Prion disease tempo determined by host-dependent substrate reduction

Charles E. Mays, Chae Kim, Tracy Haldiman, Jacques van der Merwe, Agnes Lau, Jing Yang, Jennifer Grams, Michele A. Di Bari, Romolo Nonno, Glenn C. Telling, Qingzhong Kong, Jan Langeveld, Debbie McKenzie, David Westaway, and Jiri G. Safar

Introduction

Prions are transmissible agents responsible for incurable neurodegenerative diseases in humans and animals. Infection with prions is unique in that they may manifest after a lag phase that can exceed 5 decades from the time of exposure (1). The generation of infectivity from recombinant prion protein (PrP) (2) supports the “protein-only” hypothesis, whereby prion replication corresponds to a conformational transition from the host-encoded, cellular prion protein (PrPC) substrate into a misfolded prion protein (PrPSc), which is derived from its cellular precursor (PrPSc), as well as downregulation of the PrP-like Shaddo (Sho) glycoprotein. Given the overlapping cellular environments for PrPC and Sho, we inferred that PrPC levels might also be altered as part of a host response during prion infection. Using rodent models, we found that, in addition to changes in PrPSc glycosylation and proteolytic processing, net reductions in PrPC occur in a wide range of prion diseases, including sheep scrapie, human Creutzfeldt-Jakob disease, and cervid chronic wasting disease. The reduction in PrPC results in decreased prion replication, as measured by the protein misfolding cyclic amplification technique for generating PrPSc in vitro. While PrPC downregulation is not discernible in animals with unusually short incubation periods and high PrPC expression, slowly evolving prion infections exhibit downregulation of the PrPC substrate required for new PrPSc synthesis and as a receptor for pathogenic signaling. Our data reveal PrPC downregulation as a previously unappreciated element of disease pathogenesis that defines the extensive, presymptomatic period for many prion strains.

Results

PrPSc downregulation at end stage in scrapie, Creutzfeldt-Jakob disease, and chronic wasting disease models. The procedure to couple velocity gradient centrifugation with CDI (as explained in the Introduction) has been validated previously for samples derived from hamsters (5) and humans (15). To extend this approach to the analysis of mouse PrP, we recalibrated conditions for the use of the monoclonal antibody 12B2 (mouse PrP epitope at residues 88–92) (ref. 16 and Figure 1, A and B). We then applied the recalibrated technique to the analysis of mouse brain homogenates; these homogenates were derived from WT mouse brains harvested at clinical end stage after infection with the Rocky Mountain Laboratory (RML) iso-

Conflict of interest: The authors have declared that no conflict of interest exists.

Citation for this article: J Clin Invest. 2014;124(2):847–858. doi:10.1172/JCI72241.
Figure 1
Separation of PrP isoforms and prion infectivity by sedimentation velocity in sucrose gradient. (A) The gradient fractionation profile of PrPs present in uninfected brain homogenates of WT FVB mice compared to that of those infected with the RML scrapie isolate strain after separation by ultracentrifugation in sucrose gradient. Fractions collected from the bottom of the tubes were treated with PK or left untreated and analyzed by (A) Western blot using anti-PrP mAb 12B2 and (B) CDI. Protein marker mass is shown in kDa for Western blots, and the asterisk indicates cross-reactivity between secondary antibody and IgG light chain present in the brain homogenates. The concentration of PrPC (green), total PrP\textsuperscript{Sc} (brown), and rPrP\textsuperscript{Sc} (black) in fractions from triplicate samples was determined with CDI with Eu-labeled mAb 12B2 before or after PK treatment (14). (C) Densitometry analysis of the Western blot signal for Sho was estimated with ImageJ. (D) SSCA performed in L929 cells to estimate infectivity in gradient-fractionated RML prions. Data for uninfected animals appears on the left and that for RML-infected animals appears on the right. Data represent average ± SEM.
late of mouse-adapted scrapie prions. Our analyses demonstrated that 93% of the upper gradient fractions, fractions 9 and 10, are composed of PrPSc, while rPrPSc and sPrPSc make up the remaining 7% (Figure 1B). Since PrPSc is the predominant PrP isoform, we attempted to use the protein within these gradient fractions as a substrate in an in vitro assay to make PrPSc; this assay procedure is called protein misfolding cyclic amplification (PMCA) and is conceptually similar to PCR amplification of nucleic acids. However, proteins within the upper fractions of the velocity gradients were incompatible with our PMCA conditions and showed no detectable amplification, even though the levels of PrPc and concentrations of PrPSc after seeding with infected brain homogenate were comparable to the PMCA control experiment with nonfractionated material (Supplemental Figure 1; see complete unedited blots in the supplemental material; supplemental material available online with this article; doi:10.1172/JCI72241DS1). Nonetheless, the low infectivity of these fractions, as determined by the standard scrapie cell assay (SSCA; a cell monolayer assay to determine the infectious titer for prions), supports the notion that the major component of these upper fractions is indeed PrPc (Figure 1D).

