Normal levels of the antiprion proteins Btn2 and Cur1 cure most newly formed [URE3] prion variants

Reed B. Wickner¹, Evgeny Bezsonov, and David A. Bateman

Laboratory of Biochemistry and Genetics, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892-0830

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[URE3] is an amyloid prion of the *Saccharomyces cerevisiae* Ure2p, a regulator of nitrogen catabolism. Overproduction of Btn2p, involved in late endosome to Golgi protein transport, or its paralog Cur1p, cures [URE3]. Btn2p, in curing, is colocalized with Ure2p in a single locus, suggesting sequestration of Ure2p amyloid filaments. We find that most [URE3] variants generated in a *btn2 cur1* double mutant are cured by restoring normal levels of Btn2p and Cur1p, with both proteins needed for efficient curing. The [URE3] variants cured by normal levels of Btn2p and Cur1p all have low seed number, again suggesting a seed sequestration mechanism. Hsp42 overproduction also cures [URE3], and Hsp42p aids Btn2 overproduction curing. Cur1p is needed for Hsp42 overproduction curing of [URE3], but neither Btn2p nor Cur1p is needed for overproduction curing by the other. Although *hsp42Δ* strains stably propagate [URE3-1], *hsp26Δ* destabilizes this prion. Thus, Btn2p and Cur1p are antiprion system components at their normal levels, acting with Hsp42. Btn2p is related in sequence to human Hook proteins, involved in aggresome formation and other transport activities.

Bmh1 | Sis1 | prion seed sequestration

The yeast prion [URE3] is a self-propagating amyloid of Ure2p (1–4). Ure2p normally functions as a soluble regulator of nitrogen catabolism (5, 6), and its conversion to the aggregated amyloid prion form produces inappropriate derepression of many genes of nitrogen catabolism, resulting in slightly slowed growth (7). Many [URE3] isolates have a severe toxic effect, producing extremely slowed growth (8). A single protein sequence can assume any of many different heritable/infectious forms with different biological properties and, one infers, different protein conformations. The parallel in-register β -sheet architecture of the [URE3] prion amyloid (9) can explain the templating properties of this prion (10) and the ability of the Ure2p amyloid to act as a gene with multiple alleles (4).

[PSI⁺] is similarly an amyloid prion of Sup35p, a subunit of the translation termination factor [reviewed by Liebman and Chernoff (11)], and [PIN⁺] is a prion of Rnq1p, a protein of unknown function (12–14).

Eukaryotic cells deal with protein aggregates in several ways, including resolubilization, degradation, sequestration, and combinations of these processes. Several organelles and systems have been recognized to play a role in these processes, including vacuoles (the yeast equivalent of the mammalian lysosomes), the ubiquitin-proteasome systems, the autophagy system, and the various chaperones.

In mammalian cells the aggresome is a centrosomal structure to which aggregates are brought in a microtubule-dependent process (15). A yeast site near the spindle pole body (the yeast centrosome) collects huntingtin/polyQ aggregates in a process that depends on microtubule function, suggesting that this is the yeast aggresome (16). Bmh1p, a 14-3-3 protein, was found associated with huntingtin in yeast aggresomes, and *bmh1Δ* prevented aggresome accumulation of huntingtin (16).

Btn2p and Cur1p were found to cure the [URE3] prion when overproduced (17). These proteins are paralogs, members of the Hook family, consistent with their similar effects, but Btn2 localized to a site next to the nucleus, whereas Cur1 was localized

largely within the nucleus (17). During the curing process, those [URE3] cells having both Ure2p-GFP aggregates and Btn2-RFP dots show striking colocalization (17–19). Partial colocalization of Btn2-RFP with Sup35NM-GFP in a [PSI⁺] strain or with the huntingtin-like Q103-GFP were also observed (17), but overexpression of Btn2p or Cur1p did not cure [PSI⁺]. Doubly deleted *btn2Δ cur1Δ* strains show an elevated seed number of [URE3] and partial resistance to prion curing by dimethyl sulfoxide, overexpression of *Ssa1p*, overexpression of Hsp104, and other agents (17). These results suggested that Btn2p and Cur1p expressed at their normal levels affect [URE3] propagation. It was suggested that at least Btn2p collects Ure2p amyloid aggregates at the Btn2 site near the nucleus, thereby decreasing the operative seed number and destabilizing the prion (17).

Another study described two morphological sites of accumulation of nonspecific aggregates, one near the nucleus (JUNQ) and the other peripheral to the nucleus (IPOD), neither localized at the spindle pole body (20). Ubiquitinated proteins accumulated at the juxtanuclear site, whereas aggregates of overproduced “amyloidogenic” prion proteins Ure2p and Rnq1p, in strains not carrying prions, were reported to be moved to the peripheral site (20). Prion formation by Ure2p is induced 20- to 200-fold by overproduction of the prion protein (2), but still only a relatively small minority of cells become [URE3], although most cells may have aggregates of Ure2p while it is overproduced. The same is true of induction of [PSI⁺], a prion of Sup35p (2, 21, 22) and of [PIN⁺], a prion of Rnq1p (14). Thus, aggregates of overproduced Ure2p or Rnq1p in cells not carrying the corresponding prion will be largely nonprion aggregates, and there is no reason to assume that they are amyloid, even though those proteins are indeed “amyloidogenic.” The formation of both

Significance

The [URE3] prion (infectious protein) is an amyloid (filamentous polymer) of Ure2p. Overproduction of Btn2p or Cur1p cure this prion, and Btn2p colocalizes with Ure2p aggregates in the curing process. We show that most [URE3] variants arising in the absence of Btn2p and Cur1p are cured by restoring the normal levels of these two proteins. The variants cured by normal levels of Btn2p and Cur1p are those with low seed number, a low number of heritable particles, consistent with seed aggregation, and sequestration as the curing mechanism. These Btn2p and Cur1p antiprion proteins are members of the Hook protein family, metazoan members of which have roles in microtubule-dependent movement of organelles and aggregates in the cell.

