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Scrapie Infectivity Correlates with Converting Activity, Protease Resistance, and Aggregation of Scrapie-Associated Prion Protein in Guanidine Denaturation Studies

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Denaturation studies with guanidine HCl (GdnHCl) were performed to test the relationship between scrapie infectivity and properties of scrapie-associated prion protein (PrP^{Sc}). Large GdnHCl-induced reductions in infectivity were associated with the irreversible elimination of both the proteinase K resistance and apparent self-propagating converting activity of PrP^{Sc}. In intermediate GdnHCl concentrations that stimulate converting activity and partially disaggregate PrP^{Sc}, both scrapie infectivity and converting activity were associated with residual partially protease-resistant multimers of PrP^{Sc}.

The nature of the infectious agent of the transmissible spongiform encephalopathies (TSEs) or prion protein (PrP) diseases remains unclear. There is evidence suggesting that the agent is partly or entirely composed of an abnormal, protease-resistant form of prion protein (PrP-res) (reviewed in reference 25). It has been postulated that if the infectious TSE agent is composed only of protein, it might propagate itself by inducing conversion of a normal host-encoded protein to the infectious form (7, 9, 11, 20). We have recently reported evidence that the scrapie-associated form of PrP-res (PrP^{Sc}) has self-propagating activity (converting activity) in that it can induce in vitro conversion of the normal, protease-sensitive isoform of PrP (PrP-sen) into a protease-resistant form that appears to be indistinguishable from brain-derived PrP-res (13). Striking species specificity has been observed in this cell-free conversion reaction, providing a possible molecular basis by which PrP itself could mediate species barrier effects in transmission of TSE agents (15). The propagation of TSE strain-specific properties of PrP-res has also been documented in this same cell-free conversion reaction, suggesting that the self-propagation of PrP-res variants with distinct three-dimensional structures could be the molecular basis of TSE strains (1).

Recent observations indicate that the converting activity depends upon the aggregation state and conformation of PrP^{Sc} (4, 13, 14). Pretreatment of PrP^{Sc} with >3.5 M guanidine HCl (GdnHCl), a chaotropic salt which affects the physical properties of PrP-res preparations (21, 24), eliminates PrP^{Sc}'s proteinase K (PK) resistance and converting activity (4, 13, 14). In contrast, pretreatment of PrP^{Sc} with intermediate concentrations of GdnHCl (2.5 to 3.0 M) can enhance the converting activity of PrP^{Sc}. Under the latter conditions, converting activity is associated with partially PK-resistant aggregates of PrP^{Sc} that can be separated by centrifugation from PK-sensitive, GdnHCl-disaggregated forms of PrP that can copurify with PrP^{Sc} preparations from brain tissue (4). The fact that converting activity is associated only with PK-resistant PrP^{Sc} aggregates under these and all of the other conditions analyzed so far is consistent with the idea that the primary mechanism of PrP-res formation is nucleated polymerization. This model holds that efficient conversion of PrP-sen to PrP-res requires an ordered oligomeric nucleus or seed (7, 9, 11, 16). Considering the possibility that PrP-res is the infectious agent which depends on the converting activity for its self-propagation, we investigated whether the effects of GdnHCl on the converting activity, PK resistance, and aggregation of PrP^{Sc} also affect scrapie infectivity.

A pilot experiment indicated that the scrapie infectivity associated with PrP^{Sc} isolated from 263K scrapie-infected hamsters was not significantly affected by 3.0 M GdnHCl pretreatment but was reduced ~10,000-fold by pretreatment with 6 M GdnHCl (data not shown). Furthermore, analysis of the same samples for cell-free converting activity revealed that, consistent with our previous results (13), cell-free converting activity was retained in the 3 M GdnHCl-treated samples but eliminated by the 6 M GdnHCl pretreatment (data not shown).

Based on these initial observations, we undertook a more detailed analysis of the effects of GdnHCl on scrapie infectivity, cell-free converting activity, and the PK resistance of PrP^{Sc} (Tables 1 and 2 and Fig. 1 and 2). PrP^{Sc} (1.4 μg/μl) was incubated in several concentrations of GdnHCl in 0.12% sulfobetaine 3-14–1.2 mM sodium phosphate (pH 7.2)–16 mM NaCl for 1 h at 37°C. Aliquots were diluted 10-fold in phosphate-buffered balanced salts solution with 1% fetal bovine serum and incubated for 1 to 3 h. The samples were then further diluted by the amount designated in Table 1 and, within 5 min, inoculated intracerebrally into Syrian golden hamsters for endpoint dilution bioassay. Fifty percent lethal doses (LD_{50}) were estimated from the number of scrapie-afflicted animals versus the number of unaffected animals at each serial 10-fold dilution (6). Table 1 shows that the infectivity level was not adversely affected by treatment with 2.5 or 3.0 M GdnHCl. In contrast, 4.0 M GdnHCl treatment caused an ~500-fold reduction in scrapie infectivity.

