JOURNAL OF VIROLOGY, Feb. 1991, p. 1031–1034
0022-538X/91/021031-04$02.00/0

Vol. 65, No. 2

Protease Sensitivity and Nuclease Resistance of the Scrapie Agent Propagated In Vitro in Neuroblastoma Cells

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Received 26 June 1990/Accepted 9 November 1990

The scrapie agent has been propagated in vitro in mouse neuroblastoma cells. To further characterize the tissue culture-derived scrapie agent, we studied the effects of protease and nuclease digestion on the agent derived from these cells. The scrapie agent in these cells was found to be resistant to protease digestions for short times but was inactivated by prolonged digestion at high protease concentrations. In contrast, digestion with a variety of nucleases did not alter the agent titer. These results demonstrate that the agent requires an essential protein or proteins for infectivity. If the agent also contains a nucleic acid genome, it must be more nuclease resistant than the majority of cellular DNA and RNA. These properties of the tissue culture-derived scrapie agent were identical to those of brain-derived scrapie agent and thus cannot be attributed to secondary effects of tissue pathology, since the infected cell cultures show no cytopathic effects as a result of infection.

Scrapie is a slow transmissible disease of sheep and goats that has been experimentally transmitted to several animal species, including hamsters and mice. The precise biochemical nature of the transmissible agent isolated from infected animals is unknown (7). Many investigators attempted to characterize the nature of the scrapie agent isolated from the brains of infected animals by treating brain homogenates with reagents that denature or digest proteins or nucleic acids. Infectivity from brain tissue was found to be resistant to protease digestion, except in the presence of denaturants or after prolonged digestions with high concentrations of enzyme (8, 12, 19, 20, 27, 29). Nuclease digestions of crude or purified brain samples from scrapie-infected animals had no effect on the titer of the scrapie agent (8, 16, 17, 28). In the absence of precise biochemical knowledge of the scrapie agent, these properties of partial protease resistance and nuclease resistance of the scrapie agent led some investigators to conclude that the agent lacked a conventional nucleic acid genome and consisted primarily, if not exclusively, of protein (1, 14, 24, 25). However, the interpretation of these results is hampered by the fact that the preparations containing scrapie agent were derived from infected tissues. These tissues contain a complex mixture of cell types and often include severe degenerative lesions. Thus, properties attributed to the scrapie agent could be due to the association of the agent with by-products of these cells or to the disease pathology.

We and others have developed scrapie-infected mouse neuroblastoma cell clones that have a high percentage of infected cells and maintain the scrapie agent through many passages in culture (5, 30, 31). No cytopathic effects have been observed in these cells. It is possible that the agent propagated in a homogeneous cell population in the absence of obvious cytopathic effects might exhibit characteristics different from those previously attributed to the agent derived from infected animals. Therefore, we examined the properties of the scrapie agent produced in these cells to determine whether the protease sensitivity and nuclease resistance previously reported for brain-derived agent were actual properties of the agent itself or were a consequence of association of the agent with by-products of diseased brain tissue.

We first analyzed the proteinase K (PK) sensitivity of the agent in cell lysates. Whole cell lysates, rather than partially purified fractions, were examined to ensure that the results obtained would reflect the characteristics of a large percentage of the scrapie agent particles. Infected cells were lysed in a detergent buffer (0.3 M sucrose, 0.2% Sarkosyl, 10 mM Tris hydrochloride, pH 7.4), and aliquots of the lysate were treated with 100 or 500 μg of PK per ml for 1, 4, or 16 h at 37°C. Control samples were treated identically except that PK was not added. The PK was inactivated with 1 mM phenylmethylsulfonyl fluoride, and the scrapie agent was titered in RML mice by using an endpoint dilution assay (Table 1). After 1 h of incubation with either 100 or 500 μg of PK per ml, over 90% of cellular proteins were hydrolyzed, as detected by reduction in protein staining in sodium dodecyl sulfate (SDS)-polyacrylamide gels (data not shown); however, there were no decreases in the scrapie agent titers of the PK-treated lysates compared with the control sample. In contrast, incubation of the lysate with 500 μg of PK per ml for 4 h resulted in a 100-fold decrease in the scrapie agent titer. Further incubation for 16 h with 500 μg/ml produced a decrease of 10^4 50% lethal dose (LD_{50}). These results indicated that the scrapie agent in neuroblastoma cells required a protein or proteins for infectivity. However, this component was more resistant to digestion than were the majority of cellular proteins.

