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Conversion of PrP to a Self-Perpetuating PrPSc-like Conformation in the Cytosol

Jiyan Ma and Susan Lindquist

Finally, alterations in PrP trafficking, such as that observed here with moPrP cells (*48–50*), can prevent toxic accumulation of PrP in the cytosol without compromising viability. This provides a potential therapeutic strategy for prion disease.

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Conversion of PrP to a Self-Perpetuating PrP^{Sc}-like Conformation in the Cytosol

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A rare conformation of the prion protein, PrP^{Sc}, is found only in mammals with transmissible prion diseases and represents either the infectious agent itself or a major component of it. The mechanism for initiating PrP^{Sc} formation is unknown. We report that PrP retrogradely transported out of the endoplasmic reticulum produced both amorphous aggregates and a PrP^{Sc}-like conformation in the cytosol. The distribution between these forms correlated with the rate of appearance in the cytosol. Once conversion to the PrP^{Sc}-like conformation occurred, it was sustained. Thus, PrP has an inherent capacity to promote its own conformational conversion in mammalian cells. These observations might explain the origin of PrP^{Sc}.

Changes in the trafficking and conformation of the mammalian prion protein (PrP) are associated with a group of fatal neurodegenerative diseases (*1*). Some PrP-associated diseases involve an infectious agent hypothesized to be an altered conformation of PrP known as PrP^{Sc}. PrP^{Sc} is thought to propagate by converting other PrP molecules to the same conformation (*1*). Alternatively, PrP^{Sc} may propagate by association with an as-yet-unidentified infectious agent (*2, 3*). In either case, conversion of PrP to PrP^{Sc} is a central event in the etiology of transmissible PrP-associated diseases, yet the mechanism that initiates conversion is a complete mystery.

When PrP is expressed in the cytosol of yeast cells, a fraction converts to a form with the biochemical properties of PrP^{Sc}. This might be an aberration of heterologous expression. Alternatively, some general features of the cytosol (e.g., chaperones, the reducing environment) may promote conversion (*4*). A natural route by which PrP enters the cytosol of mammalian cells is retrograde transport (*5, 6*), which delivers proteins that misfold during maturation in the endoplasmic reticulum (ER) to the cytosol for degradation by proteasomes (*7, 8*). When proteasome activity is blocked, PrP accumulates in the cytosol (*5*). If the number of PrP molecules delivered to the cytosol ever exceeds its quality-control capacity, some PrP might convert to a PrP^{Sc} form.

Spontaneous prion diseases are very rare. Even in individuals with the most virulent PrP mutations, several decades elapse before chance and circumstance produce conversion. We increased the likelihood of detecting conversion events by assessing the conformational state of the PrP that accumulated in the cytosol when proteasome activity was compromised (Fig. 1) (*9*). Several cell types were transfected with a wild-type mouse PrP gene (*10*), treated with one of three different proteasome inhibitors, lysed with detergents, and subjected to centrifugation (*10*).

An increase in PrP accumulation was

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observed with each proteasome inhibitor in each cell type (Fig. 1A) (9). PrP^C, the normal cellular form of PrP, is soluble in mild detergents. The PrP that accumulated after proteasome inhibition was mostly insoluble in detergent (Fig. 1A) and migrated with the 27-kD unglycosylated form of PrP that has had its NH$_2$- and COOH-terminal signal sequences removed by ER processing enzymes (5). This is the state of PrP that misfolds in the ER, is retrogradely transported, and accumulates in the cytosol with proteasome inhibition (5, 11). In all cells, immunofluorescent analysis (5, 9) confirmed that PrP had accumulated in the cytosol.

PrP^{Sc} is distinguished from other aggregated forms of PrP by an unusual pattern of protease resistance: The first ~90 amino acids remain very sensitive to proteinase K (PK) digestion, whereas the rest are extremely resistant (12). When detergent lysates of unfractionated cells were digested with PK (10), some yielded a major 21-kD PK-resistant fragment (Fig. 1B), the same size as the PK-resistant fragment of unglycosylated PrP^{Sc} (13). The size of this fragment, and the presence of both 3F4 (amino acids 108 to 111) and R20 [amino acids 218 to 232 (14)] epitopes (Fig. 1C), confirmed that it had the same distinctive cleavage pattern as PrP^{Sc} (12). Also, like PrP^{Sc}, its resistance to digestion was remarkable. The 21-kD PrP band remained after virtually all Coomassie-stainable material was digested away (9).