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¹To whom correspondence should be addressed. E-mail: wickner@helix.nih.gov.

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IPOD and JUNQ foci were inhibited by benomyl (20), a drug that depolymerizes microtubules.

Specht et al. (23) found that Hsp42 was necessary for aggregate accumulation at the peripheral site, but not at the juxtanuclear site. They found that Hsp42 did not affect the localization of “amyloidogenic” aggregates of Rnq1p, but again, the cells were not carrying the corresponding prion, [PIN⁺], so these aggregates were likely nonamyloid. Specht et al. found that although benomyl inhibited formation of both peripheral and juxtanuclear aggregate foci, a benomyl-resistant tubulin2 mutant showed the same effect. In contrast, LatrunculinA, which depolymerizes the actin cytoskeleton, inhibited accumulation of aggregates at both sites, but a mutation in actin-1 that prevents LatrunculinA action prevented this action of the drug (23). Thus, the actin cytoskeleton is involved in IPOD and JUNQ focus formation, distinguishing both from the aggresome. Overproduction of either Hsp26 or Hsp42 were reported to cure the [PSI⁺] prion (24), but *hsp26Δ* did not affect IPOD or JUNQ aggregate focus formation (23). Hsp42p associates with Btn2p in vivo (25).

The prion-curing ability of overproduced Btn2p and Cur1p was confirmed by Malinowska et al. using an artificial prion (25). As in other studies cited above, this group overproduced the RFP-labeled prion domains of Ure2, Sup35, Rnq1, or Nrp1 in a strain carrying none of the corresponding prions. There was no significant colocalization with Btn2-GFP, from which it was inferred that Btn2p was not directly associating with prion amyloid in the curing process (25). As discussed above, only a tiny minority of cells overexpressing these prion domains form prions, although most cells show aggregates of the overproduced protein, so this inference is not justified. Indeed, Btn2p does colocalize with amyloid aggregates of Ure2p in a [URE3] strain early in the curing process (17–19). Btn2p is also partially colocalized with amyloid aggregates of Sup35p in a [PSI⁺] strain (17). Btn2p also colocalizes with nonamyloid aggregates of optineurin, PrP, Rnq1p, and Q103P (19). Malinowska et al. further showed that Btn2p and Cur1p each interact physically with the Hsp40 family member Sis1p, and that both Btn2p and Cur1p are drawn into the nucleus as a result of this association (25). It was suggested that overproduced Btn2p or Cur1p cure prions by sequestering the Sis1p that is needed for prion propagation (25).

Here we show that normal levels of Btn2p and Cur1p can cure most [URE3] variants despite being 20–400 times less abundant in the cell than Sis1p. [URE3] variants cured by normal levels of Btn2 and Cur1 are much lower in copy number than those not cured under this condition, suggesting that sequestration of seeds is the mechanism of prion curing. Btn2p and Cur1p are thus elements of normal prion-curing systems, suggesting that yeast does not welcome [URE3] but has systems in place to rapidly eliminate it. We find that Hsp42 is important for curing by overproduction of Btn2p or Cur1p and that overproduction of Hsp42 itself cures [URE3] in a process requiring Cur1p, but not Btn2p.

Results

Most [URE3] Variants Are Cured by Normal Levels of Btn2p and Cur1p.

Formation of the [URE3] prion inactivates the nitrogen regulation function of Ure2p, resulting in the derepression of *DAL5* (among many genes). We used strains having the Ure2p-regulated *DAL5* promoter linked to the *ADE2* gene (4, 26), so that [URE3] cells become Ade⁺, but cells lacking the prion are Ade[−]. To determine whether normal levels of Btn2p or Cur1p can cure [URE3] variants, we induced prion formation in a *btn2Δ cur1Δ* strain by overproduction of the Ure2p prion domain (27), selecting Ade⁺ clones. As a simple screening method, we restored Btn2p and Cur1p by mating isolates with the *BTN2 CUR1* [ure-o] strain 4592. We found that 38 of 48 Ade⁺ isolates produced only Ade[−] diploids. All 48 haploid candidates became uniformly Ade[−] when grown to single colonies on 4 mM guanidine hydrochloride, indicating that they carried a [URE3] prion.

To determine whether the loss of the Ade⁺ phenotype in the *btn2Δ/+ cur1Δ/+* diploids was due to restoration of Btn2 and Cur1, we mated another series of Ade⁺ haploid isolates with the *btn2Δ cur1Δ* [ure-o] strain 5197 carrying either the vector or p1482, a plasmid that expresses Btn2p and Cur1p at their normal levels (Fig. 1A). Diploids with Btn2p and Cur1p restored were cured of [URE3] in 44 of 48 cases, but not in the diploids with the empty vector that remained *btn2Δ cur1Δ* (Fig. 1A). In addition, those haploid isolates (six tested) that became Ade[−] on mating with a wild-type strain each also became Ade[−] on transformation with p1482 (*BTN2* and *CUR1* expressed at normal levels) (Fig. 1B). This plasmid (p1482) destabilized neither the [URE3] of an isolate that produced Ade⁺ diploids nor [URE3-1] in the same background. We denote these frequently arising [URE3] variants as [URE3-bcs], for Btn2-Cur1-hypersensitive.

