Topic Introduction

Prions

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Infectious proteins (prions) are usually self-templating filamentous protein polymers (amyloids). Yeast prions are genes composed of protein and, like the multiple alleles of DNA-based genes, can have an array of “variants,” each a distinct self-propagating amyloid conformation. Like the lethal mammalian prions and amyloid diseases, yeast prions may be lethal, or only mildly detrimental, and show an array of phenotypes depending on the protein involved and the prion variant. Yeast prions are models for both rare mammalian prion diseases and for several very common amyloidoses such as Alzheimer’s disease, type 2 diabetes, and Parkinson’s disease. Here, we describe their detection and characterization using genetic, cell biological, biochemical, and physical methods.

BACKGROUND

[PSI+] and [URE3] were discovered as nonchromosomal genetic elements (Cox 1965; Lacroute 1971), and then found to be prions based on their unusual genetic properties (Wickner 1994, for yeast prion nomenclature see Box 1). Soon thereafter, the [Het-s] prion of *Podospora anserina* (Coustou et al. 1997) and the yeast [PIN+] prion (Derkatch et al. 2001) were found and there are now 10 well-documented prions of *S. cerevisiae* (Table 1; reviewed in Wickner et al. [2013]). Although a prion can be a self-modifying protein, such as the vacuolar protease B that cleaves/activates its own inactive precursor (Roberts and Wickner 2003), most are filamentous ordered polymers of a specific protein, similar to the disease-associated amyloids of mammals. Like the mammalian pathologic amyloids, the yeast and fungal prion amyloids have a cross- β structure, meaning that the β strands run essentially perpendicular to the long axis of the filaments. The [URE3], [PSI+], and [PIN+] prions are based on in-register parallel β sheet amyloid filaments of Ure2p, Sup35p, and Rnq1p, respectively (reviewed in Wickner et al. 2013). Once one of these proteins forms amyloid, it acts catalytically to convert the same protein to the amyloid form. Passage of amyloid filaments to another cell results in that cell, and its progeny, being “infected” with the prion.

GENETIC APPROACHES USED IN STUDYING YEAST PRIONS

Yeast prions began as phenomena in classical genetics, nonchromosomal genetic elements showing 4 prion : 0 segregation in meiosis (Cox 1965; Lacroute 1971). Cytofusion, mating without nuclear fusion followed by separation of the parental nuclei with mixed cytoplasm into the daughter cells (Conde and Fink 1976), is now usually used to show nonchromosomal inheritance, and is an impor-

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BOX 1. YEAST PRION NOMENCLATURE

Yeast prions are in square brackets like other nonchromosomal genes. Capitals indicate the dominant allele (the prion), and lower case shows the recessive allele (the normal protein). Thus, [URE3] or its absence [ure-o] (as [rho-o] means no mitochondrial DNA and [kil-o] means no M dsRNA—the “killer factor”). The presence of [PSI] is shown as [PSI+] and its absence [psi-].

tant method in most prion studies (see Protocol: **Genetic Methods for Studying Prions** [Wickner et al. 2015]). That the nonchromosomal genes [URE3] and [PSI+] were actually prions was first shown based on their unusual genetic properties (Wickner 1994).

Three Genetic Criteria for a Yeast Prion

Reversible Curing

If a certain treatment of cells carrying a nonchromosomal genetic element results in its elimination from the entire population, one says cells are “cured” and it is known in many such cases that the entire replicon is lost, and not simply mutated. Even if a prion can be cured, it should arise again at some low frequency in the cured strain (Wickner 1994). This is unlike a nucleic acid replicon (such as mitochondrial DNA) that, once cured, does not arise again *de novo*. The protein capable of forming the prion is still present and can again form amyloid. Reversible curability is frequently misinterpreted to mean just curability by the Hsp104 inhibitor, guanidine (see below). Note that in many strains, guanidine cures or induces mutation of mitDNA (Villa and Juliani 1980), so a trait altered by guanidine cannot be assumed to be caused by a prion (e.g., Halfmann et al. 2012).

Prion Protein Overproduction Increases Prion Generation

The *de novo* formation of a nonchromosomal genetic element in itself suggests a prion. However, because prions are altered protein forms that have the ability to catalyze their own formation, the increase in the frequency of a prion arising on overproduction of the prion-forming protein is particularly strong evidence that one is dealing with a prion (Wickner 1994).