In the brains and spleens of scrapie-infected animals, a PK-resistant form of an endogenous protein, PrP, accumulates. This PK-resistant PrP can aggregate into amyloidlike fibrils (9–11, 21, 28, 34) and plaques (2, 18, 32, 34) and has been postulated to be a component of the transmissible agent itself (4, 11, 20). We have shown that a protease-resistant form of PrP accumulates in our infected cell lines (6). Having shown that the infectivity in these cell lines was sensitive to prolonged PK digestion, we sought to determine whether the degree of PK resistance of PrP correlated with the PK resistance of the agent infectivity. The PK-resistant PrP

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TABLE 1. Scrapie infectivity of neuroblastoma cell lysate treated with PK[a]

PK (μg/ml)	Time (h)	$\log_{10}$ LD_{50}/ 5×10^5 cell equivalents[b]
Treatment 1		
0	1	4.2
100	1	3.6
500	1	4.1
Treatment 2		
0	4	3.9
500	4	1.9[c]
Treatment 3		
0	16	4.2
500	16	0[c]

[a] The scrapie-infected neuroblastoma cell clone 161-7 used is a subclone of the 29-161 clone described previously (30, 31).

[b] Determined by endpoint dilution assay on 10 mice per group (30). The LD_{50}s were determined by the Spearman-Karber method (13), and estimated standard errors ranged from 0.1 to 0.3 $\log_{10}$ LD_{50}. However, this was an estimate of the error within a single dilution series in each assay and appeared to be an underestimate of the reproducibility of the assay, as previous comparisons among repeated assays of the same material in our laboratory showed variability ranging from 0.3 to 1.0 $\log_{10}$ LD_{50} (data not shown).

[c] Significantly different from the value for the no-PK control, based on analysis of the titers as determined by using the normal distribution as the statistic.

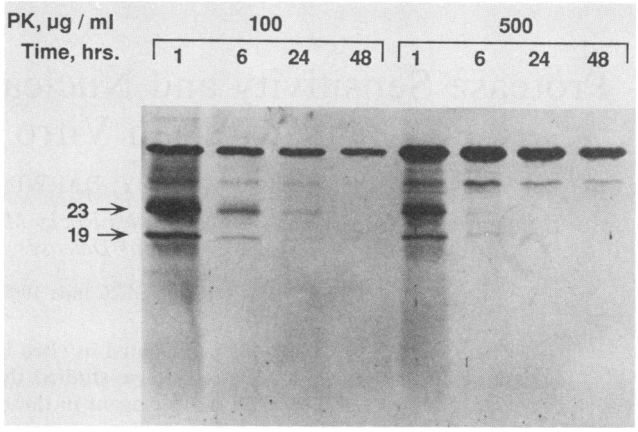

FIG. 1. PK-resistant PrP levels in cell lysates treated with PK. Lysate from clone 161-7 neuroblastoma cells was treated with 100 or 500 μg of PK per ml for the indicated times. Proteins were extracted, and extracts were subjected to SDS-polyacrylamide gel electrophoresis and immunoblotting as described previously (6). The positions of the specific PrP bands are designated on the left in kilodaltons. The 23- and 19-kDa bands can be specifically competed for by preincubating the antibody with the peptide to which it was raised (6). The dark band at the top in each lane is PK, which reacts nonspecifically in this detection system.

could be digested under prolonged PK treatment, and this digestion resulted in the loss of infectivity in the sample (Table 2 and Fig. 1). This interpretation was in agreement with previous data from studies of hamster scrapie-infected brain homogenates (20). However, the correlations in both of these studies are somewhat imprecise because of the large errors associated with the scrapie infectivity assay (±0.5 $\log_{10}$ LD_{50}).

Experiments were also done to determine whether the scrapie agent from total cell lysates prepared from infected neuroblastoma cells contained an essential nucleic acid. A detergent lysate of the cells was prepared and treated with PK at 100 μg/ml for 1 h. We showed previously that this protease digestion had no measurable effect on the scrapie agent titer, and it was thought that mild protease treatment might facilitate digestion of nucleic acids complexed with proteins. The PK was inactivated with phenylmethylsulfonyl fluoride, and the lysate was treated for 4 h at 37°C with a mixture of nucleases to hydrolyze both DNA (DNase I and micrococcal nuclease) and RNA (RNases A, T_1, and T_2). After treatment, an aliquot was assayed for scrapie infectivity and the remaining DNA and RNA levels were measured by the method of Schneider (33). The amount of DNA remaining in the sample was less than 0.2% of the starting concentration, and the RNA content was reduced to 0.7% of the initial concentration, indicating the enzymes were active in this reaction. However, the nuclease digestion had no measurable effect on the scrapie agent titer of the lysate (Table 3, experiment 1). To test the possibility that nucleic acids might themselves be protecting a protein essential for infectivity (23), we further digested the nuclease-treated sample with an additional 100 μg of PK per ml for 1 h. This treatment also did not significantly reduce the scrapie agent titer (Table 3, experiment 1).