To distinguish the roles of Btn2 and Cur1 in eliminating [URE3-bcs], we transformed several such isolates with pRS316 (*CEN* vector), p1478 (pRS316-*CUR1*), p1479 (pRS316-*BTN2*), or p1482 (pRS316-*BTN2-CUR1*), single-copy plasmids with the genes controlled by their native promoters. Each gene increased the loss of each of several [URE3-bcs] variants examined, but both were needed for maximal curing (Table 1). As is evident

Fig. 1. Normal expression of Btn2p and Cur1p cures Btn2-Cur1 hypersensitive [URE3]s ([URE3-bcs]). (A) Strain 5190 (*btn2Δ cur1Δ pGAL:URE2* prion domain) was grown for 2 d in 2% (wt/vol) raffinose, 2% (wt/vol) galactose minimal medium and plated for Ade⁺ ([URE3]) cells. Candidates were picked to an array on −Ade medium, grown for 1 d, replica-plated to YPAD, and mated for 1 d with strain 5197 (also *btn2Δ cur1Δ*) carrying either the vector (pRS316) or *pBTN2-CUR1* (p1482) and replica-plated to minimal medium with and without adenine. Diploids formed with 5197 carrying the vector are still *btn2Δ cur1Δ*, grow on −Ade, and are pink, owing to a weak phenotype and frequent loss of the prion. Diploids formed with 5197 carrying *pBTN2-CUR1* are Ade[−] owing to curing of the [URE3-bcs] prion. Four isolates are not efficiently cured by mating with 5197 carrying *pBTN2-CUR1*. (B) Strain dk133 (*btn2Δ cur1Δ*) carrying [URE3-1] (28) or [URE3-bcs106] was transformed with the single-copy vector pRS316 or the same plasmid carrying *BTN2* and *CUR1* controlled by their own promoters. Transformants were selected in the presence of adenine and replica plated to plates without adenine. Growth of Btn2-Cur1 insensitive papillae derived from [URE3-bcs106] can be seen.

Table 1. Btn2p and Cur1p both needed (at normal levels) for maximal elimination of [URE3-bcs]

[URE3] variant	Transformant clones												Phenotype of subclones from Ade ⁻ clones that lost pBTN2-CUR1	
	vector			pBTN2			pCUR1			pBTN2-CUR1			Ade ⁺	Ade ⁻
	% Ade ⁺ / sectored / Ade ⁻													
[URE3-1]	100	0	0	100	0	0	100	0	0	100	0	0		N.A.
[URE3-bcs106]	92	8	0	27	48	25	0	65	35	0	3	97	0	63
[URE3-bcs60]	80	20	0	58	33	9	48	40	12	40	42	18	0	39
[URE3-bcs66]	25	75	0	12	73	15	13	68	19	13	15	72	0	96
[URE3-bcs121]	43	52	5	34	60	16	26	52	22	0	68	32		

Strain dk133 (*btn2Δ cur1Δ*) carrying the indicated [URE3-bcs] or [URE3-1] (28) was transformed with the *CEN* vector pRS316, or the vector carrying *BTN2* and/or *CUR1* under their native promoters (p1478, p1479, or p1482). Transformants were selected in the presence of adenine and replica plated to a plate lacking adenine. More than 100 transformant clones were examined in each case. Results are expressed as percent Ade⁺/sectored/ Ade⁻. From each of four Ade⁻ transformants with p1482 (*pBTN2-CUR1*) of cells carrying [URE3-bcs106], [URE3-bcs60], and [URE3-bcs66], Ura⁻ subclones that had lost p1482 were tested for adenine auxotrophy by replica-plating. The results, summed and shown in the rightmost column, show that [URE3-bcs] had been cured by *pBTN2-CUR1*. N.A., not applicable.

from Table 1 for the four [URE3-bcs] isolates transformed with the vector, all of the more than 50 [URE3-bcs] variants examined were quite unstable, compared with either [URE3-1] (28) or non-bcs [URE3] variants isolated in the same experiment. The inverse correlation of seed number and instability well known from previous work (29–31) suggests that these Btn2/Cur1 hypersensitive [URE3] variants are characterized by low seed number. This question is further addressed below.

Does restoring Btn2p and Cur1p cure [URE3-bcs] or simply suppress the Ade⁻ phenotype? The presence of frequent sectoring on introduction of *BTN2*, *CUR1* or both (Table 1) suggests that curing is occurring. Loss of *pBTN2-CUR1* (p1482) from Ade⁻ transformants of several [URE3-bcs] isolates did not restore the Ade⁺ phenotype, showing that curing had occurred (Table 1). Further, sporulation of Ade⁻ diploids formed by mass mating each of three [URE3-bcs] isolates with wild-type strain 4592 produced exclusively 4 Ade⁻: 0 segregation. In particular, all of the *btn2Δ cur1Δ* segregants (10 or more in each cross) were Ade⁻, implying that the [URE3] prion was gone and not simply phenotypically suppressed by Btn2p and Cur1p.

Btn2/Cur1-Hypersensitive [URE3] Variants Have Low Seed Number.

The increase in [URE3] seed number in *btn2Δ cur1Δ* strains (17), and the colocalization of Btn2p and Ure2p in [URE3] strains undergoing curing by overproduced Btn2p (17–19), along with our observation above that [URE3-bcs] variants are all unstable, suggest that [URE3] variants with particularly low seed number might be those that are hypersensitive to Btn2p and Cur1p at their normal levels. Guanidine is a specific inhibitor of Hsp104 (32–34) in its filament-cleaving activity (35), and Cox et al. (29) have used these facts to develop methods of measuring prion seeds. As a prion-containing cell grows on medium containing guanidine, new seeds are not generated, but seeds already present segregate until there is only one seed per cell. The number of prion-containing cells in the colony is presumed to equal the number of seeds originally present in the founder cell of the clone. Plating the entire colony on media selective for the prion (–Ade plates) without guanidine gives a measure of the seed number (29). Using this method, we found that each [URE3-bcs] isolate had seed numbers significantly lower than that of [URE3-1] (Table 2). Different experiments gave significantly different seed numbers for [URE3-1], a variability similar to that seen by Cox et al. for [PSI⁺] (29), but perhaps owing to the slightly different guanidine concentrations used. Data within a single experiment

show clear differences, with all bcs variants having consistently lower seed numbers.

A second method for measuring seed number is to grow a culture in the presence of guanidine, measure the kinetics of loss of the prion, and use those data to calculate seed number (29). Again, this method showed that [URE3-bcs] isolates had significantly lower seed numbers than a [URE3] that is maintained in the presence of normal levels of Btn2 and Cur1 (Fig. 2).

