Protease-resistant PrP and Nonprotein Components of Prion Rods*

Reconstitution of Prion Infectivity from Solubilized Protease-resistant PrP and Nonprotein Components of Prion Rods*

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The scrapie isoform of the prion protein, PrPSc, is the only identified component of the infectious prion, an agent causing neurodegenerative diseases such as Creutzfeldt-Jakob disease and bovine spongiform encephalopathy. Following proteolysis, PrPSc is trimmed to a fragment designated PrP 27–30. Both PrPSc and PrP 27–30 molecules tend to aggregate and precipitate as amyloid rods when membranes from prion-infected brain are extracted with detergents. Although prion rods were also shown to contain lipids and sugar polymers, no physiological role has yet been attributed to these molecules. In this work, we show that prion infectivity can be reconstituted by combining Me2SO-solubilized PrP 27–30, which at best contained low prion infectivity, with nonprotein components of prion rods (heavy fraction after deproteination, originating from a scrapie-infected hamster brain), which did not present any infectivity. Whereas heparanase digestion of the heavy fraction after deproteination (originating from a scrapie-infected hamster brain), before its combination with solubilized PrP 27–30, considerably reduced the reconstitution of infectivity, preliminary results suggest that infectivity can be greatly increased by combining nonaggregated protease-resistant PrP with heparan sulfate, a known component of amyloid plaques in the brain. We submit that whereas PrP 27–30 is probably the obligatory template for the conversion of PrPSc to PrPSc, sulfated sugar polymers may play an important role in the pathogenesis of prion diseases.

PrPSc, the abnormal isoform of prion protein, is the only known component of the prion, an agent causing fatal neurodegenerative disorders such as bovine spongiform encephalopathy and Creutzfeldt-Jakob disease. It has been postulated that prion diseases propagate by the conversion of PrPSc molecules into protease-resistant and insoluble PrPSc molecules by a mechanism in which PrPSc serves as a template. Whereas some PrPSc may be insoluble in vivo, it is well documented that most PrPSc, as well as its protease-resistant core denominated PrP 27–30, precipitate into insoluble aggregates (also known as prion rods) when membranes from scrapie-infected brains are extracted with detergents such as sarkosyl. In addition to PrPSc, prion aggregates were shown to contain nonprotein components, which include sphingolipids as well as polysaccharides. The traces of nucleic acids present in prion rods are believed to be too small to function as coding tools. No physiological role has ever been attributed to any nonprotein components of prion rods.

Disruption of prion rods into detergent protein lipid complexes resulted in the retention of their protease resistance property concomitantly with an increase in their prion infectivity, suggesting that solubilized PrPSc is more infectious than the aggregated prion protein. Contrarily, disruption of prion rods by sonication and SDS resulted in a protease-sensitive PrP with complete loss of infectivity.

As opposed to methods to disrupt prion aggregates, we have recently introduced a new experimental procedure that results in the production of nonaggregated PrPSc or PrP 27–30 molecules by inhibition of the primary detergent-induced aggregation used for rod formation. When membranes from brains of hamsters terminally ill with scrapie were incubated in the presence of Me2SO in addition to sarkosyl and subsequently applied to a sucrose density gradient, the protease-resistant PrP molecules (PrP 27–30) were divided between the light fractions, containing soluble or poorly aggregated PrP 27–30 molecules, and the heaviest fractions, containing insoluble and heavily aggregated PrP 27–30 molecules. Interestingly, when light and heavy fractions of such gradients, containing similar concentrations of protease-resistant PrP, were inoculated into hamsters, the infectivity of the light fractions was lower by more than 2 logs than the infectivity of the heavy fractions. Light fractions produced in parallel in the absence of Me2SO, which did not contain any detectable PrP, presented the same low infectivity as the Me2SO light fractions, suggesting that this residual infectivity was not due to the presence of Me2SO-solubilized PrP 27–30 molecules. We attribute the low infectivity present in both light fractions to the fact that the brain extracts were applied to the sucrose gradient from the top, and therefore some small prion aggregates, containing undetectable PrP 27–30, may not have sedimented.

