De novo generation of a PrP^{Sc}-like conformation in living cells

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Conformational conversion of the cellular PrP^{C} protein to PrP^{Sc} is a central aspect of the prion diseases, but how PrP initially converts to this conformation remains a mystery. Here we show that PrP expressed in the yeast cytoplasm, instead of the endoplasmic reticulum, acquires the characteristics of PrP^{Sc}, namely detergent insolubility and a distinct pattern of protease resistance. Neuroblastoma cells cultured under reducing, glycosylation-inhibiting conditions produce PrP with the same characteristics. We therefore describe what is, to our knowledge, the first conversion of full-length PrP in a heterologous system, show the importance of reducing and deglycosylation conditions in PrP conformational transitions, and suggest a model for initiating events in sporadic and inherited prion diseases.

The transmissible spongiform encephalopathies (TSEs) — the prion diseases—are a group of fatal neurodegenerative disorders that uniquely occur in transmissible, dominantly heritable and sporadic forms. The origin of the TSEs remains controversial, but it is universally accepted that a conformationally altered form of a normal cellular protein, PrP, is crucial to the infectivity of the disease agent (the prion)[1–3]. This conformer, PrP^{Sc}, is the most abundant component of infectious material[4]. Moreover, mice carrying deletions of PrP are immune to these diseases[5] and mutations in PrP are linked to heritable forms of the diseases[1].

According to the widely held protein-only prion hypothesis, PrP^{Sc} is itself the infectious agent. Specifically, PrP^{Sc} causes disease and creates new infectious material by interacting with the normal cellular form of PrP and promoting its conversion to the same pathogenic state[1–3,6]. Alternative hypotheses hold that the infectious agent also includes an as-yet-undiscovered nucleic acid, for which PrP^{Sc} serves as a receptor and/or packaging substance[2,7]. In either case, PrP mutations might promote disease by facilitating the conversion of PrP to PrP^{Sc}. How this might occur is unclear[1–3]. Furthermore, 90% of cases of the human Creutzfeldt–Jacob disease (CJD) and some Gerstmann-Sträussler-Scheinker syndrome (GSS) cases are sporadic: patients carry wild-type PrP genes and have had no known contact with contaminating sources[1]. How wild-type PrP^{C} might give rise to PrP^{Sc} in such circumstances also remains a mystery. Here, we describe conditions in two very different systems, the yeast *Saccharomyces cerevisiae* and murine neuroblastoma cells, that cause PrP to convert spontaneously, and with high efficiency, to a form with all the signature characteristics of PrP^{Sc}.

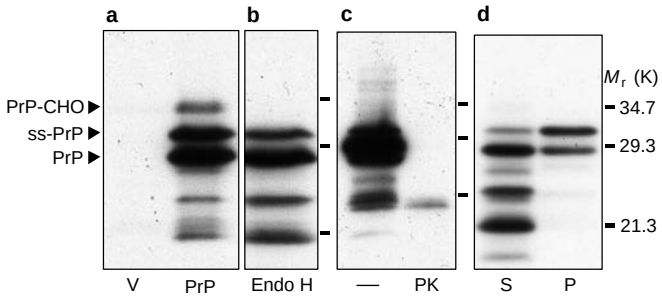

Figure 1 **Analysis of the Kar2–PrP fusion protein in yeast.** Proteins resolved by SDS–PAGE were probed with monoclonal antibody 3F4. Bars represent M_r standards. PrP-CHO, glycosylated PrP; ss-PrP, unglycosylated PrP with the signal peptide of the yeast protein Kar2 attached; PrP, unglycosylated PrP with the signal peptide cleaved. **a**, Proteins from cells carrying the vector alone (V) or the *KAR2–PrP* fusion gene on plasmid PrP-pDN (PrP). **b**, Endoglycosidase-H-treated proteins from cells with the *KAR2–PrP* fusion gene. **c**, Total cell lysates incubated without (–) or with proteinase K (PK). **d**, Supernatant (S) and pellet (P) fractions.

