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Molecular Basis of a Yeast Prion Species Barrier

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Summary

The yeast [PSI$^+$] factor is inherited by a prion mechanism involving self-propagating Sup35p aggregates. We find that Sup35p prion function is conserved among distantly related yeasts. As with mammalian prions, a species barrier inhibits prion induction between Sup35p from different yeast species. This barrier is faithfully reproduced in vitro where, remarkably, ongoing polymerization of one Sup35p species does not affect conversion of another. Chimeric analysis identifies a short domain sufficient to allow foreign Sup35p to cross this barrier. These observations argue that the species barrier results from specificity in the growing aggregate, mediated by a well-defined epitope on the amyloid surface and, together with our identification of a novel yeast prion domain, show that multiple prion-based heritable states can propagate independently within one cell.

Introduction

Amyloid protein aggregates have been increasingly implicated in human diseases, including prion-based encephalopathies, noninfectious neurodegenerative diseases, and systemic amyloidoses (Koo et al., 1999). Amyloids are β sheet rich, ordered structures consisting of protofibrils (Sunde and Blake, 1997) that coalesce in vitro to form extended fibrils that bind the dye Congo red. Fibrils are also found under some conditions in vivo, although their role in pathogenesis remains unresolved (Lansbury, 1999). Despite having similar aggregated structures, sequence comparison of amyloidogenic proteins fails to reveal any obvious similarities.

A striking property of most amyloids is the ability to catalyze their own propagation. In prion diseases, this self-propagation is thought to be the basis of protein-mediated infectivity. Here, the abnormal β sheet–rich prion form (PrP^{SC}) can convert the normal cellular α-helical protein (PrP^{C}) into the prion isoform (reviewed in Prusiner et al., 1998). Even in the noninfectious amyloid diseases, such as Alzheimer's disease, amyloid self-propagation may be critical to disease progression (Lansbury, 1999).

Studies of the mammalian prion have highlighted the importance of specificity in amyloid propagation. Here, a species barrier limits PrP^{SC} derived from one species from infecting another preventing, for example, the transmission of scrapie from sheep to man (Prusiner et al., 1998). The recent description of variant Creutzfeld-Jacob disease, however, suggests that in rare instances bovine prions can cross the species barrier to infect humans. Extensive transgenic mouse and chimera analyses indicate that the species barrier is largely due to differences in sequence of the prion proteins. However, the extent to which the species barrier is mediated by direct interaction between prion particles as opposed to species-specific interactions with cellular factors is unresolved (Kocisko et al., 1995; Telling et al., 1995). Even in nonprion amyloid diseases, the ability of amyloids to incorporate other types of protein has been implicated in the disease process (Han et al., 1995), although other studies have failed to observe cross-seeding between different amyloid-forming peptides (Come et al., 1993).

The prion-like phenomenon [PSI$^+$] of *Saccharomyces cerevisiae* offers a powerful system to study the molecular basis of amyloid propagation and specificity. Identified as a non-Mendelian trait that confers suppression of nonsense mutations, [PSI$^+$] arises from conversion of the translational termination factor Sup35p from a soluble and active state into an insoluble and inactive amyloid (Wickner et al., 1995; Lindquist, 1997). The ability of Sup35p amyloids to incorporate newly made soluble proteins is thought to be the basis of [PSI$^+$] propagation. As Sup35p aggregation increases translational readthrough, the presence of the [PSI$^+$] prion can be readily monitored.

To investigate the requirements for prion formation and amyloid specificity and to examine if there is evolutionary pressure to retain prion function, we cloned and characterized Sup35p from a spectrum of Saccharomycetales (budding yeasts). Interestingly, we find that the ability to support a prion mechanism of inheritance is broadly conserved. Moreover, as with mammalian prions, a species barrier prevents cross-species prion induction. We have taken advantage of these phenomena to elucidate the requirements for prion formation and the molecular basis of this species barrier.

Results

The Prion Domain Is Conserved in Yeast Evolution

The N terminus of Sup35p is necessary and sufficient for prion formation and propagation. This prion domain (PrD) is connected to the C-terminal translation termination domain (EF) by a highly charged middle domain (M) of unknown function (Ter-Avanesyan et al., 1993). Deletion of the PrD allows Sup35p to remain soluble and functional even in [PSI$^+$] yeast, whereas transient overexpression of this domain induces conversion of [psi$^-$] yeast to [PSI$^+$] (Patino et al., 1996; Paushkin et al., 1996). Mutational analyses have begun to define the sequence requirements for prion formation and propagation (DePace et al., 1998; Liu and Lindquist, 1999).

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Although the PrD is generally tolerant to amino acid changes, several unusual features were found to be important. In particular, it has a high glutamine (Gln) and asparagine (Asn) and low charge content. In addition, the PrD has a set of imperfect oligopeptide repeats, deletion and expansion of which modulate its ability to induce the conversion to [PSI$^+$].

To determine whether these features are conserved, we cloned and characterized Sup35p PrDs from a variety of yeasts. Taking advantage of the conservation of the EF domain, we used one-sided PCR (Frohman, 1993) as well as available *SUP35* sequences (Kushnirov et al., 1990) to clone sequences upstream of this region. In total, we examined *SUP35* genes from seven non–*S. cerevisiae* budding yeast species (*Candida albicans*, *Kluyveromyces lactis* and *marxianus*, *Pichia methanolica* and *pastoris*, *Saccharomycodes ludwigii*, and *Zygosaccharomyces rouxi*) (Figure 1A).

Significantly, all of the Sup35 proteins examined have N-terminal regions similar in composition to the PrD and M domains of the *S. cerevisiae* protein (Figures 1B and 1C). Although there is little exact sequence homology, all of the PrDs have a high Gln/Asn (36% to 43%) and a low charge (2% to 10%) content. This composition resembles that of a modular prion domain from another *S. cerevisiae* prion protein, Ure2p (Edskes et al., 1999), but is very different from that of full-length proteins from the *S. cerevisiae* genome, which have on average 9% Gln/Asn and 23% charged residues (Figure 1B). Finally, the imperfect oligopeptide repeats of QGGYQQYN, although highly divergent, are clearly detectable (Figure 1C).

Foreign PrDs Aggregate, but Do Not Interact with *S. cerevisiae* Sup35p

The unusual sequence composition common to all Sup35p N-terminal domains prompted us to examine if the ability to support prion-based inheritance is also conserved. Focusing on *C. albicans*, *K. lactis*, and *P. methanolica*, we asked whether the foreign Sup35p N-terminal domains could be recruited efficiently into the *S. cerevisiae* [PSI$^+$] aggregate using both functional and visual assays. In the functional assay, foreign PrDM fused to *S. cerevisiae* EF domain, termed PrDM-EF, is ectopically expressed in the [PSI$^+$] yeast. The PrDM-EF gene is under control of the *S. cerevisiae SUP35* promoter, resulting in a moderate, constitutive level of expression. If the foreign PrDs were not incorporated in the endogenous [PSI$^+$] aggregate, soluble fusion protein would provide functional translation termination activity, thereby leading to an antisuppressed phenotype. This can be phenotypically monitored by use of yeast harboring an *ade-1* marker with a suppressible nonsense mutation (Chernoff et al., 1995). In suppressed yeast, functional Ade1p is produced, resulting in white colonies on low adenine medium and growth on adenine-free medium. By contrast, the lack of functional Ade1p in either [psi$^-$] or antisuppressed [PSI$^+$] yeast results in red colonies on low adenine and lack of growth on adenine-free media. As expected, when *S. cerevisiae* PrDM ($PrDM_{SC}$) was used in the fusion protein, [PSI$^+$] yeast retained the suppression phenotype (Figure 2A). In contrast, expression of fusion proteins containing PrDMs from *C. albicans*, *K. lactis*, and *P. methanolica*—denoted $PrDM_{CA}$, $PrDM_{KL}$, and $PrDM_{PM}$, respectively—conferred antisuppression to [PSI$^+$] cells, suggesting that they are not inactivated by the endogenous *S. cerevisiae* aggregate.