In this work, we investigated whether molecules other than protease-resistant PrP might have a physiological role in prion infectivity. To this effect, we combined the low infectious protease-resistant Me2SO-solubilized PrP described above with nonprotein components that remain in prion aggregates subsequent to denaturation and harsh protease digestion (NPHSc). In some experiments we substituted the NPHSc frac-

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1 The abbreviations used are: NPH, nonprotein heavy; NPHSc, NPH fraction originating from a scrapie-infected hamster brain; PK, proteinase K; l, light; H, heavy; GdnSCN, guanidium thiocyanate; HSc, heavy fraction after deproteination, originating from a normal hamster brain; LSc, light fraction from a scrapie-infected hamster brain; HScMe2SO, light fraction from a scrapie-infected hamster brain solubilized with Me2SO.
tion for heparan sulfate, a known component of prion rods. Our results show that the addition of the deproteinized sedimented fraction (NPHSc) to low infectious solubilized protease-resistant PrP restores the prion infectivity to its original values. We therefore propose that in addition to PrPSc, prion infectivity may depend upon, or at least be largely facilitated by, the presence of other components of prion rods.

**EXPERIMENTAL PROCEDURES**

**Sucrose Gradients**—Three hundred μl of 10, 15, 20, 25, 30, and 60% sucrose in phosphate-buffered saline were loaded into centrifuge tubes adapted for the TLS-55 rotor of the TL 100 ultracentrifuge (Beckman Instruments) to form a zonal gradient. Normal or scrapie brain membranes (25 μl containing 15 μg/ml protein) were diluted with STE buffer (100 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA) containing 2% sarkosyl to a final volume of 240 μl. When appropriate, Me2SO (10%) was added to the brain extract and incubated for 16 h at 4 °C before the extract was centrifuged (Beckman) at 55,000 rpm (loaded on top of the gradient and centrifuged in a TLS-55 tube) until the top fractions of each gradient were pooled and denominated fraction L. The three pooled bottom fractions (H) were tested either directly or after a deproteinization treatment that included the following: 1, denaturation with 4.5 M GndSCN (final concentration) for 15 min; 2, precipitation with methanol to wash out the GndSCN; 3, resuspension in 2% sarkosyl STE buffer before digestion with PK (100 μg/ml for 60 min at 37 °C) to form NPH fractions. When applicable, NPH samples were digested by heparanase (25 units/ml of heparinase III (Sigma) for 16 h at 37 °C). To form the combined fractions, original samples (L, NPH, or H) were mixed at equal volumes and incubated for 16 h at 4 °C. The HS concentration was 4 mg/ml. Original samples as well as combinations (detailed in Fig. 2) were assayed for the presence of PrPSc, infectivity, and structure by electron microscopy. NPHSc, NPH, sample digested with heparanase; L(N), light fraction from a normal hamster brain.

**Sample Preparation**—The three top fractions of each gradient were pooled and denominated fraction L (light). The three pooled bottom fractions (H, heavy) were tested either directly or after a deproteinization treatment that included the following: 1) denaturation with 4.5 M guanidium thiocyanate (GdnSCN) (final concentration) for 15 min; 2) precipitation with methanol to wash out the GdnSCN; 3) resuspension in 2% sarkosyl STE buffer before digestion with PK (100 μg/ml for 60 min at 37 °C) to form NPH (non-protein heavy) fractions. To form the combined fractions, original samples (L, NPH, or HS (heparan sulfate)) were mixed at equal volumes and incubated for 16 h at 4 °C. Original samples as well as combinations (detailed in Fig. 2) were assayed for the presence of PrP and infectivity. All volumes of original samples were adjusted before inoculation to contain the same concentration of L or NPH samples present in the mixtures.