Phenotype Relationship

The prion protein structural gene is necessary for the propagation of the prion, just as many chromosomal genes are needed for propagation of nucleic acid replicons. Generally, the prion phenotype resembles the phenotype that results from loss of function of the prion protein-coding gene. For example, the prion phenotypes of [PSI+], [URE3], [SWI+], [OCT+], [BETA], [MOT+], and [MOD+] are attributable to deficiency of the normal form of their respective prion proteins. Thus phenotypic similarity between nonchromosomal genes and recessive mutants in the genes that are required for their propagation is evidence that the nonchromosomal gene is a prion (Wickner 1994).

[PIN+] is a prion of Rnq1p that was discovered as a nonchromosomal genetic element necessary for the induction of the [PSI+] prion by overexpression of Sup35p (Derkatch et al. 1997, 2001). The amyloid of Rnq1p in [PIN+] strains occasionally cross-seeds Sup35p amyloid formation (as well as Ure2p amyloid formation) thus facilitating prion formation (Derkatch et al. 2001). Derkatch et al. showed that overproduction of any of several Q/N-rich proteins could seed generation of [PSI+]. Rnq1p is rich in Q and N residues (hence its name) and had been shown capable of self-propagating aggregation (Sondheimer and Lindquist 2000). Derkatch et al. showed that amyloid of Rnq1p was the basis of [PIN+] by applying the above three genetic criteria (Derkatch et al. 2001). Several of the

TABLE 1. Prions of *S. cerevisiae* and *Podospora anserina*

Prion	Affected protein	Normal function	Phenotype	References
[URE3]	Ure2p	Nitrogen catabolite repression	Inappropriate derepression of genes for catabolism of poor nitrogen sources; very slow growth	Wickner 1994; McGlinchey et al. 2011
[PSI+]	Sup35p	Translation termination	Read-through of translation termination codons; lethality; very slow growth	Wickner 1994; McGlinchey et al. 2011
[PIN+]	Rnq1p	Unknown	Increased generation of [PSI+] or [URE3]	Derkatch et al. 1997, 2001
[SWI+]	Swi1p	Chromatin remodeling	Inability to use nonfermentable C sources; slow growth	Du et al. 2008
[OCT+]	Cyc8p	Transcription repression	Derepressed transcription; flocculence	Patel et al. 2009
[MOT+]	Mot3p	Repressor of genes for anaerobic growth	Derepressed anaerobic genes	Alberti et al. 2009
[ISP+]	Sfp1p	Transcription factor	Decreased translation termination codon read-through	Rogoza et al. 2010
[MOD+]	Mod5p	tRNA isopentenyl—transferase	Slow growth; resistance to azole antifungal drugs	Suzuki et al. 2012
[Het-s]	HET-s	Heterokaryon incompatibility	Heterokaryon incompatibility (<i>Podospora anserina</i>)	Coustou et al. 1997
[BETA]	Prb1p	Vacuolar protease B	Necessary for normal sporulation, survival in stationary phase.	Roberts and Wickner 2003

other proteins identified in this screen, or similar screens, have also proven to be prions, including Swi1p ([SWI+]), Cyc8p ([OCT+]), and Mod5p ([MOD+]) (Du et al. 2008; Patel et al. 2009; Suzuki et al. 2012). It is expected that this method will be useful in finding prions of other organisms, particularly because the prion domain of Mod5p is not Q/N rich, unlike those of other yeast prions (Suzuki et al. 2012).

Curing Yeast Prions

Although there are no effective treatments for mammalian prion diseases, there are many ways to cure yeast prions. This has been a particularly useful method of detecting genes other than the prion protein structural gene that are involved in prion generation or propagation. Curing of [PSI+] and other prions with millimolar concentrations of guanidine (Tuite et al. 1981) has been an extremely useful tool. Overproduction of the disaggregase Hsp104 cures [PSI+] (Chernoff and Ono 1992), which led to the finding that Hsp104 is also necessary for propagation of [PSI+] (Chernoff et al. 1995) and most other yeast prions. Indeed, it is by inhibition of Hsp104 that guanidine cures prions (Ferreira et al. 2001; Jung and Masison 2001; Jung et al. 2002). Studies of other genes affecting the Hsp104-overproduction curing of [PSI+] have identified many other chaperones and nonchaperone components (Chernoff et al. 1999; Newnam et al. 1999; Allen et al. 2007; Reidy and Masison 2010; Kiktev et al. 2012). Curing of [URE3] by overproduced Ydj1p showed that Hsp40s could be involved in prion propagation (Moriyama et al. 2000). Curing of [URE3] by overproduced nucleotide exchange factors for Hsp70s (Kryndushkin and Wickner 2007) supported Masison's model of nucleotide regulation of the Hsp70 role in prion propagation (reviewed by Sharma and Masison [2009]). A general screen for factors whose overproduction cures [URE3] revealed the somewhat homologous Btn1p and Cur1p, two proteins that, at normal levels, cure most variants of the [URE3] prion arising (Kryndushkin et al. 2008; Wickner et al. 2014).