The nuclease sensitivity of the agent was also determined after pelleting at 215,000 × *g* for 2 h, since this is a common step in PrP and scrapie agent purification protocols (3, 15). The high-speed pellet was resuspended in the detergent lysis buffer, digested for 1 h with PK at 100 μg/ml, and then treated with the mixture of nucleases. Again, despite significant nucleic acid digestion (Table 3, experiment 2), no reduction in the scrapie agent titer was observed. Digestion of the pelleted sample with DNase I alone, or with the RNases alone also had no effect on the titer. Digestion with RNase H, a nuclease specific for RNA-DNA hybrids, also did not significantly reduce the titer. Thus, even in samples in which greater than 99% of the total nucleic acid was digested, no reduction of the scrapie agent titer was observed. It should be noted, however, that even though a large percentage of the total nucleic acid was digested, the

TABLE 2. Susceptibility of PK-resistant PrP to digestion with PK

PK treatment	% Remaining PK-resistant PrP[a]	Estimated LD_{50}[b]
100 μg/ml		
1 h	100	$10^{2.0}$
6 h	20	$10^{2.1}$
24 h	4	0
48 h	<4	0
500 μg/ml		
1 h	48	$10^{2.0}$
6 h	5	10^{0}
24 h	<4	0
48 h	<4	0

[a] Determined from densitometric scanning of a Western immunoblot (Fig. 1). All values are relative to signal from the 100-μg/ml PK, 1-h sample. Resistance was not determined in samples not treated with PK because PrP detection by Western blotting of such samples with many non-PrP proteins gives a barely detectable signal. The limit of detection of PK-resistant PrP was 4% of the signal from the 100-μg/ml, 1-h sample. Treatment with PK for 1 h gave a strong PK-raesistant PrP signal and no reduction in infectivity (Table 1).

[b] Estimated from the average incubation period until death of 10 inoculated mice per group compared with the values obtained for samples previously titered by using an endpoint dilution assay. The titers are accurate to within approximately 10-fold of the value shown.

TABLE 3. Scrapie infectivity of cell lysates treated with nucleases

Treatment[a]	% Remaining (μg/ml)		Infectivity (log_{10} $LD_{50}/5 \times 10^5$ cell equivalents)
	DNA	RNA	
Expt 1			
PK	100 (552)	100 (208)	3.6
PK + nucleases[b]	<0.2 (<1.1)	0.7 (1.4)	3.6
PK + nucleases + PK	ND[c]	ND	3.7
Expt 2			
PK	100 (866)	100 (337)	3.6
PK + nucleases	<0.2 (<1.1)	2 (7)	3.6
PK + DNase I	0.2 (1.7)	118 (400)	4.2
PK + RNases[d]	92 (799)	<0.04 (<0.13)	3.5
PK + RNase H	115 (999)	97 (325)	3.5

[a] Cells were lysed in 0.3 M sucrose–0.2% Sarkosyl in 10 mM Tris hydrochloride (pH 7.4) at a concentration of 10^7 cells per ml. In experiment 1, this lysate was digested directly with the enzymes indicated. In experiment 2, the lysate was centrifuged at 215,000 × *g* for 2 h, and the pellet was resuspended in the starting volume of lysis buffer before digestion with enzymes.

[b] Nucleases were DNase I (100 μg/ml), micrococcal nuclease (100 μg/ml), and RNases A (100 μg/ml), T_1 (1,000 U/ml), and T_2 (30 U/ml).

[c] ND, Not determined.

[d] RNases were RNases A, T_1, and T_2.

remaining RNA and DNA concentration (0.1 to 7 μg/ml) represented a significant pool of nuclease-resistant DNA or RNA.

The results of this study indicated that the scrapie agent propagated in neuroblastoma cells had properties of nuclease resistance and partial protease resistance similar to those of the agent derived from brain homogenates. Thus, these properties appeared to be intrinsic to the agent itself and did not result from its association with or modification by molecules present solely in homogenates of diseased brain tissue. These results support the concept that the scrapie agent requires a protein or proteins for infectivity. However, the required proteins appeared to be relatively resistant to protease digestion. The PK-resistant PrP in the infected cells might be one of the proteins necessary for infectivity of the scrapie agent. The protease resistance of PrP or other essential proteins could be due to conformational or chemical modification of the proteins or to tight association of the proteins with other cell components. One of the hallmarks of scrapie infection in animals is the accumulation of aggregates of PrP molecules at or near areas of pathology in the brain (11, 21, 22, 26, 28, 34). Similar aggregation of PK-resistant PrP in the cell lysates might also result in PK resistance.