In comparing RML prion-infected and noninfected animals, we discovered that total PrPc levels were decreased by 41% (P < 0.001) (Figure 1B). Interestingly, the PrP-like protein Sho was found in the same gradient fractions as PrPc, where it was also reduced in the RML-infected animals by approximately 48% of control values (P < 0.001) (Figure 1, A and C).

To determine whether PrPc downregulation is a broad-spectrum characteristic of prion infection, we applied similar analysis to end-stage brain material derived from WT mice infected with different strains of prions. Prion strains are microbiological isolates with distinct pathogenic properties that “breed true”; in molecular terms, they are thought to correspond to differently folded isoforms of PrPSc (17). For this purpose, we examined WT mice challenged with the 22L or 139A strain of scrapie prions, Syrian hamsters inoculated with the Sc237 scrapie prion strain, Tg cervid PrP (TgCe) mice (18) inoculated with chronic wasting disease (CWD) prions, Tg human PrP (TgHu) mice (19) challenged with a strain of human sporadic Creutzfeldt Jakob Disease (sCJD) prions, and bank voles inoculated with bank vole-adapted CWD prions. Our further analyses also included mice with a different genetic makeup. The most common allele of the mouse PrP gene (Prnp) is the Prnpa allele encoding PrPc-A. Here, we performed RML bioassays using mice that produce different levels of PrPc-A, which correspond to Tg mice expressing 0.53 times endogenous PrPc-A levels of WT mice (TgPrnp-A; AL mice) and TgPrnpa mice (a widely used Tg, commonly referred to as tga20) overexpressing PrPc-A by approximately 6-fold (20). Excluding TgPrnp-A mice and bank voles, marked decreases (P < 0.001) were noted in the net PrPc (primarily present in fractions 9 and 10 in infected brains; Supplemental Figures 1–6), and these decreases ranged from 31% ± 3.9% to 68% ± 8.4% (± SEM) (Figure 2). As described for analyses with the RML prion isolate, Sho protein colocalized in fractions 9 and 10 with PrPc in 22L- and 139A-infected brain material from WT mice at levels 42%–48% of the control values (22L strain, P < 0.001; 139A strain, P < 0.001) (Supplemental Figure 1C).

PrPc quality is altered by prion infection. For the 3 scrapie strains analyzed in WT mice (Figure 1A and Supplemental Figure 1A), we were surprised to discover that residual PrPc present in infected animals exhibited an altered glycosylation profile. Generally, diglycosylated forms were underrepresented, and there were increased levels of monoglycosylated and unglycosylated PrPc bands in fractions 9 and 10 (versus the abundance of diglycosylated, monoglycosylated, and unglycosylated protein found in disease-free animals; Figure 3A).

In the next experiment, we used gel analysis to examine the upper velocity gradient fractions (fractions 10, 9, and 8) from the 3 mouse-adapted scrapie prion strains. For this purpose, samples were normalized according to PrP immunoblot signal when electrophoresed alongside equivalent gradient fractions from uninfected control animals. Protein samples were examined with or without PNGaseF deglycosylation (Figure 3, B and C). Although low levels of proteinase K-resistant (PK-resistant) PrPSc (~8% of total rPrPSc and 2% of PrPc) were detected in each strain/host combination in the upper fractions of velocity gradients (Figure 1 and Supplemental Figure 1), unexpectedly high amounts of the PrPc2 carboxyl-terminal fragment colocalized in the upper gradient fractions with protease-sensitive PrPc (Figure 3, C and D). This is of interest because the C2 cleavage fragment is generally considered to be a rPrPSc product, not a PrPc product (21). Furthermore, comparison with the untreated samples indicates that this C2 fragment is primarily in its unglycosylated form (Figure 3B).

Preclinical PrPc downregulation in infected WT mice. As Sho downregulation occurs preclinically (10, 11), we next assessed a time course of WT mice challenged with RML prions or negative control inoculum. Levels of Sho and the different PrP species were analyzed, showing a coincident appearance of the disease-associated PrPc2 fragment and Sho reduction by 120 days after inoculation (dpi), a finding in line with previous reports (10) (Figure 4, A and B). Using velocity gradient centrifugation plus CDI analysis on the time course series, rPrPSc began to increase by day 30 and continued to accumulate throughout the disease course (Figure 5A), a trend that paralleled a determination of infectious titer by scrapie cell assay in L929 cells (Figure 4C). However, these data illustrated a downturn for the rate of PrPSc replication at 60 dpi (Figure 5A). A biphasic profile was seen for PrPc in the infected mice, with a marked decrease beginning 60 dpi (Figure 5B), suggesting an inverse relationship to the aforementioned rPrPSc levels. This effect was even more noticeable, as it diverged from the slow rise in PrPc measured in aging mock-inoculated controls (Figure 5B).