Results

Expression of PrP in the yeast secretory pathway. PrP^{C} is a cell surface glycoprotein with a glycophosphatidylinositol (GPI) anchor. The 22-amino-acid amino-terminal signal sequence is cleaved as the protein translocates into the endoplasmic reticulum (ER). The 23 carboxy-terminal amino acids are removed when the GPI anchor is added[1,2]. To express PrP in the yeast endoplasmic reticulum (ER), we fused the coding sequence of mouse PrP (amino acids 23–231, carrying the convenient 3F4 epitope from hamster PrP) to the signal-peptide sequence of Kar2, a yeast ER protein.

Several forms of PrP were detected in yeast lysates (Fig. 1a,b) before and after incubation with endoglycosidase H (to remove *N*-linked oligosaccharides added in the secretory pathway). The endoglycosidase-H-sensitive form of PrP (relative molecular mass ~33,000 (M_r ~33K)) represents PrP molecules that have entered the ER and been glycosylated (PrP-CHO). The endoglycosidase-H-resistant species (M_r 31K) is of the size expected for the uncleaved fusion protein (ss-PrP). It might represent proteins that translocated to the ER without removal of their signal sequences or proteins mistargeted to the cytoplasm that never entered the ER. The other major endoglycosidase-H-resistant species (M_r ~27K) corresponds to protein that translocated into the ER and had its signal peptide (M_r ~4K) removed, but is not glycosylated (PrP)[8,9].

Two hallmarks of PrP^{Sc} that distinguish it from PrP^{C} are the production of a specific protease-resistant fragment on digestion with proteinase K and insolubility in detergents[1–3]. When total cell lysates were digested with proteinase K, a small fraction of PrP was converted to 19–20K (Fig. 1c), the size of the specific digestion product of unglycosylated PrP^{Sc} (refs 8, 9). When yeast lysates were extracted with the detergent Sarkosyl and subjected to centrifugation, the ss-PrP species (with the signal sequence) was enriched in the pellet fraction (Fig. 1d). Furthermore, the pellet contained most of the 19–20K proteinase-K-resistant species (data not shown). These observations led us to suspect that a fraction of the PrP fusion protein might be cytoplasmic, either because of inefficient import to the ER or because of the retrograde transport of misfolded protein from the ER, and be converted to a PrP^{Sc}-like conformation in the cytoplasm.

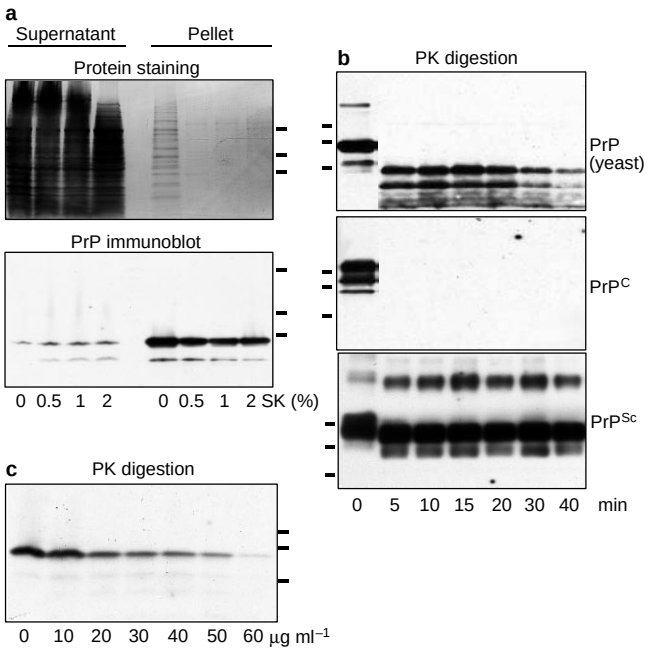

Figure 2 **Analysis of PrP proteins expressed in the yeast cytoplasm. a**, Solubility of PrP in Sarkosyl (SK; 0–2%). Supernatant and pellet fractions were resolved by SDS–PAGE and visualized by Coomassie staining or by immunoblotting with antibody 3F4. Bars represent M_r standards, 51.2K, 33.6K and 28.6K from top to bottom. **b**, Proteins from yeast and mammalian sources digested with proteinase K (PK) for the indicated times. Top, pellet fraction from yeast lysates prepared with 10% Sarkosyl. Middle, total cell lysates from fibroblast cells expressing wild-type hamster PrP in its normal cellular form, PrP^C. Bottom, PrP^{Sc} isolated from diseased hamster brains. **c**, Proteins from yeast cells expressing PrP from a single-copy vector were digested with proteinase K at the indicated concentrations at 37 °C for 1 h. For **b**, **c**, PrP protein was detected with antibody 3F4; bars represent M_r standards, 33.4K, 28.4K and 20.1K from top to bottom.