In the visual assay, the in vivo aggregation state is observed directly using an inducible PrDM fused to GFP (Patino et al., 1996). As expected, upon $PrDM_{SC}$-GFP induction, punctate foci appear rapidly in the majority of [PSI$^+$] cells (Figures 2B and 2C). In contrast, [psi$^-$] cells show a prolonged diffuse cytoplasmic fluorescence, with foci forming slowly. Expression of the fusion of the N-terminal regions of *C. albicans*, *K. lactis*, and *P. methanolica* to GFP also resulted in formation of foci. Indeed, de novo formation of foreign PrDM foci in [psi$^-$] cells, especially of $PrDM_{PM}$-GFP, is faster than that of *S. cerevisiae* PrDM. In contrast to $PrDM_{SC}$-GFP, however, the in vivo kinetics of foreign PrDM aggregation are similar in [psi$^-$] and [PSI$^+$] cells (Figure 2C). Together, these assays show that although the ability of these PrDs to aggregate is conserved, the foreign prion domains are not incorporated into the endogenous Sup35p aggregate present in [PSI$^+$] yeast.

Conversely, we asked if foreign PrD aggregates could incorporate soluble *S. cerevisiae* Sup35p present in [psi$^-$] yeast. Aggregates formed by transient overexpression of $PrDM_{SC}$, either by itself or fused to GFP, incorporate native full-length Sup35p, leading to a permanent conversion to [PSI$^+$] (Chernoff et al., 1993; Patino et al., 1996). After 24 hr of induction, ~1.5% of [psi$^-$] cells convert to [PSI$^+$] (Figure 2D). In contrast, similar levels of overexpression of foreign PrDM-GFPs, even at timepoints when foci are readily observable, fail to induce [PSI$^+$] conversion (Figure 2D and inset). Thus, the foreign PrD aggregates are unable to seed the in vivo aggregation of *S. cerevisiae* Sup35p.

Hereafter, we designate the aggregation state of the foreign PrDs by [CHI]. For example, in [psi$^-$ CHI$^+$] yeast, endogenous Sup35p is soluble and the foreign PrD is aggregated, whereas in [psi$^-$ chi$^-$] yeast, both the endogenous and foreign Sup35p are soluble.

Foreign PrDs Form Stable Prions that Are Limited by a Species Barrier

Given the long evolutionary distance separating these yeast species, the failure of the foreign PrDs to interact with *S. cerevisiae* prions is not surprising, but leaves unresolved the question of whether the foreign PrDs behave as prions. To address this question, we devised a novel genetic system that allowed us to monitor the induction and propagation of [CHI$^+$] (Figure 3A). Here, two plasmids were introduced into yeast. The first, termed the maintainer plasmid, encodes an HA epitope–tagged PrDM-EF fusion protein under control of the *S. cerevisiae SUP35* promoter. The second inducer plasmid encodes a PrDM-GFP fusion protein under control of the inducible *CUP1* promoter. Transient overexpression of the inducer protein, either from the same (homotypic) or different (heterotypic) species as the maintainer PrD, results in de novo formation of the GFP fusion aggregates, thus mimicking infection experiments used in studies of mammalian prions. The ability of the inducer aggregates to "infect" the maintainer PrD is monitored by the permanent change in suppression phenotype.

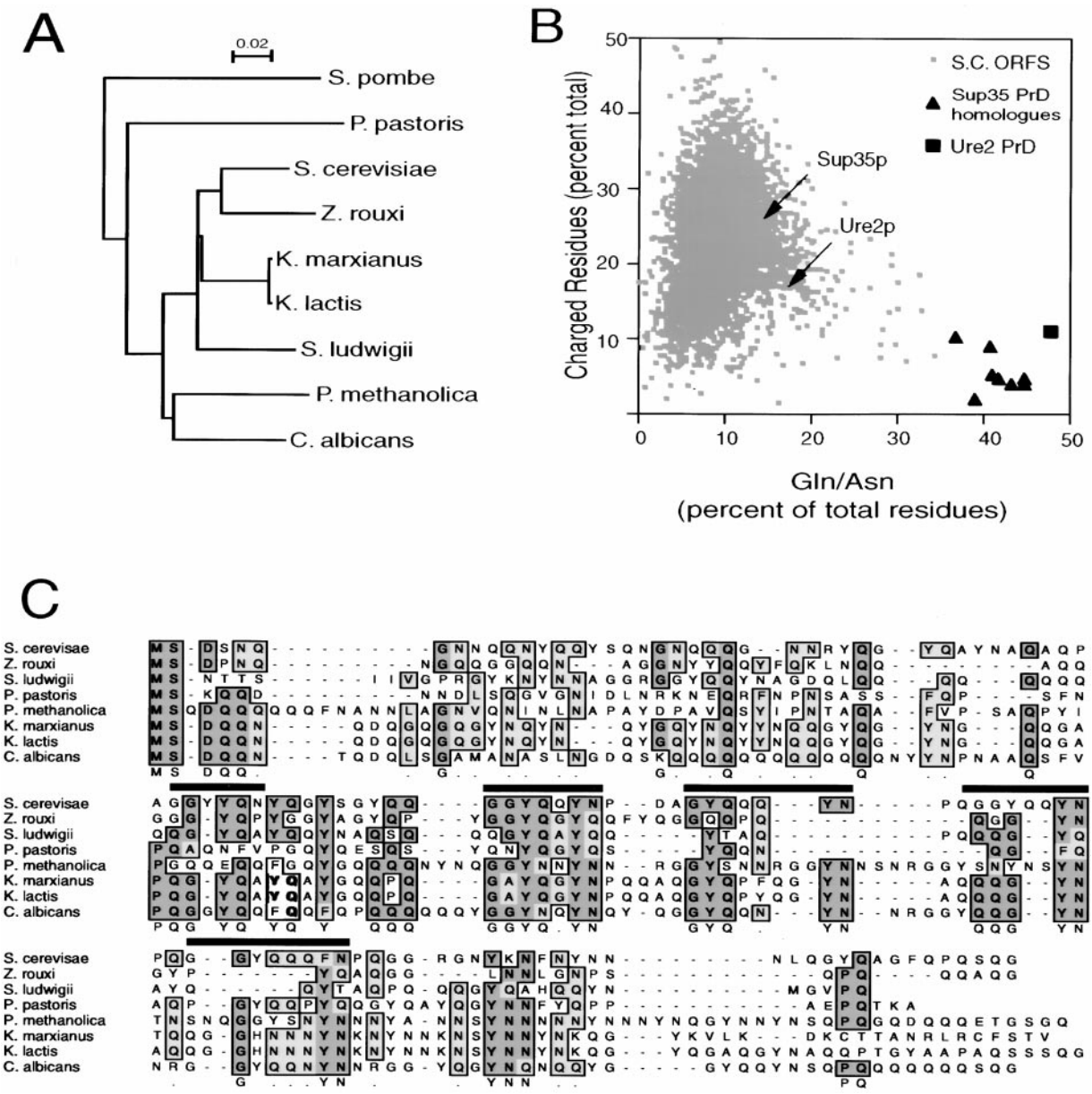

Figure 1. Evolutionary Analysis of Sup35p PrDs

(A) Phylogenetic relationship of yeast species based on 26S RNA sequences (Kurtzman, 1994). For comparison, the fission yeast *S. pombe*, whose Sup35p does not have a PrD (Ito et al., 1998), is shown. Scale (percent divergence) is denoted on top. Resende et al. have previously submitted a full-length sequence of *SUP35* from *C. albicans* (AF020554) to public databases.

(B) Plot of percent of charged (Arg, Lys, Asp, Glu) versus Gln/Asn residues for the Sup35 PrD homologs as well as the *S. cerevisiae* Ure2p prion domain. For comparison, the full-length *S. cerevisiae* ORFs, including specifically the entire Sup35p and Ure2p proteins are also shown.

(C) Amino acid sequence comparison of PrD homologs. Amino acid identities and similarities are indicated by dark gray and light gray boxes, respectively. Sequences were aligned using the ClustalW algorithm (Higgins et al., 1996). The black bar denotes the approximate location of the oligopeptide repeats. The GenBank accession numbers are as follows: *Z. rouxii*, AF206292; *S. ludwigii*, AF206291; *P. pastoris*, AF206290; *K. marxianus*, AF206289; *K. lactis*, AF206288; *C. albicans*, AF206287.