Another set of aliquots of the same GdnHCl-treated PrP^{Sc} samples, each containing 2.8 μg of PrP^{Sc}, were assayed in duplicate for cell-free converting activity essentially as described previously, by using [^{35}S]methionine-labelled glycophosphatidylinositol-minus hamster PrP-sen (4). Although the con-

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TABLE 1. Endpoint dilution bioassay for scrapie infectivity in GdnHCl-treated PrP^{Sc} samples

GdnHCl concn (M) used for pretreatment (fraction)	No. of diseased hamsters/total (avg incubation time [days] ± SD)[a]							Log LD_{50} ± SE[c]
	10^{-3}[b]	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	
0		6/6 (104 ± 7)	6/6 (117 ± 15)	5/6 (132 ± 12)	3/6 (175 ± 46)	1/6 (137)	0/5	7.0 ± 0.3
2.5		6/6 (105 ± 9)	6/6 (109 ± 4)	5/6 (125 ± 7)	6/6 (169 ± 31)	2/4 (151 ± 6)		7.8 ± 0.3
2.5 (supernatant)	6/6 (183 ± 39)	1/5 (207)	0/3	2/3 (182 ± 5)	0/3	0/4		4.4 ± 1.0
2.5 (pellet)		4/4 (107 ± 3)	5/5 (115 ± 4)	5/5 (122 ± 10)	4/5 (146 ± 27)	0/5		7.3 ± 0.2
3.0		5/6 (116 ± 5)	5/6 (150 ± 29)	5/6 (199 ± 40)	4/4 (223 ± 25)	0/5	0/3	7.0 ± 0.3
4.0	6/6 (172 ± 27)	5/6 (193 ± 33)	0/3					4.3 ± 0.2

[a] Although each of the designated sample dilutions was originally inoculated intracerebrally into five or six Syrian golden hamsters, intercurrent nonscrapie deaths caused the loss of some of the hamsters. Incubation times are from the time of inoculation to sacrifice, when the animals were no longer able to nourish themselves following a clinical diagnosis of scrapie. The average incubation period was calculated by using only those animals that became clinically ill within ~250 days of inoculation.

[b] Dilution of sample prior to inoculation into hamsters.

[c] The LD_{50} is the dose that would kill 50% of the animals inoculated. Log_{10} LD_{50} were calculated from the numbers of diseased animals divided by the total number of animals inoculated at each dilution by using the method of Spearman and Karber (6).

verting activity was stimulated 1.5- to 3-fold relative to the control by the 2.5 and 3.0 M GdnHCl pretreatments, it was reduced below the level of detection by 4.0 M GdnHCl (Fig. 1 and Table 2). Thus, converting activity was detected in all of the samples with $\geq 10^7$ LD_{50} but was not detected in the 4.0 M GdnHCl-treated samples containing ~500-fold less infectivity. Clearly, the infectivity bioassay is more sensitive than the converting activity assay because infectivity can be readily detected in 10^7-fold dilutions of the control samples (Table 1), but the lower limit of detection of the converting activity would be reached by an ~10^2-fold dilution (data not shown). Nonetheless, these results indicate that the major losses in both scrapie infectivity and converting activity occurred when the concentration of GdnHCl in the pretreatment step was increased from 3 to 4 M.

Aliquots of the PrP^{Sc} samples used in the infectivity test described in Table 1 were also tested for resistance to 50 μg of PK per ml in the original concentration of GdnHCl in the pretreatment step or after dilution to 0.8 M GdnHCl and incubation for 18 h at 37°C to allow refolding as described previously (14) (Fig. 2 and Table 2). Immunoblot analysis of PK-digested samples indicated that the 2.5 and 3.0 M GdnHCl treatments reduced the PK resistance of the monoclonal antibody 3F4 epitope (2, 12) of PrP^{Sc}, but this reduction was reversed upon dilution of the GdnHCl. In contrast, the 4.0 M GdnHCl treatment eliminated the PK resistance of the 3F4 epitope and this effect was irreversible. We have shown in other studies that the reversible unfolding of PrP^{Sc} induced by 2.5 to 3.0 M GdnHCl is only partial; an ~3-kDa N-terminal region containing the 3F4 epitope becomes PK sensitive, but a C-terminal domain (~16 kDa in the aglycosyl form) remains resistant to PK (14). In 4 M GdnHCl, virtually the whole PrP^{Sc} molecule is PK sensitive and does not recover PK resistance upon dilution of the GdnHCl. When the region containing the 3F4 epitope is able to refold, it occurs within minutes of dilution of the GdnHCl (14). Therefore, we assume that there was ample time for any such refolding of PrP-res to occur both in the aliquots that were diluted and incubated for 18 h prior to PK treatment (Fig. 2) and in the aliquots that were diluted and incubated for 1 to 3 h prior to the hamster infectivity bioassay (Tables 1 and 2). Thus, we conclude that the large GdnHCl-induced loss of scrapie infectivity in 4.0 M GdnHCl was accompanied by an irreversible, rather than reversible, loss of the PK resistance of PrP^{Sc}.