In the interval of 60 to 140 dpi, PrPc levels in infected mice fell by over 40%, from 1,200 to 700 ng/ml. To ascertain whether the preclinical drop in PrPc levels was merely attributable to depletion of PrPc substrate to make new PrPSc, we fit the data deriv-
PrPC downregulation as a common feature of prion disease. (A) Concentration of PrPC (green), total PrPSc (brown), and PK-resistant PrPSc (black) in brains of end-stage infected animals as follows: WT FVB mice challenged with RML, 22L, or 139A scrapie prions; RML-infected (black) in brains of end-stage infected animals as follows: WT FVB mice; RML-infected TgPrnpa mice overexpressing PrPC; Syrian hamsters (SHa) inoculated with Sc237 scrapie prions; TgCe “cervidized” mice inoculated with CWD prions; TgHu “humanized” mice challenged with human sCJD prions; and bank voles (Bv109l Prnp) inoculated with CWD prions. (B) Percentage reduction in PrPC relative to uninfected animals for data represented in A. Healthy mock-infected controls (0; white columns) were compared to inoculated animals (green columns). The columns and error bars represent average ± SEM measured in 3 brains, each sample tested in duplicate or triplicate by CDI. ***P < 0.001, **P < 0.01, determined by ANOVA.

Figure 2

PrPC downregulation in models with different levels of starting PrPC. Although prior studies have defined a profound effect of PrPC levels in healthy mice on disease incubation times subsequent to inoculation (24, 25), a downregulation effect was never considered in the interpretation of these studies. Consequently, we investigated prion infections in mice underexpressing or overexpressing PrPC.

To evaluate underexpression, we conducted a time course for RML prion-infected FVB/N hemizygous knockout (Prnp0+/+) mice and controls inoculated with normal brain homogenate. Hemizygous Prnp0+/+ mice are established as generating high levels of infectivity and PrPSc months before exhibiting symptoms of prion disease (26). To this effect, we observed that PrPSc began to accumulate in the brains of these animals between 60 and 100 dpi (Figure 5C). As in the case of WT animals, a parallel preclinical decrease in PrPC levels and PrPSc replication rate was observed in infected hemizygotes beginning at 100 dpi (nearly 300 days before the expected time in which they succumb to disease; ref. 26), and both parameters continued dropping throughout the time course, reaching levels approximately 40% lower than those of the controls (P < 0.001) (Figure 5, C and D). This was accompanied by a gradual decrease in Sho throughout the duration of the time course (Figure 5E). While this cohort of animals has yet to reach disease end point, the effects of underexpression later in the disease course were explored using the TgPmpn0+/+ AL mouse line made on a Prnp0+/+ background and expressing 53% of WT levels of PrP0−/− (Supplemental Figure 8). When measured in RML prion-infected mice at disease end point (400–430 dpi), the absolute PrPC levels in ng/ml lysate were downregulated to the lowest in our survey (Figure 2).

Uninfected adult TgPnp mice overexpress 6 to 7 times PrPC levels of WT mice and have incubation periods of 60 to 80 dpi (20, 27). By CDI, the highest PrPC levels in uninfected TgPnp mice (C57BL6 background) were approximately 3 times those seen in WT FVB/N mice (Figure 2A). Infected TgPnp mice and normal brain homogenate inoculated controls were investigated in a time course series (Supplemental Figure 9, A–C). TgPnp mice were also discovered to exhibit a biphasic PrPC profile, albeit with a smaller effect size (11%, measured between 45 and 60 dpi), that was not significant due to the interindividual variability (±12%) (Supplemental Figure 9B). Nonetheless, a downturn in rPrPSc replication rate was noted when comparing the same 2 time points (Supplemental Figure 9A), offering an analogous trend to the effect noted in WT and hemizygous Prnp0+/+ mice (Figure 5, A and C). Like PrPC, Sho levels were affected little during the disease process in these mice (Supplemental Figure 9C).
These data suggest that notable effect sizes for PrPC downregulation are associated with long prion incubation times and vice versa. By plotting the incubation times of RML prions in these 3 hosts (all with the same Prnpa genotype but expressing different levels of PrPC) versus terminal concentrations of PrPC and rPrPSc, we found that prion infection caused a highly significant shift in the slope of the linear regression between incubation time of the disease and end point levels of PrPC (Figure 6A and ref. 4). This shift in slope reflects a correlation for PrPC downregulation in the infected animals, with the largest difference in the data with or without infection occurring in the TgPrnpa-AL animals. A correlation was also apparent after plotting incubation time and the ratio between end point PrPC and rPrPSc (Figure 6B). Cumulatively, downregulation of PrPC was linked to the low expression levels of PrP C and high levels of the accumulated rPrPSc at disease end stage and is associated with prolonged incubation time (R = 0.99, P < 0.001). We next applied a similar plotting scheme to the spectrum of prion strains and disease models inoculated in our experiments. We found that this effect is broadly applicable despite independent variables that included 4 different Prnp genotypes, different starting PrPC levels, and 6 different strains of prions (Figure 6C; R = 0.77, P < 0.05).