This system allowed us to test whether the foreign PrD aggregates behave as prions, and if so, whether divergence in sequences leads to a species barrier to prion propagation (Figure 3A). In support of the species barrier model, we found that the conversion of the maintainer PrD is induced only upon overexpression of the homotypic inducer PrD. For example, overexpression of $PrDM_{CA}$-GFP in the presence of $PrDM_{CA}$-EF maintainer resulted in ~7.5% adenine-prototrophic [PSI^+ CHI_{CA}^+] colonies (Figure 3B). By contrast, less than 0.005% [chi_{CA}^-] cells were converted when an empty control plasmid or any of the heterotypic inducers were used. Likewise, in the presence of $PrDM_{KL}$-EF and $PrDM_{PM}$-EF maintainers, only the overexpression of the homotypic inducers caused efficient conversion to the [CHI^+] state. Finally, double fluorescence experiments using fusions

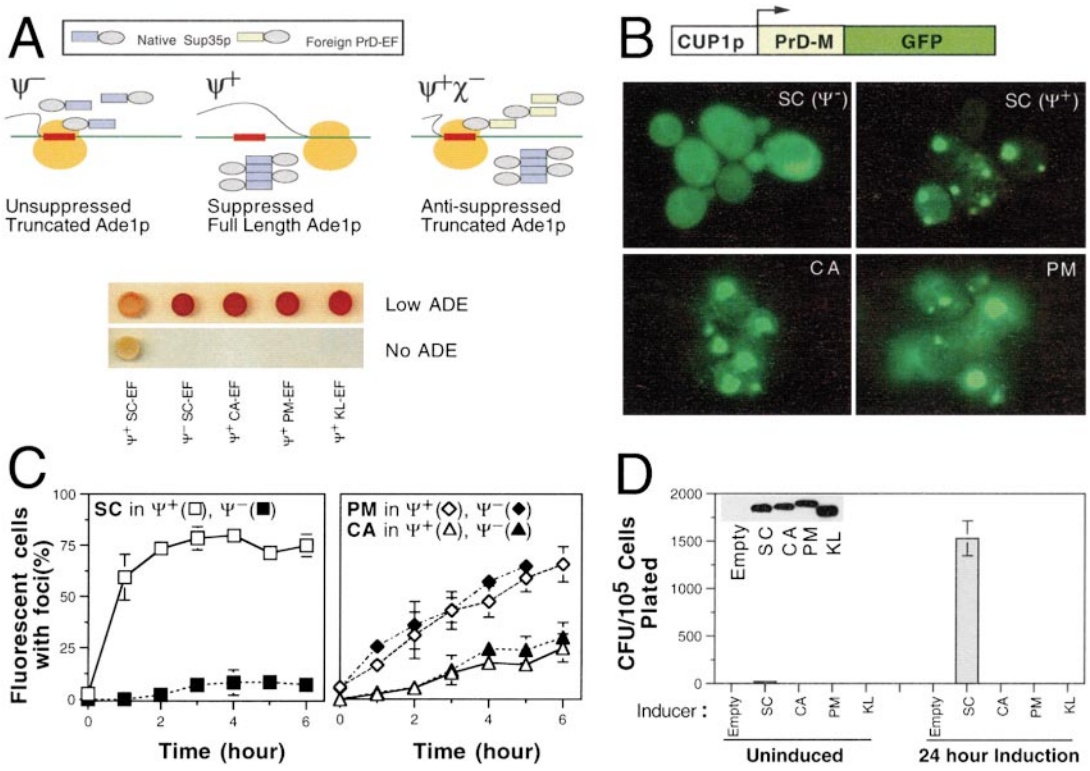

Figure 2. Examination of the Ability of Foreign PrDs to Interact with *S. cerevisiae* PrD

Throughout the figures CA, KL, PM, and SC refer to $PrDM_{CA}$, $PrDM_{KL}$, $PrDM_{PM}$, and $PrDM_{SC}$, respectively. ψ^+ and ψ^- indicate the presence or absence of the *S. cerevisiae* PrD aggregate. χ^+ and χ^- indicate the presence or absence of the foreign PrDM aggregates. All quantitative experiments were conducted in triplicate and errors are indicated.

(A) Effect of foreign PrDM-EF fusion proteins on the [PSI^+] suppression phenotype. [PSI^+] yeast expressing the indicated species of PrDM-EF fusion were plated on low or no ADE medium. For comparison, [psi^-] yeast are also shown. On top is shown a schematic model explaining the antisuppression phenotype (red colonies on low ADE and lack of growth on no ADE) resulting from expression of foreign PrDM. In [psi^-] cells, soluble Sup35p mediates translational termination at the ade1 nonsense mutation (red bar). In suppressed [PSI^+] yeast, absence of soluble Sup35p results in translational readthrough. Failure of foreign PrDM-EF to be incorporated into the [PSI^+] amyloid leads to soluble EF and antisuppression.

(B) Illustration of GFP visual assay. On top is shown a schematic of the copper-inducible PrDM-GFP plasmid. From left to right are examples of diffuse GFP fluorescence in [psi^-] cells, discrete foci in [PSI^+] cells and de novo aggregates formed by overexpression of $PrDM_{CA}$-GFP, and $PrDM_{PM}$-GFP as observed by fluorescence microscopy.

(C) Quantitative foci formation kinetics. [PSI^+] and [psi^-] yeast, as indicated, containing either $PrDM_{SC}$-GFP (left) or the indicated foreign PrDM-GFP (right) were induced in early log phase, and results are plotted as the percentage of fluorescent cells with visible foci as a function of induction time.

(D) Induction of [PSI^+] by overexpression of PrDM-GFP. [psi^-] yeast containing a plasmid encoding the indicated species of PrDM-GFP or an empty control plasmid were plated on media lacking adenine either before (uninduced) or after 24 hr of copper induction. The number of [PSI^+] colonies (CFUs) per 10^5 cells is plotted. (Inset) Immunoblots of inducer PrDM expression following induction.

between PrDM and two color variants of GFP showed that prion aggregates from two different PrD species do not colocalize (Figure 3C).

We next asked whether the [CHI^+] prion state could propagate stably. [PSI^+ CHI^+] yeast were sequentially patched onto medium that selects for the maintainer plasmid but not for the inducer plasmid or the prion state. Interestingly, some [PSI^+ CHI^+] isolates rapidly reverted back to unsuppressed [chi^-] state, whereas other strains propagated the [CHI^+] aggregate robustly. After three successive patches, corresponding to ~60 generations, a typically strong [PSI^+ CHI_{CA}^+] strain had lost the inducer plasmid but could still grow on medium lacking adenine. Even more remarkably, no red sectors were observed on low adenine media, demonstrating that even in the absence of selection, yeast uniformly retained the [CHI^+] prion (Figure 3D).

The reversible loss of the [PSI^+] factor, by overexpression of the molecular chaperone HSP104 or by exposure to guanidine hydrochloride, provided critical evidence that [PSI^+] inheritance is mediated by a change in protein conformation rather than by a DNA element (Wickner, 1994; Chernoff et al., 1995). HSP104 overexpression and guanidine caused solubilization of the [CHI_{CA}^+] aggregate, suggesting that both [CHI^+] and [PSI^+] prions are cured by similar mechanisms (Figure 3E and data not shown). Furthermore, the [CHI_{CA}^+] prion can propagate even in yeast lacking the endogenous sup35 gene (Figure 3F). As with [PSI^+ CHI^+] yeast, transient exposure to guanidine also cures the [CHI^+] state. Together, these data provide strong genetic evidence that foreign PrDs can support prion-based inheritance and that a barrier prevents cross-seeding between different species of PrDs.

Using a centrifugation assay (Patino et al., 1996;