Mutation and Segregation of [URE3-bcs] Isolates. Subclones of [URE3-bcs] isolates were analyzed by mating with wild-type strain (4592) or by mating with strain 5197 carrying p1482 (*CEN URA3 BTN2 CUR1*). Although many subclones retained the sensitivity to normal levels of Btn2p and Cur1p, others became completely insensitive to curing by these proteins, and a few produced no diploids (with or without adenine present) (Table S1). Further subcloning of each type of variant showed that each

Table 2. Seed number of [URE3] variants

Prion variant	Seed number
Experiment 1	
[URE3-1]	708 ± 338
[URE3-bcs6]	323 ± 93
[URE3-bcs2]	260 ± 147
[URE3-bcs3]	306 ± 66
[URE3-bcs4]	70 ± 23
Experiment 2	
[URE3-1]	274 ± 138
[URE3-bcs101]	49 ± 9
[URE3-bcs102]	97 ± 87
[URE3-bcs103]	101 ± 24
[URE3-bcs104]	136 ± 96
[URE3-bcs105]	63 ± 12
[URE3-bcs106]	82 ± 14
[URE3-bcs107]	48 ± 8
[URE3-bcs108]	40 ± 7
[URE3-bcs109]	60 ± 20
[URE3-bcs110]	92 ± 37

Strains were streaked for single colonies on YPAD containing 4 mM guanidine HCl (experiment 1) or 5 mM guanidine HCl (experiment 2). Single colonies were suspended in water and plated on –Ade plates. Ade⁺ colonies are a relative measure of the number of prion seeds in the cell founding the colony (29). Seed number ± SD is shown.

Fig. 2. Btn2-Cur1 hypersensitive [URE3] variants have significantly fewer seeds than an insensitive [URE3] variant arising in the same *btn2Δ cur1Δ* background. Btn2-Cur1 insensitive [URE3] strain 5W (black and white squares) and Btn2-Cur1 hypersensitive [URE3] variants 124, 116, and Dr2 were selected from $-Ade$ plates and added to either YPD or YPD containing 3.3 mM guanidine hydrochloride at 1×10^4 cells per mL initial density. Aliquots were removed at time 0 and every hour for 24 h, plating 100 cells per 1/2 YPD plate. After 6 d growth at 30 °C, plates were photographed and colonies were counted using OpenCFU Version 3.8.11, selecting cured red colonies with color filter: hue 16 plus and minus 4 units. Total colonies on the plates and volumes added each hour to the 1/2 YPD plates from the master stock were used to calculate generation number. The values shown are corrected for spontaneous prion loss at each time point by the formula: Fraction [URE3] shown = Fraction [URE3] in guanidine/Fraction [URE3] without guanidine. Uncorrected data are shown in Fig. S1. The shift to the left shows that the [URE3-bcs] isolates have lower seed numbers than the variant 5W not cured by restoring normal levels of Btn2p and Cur1p.

could give rise to the other types at some frequency. Subclones that became insensitive to curing often remained insensitive. Thus, in addition to frequent loss of [URE3-bcs], the prion can change (mutate) to different variants, and these can segregate. We suggest that continued selection for prion maintenance selects for a more stable variant arising in the population, such variants are stable because they have higher seed number, and higher seed number makes them insensitive to curing by normal levels of Btn2p and Cur1p. Specifically variant 5W, whose seed number is shown in Fig. 2 to be higher than several bcs variants, was a Btn2/Cur1-insensitive variant derived from a hypersensitive variant (Table S1).

Spontaneous Prion Generation Elevated in *btn2Δ cur1Δ* Strains. If Btn2p and Cur1p are eliminating many [URE3] variants as they arise, the frequency of spontaneous [URE3] should be higher in a doubly deleted strain than in an isogenic wild type. Indeed we found that strain dk133 (*btn2Δ cur1Δ*) carrying the vector pRS316 produced 106 ± 15 [URE3] clones per 10^6 cells plated, whereas the same strain carrying p1482 (a *CEN* plasmid expressing both *BTN2* and *CUR1*, each from their native promoter) produced 19 ± 6 [URE3] clones per 10^6 cells.

Curing by Overproduction of Btn2p (Cur1p) Does Not Require Cur1p (Btn2p). Overexpression of Btn2p in a *cur1Δ* strain gave similar curing efficiency as in a wild-type host. Similarly, overexpression of Cur1p in a *btn2Δ* strain cured [URE3-1] as well as in the wild type (Fig. 3). This indicates that neither protein depends on the other for curing. It is possible that Btn2p and Cur1p participate in completely different systems, but their sequence similarity suggests that they may be alternative components of the same antiprion apparatus.

Hsp42 Overproduction Cures [URE3-1], Requires Cur1p. Hsp42 is known to associate with some aggregates (23), to interact with Btn2p (25), and is reported to cure [PSI⁺] when overproduced (24). We found that overexpression of Hsp42 cures [URE3-1] and that this curing is essentially eliminated in a *btn2Δ cur1Δ* strain (Fig. 4A). Comparing Hsp42 overproduction curing in single *btn2Δ* and *cur1Δ* mutants showed that Btn2p is dispensable for Hsp42 overproduction curing but that Cur1p is necessary (Fig. 4B).

The same Hsp42 overproduction plasmids that efficiently cured [URE3-1] did not detectably cure [PSI⁺] from strain 779-6A (Fig. 4C). We also found that similar overproduction of Hsp26 did not cure either [URE3-1] (not shown) or [PSI⁺] (Fig. 4C). It is possible that our results differ from those of Duennwald et al. (24) because of prion or host strain differences.

Although *hsp42Δ* did not prevent [URE3-1] propagation, *hsp26Δ* destabilized this prion. Two *hsp26Δ* isolates from strain BY241 were generated, and [URE3-1] was introduced by cytoduction from strain 1735 into the parent and both *hsp26Δ* isolates. Wild-type cytoductants were *Ade*⁺ and white on 1/2 YPD, whereas all *hsp26Δ* cytoductants were pink and weakly *Ade*⁺. Wild-type and *hsp26Δ* cytoductants were then used as donors to the [ure-o] strain 4591. All cytoductants from the wild type carried [URE3-1], but most of those from the *hsp26Δ* cytoductants did not (Table 3). Thus, *hsp26Δ* destabilizes [URE3-1].