In 2.5 M GdnHCl, approximately half of the PrP in our typical PrP^{Sc} preparation is disaggregated in a PK-sensitive form, but converting activity is associated only with the remaining partially PK-resistant PrP^{Sc} aggregates that can be separated from the soluble PrP by centrifugation (4, 14). To test whether scrapie infectivity is also associated with PrP^{Sc} aggregates rather than GdnHCl-solubilized PrP, a 20-μl aliquot of the PrP^{Sc} suspension in 2.5 M GdnHCl was layered over a 40-μl layer of 5% sucrose and centrifuged at 259,000 × *g* for 30 min in a Beckman TLS 55 rotor. The upper 20-μl sample layer was drawn off as the supernatant fraction. The next 30 μl of the sucrose layer was drawn off the top and discarded. The residual liquid was brought up to 20 μl and sonicated for 2 to 4 s in a cuphorn probe to resuspend the pellet. Aliquots of the pellet and supernatant fractions were assayed for scrapie infectivity, converting activity, and PK resistance of PrP^{Sc} as described above. Although both fractions contained similar total amounts of PrP (Fig. 2, lanes 4 and 5), ~1,000-fold more infectivity and all of the detectable converting activity and PK-resistant PrP were recovered in the pellet fraction (Table 1 and Fig. 1 and 2). These results indicated that scrapie infectivity, converting activity, and the PK resistance of PrP^{Sc} were associated with PrP multimers or aggregates that pelleted at 259,000 × *g*.

In summary, we have shown that at concentrations of GdnHCl between 3.0 and 4.0 M there are coincident large-scale (orders of magnitude) losses in scrapie infectivity, converting activity, and the PK resistance of PrP^{Sc}. It is difficult to assess how smaller-scale (severalfold) changes in converting activity, e.g., those associated with the 2.5 and 3.0 M GdnHCl treatments, relate to changes in infectivity because of the inaccuracy of the

TABLE 2. Comparison of effects of GdnHCl pretreatment on scrapie infectivity, cell-free converting activity, and PK resistance of PrP^{Sc} after GdnHCl dilution

GdnHCl concn (M) used for pretreatment (fraction)	Scrapie infectivity[a]	Converting activity (avg % conversion ± SD)[b]	PK resistance of PrP-res[c]
0	1.0×10^7	8.5 ± 3.5	+
2.5	6.3×10^7	25.0 ± 2.8	+
2.5 (supernatant)	2.5×10^4	—	−
2.5 (pellet)	2.0×10^7	7.5 ± 0.7	+
3.0	1.0×10^7	13.0 ± 4.2	+
4.0	2.0×10^4	—	−

[a] Recalculated in scientific notation from log LD_{50} data in Table 1.

[b] Calculated from PhosphorImager analysis of data in Fig. 1. —, below the level of detection.

[c] Summarized from Fig. 2. +, PK resistance detected after dilution of GdnHCl to allow renaturation of PrP-res. −, PK resistance was below the level of detection.

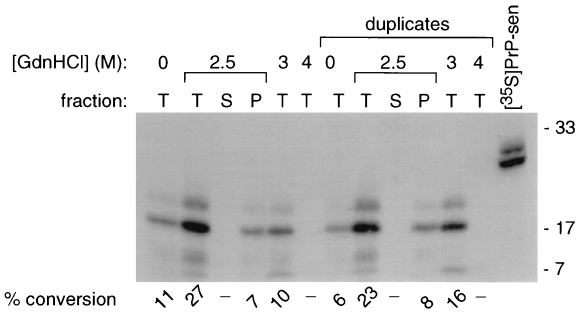

FIG. 1. Cell-free conversion reactions using PrP-res pretreated with various concentrations of GdnHCl. Duplicate aliquots were taken from the total (T), supernatant (S), and pellet (P) fractions of the same pretreated PrP-res samples that were assayed for scrapie infectivity (Table 1). The aliquots were diluted, incubated for 1 day with ^{35}S-labelled PrP-sen, treated with PK, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described previously (4). Residual ^{35}S-labelled PrP bands were detected by PhosphorImager analysis of dried gels. The lane farthest to the right shows one-third of the ^{35}S-labelled PrP-sen used in each conversion reaction without PK treatment. PhosphorImager analysis of all bands of ≥17 kDa was used to quantitate the percentage of the total ^{35}S-labelled PrP-sen converted to PK-resistant forms as designated at the bottom. The positions of molecular mass markers (in kilodaltons) are shown at the right.

infectivity bioassay; as indicated in Table 1, the infectivity estimates can have standard errors of ± 1 $\log_{10}$. We have also shown that in 2.5 M GdnHCl, scrapie infectivity, converting activity, and the PK resistance of PrP^{Sc} are associated predominantly with the forms of PrP in the pellet rather than with those in the supernatant fractions from a 259,000 × *g* centrifugation. Taken together, these results are consistent with the concept that scrapie infectivity is functionally associated with an abnormal form of PrP that is multimeric, PK resistant, and capable of inducing the conversion of normal PrP to the PK-resistant form.