Transfection

Transfection of yeast cells with amyloid formed from recombinant prion proteins was viewed as a final proof of the prion model, and showed that amyloid was indeed the infectious material (King and Diaz-Avalos 2004; Tanaka et al. 2004; Brachmann et al. 2005). This approach has since become an important method for characterizing prion amyloids (Brachmann et al. 2006); see Protocol: **Prion Transfection of Yeast** (Edskes et al. 2015).

BIOCHEMICAL METHODS

The protease-resistance of Ure2p in extracts of [URE3]-carrying strains, like that of PrP in scrapie-infected tissues, provided the first biochemical evidence for the yeast prions (Masison and Wickner 1995). The rapid sedimentation of Sup35p in [PSI⁺] cells was the first evidence for aggregation as the mode of prion formation (Paushkin et al. 1996). Cold sodium dodecyl sulfate (SDS) solubilizes most cellular components, but not amyloid fibers, so agarose gels of cold SDS-treated extracts, the semi-denaturing detergent agarose gel electrophoresis method, have been widely used to characterize different prion variants, and even to screen for prions (Kryndushkin et al. 2003, 2013), and this method is detailed by its developer in an associated protocol (see Protocol: **Isolation and Analysis of Prion and Amyloid Aggregates from Yeast Cells** [Kryndushkin et al. 2015]).

CELL BIOLOGICAL METHODS

Most prions are aggregated forms of normally evenly dispersed soluble proteins. Prion proteins have a special domain that determines the prion properties of the protein, and constitutes the part that forms amyloid. Fusions of the full-length prion protein, or just the prion domain, with green fluorescent protein have been used in yeast to study prion behavior, and to document protein aggregation in vivo (Patino et al. 1996). Fluorescence recovery after photobleaching has been used to measure mobility of prion aggregates in vivo (Wu et al. 2006).

COMPUTATIONAL METHODS

The most successful method for predicting prion-forming ability is the PAPA program developed for Q/N-rich regions such as those that are the prion domains of most yeast prions (Toombs et al. 2012). The method is based solely on the amino acid composition of the domain in question because it is known that shuffling the sequence of the Ure2p or Sup35p prion domains does not adversely affect their ability to become prions (Ross et al. 2005).

PHYSICAL STUDIES OF YEAST PRION AMYLOIDS

Electron Microscopy

The filamentous nature of prion amyloids must be verified by electron microscopy. Their morphological properties are also important as they may differ with prion variants. Amyloid filaments can be uniform or heterogeneous with various widths and lengths. Filaments may remain solitary or laterally bundle and can have a variety of morphologies. Filaments may be of spiral appearance (or not), straight, curved, and helical. Infectious amyloid made from recombinant yeast prion proteins can be examined by making a suspension in water, placing 5 μ L on a carbon-coated grid (commercially available) for 2 min, wicking off the excess water, washing once with 5 μ L of water, applying 5 μ L of 2% uranyl acetate for 1 or 2 min, wicking off the excess, and allowing the grid to air-dry. The details of insertion of the grid into the microscope, focusing the beam, and collection of images are machine-specific.

Measurement of Filament Mass-per-Length

This is important for distinguishing different amyloid architectures (Paravastu et al. 2008). For example, a β helix has ≤ 0.5 molecules per 4.7 Å, while parallel in-register architectures may have 1 (for the yeast prion amyloids of Sup35p, Ure2p, or Rnq1p), 2 (for one kind of Abeta fiber), or 3 (for

another Abeta fiber variant). This is generally performed using a scanning transmission electron microscope (Wall and Hainfeld 1986), but can also be done using a transmission electron microscope in tilted beam (dark field) mode (Chen et al. 2009). Using thin-layer graphene as a sample support enables using lower beam intensities to preserve sample integrity and reduces background scattering for more accurate mass measurements (Jeon et al. 2013).