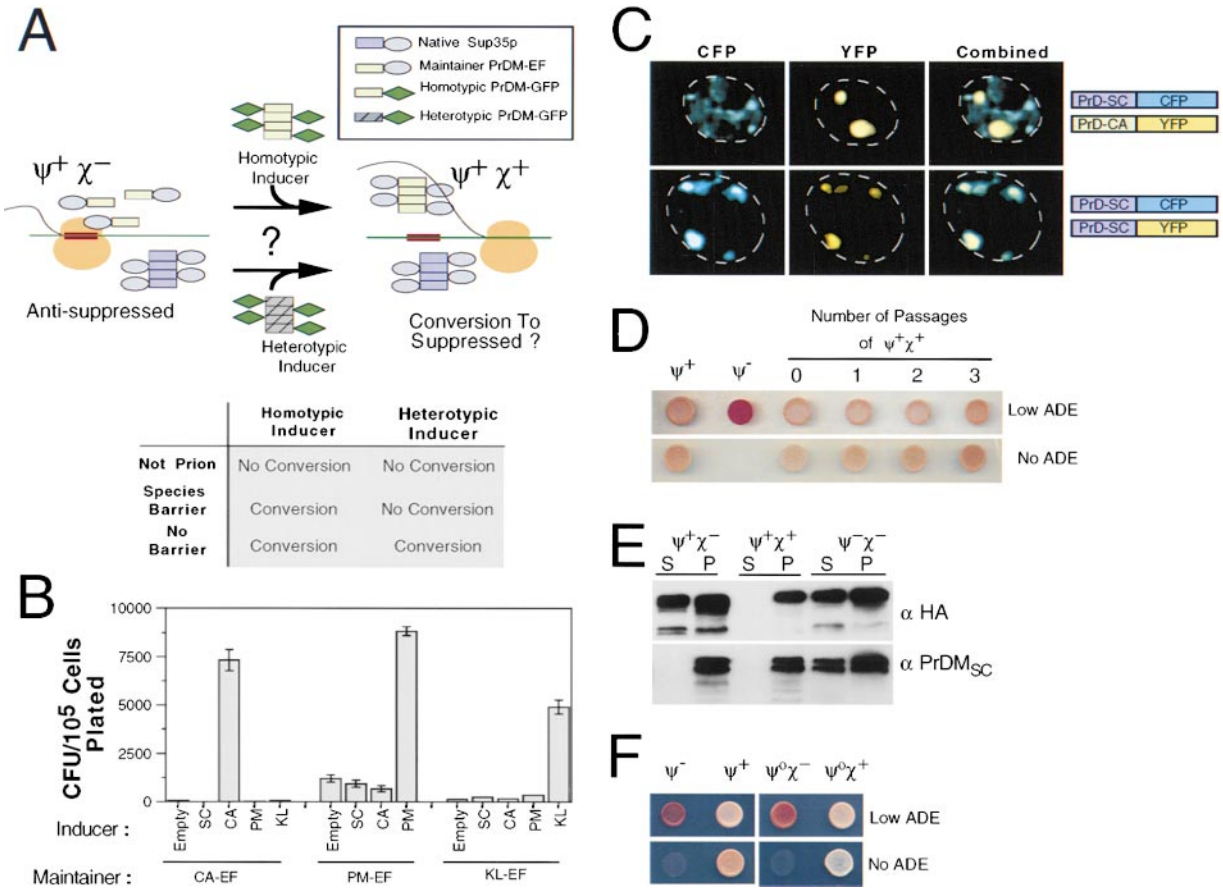

Figure 3. Examination of the Ability of Homotypic or Heterotypic PrD Overexpression to Induce [CHI$^+$] Prions

(A) Schematic of [CHI$^+$] two-plasmid induction assay. Expression of foreign PrDM-EF from the maintainer plasmid in [PSI$^+$ chi$^-$] cells results in soluble EF activity, leading to antisuppression. Aggregates are introduced by transient overexpression of the PrDM-GFP fusion either from the same (homotypic) or different (heterotypic) species as the maintainer. The ability of the introduced aggregates to convert the PrDM-EF fusion yielding [PSI$^+$ CHI$^+$] yeast can be monitored by a persistent change in suppression phenotype. Below, for each of the three indicated models, the predicted effect of homotypic and heterotypic inducers on [CHI$^+$] conversion is shown.

(B) Induction of [CHI$^+$] by overexpression of homotypic and heterotypic PrDM-GFP inducers. [PSI$^+$ chi$^-$] yeast containing HA epitope–tagged *C. albicans*, *P. methanolica*, or *K. lactis* maintainer plasmid indicated by CA-EF, PM-EF, and KL-EF, respectively, and the indicated inducer plasmid were grown in selective medium to early log phase. Following 40 hr of copper induction, the number of [PSI$^+$ CHI$^+$] colonies (CFUs) was determined by plating onto no ADE medium.

(C) In vivo observation of species-specific aggregation. Shown are fluorescence images of [PSI$^+$] yeast coexpressing fusion proteins between the indicated species of PrDM and a yellow (YFP) and cyan (CFP) variant of GFP driven by an inducible copper promoter. The left, middle, and right panels display the cyan, yellow, and combined fluorescence, respectively. Schematics on the right indicate the identity of the PrDM-GFP variant fusion proteins. Dotted lines denote the cell outlines.

(D) Stability of propagation of a [CHI$^+$] prion. A robust [PSI$^+$ CHI$_{CA}^+$] isolate was patched serially onto complete medium. Following the indicated number of passages, an aliquot was patched on either low or no ADE medium, as indicated, to test for the presence of the suppression phenotype caused by [CHI$^+$]. For comparison, [psi$^-$] and [PSI$^+$] yeast are shown.

(E) Centrifugation assay to examine the solubility of foreign PrDM-EF. Extracts from the indicated yeast strains were centrifuged at 100,000 g, and soluble (S) or pelleted (P) fractions were assayed by immunoblots with antibodies (α-HA) specific to the epitope-tagged PrDM$_{CA}$-EF or with antibodies (a PrDM$_{SC}$) specific to the *S. cerevisiae* Sup35p.

(F) Formation and propagation of [CHI$^+$] in the absence of *S. cerevisiae* Sup35p. A yeast strain was constructed in which the chromosomal *SUP35* gene was deleted and replaced with an episomal copy PrDM$_{CA}$-EF. Shown are examples of this strain either prior to or after induction of the [CHI$^+$] prion, denoted [ψ° χ^-] and [ψ° χ^+], respectively, plated on either low or no ADE medium. For comparison [PSI$^+$] and [psi$^-$] yeast are also shown.

Paushkin et al., 1996), we confirmed biochemically that the [CHI$^+$] suppression phenotype results from a heritable aggregation of the foreign PrDM-EF protein. Aggregated Sup35p from [PSI$^+$] yeast extract fractionates to the pellet following high-speed centrifugation, whereas soluble Sup35p from [psi$^-$] yeast extract remains largely in the supernatant. When subjected to this centrifugation assay, PrDM$_{CA}$-EF protein from [CHI$_{CA}^+$] yeast fractionates to the pellet (Figure 3E). By contrast, it remains largely in the supernatant both in the unconverted and the guanidine- or HSP104-cured [chi$^-$] strains (Figure 3E and data not shown).

Selective Seeding of Fibril Formation Recapitulates the Species Barrier In Vitro

Purified Sup35p forms self-seeding amyloids in vitro, thereby providing a simple biochemical system to examine the molecular basis of the observed species barrier

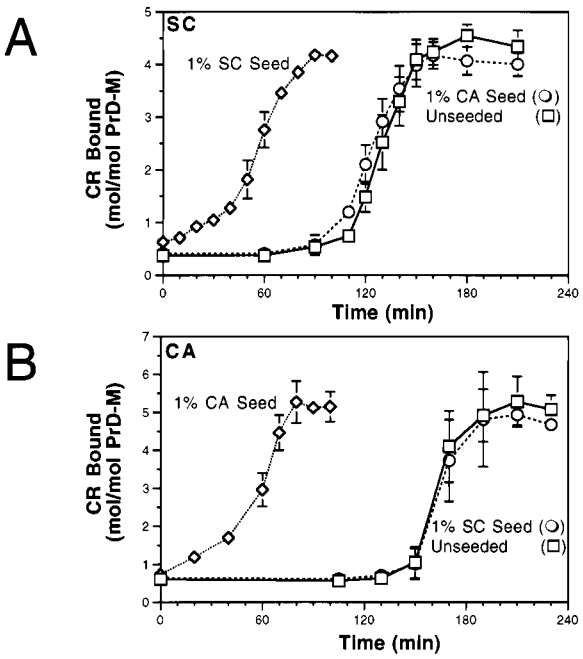

Figure 4. In Vitro Amyloid Fibril Formation of $PrDM_{SC}$ and $PrDM_{CA}$

To initiate conversion, concentrated pure PrDM protein in urea was diluted into conversion buffer and subjected to continuous slow rotation. At indicated times, the extent of fibril formation was assayed by Congo red binding. Each curve was conducted in triplicate. Bars indicate errors larger than the symbol size.

(A) Conversion kinetics of 2.5 μM *S. cerevisiae* PrDM in the absence (square) and presence of 1% (wt/wt) preformed $PrDM_{SC}$ (diamond) and $PrDM_{CA}$ (circle) fibrils.

(B) Conversion kinetics of 2.5 μM *C. albicans* PrDM in the absence (square) and presence of 1% (wt/wt) preformed $PrDM_{CA}$ (diamond) and $PrDM_{SC}$ (circle) fibrils.

(Glover et al., 1997; King et al., 1997; Paushkin et al., 1997; DePace et al., 1998). After dilution of purified $PrDM_{SC}$ from denaturant, there is an initial lag phase of ~120 minutes, followed by a cooperative conversion from random coil to β sheet–rich amyloid fibrils. This conversion can be readily monitored by selective binding of the fibrils to the dye Congo red (Figure 4A). Importantly, the addition of preformed fibril catalyzes this conversion by eliminating the lag phase, thus recapitulating in vitro the self-propagation of the prion state.