These results also showed that the agent from tissue culture cells, like that isolated from infected brain samples, was resistant to inactivation by nuclease digestion. Therefore, if the agent has a nucleic acid genome, it is more resistant to nuclease digestion than is most of the cellular nucleic acid. This might occur if the scrapie genome were complexed to other molecules that interfere with the nuclease digestion, although pretreatment of the lysate with PK did not make the agent nuclease sensitive. The scrapie agent genome could also have an unusual secondary structure that renders it resistant to nuclease digestion.

The availability of scrapie-infected tissue culture cells has allowed the biochemical analysis of the scrapie agent in the absence of gross pathological changes associated with the disease in animals, and further characterization of these cells should provide information regarding the genetic makeup of the infectious agent.

We thank Robert Evans and Gary Hettrick for graphics assistance and Irene Cook Rodriguez for manuscript preparation.

REFERENCES

1. **Alper, T., W. A. Cramp, D. A. Haig, and M. C. Clarke.** 1967. Does the agent of scrapie replicate without nucleic acid? Nature (London) **214**:764–766.
2. **Bendheim, P. E., R. A. Barry, S. J. DeArmond, D. P. Stites, and S. B. Prusiner.** 1984. Antibodies to a scrapie prion protein. Nature (London) **310**:418–421.
3. **Bolton, D. C., P. E. Bendheim, A. D. Marmostein, and A. Potempska.** 1987. Isolation and structural studies of the intact scrapie agent protein. Arch. Biochem. Biophys. **258**:579–590.
4. **Bolton, D. C., M. P. McKinley, and S. B. Prusiner.** 1982. Identification of a protein that purifies with the scrapie prion. Science **218**:1309–1311.
5. **Butler, D. A., M. R. D. Scott, J. M. Bockman, D. R. Borchelt, A. Taraboulos, K. K. Hsiao, D. T. Kingsbury, and S. B. Prusiner.** 1988. Scrapie-infected murine neuroblastoma cells produce protease-resistant prion proteins. J. Virol. **62**:1558–1564.
6. **Caughey, B., K. Neary, R. Buller, D. Ernst, L. Perry, B. Chesebro, and R. Race.** 1990. Normal and scrapie-associated forms of prion protein differ in their sensitivities to phospholipase and proteases in intact neuroblastoma cells. J. Virol. **64**:1093–1101.
7. **Chesebro, B.** 1990. Spongiform encephalopathies: the transmissible agents, p. 2325–2336. *In* B. N. Fields, and D. M. Knipe (ed.), Virology, vol. 2. Raven Press, New York.
8. **Cho, H. J.** 1980. Requirement of a protein component for scrapie infectivity. Intervirology **14**:213–216.
9. **Cho, H. J.** 1986. Antibody to scrapie-associated fibril protein identifies a cellular antigen. J. Gen. Virol. **67**:243–253.
10. **DeArmond, S. J., M. P. McKinley, R. A. Barry, M. B. Braunfeld, J. R. McColloch, and S. B. Prusiner.** 1985. Identification of prion amyloid filaments in scrapie-infected brain. Cell **41**:221–235.
11. **Diringer, H., H. Gelderblom, H. Hilmert, M. Ozel, C. Edelbluth, and R. H. Kimberlin.** 1983. Scrapie infectivity, fibrils and low molecular weight protein. Nature (London) **306**:476–478.
12. **Diringer, H., H. Hilmert, D. Simon, E. Werner, and B. Ehlers.** 1983. Towards purification of the scrapie agent. Eur. J. Biochem. **134**:555–560.
13. **Dougherty, R. M.** 1964. Animal virus titration techniques, p. 183–186. *In* R. J. C. Harris (ed.), Techniques in experimental virology. Academic Press, Inc., New York.
14. **Griffith, J. S.** 1967. Self-replication and scrapie. Nature (London) **215**:1043–1044.
15. **Hope, J., G. Multhaup, L. J. D. Reekie, R. H. Kimberlin, and K. Beyreuther.** 1988. Molecular pathology of scrapie-associated fibril protein (PrP) in mouse brain affected by the ME7 strain of scrapie. Eur. J. Biochem. **172**:271–277.
16. **Hunter, G. D.** 1979. The enigma of the scrapie agent: biochemical approaches and the involvement of membranes and nucleic acids, p. 365–385. *In* S. B. Prusiner and W. J. Hadlow (ed.), Slow transmissible diseases of the nervous system, vol. 2. Academic Press, Inc., New York.
17. **Hunter, G. D., and G. C. Millson.** 1967. Attempts to release the scrapie agent from tissue debris. J. Comp. Pathol. **77**:301–307.
18. **Kitamoto, T., J. Tateishi, T. Tashima, I. Takeshita, R. A. Barry, S. J. DeArmond, and S. B. Prusiner.** 1986. Amyloid plaques in Creutzfeldt-Jakob disease stain with prion protein antibodies. Ann. Neurol. **20**:204–208.
19. **Manuelidis, L., T. Sklaviadis, and E. E. Manuelidis.** 1987. Evidence suggesting that PrP is not the infectious agent in Creutzfeld-Jakob disease. EMBO J. **6**:341–347.
20. **McKinley, M. P., D. C. Bolton, and S. B. Prusiner.** 1983. A protease-resistant protein is a structural component of the scrapie prion. Cell **35**:57–62.
21. **Merz, P. A., R. J. Kascsak, R. Rubenstein, R. I. Carp, and H. M. Wisniewski.** 1987. Antisera to scrapie-associated fibril protein and prion protein decorate scrapie-associated fibrils. J. Virol. **61**:42–49.
22. **Merz, P. A., R. A. Somerville, H. M. Wisniewski, and K. Iqbal.**