Surprisingly, the fraction of PrP that converted to this species varied greatly but reproducibly with even modest differences in conditions (Fig. 1B). For example, aliquots of cells treated with 5 μM epoxomicin yielded much more of the 21-kD PK-resistant fragment than did identical aliquots treated with 1 μM epoxomicin, even though both had accumulated similar quantities of aggregated PrP (compare the samples in Fig. 1B, left, with those immediately above them). Similarly, aliquots treated with 50 μM MG132 and 10 μM lactacystin (15) accumulated comparable levels of aggregated PrP, but the former consistently yielded much more of the 21-kD PK-resistant band (Fig. 1B; Fig. 1A, middle). These treatments did not kill COS cells or NIH 3T3 cells. In contrast, many N2A cells were killed by the treatments; however, death did not increase with conversion to the PrP^{Sc}-like form (9, 16).

Various models of prion formation suggest that conversion to a specific ordered, rather than an amorphous, aggregate requires a critical number of PrP molecules in a susceptible conformation interacting to form a nucleus (17–19). This would impose a strong concentration dependence on conversion.

Indeed, higher rates of conversion were associated with higher initial rates of accu-

mulation. In the first few hours, PrP accumulated more rapidly in cells treated with 5 μM epoxomicin than with 1 μM epoxomicin and accumulated more rapidly with MG132 than with lactacystin (Fig. 1D). (At later times, accumulation might be equalized by changes in synthesis or degradation.) Also, transiently transfected N2A cultures produced much less PrP than did stably transfected cultures, but they consistently yielded much more of the PrP^{Sc}-like PK-resistant band. (In Fig. 1, total-protein blots of stably transfected cells were exposed for one-tenth the time of transiently transfected blots, but PK digestion blots of stably transfected samples were exposed 10 times as long.) This large difference in

conversion efficiency presumably relates to the highly concentrated expression in a small number of cells that is characteristic of transient transfections; all stably transfected cells expressed the protein but at a lower level. Finally, a mutant, PrP^{D177N} (Asp^{177} → Asn), which causes heritable and transmissible forms of prion disease, exhibits a higher rate of misfolding in the ER and displays a higher rate of retrograde transport (5). In samples matched for transfection efficiency, cells expressing PrP^{D177N} produced more of the PrP^{Sc}-like form than did cells expressing wild-type PrP (Fig. 1E, last two lanes).

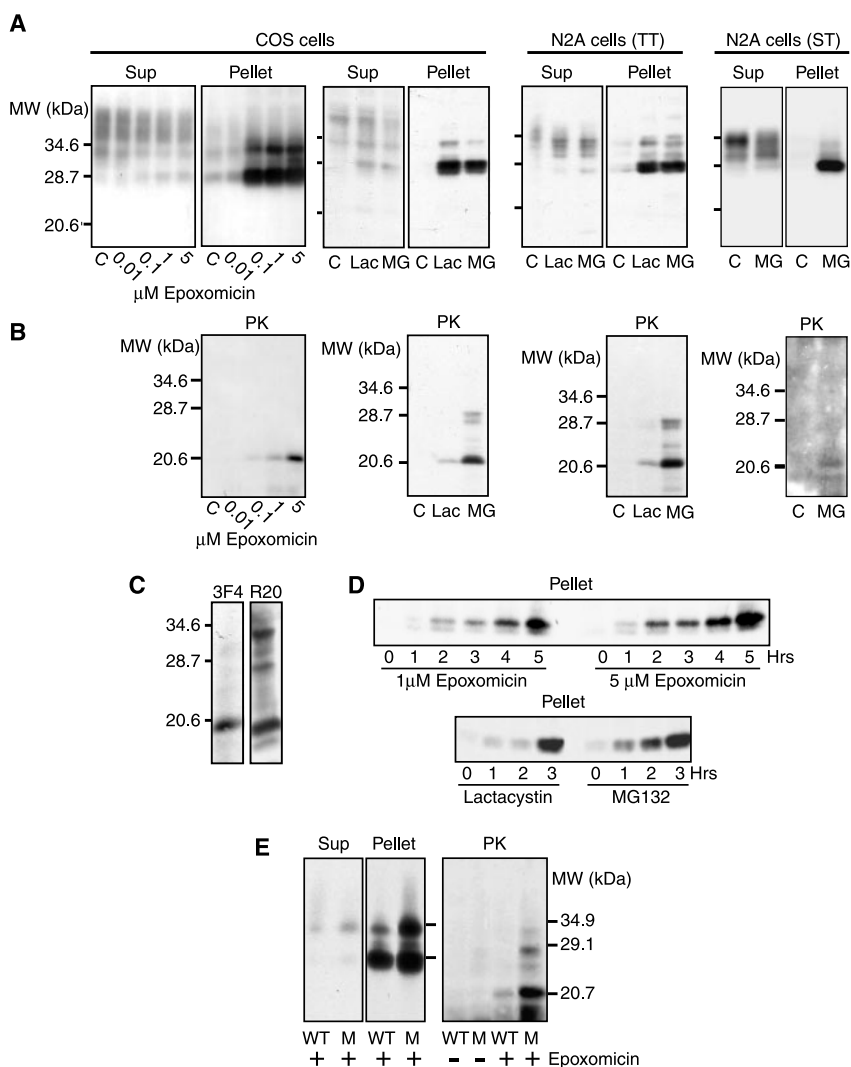The seminal characteristic of PrP^{Sc} is that, in some way, it promotes conversion of addi-