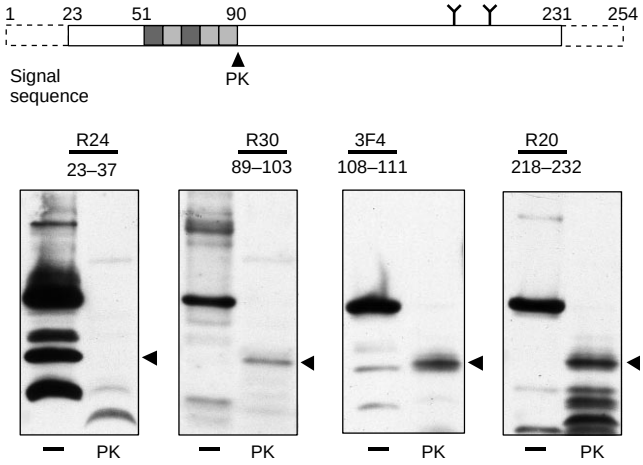

Figure 3 **Analysis of proteinase-K-resistant fragments from the yeast cytoplasm with epitope-specific antibodies.** Top, diagram of PrP, showing the positions recognized by antibodies R24, R30, 3F4 and R20. Dotted lines, sequences removed during the normal maturation of PrP and not present in the yeast cytoplasmic expression construct; shaded boxes, oligopeptide repeats; Y, glycosylation sites; arrowhead, approximate position of cleavage of Prp^{Sc} by proteinase K (PK). Bottom, pellet fractions prepared as in Fig. 2 without (–) and with (PK) digestion were resolved by SDS and visualized by immunoblotting with, from left to right, antibodies R24, R30, 3F4, and R20. The R24 blot was overexposed to confirm the absence of reactivity. Arrowheads represent the position of proteinase-K-resistant fragment.

PrP expressed in the yeast cytoplasm is insoluble and proteinase K resistant. To determine directly whether the cytoplasmic environment favours the spontaneous generation of this conformation, we expressed mouse PrP without a signal sequence (PrP(23–231)) in yeast. In this case, whereas most yeast proteins were recovered in the detergent-soluble fractions, most of the cytoplasmic PrP protein was recovered in the pellet fractions (Fig. 2a). Similar results were obtained with wild-type mouse PrP (without the 3F4 epitope) and with Syrian hamster PrP (data not shown).

PrP^{Sc} forms ordered aggregates that are resistant to solubilization with Sarkosyl at concentrations as high as 10% (ref. 10). When exposed to proteinase K, PrP shows an unusual pattern of protease digestion in which a specific portion of the N-terminal region is extremely sensitive to proteolytic digestion, and is quantitatively cleaved, while the rest of the molecule remains extremely resistant to further digestion[1,2]. To test whether aggregates of PrP expressed in the yeast cytoplasm exhibit similar properties, cell lysates were extracted with 10% Sarkosyl and subjected to differential centrifugation. Pellets were collected and digested with 50 μg ml^{-1} of proteinase K at 37 °C. To provide positive and negative controls, authentic PrP^{Sc} isolated from the brains of diseased hamsters and lysates of fibroblast cells expressing hamster PrP^C were digested with proteinase K under the same conditions.