Sis1p Overproduction Prevents Overproduction Curing by Btn2, Cur1, or Hsp42. Sis1p is an essential Hsp40 whose depletion results in rapid loss of [PIN⁺] and [URE3] and slower loss of [PSI⁺] (36). Malinowska et al. (25) found that Sis1p overproduction largely prevented Btn2 or Cur1 overproduction curing of their Nrp1-Sup35C-based prion. We found that constitutive overproduction of Sis1p blocked curing of [URE3-1] by overproduced Btn2p, Cur1p,

Fig. 3. Btn2p and Cur1p work independently to cure [URE3-1]. High-copy plasmids expressing Btn2p (p1475), Cur1p (p1488), or the vector (pRS426) were transformed into the wild-type [URE3-1] strain 4648 or the isogenic *btn2Δ*, *cur1Δ*, or *btn2Δ cur1Δ* strains. After growth to single colonies on media selective for the presence of the plasmids but containing adenine, a mixture of colonies was streaked on 1/2 YPD.

Fig. 4. Hsp42 overexpression curing of [URE3]. (A) Wild-type strain 4648 or the isogenic strain dk133 (*btn2Δ cur1Δ*), each bearing [URE3-1], were transformed with vector p1062 or p1062-HSP42 (p1505, *CEN URA3 GAL1:HSP42*), grown in galactose medium for 3 d, and plated on 1/2 YPD. (B) Cur1p, but not Btn2p, is needed for curing of [URE3-1] by overexpression of Hsp42. (C) Hsp42 and Hsp26 overproduction do not cure [PSI+] of strain 779-6A. 779-6A ([PSI+]) transformed with vectors p1062 and p896, p1505 and p1508 (*GAL1:HSP42*), and p1511 and p1510 (*GAL1:HSP26*) were grown 100-fold twice in Gal-Raf medium selecting for the plasmids. From the second growth (4 d later) aliquots were streaked for single colonies on 1/2YPD and grown for 3 d at 30 °C.

or Hsp42 (Fig. 5). Malinowska et al. showed that overproduction of Sis1p does not substantially affect total levels of Btn2p or Cur1p (25).

Curing of [URE3-1] by Overproduction of Btn2p and Cur1p Is Promoted by Hsp42, but Not Bmh1p. Isogenic wild-type, *hsp42Δ*, and *bmh1Δ* strains carrying [URE3-1] were used as hosts for curing. Overproduction of Btn2p and Cur1p efficiently cured [URE3-1] from the wild-type host, but only slightly from the *hsp42Δ* cells (Fig. 6). When Btn2p or Cur1p were overexpressed individually, curing by Cur1p was still effective in *hsp42Δ* cells, but curing by Btn2p was largely blocked (Fig. S2). In contrast, the *bmh1Δ* mutation did not affect curing by either Btn2p or Cur1p (Fig. 6 and Fig. S3), suggesting that Btn2p and Cur1p curing does not involve the yeast aggresome as defined by Wang et al. (16).

Overproduction Curing by Btn2p or Cur1p Is Independent of the E3 Ubiquitin Ligases San1p and Urb1p. Two ubiquitin E3 ligases, San1p and Urb1p, have been identified as responsible for marking misfolded cytoplasmic proteins for degradation in nuclear proteasomes (37, 38). We find that neither Btn2 nor Cur1 overproduction curing depends on San1p or Urb1p (Fig. 7). Curing also proceeds in *san1Δ urb1Δ* double mutants. This suggests that

the mechanism of curing does not involve this part of the ubiquitin-proteasome system.

[PIN+] Is Not Cured by Overproduced Btn2p or Cur1p. The [PIN+] strain 4970 was transformed with pRS425 (2micron DNA *LEU2* vector), p1486 (pRS425 *CUR1*), or p1476 (pRS425 *BTN2*). Strain 4970 cured of the prion by growth in the presence of guanidine was used as a [pin-] control. Transformants were grown selecting for the plasmid for 5 d on solid medium, mated with the [pin-] strain 5075 carrying a plasmid expressing an *RNQ1-GFP* fusion protein. Diploids were selected and examined by fluorescence microscopy. [PIN+] cells have a single bright dot of Rnq1-GFP fluorescence, whereas [pin-] cells (such as the diploids formed with the [pin-] control here) show even, bright fluorescence of the cytoplasm. Diploids formed with 4970 carrying the vector, or overexpressing Btn2p or Cur1p each showed >95% of cells with a single bright dot of Rnq1-GFP, indicating that no detectable curing of [PIN+] was occurring. The same plasmids produce >90% curing of [URE3] under similar conditions.

Discussion

We generated and isolated a large series of [URE3] variants in a *btn2Δ cur1Δ* strain and restored normal levels of both Btn2p

Table 3. *hsp26Δ* destabilizes [URE3-1]

Donor	Recipient	Cytoductants	
		Ade ⁺	Ade ⁻
BY241 w.t. 1	4591 ρ^o	37	0
5		31	0
8		31	0
BY241 <i>hsp26Δ</i> 1-1		16	40
1-1		16	66
1-5		0	55
1-6		3	112
BY241 <i>hsp26Δ</i> 2-4		22	130
2-4		5	42
2-5		12	102
2-6		19	83
2-6		11	74

[URE3-1] was transferred by cytoduction (cytoplasmic mixing) from strain 1735 into the parent and each of two *hsp26Δ* isolates. Several cytoductants of each were used as cytoduction donors to strain 4591 ([ure-*o*] ρ^o), and cytoductants scored for [URE3] by their Ade^{+/−} phenotype.

and Cur1p either by mating with a wild-type strain or by transformation with a *CEN* plasmid carrying both genes with their native promoters. We found that the majority of [URE3] variants (called Btn2-Cur1 hypersensitive, bcs) are cured by restoration of normal levels of Btn2p and Cur1p, levels that do not cure [URE3-1], the original isolate of Lacroute (28). This indicates that Btn2p and Cur1p, without being overproduced, are antiprion components. Each shows some [URE3-bcs]-curing activity without the other, but they are most effective when both are expressed. Likewise, overexpressed Btn2p and Cur1p can each cure [URE3-1] in the absence of the other. Thus, Btn2p and Cur1p do not depend on each other, but their sequence similarity suggests that they may, in some sense, do the same thing.