Fig. 1. The state of PrP in cells exposed to proteasome inhibitors. (**A**) Supernatant (Sup) and pellet fractions of detergent lysates from control cells (C) or cells treated with the proteasome inhibitors epoxomicin, MG132 (MG), or lactacystin (Lac) for 16 hours. TT, transient transfection; ST, stable transfection. (**B**) PK digestion (20 μg/ml for 30 min at 37°C) of total cell lysates from (A); corresponding panels in (A) and (B) are aligned. (**C**) PK-resistant PrP fragments reacted with antibodies to the 3F4 and R20 epitopes. (**D**) Time course of PrP accumulating in the pellet fraction of COS cells after proteasome inhibitor treatments. Two independent experiments are shown, each exposed to provide the best comparison of early samples within the experiment. (**E**) COS cells were transfected with wild-type PrP (WT) or the D177N mutant (M) and treated with 5 μM epoxomicin (10).

tional PrP to the same state (*1, 20*). To determine whether the PrP^Sc-like conformer that arises de novo after proteasome inhibition has this property, we investigated the possibility that conversion initiated by a transient loss of proteasome activity would be sustained after activity was restored. Transiently transfected COS cells were incubated with the reversible inhibitor MG132 for just 2 hours, rinsed, and cultured in media without the inhibitor for 21 hours. Use of a fluorogenic substrate, Z-Leu-Leu-Leu-AMC (Sigma), showed that proteasome activity was high in controls, undetectable after 2 hours of MG132 treatment, and restored to >70% of control levels 12 hours later (*9*).

Transient MG132 treatments (Fig. 2A) led to much greater accumulation of PrP than did continuous treatments (probably because longer treatments reduce protein synthesis). At the end of the incubation, the aggregated PrP exceeded the total quantity of PrP present initially, indicating that newly synthesized PrP continued to convert even after proteasome activity was restored. In a detailed time course, PrP had only begun to accumulate during the inhibitor treatment, and the 21-kD PrP^Sc-like PK digestion product was not yet detectable (Fig. 2B). But a process had been initiated that caused new PrP protein to continue misfolding. Only a fraction of the continuously accumulating PrP converted to the PrP^Sc-like form, whereas a substantial portion was in a form readily digested by PK. Thus, conversion is accompanied by the production of other misfolded forms.

The brief proteasome treatment did not cause general protein aggregation. No changes in fractionation were observed with β-actin, calreticulin (Fig. 2B), or Coomassie-stained total proteins (*9*). Moreover, a well-characterized proteasome substrate, the endogenous p53 protein (*21*), accumulated during proteasome treatment but disappeared as activity was restored.

Finally, we compared PrP with cystic fibrosis transmembrane conductance regulator (CFTR), another membrane protein that is subject to proteasome degradation and aggregates in response to proteasome inhibitors (*8, 22*). Immediately after proteasome inhibition, CFTR accumulated at moderate levels in an aggregated, detergent-insoluble state. No increase occurred during recovery (Fig. 2C). In contrast, detergent-insoluble PrP was barely detectable immediately after inhibition (Fig. 2C) but continued to accumulate after the inhibitor was removed.

We have demonstrated the de novo induction of a PrP^Sc-like conformation with a highly selective, self-perpetuating property in living cells. Because conversion can be induced by proteasome inhibitors in both neuronal and non-neuronal cells, even peripheral exposure might produce forms of PrP that could eventually spread to the brain. The strong concentration dependence we uncovered for conversion should make such events highly unlikely. However, caution should be taken

with proteasome inhibitors in research environments and in clinical applications.