**Heparan Sulfate**—400 μl of the LMe2SO sample were incubated with bovine kidney heparan sulfate (Sigma) for 16 h (4 mg/ml) at 25 °C. The resulting fraction was denominated HS/LMe2SO.

**Heparanase Digestion**—400 μl of the NPHsamples were precipitated in methanol and resuspended in STE buffer before the addition of 25 units/ml of heparinase III (Sigma) for 16 h at 37 °C. The resulting sample was denominated NPHH.

**In Vivo Infectivity Experiments**—Five male Syrian hamsters, 4 weeks old, were inoculated intracerebrally with 50 μl of each of the samples to be tested for prion infectivity. Animals were tested daily. Prion titers were measured by monitoring the incubation period until the appearance of symptoms (13). Before inoculation into hamsters, samples containing only L or H fractions were supplemented with 2% sarkosyl to retain similar concentrations of the components in the infectivity assay.

**Immunoblotting of Brain PrP**—10% (w/v) of brain tissue from...
Reconstitution of Prion Infectivity

In order to compare the study groups, ANOVA and the nonparametric Kruskal-Wallis test were applied. In addition, multiple pairwise comparisons were performed using the Dunnett and Scheffe methods. The tests were performed using the SPSS for Windows computer program. The sample abbreviations used are as described for Figs. 2 and 3.

### Table I

| Number of trials | Experiment 1 | Experiment 2 | Experiment 3 | Total animals | Incubation (mean ± S.E.) | Titer |
|------------------|--------------|--------------|--------------|---------------|--------------------------|-------|
| HSc              | 3            | 4 (87.5 ± 3.1) | 5 (96.6 ± 4.2) | 10 (83.9 ± 2.35) | 19 | 88.0 ± 2.1 | 6.7 |
| NPHN             | 3            | 5 (not sick)  | 5 (not sick)  | 5 (not sick)  | 15 | Not sick  | 0  |
| NPHN/Me2SO/HS    | 3            | 3 (117.7 ± 2.0) | 5 (120.6 ± 2.3) | 9 (111.4 ± 3.6) | 17 | 115.2 ± 2.2 | 4.0 |
| NPHN/Me2SO/LSc   | 3            | 5 (90.6 ± 3.1)  | 5 (94.6 ± 2.6)  | 5 (80.8 ± 0.9)  | 15 | 88.7 ± 2.0 | 6.6 |
| NPHN/Me2SO/LSc   | 1            | 5 (not sick)  | 5 (not sick)  | 5 (not sick)  | 6  | 114.0 ± 2.5 | 4.1 |
| NPHN/Me2SO/HS    | 3            | 4 (113.8 ± 1.6) | 4 (122.5 ± 1.2) | 4 (107.5 ± 1.7) | 12 | 114.6 ± 2.0 | 4.0 |
| NPHN/Me2SO/LSc   | 1            | 5 (112.0 ± 3.8) | 4 (120.0 ± 3.8) | 4 (120.0 ± 3.8) | 5  | 112.0 ± 3.8 | 4.2 |
| NPHN/Me2SO/HS    | 1            | 6 (89.5 ± 2.7)  | 6 (89.5 ± 2.7)  | 6 (89.5 ± 2.7)  | 6  | 89.8 ± 2.7  | 6.4 |
| NPHN/Me2SO/LSc   | 2            | 5 (95.3 ± 3.4)  | 5 (109.2 ± 3.3) | 5 (109.2 ± 3.3) | 10 | 102.1 ± 3.4 | 4.7 |

### Table II

**Significance of the variability among treatment groups (p values)**

Following ANOVA and the nonparametric Kruskal-Wallis test showing a significant variability between treatment groups, post hoc multiple pairwise comparisons were performed in order to define the significance (p values) of the variability between treatment groups. Significant differences (asterisks) were calculated with the Dunnett method. Numerical values (in this case representing no difference between the samples) were calculated using the method of Scheffe for multiple comparison.