The fact that the cell has a prion-curing system suggests that at least the cured prions are not generally advantageous to the cell. Indeed, population studies indicate that even the mildest forms of the [URE3], [PSI⁺], and [PIN⁺] prions confer a substantial detriment on their host cells (39, 40). Furthermore, most variants of [PSI⁺] are actually lethal or nearly so, and many [URE3] variants dramatically slow growth (8).

Like the toxic or lethal variants of [PSI⁺] and [URE3] (8), and the variants of [PSI⁺] and [URE3] affecting passage through an intraspecies or interspecies barrier (41–43), the [URE3-bcs] variants expand the range of known possibilities of yeast prion characteristics. The [URE3-bcs] variants that we describe here would not be detected in a wild-type host, because they are rapidly eliminated by the combined action of Btn2p and Cur1p. Because infectious prion formation occurs readily in vitro with purified Ure2p, and a range of variants are so generated (4), it is likely that [URE3-bcs] variants arise in wild-type cells but are rapidly eliminated. Even in a *btn2Δ cur1Δ* strain, [URE3-bcs] variants are unstable, being lost frequently and also giving rise to white (strong) variants with higher seed number that are insensitive to normal levels of Btn2p and Cur1p. Even without the selection for stability evident in the case of [URE3-bcs], the [PSI⁺] prion exists as a “prion cloud,” with distinct variants arising spontaneously and segregating during mitotic growth (44). The generation of stable, relatively Btn2-Cur1-insensitive variants from [URE3-bcs] must reflect some mutability of the prion.

How do Btn2p and Cur1p cure [URE3]? All of the many [URE3-bcs] isolates examined are unstable, unlike [URE3-1] and other non-bcs variants. Prion instability suggests low seed number (29–31), and we confirm that [URE3-bcs] variants indeed do have lower seed numbers than variants such as [URE3-1] that are not cured at normal Btn2p and Cur1p levels. In a *btn2Δ*

cur1Δ strain, the copy number of [URE3-1] was higher than in an isogenic *BTN2 CUR1* strain (17), again suggesting that these proteins lower prion copy number. The finding that Btn2p colocalizes with Ure2p amyloid aggregates in [URE3] strains undergoing curing (17–19) suggests a direct mechanism involving sequestration of prion seeds as previously proposed (17). As expected for this model, normal levels of Btn2p/Cur1p are enough to sequester a small number of seeds; higher seed [URE3] variants are only cured by overproduction. Just as curing of [URE3] by Btn2p overproduction draws together nearly all of the Ure2p amyloid aggregates into a single locus coincident with Btn2p (17), overproduction of Btn2p reduces the number of optineurin aggregates, and the collected aggregates are coincident with Btn2p (19).

Malinowska et al. (25) confirmed the prion-curing ability of overproduced Btn2p or Cur1p using an artificial prion consisting of a segment of Nrp1p and the nonprion part of Sup35p. These authors noted that overproduction of Sis1p, an essential Hsp40, prevented this curing activity. They found that Sis1p interacts

Fig. 5. Overproduction of Sis1p prevents curing of [URE3-1] by overproduction of Hsp42, Btn2p, or Cur1p. (A) Wild-type [URE3-1] strain 4648 carrying pGDP1-SIS1 or the vector pRS315 and pGAL-HSP42 or the vector p1062, was grown in raffinose-galactose medium for 3 d, and cells were streaked on 1/2 YPD. (B) [URE3-1] strain 4648 carrying pGDP1-SIS1 or the vector was transformed with high copy *BTN2*, *CUR1* or the plasmid vector, transformants were again grown on medium selecting for the plasmids and streaked on 1/2 YPD.

Fig. 6. Hsp42, but not Bmh1p, is important for curing of [URE3-1] by overproduced Btn2p and Cur1p. The [URE3-1] strain 4648 or isogenic *hsp42::kanMX* or *bmh1::kanMX* derivatives were transformed with the high-copy vector pRS426 or pRS426-*BTN2* (p1475) or pRS426-*CUR1* (p1488), grown on media selecting for the plasmids for 4 d, and streaked for single colonies on 1/2 YPD. Btn2p levels are not substantially altered by *hsp42Δ* (25).

with Btn2p, and overproduction of Sis1p draws both Btn2p and Cur1p into the nucleus (25), potentially explaining the prevention of curing of the cytoplasmic prion. We find that overproduction of Sis1p prevents overproduced Btn2p, Cur1p, or Hsp42 from curing [URE3-1]. An alternate explanation is that overproduced Btn2p or Cur1p ties up a large fraction of Sis1p, impairing its ability to help prion propagation (25). However, this cannot explain the [URE3]-curing activity of Btn2p and Cur1p at their normal levels that we report here, because there is 20-fold less Btn2p (10^3 molecules per cell) and 400-fold less Cur1p (<50 molecules per cell) than there is Sis1p (YNL007C, 2×10^4 molecules per cell) as estimated by Ghaemmamghami et al. (45). Even the doubly hemizygous *btn2Δ⁺ cur1Δ⁺* diploids, which likely have lower than normal levels of Btn2p and Cur1p, lost [URE3-bcs] variants (bcs candidates mated with strain 4592). Nor can this hypothesis explain the striking colocalization of Btn2p with Ure2p in cells with [URE3] undergoing curing, observed by two different groups (17, 18).