We attempted to recapitulate [CHI^+] prion propagation in vitro using purified *C. albicans* PrDM proteins ($PrDM_{CA}$). Upon dilution from denaturant, $PrDM_{CA}$ exhibited a cooperative transition to fibrils following a lag phase of ~140 minutes. As with $PrDM_{SC}$, addition of a small amount (1%) of preformed $PrDM_{CA}$ fibrils eliminated the lag phase (Figure 4B). Strikingly, the addition of $PrDM_{CA}$ fibrils, even at amounts that could efficiently catalyze $PrDM_{CA}$ amyloid formation, did not convert $PrDM_{SC}$ (Figure 4A). Conversely, addition of $PrDM_{SC}$ seed did not alter the kinetics of $PrDM_{CA}$ fibril formation (Figure 4B). Thus, our in vitro seeding experiments mirror a key aspect of the in vivo homotypic conversion experiments, in which prion formation is initiated only by homotypic PrD overexpression.

In the selective seeding experiments above, addition of a fibril seed failed to convert PrDM from another species. However, for yeast to stably exist in a [PSI^+ chi^-] state, the foreign PrD must remain soluble in the presence of continuous aggregation of similar levels of the endogenous Sup35p. To better simulate this condition, we tested the effect of selective seeding of a solution containing an equal concentration of $PrDM_{SC}$ and $PrDM_{CA}$ monomers. Addition of a small amount of $PrDM_{SC}$ seed (5%) shortly after the initiation of the polymerization reaction caused an immediate conversion detected by Congo red binding (Figure 5A). Consistent with the notion that ongoing $PrDM_{SC}$ polymerization does not induce conversion of $PrDM_{CA}$, the curve plateaus at a level corresponding to conversion of half of the total protein. Subsequent addition of $PrDM_{CA}$ seed (5%) initiated the conversion of the remaining protein. In contrast, if no seed or more $PrDM_{SC}$ seed was added, there was no immediate conversion. Instead, a slow rise in Congo red binding was observed with kinetics indistinguishable from that of the spontaneous conversion of $PrDM_{CA}$ (Figure 5B).

Immunoelectron microscopy confirmed that these two prions had a strong preference to form amyloid fibrils composed of a single species. We used a species-specific polyclonal antibody to label *S. cerevisiae* PrDM with 5 nm gold particles and a monoclonal epitope tag–specific antibody to label $PrDM_{CA}$ with 15 nm gold. In a reaction where equimolar amounts of $PrDM_{SC}$ and $PrDM_{CA}$ were polymerized simultaneously, individual fibrils were labeled with only one size of gold particles, suggesting that, as observed in vivo in the double fluorescence studies (Figure 3C), fibrils were composed exclusively of a single species of PrD (Figure 5C).

Prion Specificity Is Encoded in a Short Region of the PrD Domain

Taking advantage of the visual and [PSI^+] conversion assays described above, we next asked whether a specific region of the PrD is responsible for the species barrier. We created two complementary chimeric proteins, the first of which ($PrDM_{SC1\text{-}39CA}$) contained residues 1 to 39 from *S. cerevisiae* with the remaining PrD sequence from *C. albicans*, and the second ($PrDM_{CA1\text{-}39SC}$) in which the first 39 residues of $PrDM_{SC}$ were replaced with the *C. albicans* sequence (Figure 6A). Microscopic examination of GFP fusions shows that both chimeras form aggregates (Figure 6C and data not shown). However, while overexpression of $PrDM_{SC1\text{-}39CA}$ was highly effective at converting [psi^-] yeast to [PSI^+], overexpression of $PrDM_{CA1\text{-}39SC}$ had no detectable effect (Figure 6B).

A previous study had found that mutations that cause poor incorporation into or curing of wild-type [PSI^+] aggregates cluster to a short region composed of residues 8 to 26 (DePace et al., 1998). To test whether this epitope is sufficient to allow crossing of the species barrier, we constructed a third chimera consisting of residues 8 to 26 of *S. cerevisiae* replacing the corresponding *C. albicans* sequence, denoted $PrD_{SC8\text{-}26CA}$ (Figure 6A). This chimeric PrDM-GFP retains the ability to aggregate and is seeded by the endogenous Sup35p aggregate, as shown by the visual assay (Figure 6C). More remarkably, it induces conversion of [psi^-] yeast to [PSI^+] with only a modestly reduced efficiency (~2-fold) compared with wild-type $PrDM_{SC}$ (Figure 6B). These data indicate that a

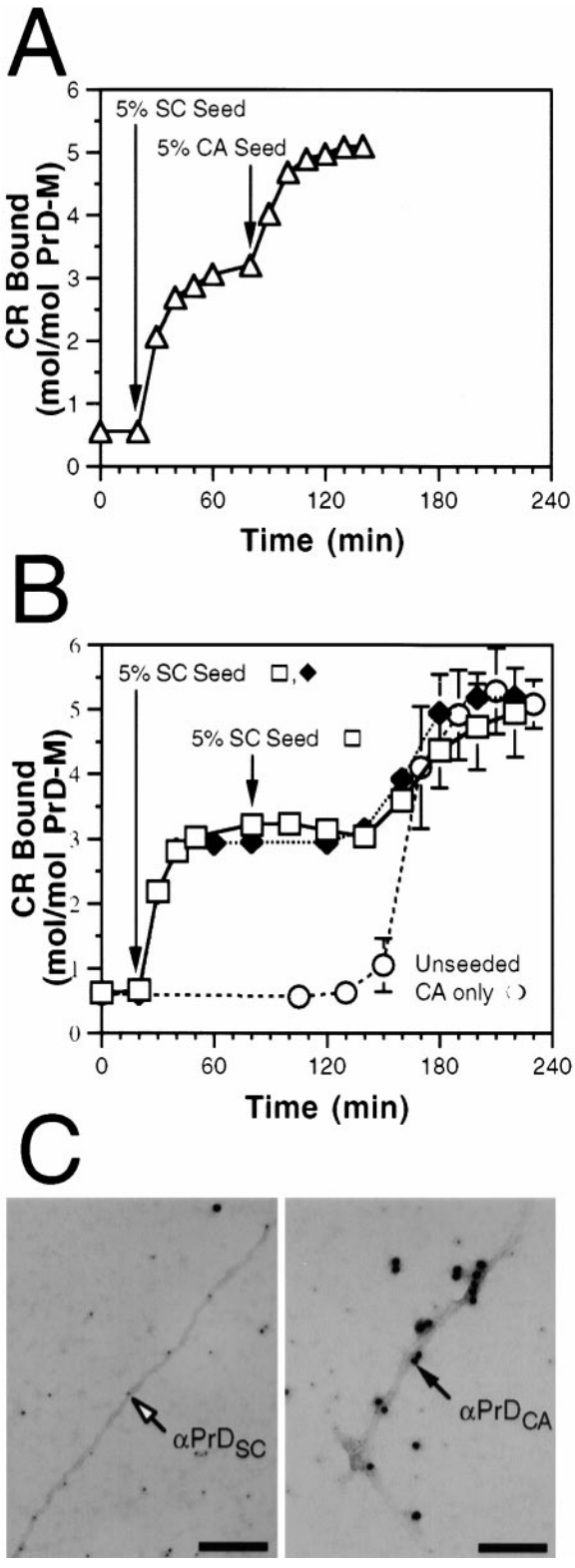

Figure 5. In Vitro Amyloid Fibril Formation of a $PrDM_{SC}$/$PrDM_{CA}$ Mixture

(A) Conversion kinetics of equimolar mixture (2.5 μM) of $PrDM_{SC}$ and $PrDM_{CA}$ seeded with 5% (wt/wt) $PrDM_{SC}$ fibril and subsequently with $PrDM_{CA}$ fibril at indicated timepoints.

(B) Conversion kinetics of an equimolar mixture (2.5 μM) of $PrDM_{SC}$ and $PrDM_{CA}$ seeded with 5% (wt/wt) $PrDM_{SC}$ fibril only (filled diamond) twice with $PrDM_{SC}$ fibril (square) compared to unseeded kinetics of $PrDM_{CA}$ (circle).

(C) Electron micrographs of the converted $PrDM_{SC}$ and $PrDM_{CA}$ mixture. $PrDM_{SC}$ and $PrDM_{CA}$ are labeled by 5 nm and 15 nm gold particles, respectively, using species-specific antibodies. Examples of fibers decorated exclusively with 5 nm (left) or 15 nm (right) gold particles from the same electron micrograph are shown. Although some fibrils were poorly labeled, no fibers were decorated by both antibodies. Scale bar corresponds to 100 nm.

short peptide epitope composed of residues 8 to 26, presumably on the growing face of the amyloid, is sufficient to mediate specificity in the incorporation of monomers into the polymerizing fibril (Figure 6D, see Discussion).