The fact that Gdn-HCl concentrations that disrupt the PK resistance and converting activity of PrP-res also diminish scrapie infectivity is consistent with previous reports that losses in the structural and conformational integrity of PrP-res correlate with losses in scrapie infectivity and vice versa (e.g., see references 8, 18, and 22). Thus, infectivity and the structure of scrapie-associated PrP that gives it PK resistance appear to be inextricably linked, at least in the case of the hamster 263K scrapie model. A more complicated question is whether or not the PK resistance of PrP and scrapie infectivity are always associated with multimeric forms of PrP. Clearly, there are aggregated forms of PrP that are neither PK resistant nor associated with infectivity (e.g., see reference 19). Thus, not all aggregates of PrP are alike or relevant to scrapie. However, those PrP aggregates (or multimers) that have the characteristic partial PK resistance of residues ~90 to ~232 have always been associated with infectivity (5, 18) and converting activity (4, 14). Conversely, no monomeric form of PrP has been shown to have either PK resistance or converting activity. There have been reports that scrapie infectivity has cofractionated with monomeric PrP in gel electrophoresis (3) and size exclusion high-performance liquid chromatography (23), but these findings have not been confirmed (10) and might be explicable as technical artifacts. Clearly, our present results show a cofractionation of scrapie infectivity with sedimentable PrP aggregates, rather than monomers, under the conditions we have explored.

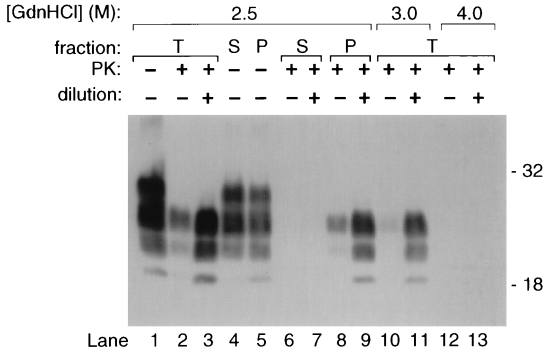

FIG. 2. PK resistance of PrP-res pretreated with the designated concentrations of GdnHCl before (−) and after (+) dilution of the GdnHCl to allow renaturation as described in the text. Aliquots were taken from the total (T), supernatant (S), and pellet (P) fractions of the same pretreated PrP-res samples that were analyzed for scrapie infectivity (Table 1). After treatment, the samples were analyzed by immunoblotting with monoclonal antibody 3F4 (12). The positions of molecular mass markers (in kilodaltons) are shown at the right.

Our observation that 2.5 M GdnHCl treatment has little effect on 263K scrapie infectivity stands in contrast to the results of a previous study with a Creutzfeldt-Jakob disease (CJD) agent passaged in hamsters in which the CJD agent was reduced >100-fold by 2.5 M GdnHCl (17). One explanation for this difference is that the CJD agent may be intrinsically more sensitive to GdnHCl inactivation than is the 263K agent strain. Another difference between these two studies is that the GdnHCl treatment of the CJD agent was done at pH 8.9, whereas our treatment of the 263K agent was done at pH 7.2. The higher pH, which has a partial disaggregating effect on both PrP-res and CJD infectivity and enhances the PK sensitivity of CJD-derived PrP-res (24), might also enhance the denaturing effects of 2.5 M GdnHCl and thereby explain the loss of CJD infectivity.

The observation that PrP-res has apparent self-propagating activity in vitro suggests that it might have similar activity in vivo. Thus, in theory, if PrP-res were transmitted from one host into the appropriate site within another host, it should be able to propagate itself by inducing the conversion of the endogenous PrP-sen of the new host to more PrP-res. In the present study, we directly tested the correlation between cell-free converting activity and scrapie infectivity. The fact that these two activities are similarly sensitive to denaturation with GdnHCl is consistent with the hypothesis that they are closely related, if not inseparable, activities. However, more definitive support for such a hypothesis, such as a demonstration of the generation of new scrapie infectivity by conversion of PrP-sen to PrP-res in the cell-free conversion reaction, is required to provide convincing evidence for this hypothesis.

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