Assessing PrP C downregulation in bank voles. Bank vole–adapted CWD prions produce the fastest prion disease in a naturally occurring animal host and hence are important in assessing the interplay between prion replication and disease pathogenesis (28). A time course series of samples was again investigated (Supplemental Figure 6). Unlike the other prion-infected specimens examined, PrPSc generated in the bank vole was primarily composed of oligomers sensitive to PK digestion (Supplemental Figure 6), a form that has been described as the most infectious PrP species (29). In this disease model, neither PrP C nor Sho levels differed between CWD- and mock-inoculated animals despite the low but continuous accumulation of rPrPSc observed in the brain 20 dpi (Supplemental Figure 9, E and F). However, a biphasic replication profile was again noted for rPrPSc, with a maximum increase from 20 to 25 dpi and steady decline during the final 5 days of disease (Supplemental Figure 9D). The short incubation period in CWD-infected bank voles may be related to the ability to rapidly generate PK-sensitive oligomers in the absence of Sho downregulation (Supplemental Figure 6 and Supplemental Figure 9E) (and PrPC downregulation) that may be typically triggered by rPrPSc (10, 11).
PrPC downregulation in cultured cells. We wanted to investigate whether preclinical PrPC downregulation observed in vivo is associated with PrPSc accumulation (as previously demonstrated for Sho; refs. 10, 11) or is a side effect of neurodegenerative change. We explored this possibility using monocultures of cells that lack pathological signs upon prion infection (30). First, we compared N2a neuroblastoma expressing mouse PrPC-A (N2a) (31) and rabbit RK13-Gag cells expressing elk PrPC (RK13 Elk21) (32) chronically infected with RML prions or elk CWD prions, respectively. In both cases, corresponding PrPC reductions were approximately 35% and 55% compared with noninfected controls (Figure 7, A and B). Next, to parallel in vivo time course experiments in the brain, we carried out de novo infections, monitoring RK13 cells stably expressing either mouse PrnpA or PrnpB for 5 weeks after treatment with RML-infected brain homogenate (Figure 7 and Supplemental Figure 10). Similar infections with mouse-adapted scrapie prions in vivo have demonstrated drastically different susceptibilities for mice with these genotypes, where mice with the Prnp genotype exhibit significantly shorter prion incubation periods than those with the Prnp genotype (33). In parallel, expression of PrP-A made RK13 cells highly susceptible to RML prion infection (Figure 7, C and D; Supplemental Figure 10A; and refs. 25, 34). In comparison to mock-inoculated cells, propagation of PrPSc resulted in a decrease in PrPC until 21 dpi, with levels then recovering to control levels by 35 dpi (Figure 7D). The converted rPrPSc represented 0.3%–0.5% of homologous PrPC expressed in these cells. In contrast, RK13 cells expressing PrPC-B were resistant to infection (Figure 7C and Supplemental Figure 10B). Since downregulation did not occur in cells expressing PrPC-B (Supplemental Figure 10C), the effect seen in Prnp-expressing cells is not a response against administration of the brain homogenate but is instead a consequence of a productive infection. Cumulatively, the cell data provide evidence that neither neuronal loss accompanying prion disease nor conversion of PrPC to PrPSc can account, per se, for the PrPC downregulatory effect.