The involvement of a natural, continuously occurring process (retrograde transport of misfolded PrP) in the conversion of PrP to a PrP^Sc-like state suggests a model to explain the rare spontaneous origin of PrP^Sc (fig. S1) (*10*). The flux of PrP into the cytosol will be influenced by trauma, toxins, aging, and disease-associated mutations in PrP (*5, 23*). In the cytosol, the chance of conversion to a PrP^Sc conformation is very small, but it increases with the rate at which PrP appears in that compartment. Differences in folding and retrograde transport could readily explain why some PrP mutations generate PrP^Sc and others do not (*24*). Once misfolding begins, it has a self-sustaining character, influencing more PrP proteins to adopt the same form. It might be infectious on its own or associate with a latent infectious agent. In either case, its unusual ability to promote its own misfolding likely con tributes to the progression and propagation of transmissible PrP diseases. Retrograde transport might be involved in the initial conversion event (*25–28*) and in generating toxic species (*29*), but need not be required for cell-to-cell propagation. Thus, the PrP^Sc that accumulates during the normal infectious cycle would include the oxidized and glycosylated species seen in transmissible PrP disease. PK-sensitive misfolded forms of PrP accumulate with the PrP^Sc-like form, which suggests that PrP^Sc may generally promote not only its own conversion but other toxic changes in PrP folding and metabolism.

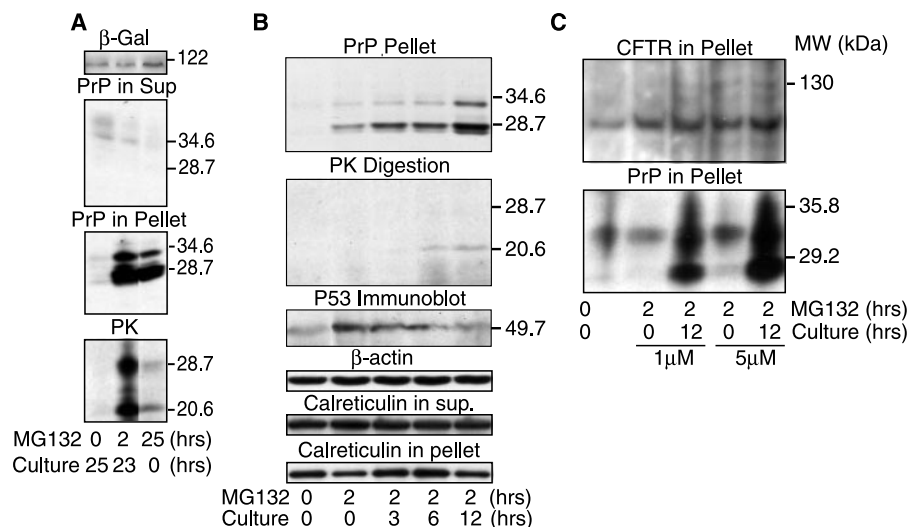**Fig. 2.** Transient proteasome inhibition is sufficient to initiate sustained PrP conversion. (**A** and **B**) COS cells expressing wild-type PrP were incubated with or without 50 μM MG132 for 2 hours. After washing, cells were cultured in normal media for the indicated times. (A) Immunoblot analysis of cotransfected β-galactosidase (top) confirmed equal levels of transfection. Cell lysates were fractionated by centrifugation (middle, supernatant and pellet fractions analyzed on same gel and exposed for same time) or digested with PK (bottom, exposed 5 times as long). (B) Identical aliquots of the same culture were incubated for the indicated times after the removal of MG132, lysed with detergents, and subjected to centrifugation or PK digestion. PrP, p53, β-actin, and calreticulin in the same samples were detected with specific antibodies. (**C**) COS cells cotransfected with PrP and CFTR. After transient treatment with dimethyl sulfoxide (left lane) or with 1 or 5 μM MG132, cells were collected immediately or after 12 hours of culture. PrP and CFTR in the pellet fractions were detected by antibodies to 3F4 or CFTR.

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17. There are two possible reasons for this phenomenon: (i) The nucleus is formed from a subset of specific

conformers, all of which must come together at the same time, or (ii) the nucleus is formed from still molten conformers that must be free to associate with each other and cannot be constrained by other interactions (e.g., bound to chaperones).