Sis1p is necessary for [URE3] propagation (36) and may bind to the amyloid. Perhaps Btn2p does not directly bind to amyloid but does bind to Sis1p bound to amyloid, and then the Btn2p (and thus the attached amyloid) is moved to the sequestration site. This model [amyloid-Sis1p-Btn2p (possibly with Hsp42)-transporter], first suggested by Kryndushkin et al. (19), could explain all of the results without invoking Sis1p depletion. Excess Sis1p could saturate the Btn2 binding sites, competing with the amyloid-bound Sis1p for transport to the sequestration site.

Despite their sequence similarity and common prion-curing ability, Btn2p and Cur1p are different in several ways. Btn2p is localized to a site near but not in the nucleus, whereas Cur1p is located throughout the nucleus (17). Colocalization of Btn2p with Ure2p aggregates is observed during curing of [URE3] by Btn2p overproduction (17, 18), but the same phenomenon was not seen with Cur1p (17). We find that [URE3] curing by overproduction of Hsp42 requires Btn2p, but not Cur1p. Moreover, curing by overproduction of Btn2p requires Hsp42, but curing by overproduction of Cur1p does not require Hsp42.

BTN2 was first identified as a gene whose transcription was elevated in cells mutant for the *BTN1* gene, the latter a homolog of the human *CLN3* involved in Batten's disease, a juvenile lysosomal disease (46). Later surveys found Btn2p is also induced by heat shock (47). Btn2p has been reported to be localized in the cytoplasm (48) and to late endosomes (49). We found that Btn2p was consistently concentrated in one or two dots close to the nucleus (17). Btn2p mediates late endosome–Golgi protein movements (49) and affects several proteins processed by this

system (49–51). Nonetheless, the relation of the different activities of Btn2p remains unclear.

We have begun to assess the roles of the various aggregate accumulation sites and pathways in the control of prion infection. Induction of autophagy by starvation does not cure [URE3], and curing of [URE3] by overproducing Btn2p is not reduced in an *atg1Δ* strain, defective in autophagy (17). The failure of *bmh1Δ* to detectably alter the efficiency of prion curing by overproduced Btn2p, Cur1p, or Hsp42p suggests that these proteins are not acting through the yeast aggresome defined by Wang et al. (16). Ubr1p and San1p are E3 ubiquitin ligases involved in targeting misfolded cytoplasmic proteins to proteasomes in the nucleus (37, 38). The [URE3] prion is certainly a misfolded form of Ure2p that is in the cytoplasm, but it seems that Btn2p and Cur1p are not working through these ubiquitin ligases.

Two sites of accumulation of nonamyloid aggregates were identified, with one being perinuclear (JUNQ) and the other peripheral (IPOD) to the nucleus (20). The IPOD site is reported to accumulate nonprion aggregates of Ure2p (20), and such nonprion aggregates were found to not coincide with Btn2p (25). However, prion aggregates of Ure2p do associate with Btn2p (17–19). Thus, Btn2p (or associated proteins) recognizes the prion form of Ure2p as distinct from nonprion aggregates of the same protein. Only accumulation of aggregates at the peripheral site requires Hsp42p, and Hsp42p is found to localize at that site (23). We find that Hsp42 is needed for Btn2p overproduction curing, raising the possibility that Btn2p may bring prion aggregates to the peripheral site. Moreover, Btn2p is not restricted to binding prion aggregates: it has been shown by others to bind to a variety of nonamyloid aggregates as well (19, 25).

Btn2p is a member of the “Hook” family (49), whose founding member is a *Drosophila* gene involved in endocytosis (52). Cur1p does not show homology to metazoan Hook proteins but is similar to Btn2p (17). The *Caenorhabditis elegans* Hook homolog, *zyg-12*, links centrosomes to the nucleus through the microtubules (53). The human Hook2 protein has a role in aggresome formation (54), very much like what we have suggested for Btn2p and Cur1p. Btn2p (410 residues) is much closer to Cur1p (252

Fig. 7. Curing of [URE3-1] by overexpression of Hsp42, Cur1p, or Btn2p is not affected by *san1Δ* or *urb1Δ*. (A) Wild-type [URE3-1] strain 4648 or isogenic *san1Δ* or *urb1Δ* strains were transformed with a plasmid carrying GAL-HSP42 (p1505) or the empty vector. Transformants were grown for 3 d on raffinose-galactose medium and then streaked for single colonies on 1/2 YPD. (B) Wild-type [URE3-1] strain 4648 or isogenic *san1Δ* or *urb1Δ* strains were transformed with high-copy (hc) plasmids overexpressing Btn2p (p1475) or Cur1p (p1488) or the empty vector. A mixture of transformant clones was regrown on medium selecting for plasmid-carrying cells, and then streaked on 1/2 YPD.

Table 4. Strains of *S. cerevisiae*

Strain	Genotype	Reference
BY241	<i>MATa leu2 trp1 ura3 P_{DAL5}:ADE2 P_{DAL5}:CAN1 kar1 [ure-o]</i>	(4)
4648	BY241 [URE3-1]	(4)
4592	<i>MATα leu2 his3 ura3 P_{DAL5}:ADE2 P_{DAL5}:CAN1 kar1 [ure-o]</i>	(4)
1735	<i>MATα ura2-60 his- [URE3-1] = MA116-8A</i>	(1)
4591	<i>MATα his3 leu2 trp1 P_{DAL5}:ADE2 P_{DAL5}:CAN1 kar1 [ure-o]</i>	(4)
dk133	BY241 <i>btn2::TRP1 cur1::kanMX [ure-o]</i>	(17)
5190	dk133 p1330	This work
dk122	BY241 <i>btn2::kanMX</i>	(17)
dk124	pBY241 <i>cur1::kanMX</i>	(17)
5197	<i>MATα leu2 ura3 trp1 his3 btn2::TRP1 cur1::kanMX P_{DAL5}:ADE2 kar1</i>	Segregants of dk133 x 4592
5200	BY241 <i>bmh1::kanMX [URE3-1]</i>	This work
5194	BY241 <i>hsp42::kanMX [URE3-1]</i>	This work
5225	BY241 <i>ubr1::kanMX [URE3-1]</i>	This work
5223	BY241 <i>san1::kanMX [URE3-1]</i>	This work
4970	<i>MATa kar1 leu2 ura3 his4 ade1-14 ho::kanMX [PIN+]</i>	This work
5075	<i>MATα lys2 leu2 ura3 trp1 rnr1::kanMX p1332</i>	(40)

residues) (32% identity in a 171-residue region of similarity, $e = 10^{-23}$) than it is to its closest human homolog, Hook1 (32% identity in an 88-residue region, $e = 10^{-3}$) (Fig. S4), a protein believed to tether cargo proteins to microtubules in the endocytosis process (e.g., refs. 55 and 56). The role shown for Btn2p in endosomal sorting (49) suggests that their functions may be similar in this respect. However, although these sequence similarities are statistically significant and indicate an evolutionary relationship, they are not close enough to ensure functional similarity.