Impact of PrPC downregulation on prion replication rate. Since prion replication rate decreased in tandem with PrPC downregulation in our time course studies, we investigated the relationship between these 2 events by simulating rPrPSc propagation during the prion infection process in vitro with PMCA (14, 35). First, we examined PMCA products for experiments seeded with increasing amounts of 139A prions in a constant amount of PrPC; here, PrPSc amplification was found to be a saturable component at PrP Sc seed concentrations of ≥1 ng/ml (Supplemental Figure 11A). This may be analogous to the decreased PrPSc replication rate at end point prion disease in the absence of PrPC downregulation, as described above for TgPrnp mice and bank voles. In experiments holding the RML PrPSc "seed" constant, dropping PrPC input in the range of 1,100 to 1,400 ng/ml (similar to the change in PrPC in early versus end stage disease in WT mice; Figure 5B) had a marked impact upon replication rate (Supplemental Figure 11B), thus indicating that PrPC is a limiting factor, as previously reported (36). To simulate conditions more precisely over the course of prion infection, RML PrPSc seeds and PrPC substrate were inversely titrated to test the impact on amplification. At PrPC concentrations below approximately 350 ng/ml and PrPSc seed concentrations of ≥1 ng/ml PrPSc, the replication rate was held below an amplification index of 20-fold, apparently due to saturation with an abundance of PrPSc (Figure 7E). However, at higher ng/ml values of PrPC, these two traces diverge and hence are associated with a concomitant increase in replication.
rate, up to a factor of 110-fold (Figure 7E). These inverse titration experiments were extended to include 139A mouse-adapted prions (Supplemental Figure 11C), human sCJD prions (Supplemental Figure 11D), and Syrian hamster Sc237 prions (Supplemental Figure 11E). In every case, a uniform biphasic response was observed for each set of PMCA reactions, in which the most efficient amplification occurred in conditions paralleling an early stage of infection, with a low concentration of PrPSc and a high concentration of PrPC. Therefore, our PMCA data indicate that rising PrPSc levels and falling PrPC levels will converge to suppress prion replication. Furthermore, these results point to an affect of PrPC downregulation beginning halfway through the incubation time interval in WT mice (Figure 5B).

Discussion

Two unaddressed factors for prion infection are their long incubation periods and observed discrepancies between PrPSc levels and clinical disease (4, 37). However, using capture and conformational antibodies to inventory the different PrP species in the brain (i.e., rPrPSc, sPrPSc, and PrPC), we have defined a new variable in disease pathogenesis, namely preclinical downregulation of PrPC. This downregulation does not emanate from changes in PrP mRNA abundance (12, 13, 38).

PrPC is indisputably required for the creation of PrPSc (2, 3, 24), but depletion by the law of mass action (i.e., a finite pool of PrPC “substrate” being consumed by a thermodynamically favorable conversion to PrPSc) is implausible as the rate of PrPSc synthesis in the brain outstrips the rate of formation of total PrPSc and rPrPSc by about 1 and 2 orders of magnitude, respectively (Table 1). It is also known from metabolic labeling of chronically infected cell cultures that only approximately 1% to 5% of PrPC converts into PrPSc (39, 40). Declining levels of PrPC in the course of prorcted prion infections were in most instances shadowed by a drop in endogenous mouse Sho (refs. 10, 11; Figures 1, 4, and 5; and Supplemental Figures 1 and 9), a cousin of PrPC that does not modulate creation of PrPSc and is not converted to a protease-resistant isoform (41). Depletion of PrPC as a simple consequence of apoptotic damage or neuronal cell death is unlikely, because PrPC downregulation occurred nearly 300 days before clinical disease in hemizygous Prnp0/0 mice and was absent in certain rapid models with an abundance of vacuolation at end stage disease (i.e., TgPrnp mice and bank voles; Figure 5D and Supplemental Figure 9, B and E). Moreover, PrPC downregulation was detected in cell culture models that are undergoing mitosis and have no synapses (Figure 7, A–D). The recovery phase noted 2 weeks later in these de novo infections may reflect selection pressures operating within the cultures of infected but mitotically active cells. From these observations, we suggest first that a host protective response is operating at the level of a cellular biological mechanism, presumably a form of proteostasis. Second, we suggest this response is triggered by the presence of rPrPSc and reduces net levels of PrPC and Sho by a “bystander” effect (Figure 8 and refs. 8, 10, 11). The reduction in net PrPC levels defined by our experiments stands in apparent contrast to a redistribution effect in persistently infected cells, in which cell surface PrP is reduced and immunohistochemical signal in a trans-Golgi network subcompartment is increased (42).

Thus far, we have not identified the tell-tale signs of a single canonical proteostatic pathway, such as eIF-2α phosphorylation (translation axis of the unfolded protein response) (43), LC3-II (autophagy), or ubiquitin (proteasome system) (Supplemental Figure 7). The largely parallel downregulation of PrPC and Sho indicates remodeling of a membrane microdomain that includes these proteins but does not include Thy-1.2 (Supplemental Figure 7A). However, the changes in the quality of PrPC (less diglycosylated, preponderance of C2 fragment) contrast with an across-the-board...
change that might be expected for a hypotheses of mass action depletion or cell death and were unexpected. The C2 aspect is especially notable as Watts and colleagues recently argued that Sho is degraded via an endocytic pathway that requires it to be physically trafficked with PrPSc (10). A component of this argument was the inverse correlation between Sho levels and the PrP C2 proteolytic fragment (10), with the latter hypothesized as a stable intermediate in the lysosomal degradation of PrPSc (44, 45). However, we have shown that models for other neurodegenerative diseases with activated lysosomes lack downregulation of Sho (11). Although our analyses of total cell lysates reaffirm a correlation between the reduction in Sho levels and the appearance of the protease-resistant PrP C2 proteolytic fragment toward disease end point (Figure 4A), our analyses of fractionated cell lysates demonstrated a surprising abundance of C2 colocalized in the upper gradient fractions. This suggests that protease-resistant C2 found toward disease end point derives from a protease-sensitive, and thus partly PrPC-like, precursor molecule. While the identity of an endoproteolytic “C2 PrPase” (like C1 PrPase; ref. 46) is unknown, progress in this area will help us to understand early events in PrPSc synthesis and early host responses. Another area of interest will be whether other parameters, such as a limited concentration of a cofactor (e.g., phosphatidylethanolamine; refs. 47, 48), can also contribute to a curtailment in the exponential phase for prion replication.