**a** The mean difference is significant at the 0.001 level.
**b** The mean difference is significant at the 0.05 level.

scrapie-infected hamsters (frozen at −80°C following flash freezing in liquid nitrogen) was homogenized in cold sucrose buffer (10 mM Tris, 0.3 M sucrose in phosphate-buffered saline). 2% sarcosyl was added to 50-µl samples before digestion with 40 µg/ml PK for 60 min at 37°C.

**Statistical Analysis—**To compare the study groups, ANOVA and the nonparametric Kruskal-Wallis test were applied. In addition, multiple pairwise comparisons were performed using the Dunnett and Scheffe methods. The tests were performed using the SPSS for Windows computer program.

**RESULTS**

Brain membranes from scrapie-infected and uninfected hamsters were extracted with sarcosyl in the presence and absence of Me2SO and, following an overnight incubation, applied to a 10–60% sucrose gradient as described (11). Gradient fractions were digested with PK and immunoblotted with nPrP monoclonal antibody 3F4 (Fig. 1a). As can be seen in the figure, the light fraction prepared in the presence of Me2SO contained a clonal antibody 3F4 (Fig. 1).

Reconstitution of Prion Infectivity

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| NPHN/Me2SO/LSc   | 3            | 5 (90.6 ± 3.1)  | 5 (94.6 ± 2.6)  | 5 (80.8 ± 0.9)  | 15 | 88.7 ± 2.0 | 6.6 |
| NPHN/Me2SO/LSc   | 1            | 5 (not sick)  | 5 (not sick)  | 5 (not sick)  | 6  | 114.0 ± 2.5 | 4.1 |
| NPHN/Me2SO/HS    | 3            | 4 (113.8 ± 1.6) | 4 (122.5 ± 1.2) | 4 (107.5 ± 1.7) | 12 | 114.6 ± 2.0 | 4.0 |
| NPHN/Me2SO/LSc   | 1            | 5 (112.0 ± 3.8) | 4 (120.0 ± 3.8) | 4 (120.0 ± 3.8) | 5  | 112.0 ± 3.8 | 4.2 |
| NPHN/Me2SO/HS    | 1            | 6 (89.5 ± 2.7)  | 6 (89.5 ± 2.7)  | 6 (89.5 ± 2.7)  | 6  | 89.8 ± 2.7  | 6.4 |
| NPHN/Me2SO/LSc   | 2            | 5 (95.3 ± 3.4)  | 5 (109.2 ± 3.3) | 5 (109.2 ± 3.3) | 10 | 102.1 ± 3.4 | 4.7 |

μg/ml PK at 37°C for 60 min. Part of each H fraction was totally denatured with 4.5 M GdnSCN and, after methanol precipitation, digested again with PK to produce the NPH fraction. Subsequently, L and NPH fractions from different sources (normal and scrapie-infected with and without Me2SO) were used to create the combined samples specified in Fig. 2.

All samples to be evaluated for infectivity were precipitated by methanol (to remove traces of Me2SO) and resuspended into inoculation buffer (1% bovine serum albumin in phosphate-buffered saline) to contain PrP27–30 at comparable concentrations (Fig. 1b). Part of each H fraction was totally denatured with 4.5 M GdnSCN and, after methanol precipitation, digested again with PK to produce the NPH fraction. Subsequently, L and NPH fractions from different sources (normal and scrapie-infected with and without Me2SO) were used to create the combined samples specified in Fig. 2.