Fibroblast PrP^C was completely digested by proteinase K after just 5 min (Fig. 2b, middle panel). Authentic PrP^{Sc} from hamster brain was quantitatively cleaved to a smaller species that then resisted further digestion (Fig. 2b, bottom panel). Similarly, the Sarkosyl-insoluble PrP protein isolated from the yeast cytoplasm (Fig. 2b, top panel) was quantitatively cleaved to a smaller species within 5 min. The primary product had an apparent molecular mass of ~19–20K. (A secondary species of ~12–14K was also present, but was variable with preparation.) These species were then very resistant to further digestion and could still be detected after 40 min at 37 °C (Fig. 2b). Similar results were obtained with wild-type mouse PrP (without the 3F4 epitope) and hamster PrP expressed in the yeast cytoplasm (data not shown).

The resistant fragment of PrP from yeast is the same as that from PrP^{Sc}. One explanation of these findings is that when PrP is expressed in the yeast cytoplasm, a significant portion of the protein is converted to a PrP^{Sc}-like conformation. The differences in the migration patterns of proteinase K digestion products from diseased hamster brains and from the yeast cytoplasm are consistent with the different glycosylation states of the proteins, as previously reported[1,8]. The somewhat greater sensitivity of the yeast-expressed PrP protein to proteolysis might reflect additional endogenous proteases in the yeast lysates and the absence of sulphydryl bonds. Alternatively, the yeast protein might form a different type of ordered aggregate that fortuitously yields a proteinase K cleavage product of the size expected for unglycosylated PrP^{Sc}.

To distinguish between these alternatives, we used four epitope-specific antibodies to determine the site of proteolytic cleavage (Fig. 3)[11,12]. PrP^{Sc} is cleaved somewhat heterogeneously within a short region ~90 residues from the N terminus[8,9,13]. The resistant product extends to the very end of the mature protein (amino acid 231). The 19–20K proteinase-K digestion product of PrP expressed in the yeast cytoplasm did not react with an antibody that recognizes amino acids 23–37 (R24), but did react with antibodies that recognize amino acids 89–103 (R30), 108–111(3F4), and 218–232 (R20) (Fig. 3). A broad band was detected with antibodies R20 and 3F4, and a much sharper band with antibody R30. These results show that the protein is cleaved in the same region as authentic PrP^{Sc}, with some cleavages interfering with recognition by R30 (refs 8, 9, 13).

PrP expressed in the yeast cytosol at lower concentrations forms a different aggregate. In transgenic mice expressing PrP at different levels, susceptibility to PrP^{Sc} increases with the concentration of the protein[14]. To determine whether conversion of PrP to the PrP^{Sc}-like