The Btn2/Cur1 pathway(s) that eliminates prions is not the first antiprion system found in yeast. Chernoff described the antiprion effects of Ssb proteins, a group of ribosome-associated Hsp70 chaperones. In *ssb1Δ ssb2Δ* strains, the frequency of [PSI⁺] arising was increased more than 10-fold, indicating that the normal levels of these proteins were blocking prion formation by Sup35p (57). Chernoff made the apt analogy of the Ssb proteins to DNA repair systems that have an antimutator effect. Overproduction of Ssb proteins can cure some [PSI⁺] variants (58) and increased the curing efficiency of overproduced Hsp104 (57). [PSI⁺] variants arising in *ssb* deletion strains are not cured by replacement of the normal genes (57).

The existence of cellular antiprion systems active without artificial gene overexpression adds to the growing lines of evidence

that at least the well-studied [URE3] and [PSI⁺] prions, and probably [PIN⁺], are pathogens (reviewed in ref. 59). Although the deletion or overexpression of any of several genes cures yeast prions, the Btn2/Cur1 system may be unique in curing prions at their normal levels.

Methods

Strains and Media. Strains are listed in Table 4. Knockout mutants were produced by PCR-amplifying the knocked-out gene from the collection strain (60), transformation of log phase BY241 cells selecting for resistance to G418, and confirmation by PCR that the normal gene was missing and the putative mutant had been formed. Media were as described by Sherman (61). Gal-Raf medium contained 2% (wt/vol) galactose, 2% (wt/vol) raffinose, 0.7% Yeast Nitrogen Base without amino acids (Difco), and required amino acids or bases.

Plasmids. Plasmids used are listed in Table 5. *BTN2*, with 500 bp upstream of the ORF, was amplified using primers 667 and 668 (Table S2), cut with XhoI and BamHI, and inserted into pRS316 (*CEN URA3*) cut with the same enzymes forming p1479. *CUR1*, with 500 bp upstream of its ORF, was amplified using primers 669 and 670 (Table S2), with XhoI and BamHI and inserted into pRS316 cut with the same enzymes forming p1478. Primers 682 and 683 (Table S2) were used to amplify *CUR1*, with 500 bp upstream of its ORF, cut with BamHI and SacI and ligated to p1479 cut with the same

Table 5. Plasmids

Name	Genes	Source
pRS316	<i>CEN URA3</i>	(62)
pRS426	2 μ m DNA <i>URA3</i>	(63)
p1479	pRS316 <i>P_{BTN2}:BTN2</i>	This work
p1478	pRS316 <i>P_{CUR1}:CUR1</i>	This work
p1482	pRS316 <i>P_{CUR1}:CUR1 P_{BTN2}:BTN2</i>	This work
p1475	pRS426 <i>P_{ADH1}:BTN2</i>	(17)
p1488	pRS426 <i>P_{CUR1}:CUR1</i>	This work
pH382 = p1330	<i>CEN LEU2 P_{GAL1}:URE2(amino acids1–65)</i>	H. K. Edskes, NIDDK, NIH, Bethesda, MD
p1332	<i>CEN URA3 RNQ1-GFP</i>	(39)
p1062 = pH392	pRS316 <i>P_{GAL1}</i>	H. K. Edskes
p1062- <i>HSP42</i> = p1505	<i>CEN URA3 P_{GAL1}:HSP42</i>	This work
p1062- <i>HSP26</i>	<i>CEN URA3 P_{GAL1}:HSP26</i>	This work
p896 = pH316	<i>CEN LEU2 P_{GAL1}</i>	H. K. Edskes
p896- <i>HSP42</i>	<i>CEN URA3 P_{GAL1}:HSP42</i>	This work
p896- <i>HSP26</i>	<i>CEN URA3 P_{GAL1}:HSP26</i>	This work
pMR294	pRS315 <i>P_{GDP1}:SIS1 CEN LEU2</i>	Michael Reidy, NIDDK, NIH, Bethesda, MD

enzymes, forming p1482. Similar methods were used for constructing p1488. For both *BTN2* and *CUR1*, the 500 bp upstream of the ORF includes all of the intergenic region, suggesting that the native promoter of each gene is included.

The *HSP42* ORF was amplified with oligos 694 and 695 (Table S2), digested with BamHI and HindIII, and inserted into the *GAL1* promoter vectors p896 and p1062 digested with the same enzymes forming p1508 and p1505, respectively. Such constructs are denoted *GAL1:HSP42*, for example. The *HSP26* ORF was similarly inserted under control of the *GAL1* promoter of these vectors forming p1510 and p1511, respectively.

Measuring Prion Seed Number. Following Cox et al. (29), we used two methods to assess prion seed number. Freshly isolated [URE3] cells growing on –Ade plates were streaked for single colonies on 1/2 YPD with 4 mM or 5 mM guanidine HCl, which is presumed to stop prion seed generation.

Single colonies were picked up in their entirety, suspended in water and plated on a –Ade plate. After 5 d the number of Ade⁺ colonies appearing was counted and assumed to represent the seed number of the cell founding that colony. A sample of Ade⁺ colonies arising from the replated cells were checked for guanidine curability. Five to ten clones from each prion variant were assayed in this manner. The details of the population test are given in the legend of Fig. 2.

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