Prior studies have invoked a hypothetical toxic subform of PrP to explain the beneficial effects of a genetic reduction in PrPC levels that cause a delay between plateauing infectivity levels and the onset of clinical symptoms (4, 49). Here, an alternative explanation emerges from the observed changes with time in PrP isoforms. We show that the ratio between residual PrPC seen in end-stage disease and the amount of PrPSc is clearly correlated with the time lag to disease onset, and present experimental manipulations strongly suggest that this ratio in fact determines time lag. Within this formula, when PrPSc and infectivity reach a plateau (4), the extent of PrPC downregulation becomes the key variable. The difference between quickly and slowly evolving disease can be seen clearly in the different slopes shown in Figure 6A, and we therefore suggest that the slowly evolving infection of the central nervous system (most typical of natural prion disease) can be seen to arise from downregulation of the very same substrate required both for de novo PrPSc synthesis and for pathogenic signaling, as defined by grafting experiments (50) and by use of regulated PrPC expression (49). Interestingly, while the need to hypothesize a toxic subform of PrPSc may be less urgent, PrPC downregulation can be reconciled with the measurements that led some to posit a 2-phase model for prion disease (4) or a form of substrate depletion predicted from analysis of prion disease kinetics (51). Cumulatively, our data show that slowly evolving pathogenesis of preclinical disease is caused by depletion of the PrPC substrate that is required for PrPSc synthesis and toxic signaling. We infer that the natural phenomenon of PrPC downregulation is partially effective in holding prion disease in check in WT animals. Drugs that enhance this protective response to further reduce PrPC may have a potent beneficial effect in treating prion disease.

Methods

Mouse lines, prion bioassays, and brain homogenates. WT mice, hemizygous Prnp<sup>+/−</sup> mice, TgPrnp<sup>−/−</sup>-AL mice expressing 53% WT levels of PrP-A and made by standard procedures using the PrP half-genomic construct (ref. 20 and see Supplemental Figure 8), TgPrnp<sup>−/−</sup> mice maintained as homozygous stock (20), humanized Tg40 mice (HuPrP-129M) (19), and cervidized Tg33 mice (Deer PrP-96G) (18) were used in this study. Mice were inoculated at 3 to 6 weeks of age with 30 to 50 μl 1% w/v brain homogenate containing mouse-adapted scrapie (RML, 22L, or 139A),
Figure 7
PrPc downregulation examined in cell culture models and by PMCA. (A) Western blot analysis was used to show PK sensitivity of cell lysates derived from uninfected N2a, ScN2a (chronically infected with RML scrapie prions), uninfected RK13 Elk21−, and RKE21+ (chronically infected with elk CWD prions). (B) CDI was used to evaluate the relative downregulation of PrPc in these cell lines. (C) Western blot analysis was used to show PK sensitivity of RK13 cells stably expressing either PrPc-A or PrPc-B following exposure to RML prions. (D) CDI was applied to quantify the PrPc levels in RK13 cells stably expressing PrPc-A following treatment with healthy (square) or RML-infected brain material (circle). Normal RK13 cells treated with RML-infected brain material (box with circle) were used as a control. (E) PrPSc replication rate was evaluated using double PMCA titrations to assess the effect of decreasing PrPc input and titrated amounts of PrPSc “seed” from mouse-adapted RML prions. The columns and data points and error bars represent average ± SEM measured in triplicate. **P < 0.01, ***P < 0.001, determined by ANOVA.
MM1 sCJD (homozygous for methionine in polymorphic codon 129 of the PRNP gene and 21-kDa fragment [type 1] of unglycosylated rPrPSc on Western blots after PK treatment), or white-tailed deer CWD prions. Bank voles (Bv1091 Prnp) were inoculated at 8 weeks of age with 20 μl brain homogenate containing bank vole–adapted elk CWD prions. For analysis, 10% w/v homogenate was made in PBS by serial passing through needles.