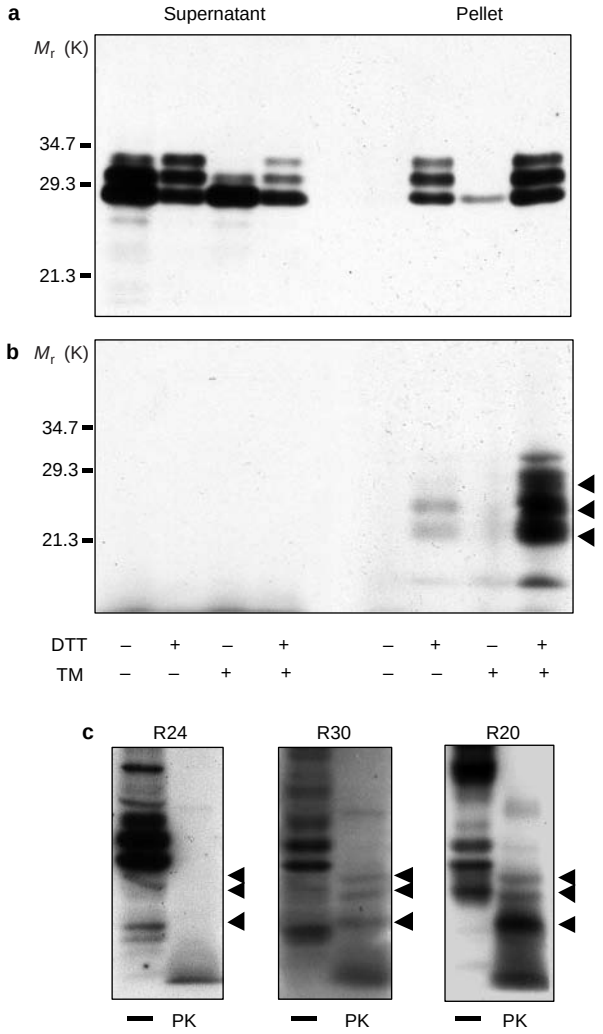

Figure 4 **Analysis of mouse PrP from neuroblastoma cells treated with DTT and tunicamycin. a**, **b**, Neuroblastoma cells were treated with DTT and tunicamycin (TM) as shown below. **a**, Differential centrifugation of cell lysates treated with 1% Sarkosyl. **b**, The same supernatant and pellet fractions digested with proteinase K. **c**, Pellet fractions prepared as from cells treated with DTT and tunicamycin, without (–) or with (PK) digestion by proteinase K, were reacted with epitope-specific antibodies. Arrowheads indicate the positions of prominent proteinase-K digestion products. PrP proteins were visualized by immunoblotting with monoclonal antibody 3F4 (**a**, **b**) or antibodies R24, R30, and R20 (**c**). The R24 blot was overexposed to confirm the absence of reactivity.

conformation in the yeast cytoplasm is also concentration dependent, we moved the construct to a single-copy vector. In this case, PrP formed smaller aggregates, which could be pelleted after centrifugation at 180,000*g* for 90 min in the presence of Sarkosyl. This PrP was very resistant to proteolytic digestion, compared with most of the yeast proteins in the lysate (Fig. 2c, and Coomassie staining, data not shown). However, it did not yield a 19–20K proteinase-K-resistant cleavage product. Rather, a variety of cleavage products were produced as the protein was digested over time. As the resulting fragments were themselves very sensitive to protease, these were only visible as multiple faint bands broadly distributed on the gel. In contrast, in reactions performed at the same time (data not shown), even at the very lowest concentration of proteinase K (10 μg ml^{-1}) all of the PrP protein produced from the high-copy vector was completely cleaved, with the primary product being a ~19–20K fragment. As in the experiment shown in Fig. 2b, this fragment was highly resistant to further digestion.

These results show that PrP can form distinct types of aggregate. Given the nonspecific nature of proteinase K, they further indicate that the aggregates that yield the specific 19–20K resistant product are highly ordered. Finally, they show that the cytoplasmic environment *per se* is not sufficient to produce the PrP^{Sc}-like form; it must be present at a high enough concentration. High concentration might be required to override inhibiting cellular factors. Alternatively, but not mutually exclusively, accumulation of the PrP^{Sc} conformer may require a high enough concentration of PrP to foster stabilizing interactions with other PrP molecules[11].

Conversion of PrP in neuroblastoma cells. In mammals, PrP^{C} is co-translationally translocated into the secretory pathway[1,2]. From the standpoint of protein folding, the two features of the secretory pathway that most strongly distinguish it from the cytoplasm are N-linked glycosylation and the oxidation of sulphydryl bonds. The conversion of mouse and hamster PrP to a PrP^{Sc}-like conformation in the yeast cytoplasm indicated that PrP^{C} protein in the secretory pathway of mammalian cells might be similarly affected if the environment were more reducing and glycosylation was inhibited. To test this possibility, we exposed Neuro 2A murine neuroblastoma cells expressing PrP to the reducing agent dithiothreitol (DTT) and/or tunicamycin. As expected for this cell line[15], three different forms of PrP (unglycosylated, mono- and di-glycosylated) were present in untreated controls (Fig. 4a). DTT alone increased glycosylation, presumably because it causes proteins to remain in the ER longer and/or in a conformation more readily subject to this modification[16]. Tunicamycin reduced the level of glycosylation.

All the PrP from untreated cells remained in the supernatant after Sarkosyl extraction. In contrast, a substantial fraction of PrP from cells treated with DTT or tunicamycin was in the pellet. The change in solubilization was strongest in cells treated with both DTT and tunicamycin (Fig. 4a).

