable Sup35-GFP aggregates in the yeast cells. Therefore, increased formation of large aggregates may be responsible for increased Ssa binding to Sup35, for example by making Sup35 more accessible to Ssa.

Certainly this model does not explain all the complexity of UPS interactions with prions. Indeed, while favoring the [PSI+] state in curing assays, ubp6Δ decreased the phenotypic manifestations of [PSI+] and the de novo [PSI+] induction in the presence of excess Sup35 (32). These effects were even more pronounced in case of the deletion of DOA4, another gene whose product is involved in deubiquitination and maintenance of Ub pool. Interestingly, doa4Δ appeared to inhibit [PSI+] curing by excess Hsp104 as well, although this effect was difficult to characterize quantitatively due to severe growth defects caused by doa4Δ (data not shown). One possible explanation for the conflicting effects of ubp6Δ and doa4Δ on [PSI+] in different assays could be drawn from the necessity of ubiquitination for initiation of endocytosis (57), a process influencing formation of the yeast aggresome-like structures (45). Indeed, Doa4 is functionally linked to the endocytic pathway (58). Thus, ubp6Δ, and especially doa4Δ, may both induce Sup35 aggregation due to UPS defects as described above and inhibit it due to endocytosis defects caused by the depletion of free Ub pool. On the other hand, ubc4Δ is not predicted to affect endocytosis, as it does not deplete the free Ub pool and is not known to be involved in endocytosis-associated Ub conjugation.

Unexpectedly, we have found that ubc5Δ does not influence [PSI+] resistance to excess Hsp104 and de novo [PSI+] formation, despite the well known functional redundancy of Ubc4 and Ubc5 (51). One possible explanation is that modes of regulation of UBC4 and UBC5 are different from each other, so that Ubc4 is primarily expressed in the conditions that are crucially important for prion formation and curing. Indeed, some evidence suggests that Ubc4 and Ubc5 are preferentially produced at the different growth phases of the yeast culture (51). Such a hypothesis would indicate that processes of prion formation and elimination preferentially occur in certain physiological state(s), a notion that is worth investigating further.

At this moment, it is not known whether the Ub system specifically influences only [PSI+] or exhibits similar effects on the other prions. Although we have not detected any increase in de novo [PIN+] formation in the ubc4Δ background, one should note that we lacked an unbiased genetic system for [PIN+] detection, and therefore all we can say is that [PIN+] is not being generated simultaneously with [PSI+]. While further experiments are needed to accurately address this question, our data have already provided one important insight into a role of the [PIN+] maintenance protein, Rnq1, in [PSI+] formation. Previously, it was shown that prion form of Rnq1 promotes de novo [PSI+] formation (18, 20). However, we have found that the presence of Rnq1 protein per se, even in its non-prion form, facilitates spontaneous [PSI+] formation at least in the ubc4Δ cells (Fig. 2B). This suggests that Rnq1 protein, independently

5 R. Wegrzyn, L. Ozolins, and Y. Chernoff, unpublished data.
of its aggregation, is playing an important role in the process of generation of other prions. Rnq1 was detected among ubiquitinated proteins in the large scale assays (50). It is therefore possible that some UPS effects on prions, e.g. increased spontaneous [PSI*] formation in ubc4Δ, could be at least in part due to variations in Rnq1 ubiquitination and, consequently, its proteolytic stability. This possibility is currently under investigation.

Taken together, our results present strong evidence for the ability of the UPS to regulate de novo prion formation and prion resistance to curing treatments in the yeast model. As the efficiency of ubiquitination and of proteasomal degradation may be influenced by various environmental and physiological conditions, this provides a potential new mechanism by which the cell may modulate protein assembly disorders and protein-based inheritance.

Acknowledgments—We thank N. Romanova for the help with the fluorescence microscopy assay, K. Gokhale and G. Newnam for help in some experiments; and D. Bedwell, E. A. Craig, D. Cyr, M. Ellison, M. Hochstrasser, and S. Lindquist for antibodies and plasmids.

REFERENCES

1. Prusiner, S. B. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 13363–13383
2. Mayer, R. J. (2003) Drug News Perspect. 16, 103–108
3. Soto, C., Estrada, L., and Castilla, J. (2006) Trends Biochem. Sci. 31, 150–155
4. Hershko, A., and Ciechanover, A. (1998) Annu. Rev. Biochem. 67, 425–479
5. Wilkinson, K. D. (2000) Semin. Cell Dev. Biol. 11, 141–148
6. Glickman, M. H., and Ciechanover, A. (2002) Physiol. Rev. 82, 373–428
7. Bence, N. F., Sampat, R. M., and Kopito, R. R. (2001) Science 292, 1552–1555
8. Patino, M. M., Liu, J. J., Glover, J. R., and Lindquist, S. (1996) Mol. Cell. Biol. 16, 4773–4781