All samples to be evaluated for infectivity were precipitated by methanol (to remove traces of Me2SO) and resuspended into inoculation buffer (1% bovine serum albumin in phosphate-buffered saline) to contain PrP27–30 at comparable concentrations (Fig. 1b). A new prion strain. Although it was repeatedly shown that manipulations performed in this work did not produce a new prion strain. Although incubation times for the different samples varied widely (see Table I), the concentration of PrP27–30, as well as the banding pattern of the protein, was the same regardless of the inocula administered to the hamsters. Histoblot analysis of all brains were also identical (data not shown). This suggests that the manipulations performed in this work did not produce a new prion strain. Although it was repeatedly shown that different strains of prions can be characterized by these parameters (14–18), end point titration analyses are required to prove this point conclusively.

All samples described in Fig. 2 (original and combined) were bioassayed for prion infectivity (Fig. 4 and Tables I and II).
Table I presents the individual and accumulative results of three infectivity experiments performed by intracerebral inoculation of Syrian hamsters with the samples described in Fig. 2. Table II presents the significance of the variability among the treatment groups (p values). Although p < 0.05 (*) is considered significant enough in this kind of test, we also noted the extremely significant comparisons where p was smaller than 0.001 (**). p values of 1 or close to 1 suggest similarity between samples. The disease incubation times for animals inoculated with similarly prepared samples in the different experiments were pooled in the general calculations because no statistically significant difference was found between them. We also calculated the titers (log ID$_{50}$) from the median of disease incubation times as described (13). However, because the accuracy of titers calculated from disease incubation times (in days) is a debatable issue, we based all the statistical analyses directly on the disease incubation times. A graphic representation of the results can be seen in Fig. 4.

Whereas very high infectivity was present in the HSc fraction, no infectivity whatsoever was observed when this fraction was first denatured with 4.5 M GdnSCN and then digested with PK, resulting in the NPHSc fraction. These results indicate that the NPHSc fraction cannot convert in vivo PrP$_C$ to PrP$_{Sc}$, because even after a long incubation time (more than 300 days), no animals inoculated with these samples present any disease symptoms. Moreover, no traces of PrP 27–30 were observed in their brains even after 300 days (Fig. 3a), suggesting that no subclinical infection was established in these animals. Preliminary experiments (data not shown) also suggest that NPHSc cannot convert PrP$_C$ to PrP$_{Sc}$ in vitro, because the brain inoculation of combined fractions containing NPHSc and light fractions from normal hamsters without PK digestion (containing large quantities of PrP$_C$) did not result in any disease symptoms or PrP$_{Sc}$ accumulation after more than 300 days.

As shown here and in our previous work (11), whereas samples HSc and LMe2SO contained similar concentrations of PrP 27–30, the aggregated fraction, HSc, presented high prion infectivity. This can be seen by comparing the titers, incubation time, and p values for both samples (Tables I and II). However, when the noninfectious NPHSc was incubated with the LMe2SO fraction to form the NPHSc/LMe2SO, infectivity was restored to the levels observed in the HSc samples. This was not the case for the NPHSc/LSc combination, suggesting that the presence of nonaggregated PrP 27–30 in the NPHSc was essential for the restoration of prion infectivity.

No increase in infectivity was detected when LMe2SO$_{Sc}$ was combined with NPH$_{N}$. This may imply that a putative second prion component is not present in normal brain. Although it may be so, it is more probable that such a second component needs to combine with protease-resistant PrP in a specific fashion to sediment into the heavy fractions of the sucrose gradient in significant quantities.

One of the candidate molecules for a prion second component is HS. This sugar polymer was found in brain amyloid deposits of Alzheimer’s disease as well as of prion diseases (19). In addition, sulfated sugars seem to have an important role in the metabolism of PrP$_{Sc}$ (20). Molecules such as pentosan sulfate and low molecular weight heparin have been shown repeatedly to inhibit the production of PrP$_{Sc}$ in scrapie-infected neuroblastoma cells (ScN2a cells). HS itself has been shown to either increase or reduce PrP$_{Sc}$ accumulation in these cells, depending on the experimental setup (21–23). These molecules have also been shown to inhibit prion disease pathogenesis in vivo (24–26). This suggests that sulfated sugars of specific size and properties may either help form prions or disrupt prion formation, probably depending on some kind of competition mechanism.