Identification of a Novel Prion-Forming Protein in *S. cerevisiae*

The observation that all known yeast prion proteins have high Gln/Asn content and few charged residues (Figure 1B) suggests that proteins with similar properties could form prions. A search of genomic databases revealed that several other proteins have domains with similar properties (Figure 7A and M. Michelitsch and J. S. W., unpublished data). We experimentally examined the ability of one such domain from a protein encoded by the uncharacterized ORF *YPL226W* (*NEW1*) to form a prion using the two-plasmid assay described above. We fused the first 153 amino acids of New1p to an HA_3 epitope–tagged EF domain and expressed this fusion protein, termed $New1p_{1–153}$-HA_3-EF, driven by the *SUP35* promoter. Initially, this fusion protein complemented the deficiency of Sup35p activity associated with [PSI^+], indicating that it is not inactivated by the [PSI^+] prion; we termed this antisuppressed state [PSI^+ nu^-]. However, transient overexpression of the same fragment fused to GFP, but not overexpression of $PrDM_{SC}$-GFP or $PrDM_{CA}$-GFP, caused ~10% of [PSI^+ nu^-] cells to convert to a suppressed state termed [PSI^+ NU^+] (Figure 7B). As with [CHI^+], we found significant variation among [NU^+] isolates, with some rapidly reverting to [nu^-] and others propagating stably (data not shown). Finally, centrifugation analysis directly demonstrated that the [NU^+] state results from aggregation of the New1p-EF fusion (Figure 7C). Taken together, these observations argue the Gln/Asn-rich N-terminal region of New1p can support a prion mechanism of inheritance.

Discussion

To investigate how prions can specifically propagate in the complex cellular milieu, we have cloned and characterized the N-terminal prion domain (PrD) from a range of budding yeasts. Despite the long evolutionary distances separating these species (Kurtzman, 1994), the Sup35p homologs examined contain PrDs capable of forming prions. In particular, upon induction of aggregates by overexpression, the foreign PrDs switch from an initially soluble [chi^-] state to an aggregated [CHI^+] prion state. This prion state can stably propagate until cured by guanidine treatment or HSP104 overexpression. As with mammalian prions (Prusiner et al., 1998), a species barrier prevents prion aggregates from one species from converting soluble PrDs of another.

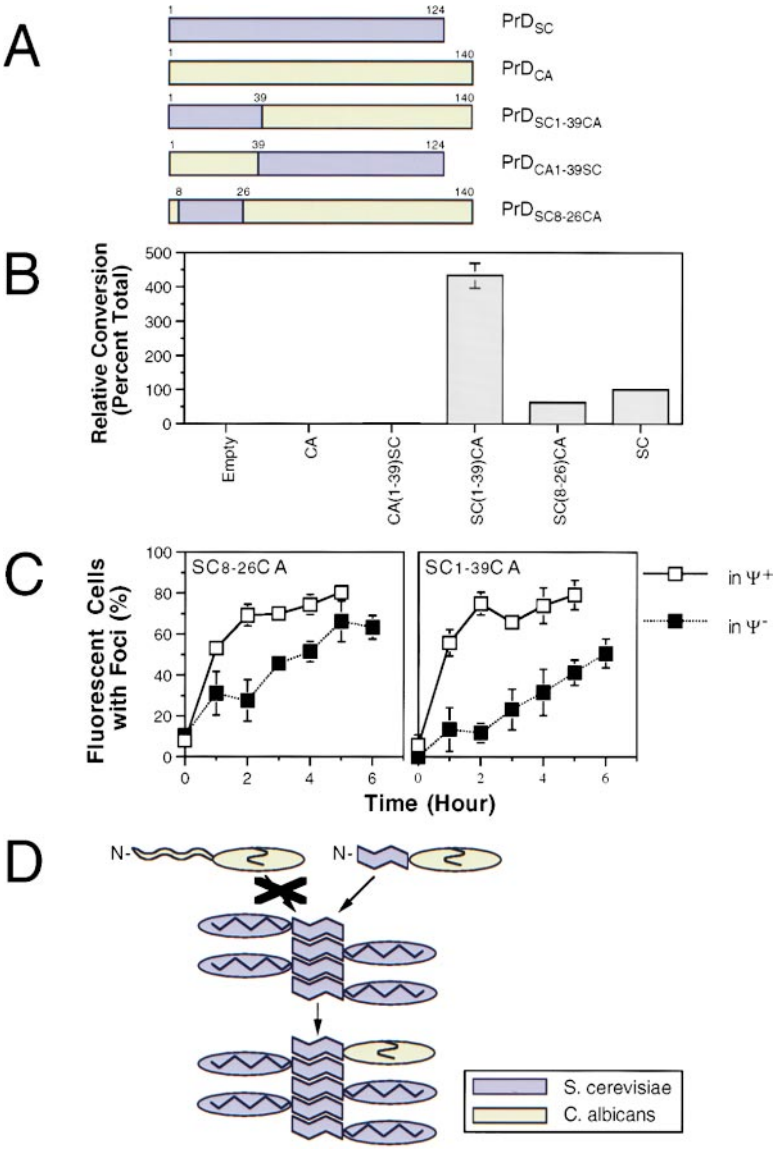

Figure 6. Chimeric Analysis of PrD

(A) Schematics of chimeric PrD sequences. Residue numbers are indicated on top. Chimeric region residue numbers are that of *S. cerevisiae* sequence. *S. cerevisiae* regions are denoted by purple and *C. albicans* by light yellow.

(B) Efficiency, relative to PrDM$_{SC}$, of conversion of [psi$^-$] to [PSI$^+$] by overexpression of the indicated PrDM-GFP fusion.

(C) Quantitative foci formation kinetics. [PSI$^+$] and [psi$^-$] yeast, as indicated, containing the indicated chimeric PrDM-GFP fusion were grown in selective medium and induced at early log phase. Shown is a plot of the percentage of fluorescent cells with visible foci as a function of induction time.

(D) Hypothetical model to explain chimera conversion data. The N-terminal region of PrD is envisioned as having many of the critical species-specific interactions involved in recruitment of new monomers to the growing amyloid, whereas the C-terminal region may be primarily involved in intramolecular interactions that stabilize the prion form.

Surprisingly, even in identical genetic backgrounds, different [CHI$^+$] isolates of the same foreign PrD species as well as different [NU$^+$] isolates show markedly different stability and levels of nonsense suppression. Analogous strain differences were found previously in [PSI$^+$] as well as in mammalian prions (Derkatch et al., 1996; Prusiner et al., 1998; Zhou et al., 1999). Initially discovered as differences in pathology between isolates of scrapie, mammalian prion strains were thought to result from nucleic acid variations, arguing against a protein-mediated mechanism of prion infectivity. It now appears that the presence of stable, distinct strains, possibly arising from different prion conformations, is an inherent property of prions.

Insights into the Molecular Architecture of the Prion Domain: Specificity and Stability Domains within the PrD

The mammalian prion species barrier, which prevents the spread of scrapie and bovine spongiform encephalopathy to man, has been the focus of intense research efforts (Prusiner et al., 1998). Many questions about the molecular basis of the prion species barrier remain. For example, in vitro experiments indicate that specific interactions between PrP^{SC} and PrP^{C} lead to a species barrier in generating the protease-resistant form (Kocisko et al., 1995; Horiuchi and Caughey, 1999). In vivo chimera analyses, however, suggest that species-dependent interactions between the prion particles and an unidentified host factor, termed Protein X, also contribute substantially to the species barrier (Telling et al., 1995). These issues have been difficult to resolve because of the inability thus far either to produce de novo infectious PrP^{SC} or to effectively recapitulate sustained prion propagation in vitro.