X-Ray Fiber Diffraction

This is important in establishing the cross- β structure (β strands oriented perpendicular to the fiber long axis) of amyloid filaments (Eanes and Glenner 1968; Stubbs 1999). The most difficult part of this method is orienting the amyloid filaments in the capillary tube used for the diffraction experiment. An alternative is electron diffraction using transmission electron microscopy and identifying a field of unstained filaments that are by chance largely bundled and oriented in one direction (Baxa et al. 2005; Shewmaker et al. 2009). The diffraction is measured for the aligned sample to determine repeated atomic spacing. Of course, even unoriented filaments can be used to show that there is a predominant β sheet secondary structure, by either X-ray or electron diffraction.

Hydrogen–Deuterium Exchange

The amide hydrogens of proteins will exchange with deuterium when the protein is placed in D_2O . In the amyloid form, the exchange rate is slowed, compared with the soluble form, by both isolation of a site from the solvent and by hydrogen bonds. Detection of exchange can be done by either nuclear magnetic resonance (NMR) or mass spectrometry. If the material is of homogeneous structure, the signal should show a single exponential decay for each amide hydrogen. As discussed below, heterogeneity of amyloid preparations is a problem in hydrogen–deuterium (HD) exchange experiments as in other physical studies.

Solid-State NMR

Because amyloids do not form crystals and are very large, neither X-ray crystallography nor solution NMR is ideal for examining their structure. However, solid-state NMR (ssNMR) is particularly suited for this problem, as illustrated by Tycko's work on structures of amyloids of Abeta peptide (Alzheimer's disease) and amylin (type II diabetes) (reviewed in Tycko 2006) and the determination of the structure of the prion domain amyloid of HET-s by Meier and coworkers (Ritter et al. 2005; Wasmer et al. 2008).

At least several milligrams of homogeneous amyloid are needed for ssNMR experiments. Proteins are labeled with ^{13}C and ^{15}N by chemical synthesis, or synthesis in *Escherichia coli* with labeled precursors. Chemical synthesis is preferable, but the prion domains of yeast prion proteins are too long for this procedure. Site-specific labeling in *E. coli* is limited to amino acids whose biosynthesis is efficiently repressed by the supplied labeled amino acid and whose metabolism does not result in shuffling of label: Leu, Ile, Val, Met, Tyr, Trp, Lys, and Phe have been used and Ala for short labeling times. Except for HET-s, which has only one prion variant in vivo, and forms a unique structure in vitro (Ritter et al. 2005; Wasmer et al. 2008), amyloid forming spontaneously in vitro from recombinant prion proteins is not generally homogeneous as shown by the spectrum of prion variants resulting from its transfection into yeast cells (e.g., Brachmann et al. 2005). Even filament preparations with a uniform appearance by electron microscopy (EM) may be a mixture of forms. Filaments of Sup35NM (the prion domain of Sup35p), which produce largely homogeneous "strong" or "weak" variants on transfection appear to be heterogeneous based on HD exchange data (Toyama et al. 2007) and may be a mixture of transmission variants (Bateman and Wickner 2013). Favoring unique amyloid structure, are seeding with filaments isolated from cells (King and Diaz-Avalos 2004), and certain filamentation conditions (Helsen and Glover 2012) including low monomer concentration and a high ratio of seeds-to-monomers so that self-seeding is not efficient (e.g., Kryndushkin et al.

2011). Nonetheless, the limited success of these approaches results in two-dimensional NMR experiments showing wide peaks that limit the possibility of complete structural determination.

The chemical shifts distinguish between helical, sheet, and random coil structures. If peaks can be assigned to specific residues, then this chemical shift data can be used to map out the secondary structure of specific amino acid residues.

Experiments to date indicate that the yeast prion amyloids have an in-register parallel folded β sheet architecture (reviewed in Tycko and Wickner [2013] and Wickner et al. [2013]). The evidence for this architecture comes from dipolar recoupling experiments that measure the distance from labeled atoms to the nearest neighbor labeled atom. Labeling specific carbonyl carbons in the prion domain of Sup35p, Ure2p, or Rnq1p with ^{13}C has generally resulted in a distance of $\sim 5 \text{ \AA}$, and the nearest neighbor has been shown to be generally on another molecule as expected for this architecture (Shewmaker et al. 2006; Baxa et al. 2007; Wickner et al. 2008). An accessible introduction to NMR is the book *Spin Dynamics* by Malcolm Levitt (2nd edition, 2008), Levitt (2008), and its application to amyloid structure is reviewed by Tycko (2011) and Tycko and Wickner (2013).

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