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Molecular Hydrogen as an Energy Source for *Helicobacter pylori*

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The gastric pathogen *Helicobacter pylori* is known to be able to use molecular hydrogen as a respiratory substrate when grown in the laboratory. We found that hydrogen is available in the gastric mucosa of mice and that its use greatly increased the stomach colonization by *H. pylori*. Hydrogenase activity in *H. pylori* is constitutive but increased fivefold upon incubation with hydrogen. Hydrogen concentrations measured in the stomachs of live mice were found to be 10 to 50 times as high as the *H. pylori* affinity for hydrogen. A hydrogenase mutant strain is much less efficient in its colonization of mice. Therefore, hydrogen present in animals as a consequence of normal colonic flora is an energy-yielding substrate that can facilitate the maintenance of a pathogenic bacterium.

The bacterial oxidation of molecular H_2 commonly occurs in nature, as hydrogen gas released by other bacteria represents a useable high-energy reductant (*1*). Once H_2 is bound and "split" by a membrane-associated hydrogenase, further oxidation-reduction and energy-generating steps are facilitated by a series of membrane-bound heme-containing electron carriers. Hydrogen is a by-product of colonic fermentation (*2*), and hydrogen has been reported to be produced (measured as excreted gas) in the gastrointestinal tract of both rodents (*3*) and humans (*4*). However, whether molecular hydrogen is used as an energy reservoir for pathogenic bacteria residing in animals is not known. To help understand the microbial communities associated with digestion, H_2 levels were determined in the termite hind-gut (*5*) and recently from the cockroach midgut (*6*), but H_2 levels in tissues of vertebrate animal hosts has not been assessed. *Helicobacter pylori* is a pathogen that solely colonizes the mucosal surfaces of the human stomach, where it gives rise to gastritis and peptic ulcers and is correlated with the development of certain types of gastric cancer (*7*). We previously reported that lab-grown *H. pylori* can express a membrane-bound "uptake-type" hydrogenase (*8*). H_2 use by *H. pylori* was accompanied by changes to other electron-carrying cell proteins that are related to energy-producing processes within cells to carry out a myriad of cell-building functions. Here we show that the mucous lining of the stomach contains ample amounts of molecular H_2. Combined with our measurements of the binding affinity of these bacteria for H_2, we conclude that hydrogenase is saturated with H_2 in the host tissues. A mutant *H. pylori* strain unable to oxidize hydrogen is severely impaired in its ability to colonize in mice. Therefore, H_2 availability and its use as an energy source is a formerly unrecognized factor in understanding how a human pathogen grows and persists in an animal host.

One hallmark of the energy-conserving uptake hydrogenases is the ability to respond positively to exogenously supplied hydrogen (*9*, *10*). Hydrogenase activity (*11*) in *H. pylori* is constitutive under all conditions we have tested, but in a chemically defined media (*12*) amperometrically determined hydrogenase activity (*13*) increases from a baseline value of 0.7 nmol H_2 oxidized/min/10^8 cells in cultures grown under micro-aerobic conditions (12% O_2, 5% CO_2, balance N_2) to 3.1 nmol H_2 oxidized/min/10^8 cells when supplemented with 10% H_2. A much milder stimulation of hydrogenase activity occurs when the cultures are grown in rich media or on blood-containing plates (BA plates) (*13*), in which hydrogenase activity is stimulated approximately twofold by the addition of 10% hydrogen (*14*). To characterize hydrogenase regulation, we used promoter fusions with the reporter gene *xylE* (*15*) from *Pseudomonas putida* to generate catechol 2,3-dioxygenase, which can be easily assayed spectrophotometrically (*13*). We assayed XylE activity in *H. pylori* strains carrying plasmids with hydrogenase structural gene promoter-*xylE* fusion (p*hyd:xylE*), a nonhydrogenase related promoter-*xylE* fusion (pHP0630:*xylE*), and a promoterless *xylE* gene (pHel:*xylE*). The results (Table 1) show that hydrogenase is regulated at the transcriptional level. The gene directly adjacent to hydrogenase (designated HP0630 and annotated as conserved; no known function in the sequenced strain 26695) (*16*) is not regulated by hydrogen,

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Table 1. XylE activities [expressed as XylE units/10^8 cells (*13*)] of *H. pylori* harboring *xylE* reporter plasmids grown under different growth conditions.

Growth condition	Plasmid		
	p*hyd:xylE*	pHP0630:*xylE*	pHel:*xylE*
− Hydrogen	1.6	9.1	<0.1
+ Hydrogen	6.6	10.4	<0.1