Our data show that for the yeast [PSI$^+$] prion, the species barrier results from a remarkable specificity in interaction between the prion protein itself, mediated by a well-defined epitope in the PrD (Figure 6D). First, for all of the species examined, only overexpression of homotypic PrD induces prion formation. Similarly, Chernoff and coworkers have recently found that the

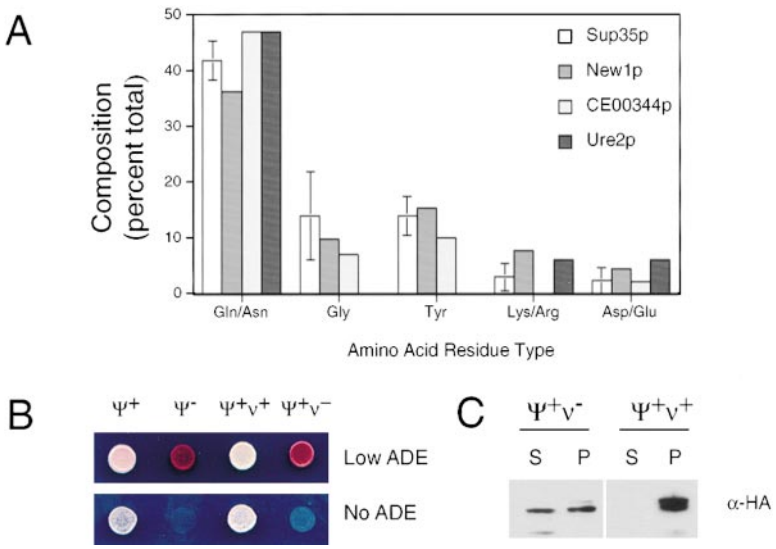

Figure 7. The N Terminus of New1p Can Act as a Prion

(A) The amino acids content of Sup35p PrD, residues 10 to 100 of New1p, residues 230 to 319 from the *C. elegans* ORF CE00344, and the first 90 amino acids of Ure2p are displayed. For the PrD plot, the average value among the various yeast species is used with error bars indicating the maximum variation.

(B) Phenotypic consequences of prion formation by the New1p$_{1-153}$-HA3-EF fusion protein. Shown are examples of [PSI$^+$ nu$^-$] and [PSI$^+$ NU$^+$] isolates grown on medium with low amounts of adenine (above) and no adenine (below), illustrating the conversion from an antisuppressed to a suppressed state following [Nu$^+$] induction. For comparison, [PSI$^+$] and [psi$^-$] strains are shown.

(C) Centrifugation assay to follow the solubility of the New1p$_{1-153}$-HA3-EF fusion, performed as in Figure 3E.

[PSI$^+$] state of the endogenous *S. cerevisiae* Sup35p can not be transmitted by expression of heterologous *P. Methanolica* Sup35p in the absence of the *S. cerevisiae* protein (Y. Chernoff et al., personal communication). Second, as with [PSI$^+$] (Tuite et al., 1981; Chernoff et al., 1995), transient overexpression of the molecular chaperone HSP104 or exposure to guanidine, which most likely acts by modulating cellular factors, cures the [CHI$_{CA}^+$] prion. Thus, at least for these components, the ability of the *S. cerevisiae* folding machinery to regulate the aggregation state of the *C. albicans* PrD is conserved. Third, in vitro selective seeding experiments faithfully recapitulate the species barrier. Strikingly, even in an equimolar solution of PrDM$_{SC}$ and PrDM$_{CA}$, seeded polymerization of the *S. cerevisiae* protein leads to rapid formation of pure PrDM$_{SC}$ fibrils without affecting the polymerization kinetics of the *C. albicans* protein. Finally, chimeric analyses reveal that prion specificity of *S. cerevisiae* can be conferred to the PrD of another species by a substitution of 19 amino acid residues near the PrD N terminus.

This localization of a species-determining region helps reconcile disparate results from previous efforts to dissect PrD function. A screen for mutant PrDs that either fail to interact with or cause curing of the endogenous [PSI$^+$] prion found that these mutations are located between residues 8 and 26 (DePace et al., 1998). However, sequences C-terminal to this region have been shown to be critical for prion function (Doel et al., 1994; Liu and Lindquist, 1999). Moreover, expansion of the imperfect oligopeptide repeats, also located outside of this species-determining domain, dramatically enhances the rate of prion formation. These observations, together with our in vitro selective seeding experiments, suggest a model (Figure 6D) in which the N terminus resides on the surface of the growing amyloid and contributes much of the specificity of PrD monomer recruitment. By contrast, the more C-terminal region might largely be involved in intramolecular interactions that stabilize the prion form. Substitution of *S. cerevisiae* residues 8 to 26 into PrD$_{CA}$ would thus change the surface of the amyloid, allowing it to incorporate PrD$_{SC}$ monomers. By contrast, expansion or deletion of the imperfect oligopeptide repeats would lead to increased or decreased stabilization of the PrD, thereby modulating its tendency to form amyloids without altering prion specificity.

An Epigenetic Switch: Prion-Based Protein Regulation?

Alleles of the mammalian prion protein prone to prion formation are rare, as would be expected given the devastating effects of these diseases. By contrast, the ability of the N-terminal domain of Sup35p to form a prion is conserved across the budding yeasts. This functional conservation is remarkable since the PrD sequence is not strongly conserved and earlier mutational analyses showed that even single point mutations in the *S. cerevisiae* PrD can inhibit prion formation (DePace et al., 1998). These observations raise the intriguing possibility that rather than being pathogenic, [PSI$^+$] might be an evolutionarily beneficial state. However, we can not rule out the possibility that the retention of prion function is a by-product of conservation of an unidentified PrD function (Bailleul et al., 1999). Consistent with a beneficial role for the [PSI$^+$] prion, Tuite and coworkers found that following exposure to high temperature or ethanol, some [PSI$^+$] yeasts show enhanced survival compared to isogenic [psi$^-$] yeasts (Eaglestone et al., 1999). In addition, we find that overexpression of PrDM$_{CA}$-GFP in *C. albicans* induces formation of punctate foci, although it remains to be seen whether these aggregates can propagate in a prion-like manner (unpublished observations).

As a mechanism of inheritance, prions provide a number of potentially advantageous features (Lindquist, 1997). Prion formation allows a cell to inhibit the activity of a specific protein and propagate this state indefinitely while retaining the potential to restore the original protein activity. Moreover, the rate of conversion to and from the prion state can be dramatically enhanced by changes in the environment (Tuite et al., 1981). Finally, because prion domains are modular (Ter-Avanesyan et al., 1993; Patino et al., 1996), fusion to prion domains

could potentially allow prion-based regulation of a broad range of proteins.

For a prion to serve as an epigenetic switch, it must propagate specifically without interfering with other proteins. Specificity of prion interactions, resulting from differences in primary sequence and manifested as a barrier to cross-species prion induction, could serve as mechanism to prevent such improper interactions. Consistent with the proposal that multiple prion states could propagate independently in the same cell, we have identified a novel prion-forming domain in *S. cerevisiae* (the N-terminal portion of New1p) and have shown that its prion state propagates independently of [PSI$^+$]. Furthermore, we find that overexpression of a Ure2p fragment that efficiently induces the [URE3] prion (Edskes et al., 1999) does not induce [PSI$^+$] nor is Ure2p incorporated into [PSI$^+$] aggregates (data not shown), indicating that the existence of one prion within a cell does not promote the appearance of others.

If multiple different prions can exist independently within a single cell, then how many different prions are there? The spectrum of Sup35p PrD sequences, together with earlier mutational analyses, provide a wealth of data to search for novel prions. Despite little strict sequence conservation, all of the examined Sup35p PrDs as well as the Ure2p prion domain contain an extremely high Gln/Asn and low charge content (Figures 1B and 7A). Moreover, in some neurodegenerative diseases, expansion of polyGln repeats leads to intranuclear aggregates in vivo and self-propagating amyloids in vitro (for review, see Bates et al., 1998). A search of genomic databases for domains with amino acid content comparable to Sup35p PrDs revealed a handful of such domains in both yeast and nematodes (Figure 7A and M. Michelitsch and J. S. W., unpublished data) at least one of which (New1p) forms a prion in yeast. The challenge now is to determine how many other Gln/Asn-rich domains can form stable, self-propagating prions and what the physiological role for such novel prions may be.

Experimental Procedures

General Procedures and Reagents

Isogenic *S. cerevisiae* [psi$^-$] and [PSI$^+$] strains 74-D694 [*Mat***a**, *ade1-14*(UGA), *his3*, *leu2*, *trp1*, *ura3*] (Chernoff et al., 1995) were used for all experiments except the Sup35p deletion studies, which used YJW541 [*Mat***a**, *ade1-14*, *his3*, *leu2*, *trp1*, *ura3*, *sup35::TRP1*]. *C. albicans* SC5314, *K. marxianus*, and *P. pastoris* were gifts from A. Johnson, E. Blackburn, and C. Craik, respectively. *K. lactis* and *S. ludwigii* were gifts from I. Herskowitz. *P. methanolica* (56509) and *Z. rouxi* (48232) were obtained from ATCC. Nucleic acid, immunoblot, and yeast manipulations were performed according to standard protocols (Ausubel, 1987). All plasmid sequences were confirmed by dye termination sequencing (Perkin-Elmer).