1981. Abnormal fibrils from scrapie-infected brain. Acta Neuropathol. **54**:63–74.
23. **Narang, H. K., D. M. Asher, and D. C. Gajdusek.** 1988. Evidence that DNA is present in abnormal tubulofilamentous structures found in scrapie. Proc. Natl. Acad. Sci. USA **85**: 3575–3579.
24. **Pattison, I. H., and K. M. Jones.** 1967. The possible nature of the transmissible agent of scrapie. Vet. Rec. **80**:2–9.
25. **Prusiner, S. B.** 1982. Novel proteinaceous infectious particles cause scrapie. Science **216**:136–144.
26. **Prusiner, S. B., D. C. Bolton, D. F. Groth, K. A. Bowman, S. P. Cochran, and M. P. McKinley.** 1982. Further purification and characterization of scrapie prions. Biochemistry **21**:6942–6950.
27. **Prusiner, S. B., D. F. Groth, S. P. Cochran, M. P. McKinley, and F. R. Masiarz.** 1980. Gel electrophoresis and glass permeation chromatography of the hamster scrapie agent after enzymatic digestion and detergent extraction. Biochemistry **19**:4892–4898.
28. **Prusiner, S. B., M. P. McKinley, K. A. Bowman, P. E. Bendheim, D. C. Bolton, D. F. Groth, and G. G. Glenner.** 1983. Scrapie prions aggregate to form amyloid-like birefringent rods. Cell **35**:349–358.
29. **Prusiner, S. B., M. P. McKinley, D. F. Groth, K. A. Bowman, N. I. Mock, S. P. Cochran, and F. R. Masiarz.** 1981. Scrapie agent contains a hydrophobic protein. Proc. Natl. Acad. Sci. USA **78**:6675–6679.
30. **Race, R. E., B. Caughey, K. Graham, D. Ernst, and B. Chesebro.** 1988. Analyses of frequency of infection, specific infectivity, and prion protein biosynthesis in scrapie-infected neuroblastoma cell clones. J. Virol. **62**:2845–2849.
31. **Race, R. E., L. H. Fadness, and B. Chesebro.** 1987. Characterization of scrapie infection in mouse neuroblastoma cells. J. Gen. Virol. **68**:1391–1399.
32. **Roberts, G. W., R. Lofthouse, R. Brown, T. J. Crow, R. A. Barry, and S. B. Prusiner.** 1986. Prion-protein immunoreactivity in human transmissible dementias. N. Engl. J. Med. **315**:1231–1232.
33. **Schneider, W. C.** 1957. Determination of nucleic acids in tissues by pentose analysis. Methods Enzymol. **3**:680–684.
34. **Wiley, C. A., P. G. Burrola, M. J. Buchmeier, M. K. Wooddell, R. A. Barry, S. B. Prusiner, and P. W. Lampert.** 1987. Immunogold localization of prion filaments in scrapie-infected hamster brains. Lab. Invest. **57**:646–655.