To test whether one of the NPH$_{Sc}$ components is an HS-like molecule, we digested the NPH$_{Sc}$ fraction with heparanase in two of the reconstitution experiments, prior to its combination with the LMe2SO$_{Sc}$ fraction. In the third experiment, we substituted HS for NPH$_{Sc}$. As can be seen in Fig. 4, the combined results of these three experiments suggest a role for HS in prion infectivity. Whereas the infectivity of the reconstituted sample containing heparanase-digested NPH$_{Sc}$ (NPH$_{Sc}$/LMe2SO$_{Sc}$) was higher than that of the light fractions presenting low infectivity, such as L$_{Sc}$ or LMe2SO$_{Sc}$, it was significantly lower.
than the infectivity of both the HSc and the NPHSc/LMe2SO fractions that present high infectivity (see Table II for p values). In addition, the HSc/LMe2SO combined sample showed a considerably higher infectivity than the LMe2SO sample alone. In addition, the clinical features of the disease as well as the neuropathology of the animals infected with HSc/LMe2SO were identical to those of the classical 263 strain in Syrian hamsters (data not shown and Ref. 27), suggesting that the HSc/LMe2SO sample did not contain a new prion strain.

Extensive experiments are required to establish whether the effect of HS is unique or whether other sugar polymers, sulfated or not, may serve as the backbone of prion rods.

DISCUSSION

The results presented here indicate that production of prion infectivity requires the presence both of protease-resistant PrP and of nonprotein components of prion rods and suggest that these components may well be sulfated sugar polymers. It remains to be established whether sulfated sugar polymers are indeed a fundamental component of prions or whether their function, although not essential, greatly facilitates prion propagation and the establishment of prion infection.

Unsuccessful attempts to dissociate and reconstitute prion infectivity were performed years ago, even before PrPsc was identified as a necessary component for infectivity (28). Prion infectivity could not yet be associated with protease-resistant PrP molecules produced by an array of in vitro conversion protocols (29–31) and has even been suggested to exist in the absence of detectable protease-resistant PrP in the inocula (32). In view of our results presented here, which suggest both prion components are required, we suggest that prion infectivity can be transmitted by a few (and therefore undetected) molecules of PrPsc, if associated with the appropriate nonprotein components. Our results also open the way to a new line of in vitro conversion experiments, which may hopefully result in full in vitro production of prion infectivity.

Would the defense of the experiment be a template in the PrPsc to PrPsc conversion stands on solid grounds (33), the pathological role of a putative second component is unclear. A possible role for any functional molecule present in the NPHsc fractions may be to anchor the PrPsc molecules associated with it to the appropriate target in the host. Without a polymer such as HS, most PrPsc molecules may be cleared from the brain before the PrPsc to PrPsc conversion reaction has been established in enough cells to establish a process of infection (34). The kind of sugar polymer used as rod backbone and anchor may play a role in modifying parameters of prion infectivity.

The fact that sulfated sugar polymers such as HS may have a crucial function in prion structure and propagation suggests several plausible explanations of the fact that small sulfated sugar polymers such as pentosan sulfate were shown to inhibit the production of PrPsc in cells (21, 22). These molecules may compete with the sugar polymer functioning as prion component for the right cell targets to which the template PrPsc molecules should be docked. Otherwise, these molecules may compete with the prion sugar component for the binding of newly formed PrPsc molecules. Interestingly, polyamines were shown recently to inhibit PrPsc accumulation from ScN2a cells (35). We suggest that these highly positively charged polymers may bind to the highly negatively charged sulfated sugars and thereby facilitate the clearance of newly formed and still non-aggregated protease-resistant PrP. The results presented here therefore provide an explanation of the fact that such disparate molecules as small sulfated sugar polymers and positively charged polymers may both prove effective in the treatment of prion diseases.

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