After digestion with proteinase K, pellets of cells treated with both DTT and tunicamycin yielded three prominent products (Fig. 4b). Cells treated with DTT alone yielded smaller quantities of the same species. The sizes of proteinase K-resistant fragments were consistent with those reported for PrP^{Sc} in its three different glycosylation states[8,9], and they reacted with epitope-specific antibodies in the expected pattern (Fig. 4c). Neither the supernatant nor the pellet fractions of untreated cells produced such products (Fig. 4b). Indeed, no proteinase K-resistant products were detected in lysates of untreated cells incubated with just 0.2 μg ml^{-1} of proteinase K for 30 min and the transient cleavage products detected with even milder treatments were of different sizes (data not shown). These results suggest that blocking glycosylation and providing a reducing environment promotes conversion of PrP^{C} to a PrP^{Sc}-like form in mammalian cells.

We also note that pellet fractions and proteinase-K-resistant species from cells treated with both tunicamycin and DTT were enriched in glycosylated forms. Separate experiments confirmed that DTT does not influence the ability of tunicamycin to block glycosylation (data not shown). Thus, these data indicate that new, poorly glycosylated proteins synthesized in the presence of DTT and tunicamycin acquire the PrP^{Sc} conformation and can then interact with previously synthesized, glycosylated proteins to promote their conversion to PrP^{Sc}. This would stabilize the glycosylated forms of PrP and allow them to participate in further conversions[3].

Discussion

We have for the first time, to our knowledge, produced PrP in a PrP^{Sc}-like conformation from full-length protein in a heterologous system. The protein is insoluble in 10% Sarkosyl, extremely sensitive to proteolysis in a specific region, and extremely resistant to further digestion. Although proteolytic digestion provides only crude structural information, it is a very sensitive method of comparing structures. Indeed, even subtle conformational changes induced by ligand

and cofactor binding can readily be detected by this technique. In innumerable studies with human and animal brains, tissue-culture cells, recombinant proteins produced in *Escherichia coli*, and synthetic peptides, many 'aggregates' have been observed. Even in our experiments, a different 'nonspecific' type of aggregate was produced in the yeast cytoplasm when PrP was expressed from a low-copy vector. However, the properties of the aggregates produced with the high-copy vector have so far been observed only in association with TSEs[1–3]. Our results show that no mammalian viruses are required to achieve this conformation initially. Other experiments can now be conducted to determine whether protein in this conformation is sufficient to cause disease.

Our results also indicate a testable model for the aetiology of sporadic and heritable prion diseases. A recent revelation of protein trafficking studies is that proteins misfolded in the ER and secretory pathway are not degraded there. Rather, they are delivered by retrograde transport to the cytoplasm for deglycosylation and degradation. This has now been demonstrated in a wide range of organisms, with both mutant and wild-type proteins[17,18]. Once proteins are transported to the cytoplasm, aggregation competes with degradation and sometimes wins[17,19]. The efficiency with which PrP converts to an insoluble, PrP^{Sc}-like form in the cytoplasm therefore suggests that mistargeting of PrP and/or retrograde transport of misfolded PrP could facilitate the initial production of PrP^{Sc}. For sporadic disease, this might occur through spontaneous misfolding events, which might be increased by transient fluctuations in the glycosylation and oxidative potential of the cell. For inherited disease, PrP mutations might simply increase the likelihood of the initial misfolding events that initiate the quality control retrograde transport mechanism. This might also apply to the transmembrane forms of PrP recently associated with TSEs[20]. The strong concentration dependence we observed for conversion in the cytoplasm accords with the extreme rarity of sporadic disease and the late onset of inherited disease; several PrP molecules must reach this state in the same place at the same time to tip the balance towards accumulation of PrP^{Sc} (ref. 11). Otherwise the protein would be degraded or produce a nonspecific aggregate. After rare transient events initiate the production of PrP^{Sc}, propagation could then proceed, as previously postulated by the protein-only hypothesis (through autophagy, lysosomal transport, cell lysis and/or endocytosis), or as postulated by the virino hypothesis (through propagation of an occult nucleic acid).