Oligonucleotide primers were as follows: P1, GGGGGATCCGT CGACACTAGTACAATGTCTGACCAACAGAATACT; P2, CCCAGAT CTTCTAGAATCCTTGACAACTTCTTCGTC; P3, GGGGGATCCGTC GACACTAGTACAATGTCTCAAGATCAACAGCAA; P4, CCCAGATC TTCTAGAATCGTTGACAATGGAGGCATC; P5, CGACGAGGATCCG TCGACATGTCAGACCAACAAAATCAAGACCAAGGG; P6, CAAAG TGAATTCAGATCTATCTTTAACGACTTCTTC; P7, GGGCGGCATAT GTCTGACCAACAGAATACTCAG; P8, GCCCGAATTCTTAGTGATGA TGGTGATGGTGGTGATCCTTGACAACTTCTTCGTC; P9, CCGGAA TTCTTAATGGTGATGATGGTGATGAGCGTAATCTGGAACGTCATA; P10, AACGGTTGGGTCATCCATCTT; P11, TTTGTTGGTATCCAT GACCCATGACAAGTACCA; P12, GGCCCAGTGAGCAGAGTGACG GAGGACTCGAGCTCAAGCTAATCCGGCGTGCATTGAC; P13, GAT CGTCAATGCACGCCGGATTTACGCC; P14, CTAATACGACTCACT ATAGGGCTCGAGCGGCCGCCCGGGCAGGT; P15, ACCTGCCC; P16, GCGCGTCGACATGCCTCCAAAGAAGTTTAAGG; P17, GCGCC GAATTCGGGAGATCTTTGATTTTTGCAATCAGTGATACTTTGACAT TCAGG; Q0, CCAGTGAGCAGAGTGACG; Q1, GACTCGAGCTCA AGCTAA; AP-1, CCATCCTAATACGACTCACTATAGGGC; AP-2, AC TCACTATAGGGCTCGAGCGGC.

Cloning of Foreign SUP35p PrDs

To clone Sup35 PrDM domains, we modified the RACE procedure for capturing cDNA 5′ ends (Frohman, 1993) to allow analysis of genomic DNA. Yeast genomic DNA from the desired species was digested to completion by a restriction enzyme that results in either 5′ GATC overhangs or blunt ends. Linkers compatible with either the GATC (annealed P12, P13) or blunt ends (annealed P14, P15) were then ligated onto the ends of the genomic fragments. PCR amplification was performed using the ligated fragments as templates, the EF-specific primer P10 and linker primer Q0 or AP1 for the GATC or blunt ended fragments, respectively. Amplified products were used as templates in a second round of PCR using nested EF-specific primer P11 and linker primer Q1 or AP2 for the GATC and blunt-ended derived fragments, respectively. For reactions resulting in a single distinct band, PCR products were purified by agarose gel electrophoresis and sequenced.

Plasmid Construction

All yeast expression vectors used a previously described (DePace et al., 1998) modular insert composed of a promoter domain flanked by 5′ Xho1 and 3′ BamHI sites, a PrDM/New1p$_{1–153}$ domain flanked by 5′ BamHI/Sal1 and 3′ BglII/EcoRI sites, and an EF/GFP/eCFP/eYFP domain flanked by 5′ EcoRI and 3′ SacI sites. For the maintainer plasmid, the insert was cloned into the XhoI–SacI sites of an URA3 marked CEN/ARS plasmid (pRS316), and a triple HA epitope was inserted between the BglII/EcoRI sites. For the inducer plasmids, the insert was cloned into the XhoI–SacI sites of a LEU2-marked 2 μm plasmid (pRS425). To create the foreign PrDM inducer and maintainer plasmids, PrDM$_{CA}$ (primers P1, P2), PrDM$_{PM}$ (P3, P4), or PrDM$_{KL}$ (P5, P6) domains were PCR-amplified from genomic DNA and inserted into the BamHI/EcoRI sites of the appropriate PrDM$_{SC}$ encoding plasmid. To create the New1p inducer and maintainer plasmids, the first 153 codons of *NEW1* were PCR amplified (P16 and P17) and inserted into the Sal1/EcoR1 sites of the appropriate PrDM$_{SC}$ encoding plasmid. The chimeras PrDM$_{SC1\text{-}39CA}$ (encoding a protein in which residues 40–124 of PrDM$_{SC}$ were replaced with residue 44–140 from PrDM$_{CA}$), PrDM$_{CA1\text{-}39SC}$ (encoding a protein in which residue 1–39 of PrDM$_{SC}$ was replaced with residue 1–44 of PrDM$_{CA}$), and PrDM$_{SC8\text{-}26CA}$ (encoding a protein in which residues 1–7 and 27–124 of PrDM$_{SC}$ were replaced with residues 1–7 and 30–140 from PrDM$_{CA}$, respectively) were all derived from the PrDM$_{SC}$ inducer plasmid by seamless cloning (Stratagene). Plasmids encoding eCFP and eYFP were obtained from Clontech. For bacterial expression, 6× His–tagged PrDM$_{CA}$ with (P7, P9) or without (P7, P8) an HA tag was PCR amplified and inserted into NdeI/EcoRI sites of a T7 expression vector.

In Vivo GFP Foci Formation

Yeast carrying the indicated species of PrDM-GFP inducer plasmid were grown to early log phase in SD-LEU and induced with 50 μM $CuSO_4$. At indicated times, cells were examined by fluorescent microscopy (Olympus B×60) and photographed by CCD camera (Photometrics). For quantitative measurement, random fields were chosen and percentage of fluorescent cells with punctate foci were calculated. Double fluorescence images were collected by wide-field 3D deconvolution microscopy (Agard et al., 1989) using filters optimized for CFP and YFP fluorescence (Chroma).

[PSI$^+$] Conversion Assay and Two-Plasmid Assay for Aggregation

For [PSI$^+$] conversion assay, [psi$^-$] yeast freshly transformed with the indicated inducer plasmids were grown in 10 ml SD-LEU to early log phase and induced with 50 μM $CuSO_4$. After 24 hr, samples

were plated onto SD-ADE. For the two-plasmid [CHI$^+$] conversion assay, [PSI$^+$] yeast freshly transformed with the indicated species of maintainer and inducer plasmids were grown in 10 ml SD-URA-LEU to early log phase and induced with 50 μM $CuSO_4$. At indicated times, samples were plated onto SD-URA-ADE. For both assays, after 5 days incubation at 30°C, visible colonies were counted. For the [CHI$^+$] induction experiments in the sup35 deletion strain, YJW541 initially carrying p316SpSupEF (Depace et al, 1998) and a HIS-marked $PrDM_{CA}$-EF maintainer plasmid were grown on 5-FOA to ensure loss of p316SpSupEF yielding the [psi° chi$^-$] strain. The [psi° CHI$^+$] was derived by transient overexpression of $PrDM_{CA}$ from an inducer plasmid, selection on medium lacking ADE and subsequent loss of inducer plasmid. For the [NU$^+$] induction experiments, [PSI$^+$] yeast freshly transformed with the New1p inducer and maintainer plasmids and treated as described above for the [CHI$^+$] experiments.

Immunoelectron Microscopy

PrDM fibrils (8 μg) produced from a conversion reaction containing equimolar $PrDM_{SC}$ and PrDM-HA_{CA} were incubated with 50 μg rabbit polyclonal antibody raised against $PrDM_{SC}$ (HTI Bio-products) and 100 μg of the mouse monoclonal anti-HA antibody 16B12 (Babco) for 20 min at room temperature. Five microliters each of 5 nm gold conjugated to anti-rabbit and 15 nm gold conjugated anti-mouse secondary antibodies (Nanoprobes) were added and incubated for an additional 20 min. The solution was added to a glow discharged carbon coated nickel grid, washed extensively with water, and stained with 2% uranyl acetate. Electron micrographs were collected with EM400 Transmission Electron Microscope (Phillips).

Other Assays

Centrifugation assays were performed as previously described (DePace et al., 1998). Recombinant PrDM was purified under denaturing conditions as described previously (Glover et al., 1997; DePace et al., 1998). Congo red binding assays were carried out as described previously (DePace et al., 1998).

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GenBank Accession Numbers

The GenBank accession numbers for the sequences reported in this paper are as follows: *Z. rouxii*, AF206292; *S. ludwigii*, AF206291; *P. pastoris*, AF206290; *K. marxianus*, AF206289; *K. lactis*, AF206288; *C. albicans*, AF206287.