Sucrose gradient and CDI. Sucrose gradient and CDI were performed as previously described (14). Briefly, the 400-μl aliquots of 10% brain homogenate in PBS, pH 7.4, containing 2% Sarkosyl were clarified by centrifugation at 30,000 g for 5 minutes and carefully layered onto the top of the 10% to 45% sucrose gradient prepared in PBS, pH 7.4, containing 1% Sarkosyl. Ultracentrifugation was performed at 237,000 g for 73 minutes at 5°C in an Optima TL ultracentrifuge (Beckman) equipped with a Beckman SW 55 Ti rotor. After the centrifugation, fractions were collected from the bottom of the tube.

The CDI assay was similar to previous descriptions (15, 52), albeit with several modifications. First, we used white Lumitrac 600 High-Binding Plates (E&K Scientific) coated with mAb 8H4 (epitope 175–185) (53) in several modifications. First, we used white Lumitrac 600 High-Binding Plates (E&K Scientific) coated with mAb 8H4 (epitope 175–185) (53) in several modifications. First, we used white Lumitrac 600 High-Binding Plates (E&K Scientific) coated with mAb 8H4 (epitope 175–185) (53) in several modifications. First, we used white Lumitrac 600 High-Binding Plates (E&K Scientific) coated with mAb 8H4 (epitope 175–185) (53) in several modifications.

Discussion), but this concept is omitted from the diagram for the sake of simplicity.

**Figure 8**

Summary of PrPSc and Sho downregulation in a prion-infected cell by an unidentified degradation pathway. (A) In a healthy cell, the balance of synthesis rate and degradation rate establish the steady-state level of PrPSc and Sho. (B) We assert that prion-infected cells are characterized by two processes. The first is the well-known biosynthetic conversion of PrPSc to PrPSc (top part of cell). We hypothesize a second process (bottom part of the cell) in which the accumulation of PrPSc triggers (open arrow) a proteostatic degradative response (Pac-Man shape). This in turn reduces the steady-state concentrations of PrPSc and Sho (indicated by smaller text) by a ‘bystander effect.’ Reduced PrPSc will have an impact on PrPSc synthesis (dotted green line). An additional effect of PrPSc reduction may be to attenuate toxic signaling (see the Discussion), but this concept is omitted from the diagram for the sake of simplicity.

The calibration and validation aspect of CDI has been reported on extensively by us and others (5, 14, 15, 56–62). Briefly, the PK-untreated sample containing PrP is divided into native and denatured aliquot, and the later is denatured with 4 M Gdn HCl for 5 minutes at 80°C. Using Eu-labeled mAb 3F4 or 12B2 for detection, the TRF signal of native sample corresponds to the epitope 107–112 that is exposed in α-helical PrPSc and hidden in PrPSc and is proportional to the concentration of PrPSc (5). The signal of denatured aliquot corresponds to the total PrP in a sample, and the concentration of PrPSc is then calculated according formula: [PrPSc] = [PrP] – [PrPSc], where D stands for denatured aliquot and N stands for native aliquot. Next, the concentration of rPrPSc is calculated in samples subjected to PK treatment followed by complete denaturation using PrP(90–231, 129M) calibration curve. The concentration of sPrPSc is calculated according formula: [sPrPSc] = [PrPSc] – [rPrPSc]. The separate calibration for the PK-treated and untreated sample is critical for correct results due to the approximately 3.5-fold lower affinity of mAb 3F4 with denatured full-length human PrP(23–231, 129M) compared to PrP(90–231, 129M) (14, 56).

SSCA. SSCA was performed as described previously (63), with some modifications. Briefly, 1,929 cells were exposed to varying concentrations of brain homogenates (0.1–0.0001% w/v) for 5 days in 96-well culture plates. The cells were passaged 3 times (1:4 and 1:7), with 20,000 cells collected at the third passage and loaded on to Multiscrifter HTS IP 96-well, 0.45-μm filter plates (Millipore). The cells were first subjected to PK digestion (5 μg/ml) followed by denaturation using 3 M guanidine thiocyanate. The Elispot reaction was performed using a mouse anti-PrP antibody (SAF83, 1:1,000) and a goat anti-mouse alkaline phosphatase–conjugated secondary antibody (1:5,000). The plates were developed using BCIP/NBT and analyzed using an Autoimmun Diagnostika GmbH Elispot plate reader (ELR07).