Forms of PrP rich in β-sheet, another attribute of PrP^{Sc}, have been produced from a smaller fragment (amino acids 90–231) of recombinant PrP *in vitro*, although this protein was proteinase K sensitive[21,22]. More recently, a conformation rich in β-sheet and capable of forming fibrillar structures was produced from a similar small fragment (amino acids 91–231)[23]. Notably, in both of these *in vitro* studies, the β-rich forms of PrP were produced from unglycosylated recombinant protein under reducing conditions. Our work indicates that a reducing environment and the absence of glycosylation favour the conversion of full-length PrP in two very different situations, in the yeast cytoplasm and in the secretory pathway of neuroblastoma cells. The dramatic effects of glycosylation and oxidative–reductive balances on the conformational state of PrP in living cells, and the possible involvement of the cytoplasm, have important implications for our understanding of the disease and for the development of new therapeutic and prophylactic strategies. □

Methods

DNA constructs and yeast strain.

Mouse PrP(23–231) coding sequence generated by the polymerase chain reaction (PCR) was cloned into a yeast expression vector, pDN182, containing the *GAL1* promoter and the Kar2 signal-peptide sequence. PCR primers were 5′-aaaatcgataaaaagcggccaaagcctgga and 5′-ccctctagattatcagctggatcttctcccgtcgta. For the cytoplasmic expression construct, PCR-generated PrP(23–231) coding sequences were cloned into the yeast 2μ expression vectors (p2UGPD and YEpFAT4) under the control of a constitutive GPD promoter. To prevent degradation in the yeast cytoplasm, a serine was included at both the N and C termini of PrP(23–231). PCR primers were 5′-cccggatcctatatgtctaaaaagcggccaaagcctggagggt and 5′-gggccgcggttatcagctggatcttctcccgtcgtaataggcct. In all experiments described here, we used the wild-type strain W303 (MATa *ade2-1 can1-100 his3-12,16 leu2-3,112 trp1-1 ura3-1*).

Analysis of PrP aggregation.

Yeast spheroplasts, obtained by incubation with zymolyase for 30–120 min at 30 °C, were lysed in 1×TNE (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 2 mM EDTA) buffer with various concentrations of Sarkosyl. For separation of supernatant and pellet fractions, lysates were layered on a 5% sucrose cushion and subjected to centrifugation at 250,000*g* for 1 h (Fig. 1) or 16,000*g* for 30 min (Fig. 2). PrP protein was detected by immunoblot analysis.

Digestion by proteinase K.

All proteinase-K digestions were performed in 1×TNE buffer with 0.2% Sarkosyl at 37 °C. No protease inhibitors were included in the proteinase-K digestions described in Fig. 1, Fig. 4 (5 μg ml^{-1} proteinase K for 30 min) and Fig. 2 (50 μg ml^{-1} proteinase K). Protease inhibitors which do not interfere with proteinase K digestion (0.5× chymostatin, pepstatin A and aprotinin) were included in the proteinase-K digestion described in Fig. 3 (80 μg ml^{-1} proteinase K for 1 h), to reduce the activity of endogenous yeast proteases. Control experiments showed that these inhibitors did not interfere with proteinase-K digestion (data not shown). For digestion of PrP generated in the yeast cytoplasm, enriched fractions of specific protease-resistant fragment (pellets from 2,000*g* and 16,000*g*) were used in the proteinase-K digestion described here.

Analysis of PrP in murine neuroblastoma cells.

Neuro 2A murine neuroblastoma cells stably expressing mPrP with the 3F4 epitope were incubated with or without 3 mM DTT and/or 5 μg ml^{-1} tunicamycin at 37 °C for 18 h, then lysed in 1×TNE buffer with 0.2% Sarkosyl. Cell lysates were disrupted by 10 passages through 21G needles on ice, and precleared by centrifugation (Eppendorf) at 5,000 r.p.m. for 5 min. Lysates, with Sarkosyl concentration adjusted to 1%, were layered on a 5% sucrose cushion and centrifuged at 315,000*g* for 1 h at 4 °C. Supernatant proteins were precipitated by methanol and resuspended in SDS sample buffer. Pellets were resuspended in sample buffer by sonication. For proteinase-K digestion, pellets were resuspended in 1×TNE buffer with 0.2% Sarkosyl by sonication.

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