PMCA. Sonication-driven PMCA was performed as described previously (14, 64) with the following modifications. The PrPSc was replicated using brains of homologous species or Tg mice expressing homologous PrPSc (14). Brain homogenates from prion-infected animals were diluted as described in the specific experiments to attain final 10% brain homogenate, and 60 μl was transferred into 0.2-ml PCR tubes equipped with 2.38-mm diameter PTFE ball (K-mac Plastics). The buffer in all PMCA reactions was PBS, pH 7.4, containing final 1% Triton X-100, 500 mM NaCl, 0.1% Sarkosyl, and 1 mM of an antioxidant α-Tocopherol. Tubes were positioned on an adapter placed on the plate holder of a microsonicator (Misonix Model 3000).
and programmed to perform cycles of 60-minute incubation at 35°C, followed by a 30-second pulse of sonication set at 80% power. Samples were incubated, without shaking, and immersed in the water of the sonicator bath for 48 cycles. The 30 μl of the amplified materials was transferred to the next tube, which was prefilled with 30 μl substrate brain homogenate for the next round, and the remaining 30-μl aliquot was analyzed with CDI and Western blot analysis.

**Cell culture and infection with prions**

All RK13- and N2a-based cell lines were maintained in Dulbecco’s Modified Eagle’s Medium (Invitrogen) containing 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C with 5% CO2. To obtain RK13 cell lines stably expressing mouse Prnp and Prnp, RK13 cells were transfected with 1 to 5 μg of specified clones in pBud.GFP (65) at approximately 90% confluence with the homogenate as previously described (66).

**Western blotting**

20–60 μg protein of each BCA-quantified (Pierce) sample was (a) deglycosylated overnight at 37°C with 250 to 500 U PNGaseF (New England Biolabs) and resuspended in gel-loading buffer (0.6 M Tris, pH 6.8; 2% SDS; 7.5% glycerol; 0.005% bromophenol blue; 2.5% mercaptoethanol); (b) digested with 100 μg/ml PK for 1 hour at 37°C, inactivated with 1 mM phenylmethylsulfonylfluoride, and resuspended in gel-loading buffer; or (c) directly mixed with gel-loading buffer. Each was boiled for approximately 10 minutes and electrophoresed through 14% Tris–Glycine SDS-PAGE gels. Proteins were transferred onto a PVDF (Immobilon-FL, Millipore) membrane. Blots were incubated with anti- Sho 06Sh1, anti-PrP SHA31 (Medicorp Inc.), anti-PrP mAb 12B2 (16), anti-PrP mAbSAF38 (Cayman), anti-PrP mAb 2D6 (a generous gift from H. Rezai; ref. 67), anti-Thy1.2 (Ascab), anti-PSD-95 (Millipore), anti-NeuN (Millipore), anti- LC3 (Millipore), anti-Ubiquitin (Santa Cruz), and anti-Phospho-ELF2α (Cell Signaling Technology). ECL Plus reagents (Amersham Pharmacia) were used for visualization by exposure of blots to film or by scanning with an ImageQuant 300 (GE Healthcare). In some instances PNGaseF (NE Biolabs) was used per the manufacturer’s recommendations prior to blot analysis, and where appropriate, blots were reprobed with anti-actin primary antibody (Sigma-Aldrich) and anti-rabbit IgG secondary antibody conjugated to horseradish peroxidase. ImageJ software was utilized for densitometry analysis of the images (68).

**Statistics**

All the statistical analyses were performed using SPSS 19 software (SPSS Inc.), and P values were calculated using Anova. P values of less than 0.05 were considered significant and are specified in the legend for each figure.

**Study approval**

All protocols and experiments were approved by the IACUC of the Case Western Reserve University and University of Alberta.

**Acknowledgments**

We thank Vitolr Surewicz for recombinant PrP used for calibration in CDI, Earl Poppitz from Cleveland Clinic Hybridoma Core Facility for production of 8H4 mAb, and R. Kascak, H. Rezai, and S. Kar for providing antibodies. We thank F. Jirik (University of Calgary, Calgary, Alberta, Canada) and V. Sim (University of Alberta) for a breeding pair of Tgprnp mice. This work was supported by grants from NINDS (NS074317), CDC (UR8/CCU515004), the Charles S. Britton Fund, the Canada Foundation for Innovation, the Canadian Institutes of Health Research (MOP36377), the Alberta Prion Research Institute, and Alberta Innovates—Health Solutions for graduate and postgraduate funding for A. Lau and C.E. Mays and a Scientist award to D. Westaway.

Received for publication July 22, 2013, and accepted in revised form November 7, 2013.

Address correspondence to: David Westaway, University of Alberta, Centre for Prions and Protein Folding Diseases, 204 Brain and Aging Research Building, Edmonton, Alberta, Canada. Phone: 780.492.9024; Fax: 780.492.9352; E-mail: david.westaway@ualberta.ca. Or to: Jiri G. Safar, Case Western Reserve University, 11100 Euclid Ave., Cleveland, Ohio 44106, USA. 216.368.4609; Fax: 216.368.4090; E-mail: jiri.safar@case.edu.


