Differences in Proteinase K Resistance and Neuronal Deposition of Abnormal Prion Proteins Characterize Bovine Spongiform Encephalopathy (BSE) and Scrapie Strains

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Abstract

Prion diseases are associated with the accumulation of an abnormal isoform of host-encoded prion protein (PrP^{Sc}). A number of prion strains can be distinguished by "glycotyping" analysis of the respective deposited PrP^{Sc} compound. In this study, the long-term proteinase K resistance, the molecular mass, and the localization of PrP^{Sc} deposits derived from conventional and transgenic mice inoculated with 11 different BSE and scrapie strains or isolates were examined. Differences were found in the long-term proteinase K resistance (50 µg/ml at 37°C) of PrP^{Sc}. For example, scrapie strain Chandler or PrP^{Sc} derived from field BSE isolates were destroyed after 6 hr of exposure, whereas PrP^{Sc} of strains 87V and ME7 and of the Hessen1 isolate were extremely resistant to proteolytic cleavage. Nonglycosylated, proteinase K–treated PrP^{Sc} of BSE isolates and of scrapie strain 87V exhibited a 1–2 kD lower molecular mass than PrP^{Sc} derived from all other scrapie strains and isolates. With the exception of strain 87V, PrP^{Sc} was generally deposited in the cerebrum, cerebellum, and brain stem of different mouse lines at comparable levels. Long-term proteinase resistance, molecular mass, and the analysis of PrP^{Sc} deposition therefore provide useful criteria in discriminating prion strains and isolates (e.g., BSE and 87V) that are otherwise indistinguishable by the PrP^{Sc} "glycotyping" technique.

Introduction

Transmissible spongiform encephalopathies (TSEs), also known as prion diseases, are fatal infectious diseases affecting humans [Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheincker syndrome (GSS), fatal familial insomnia (FFI), and kuru], sheep, goats and mouflon (scrapie), cattle [bovine spongiform encephalopathy (BSE)], mink [transmissible mink encephalopathy], and deer [chronic wasting disease]. In the course of these diseases an isoform of cellular prion protein (PrP^{C}) accumulates as a pathological prion protein (PrP^{Sc}) in the nervous and lymphatic systems (1–3). According to the prion theory, PrP^{Sc} itself constitutes the infectious agent (4). Host-encoded PrP^{C} is widely expressed in neuronal cells as a cell-membrane protein with a molecular mass of 33–36 kD in a fully matured and diglycosylated form. Its physiological function is as yet unknown. In pulse-chase experiments on neuroblastoma cells (N2a cells) infected by the scrapie strain Chandler, PrP^{C} had a half-life of less than 4 hr, whereas PrP^{Sc} was metabolically stable for 48 hr and longer (5). In contrast, PrP^{Sc} is distinguished from PrP^{C} by physicochemical features: it forms insoluble aggregates, the scrapie-associated fibrils (SAFs), also termed prion rods (6–11). Furthermore, it is partially resistant to proteinase K digestion (4,12,13); for instance, proteinase K only cleaves off 60–70 resi-

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Table 1. Incubation times of BSE and scrapie strains and isolates in rodent TSE models

Strain/ isolate	Source/host species	Rodent species or breed used for prion propagation	Incubation time (days ± SEM)
Chandler	Mouse scrapie strain	C57BL/6 mice	164 ± 4.7
		CD-1 mice	147 ± 5.1
		Tg20 mice	65 ± 0.7
22A	Mouse scrapie strain	C57BL/6 mice	481 ± 3.4
		VM95 mice	195 ± 1.2
ME7	Mouse scrapie strain	C57BL/6 mice	179 ± 7.9
79A	Mouse scrapie strain	C57BL/6 mice	169 ± 10.9
87V	Mouse scrapie strain	VM95 mice	370 ± 11.3
263K	Hamster scrapie strain	Syrian hamster	83 ± 1.8
Hessen1	Sheep scrapie isolate	C57BL/6 mice	296 ± 7.3
S833	Sheep scrapie isolate	C57BL/6 mice	142 ± 12.3
S540	Sheep scrapie isolate	C57BL/6 mice	442 ± 3.2
BSE-UK	Cattle BSE isolate	C57BL/6 mice[a]	202 ± 6.2
		VM95 mice[b]	339 ± 3.6
BSE-G	Cattle BSE isolate	C57BL/6 mice[a]	197 ± 7.0
		VM95 mice[b]	353 ± 12.1

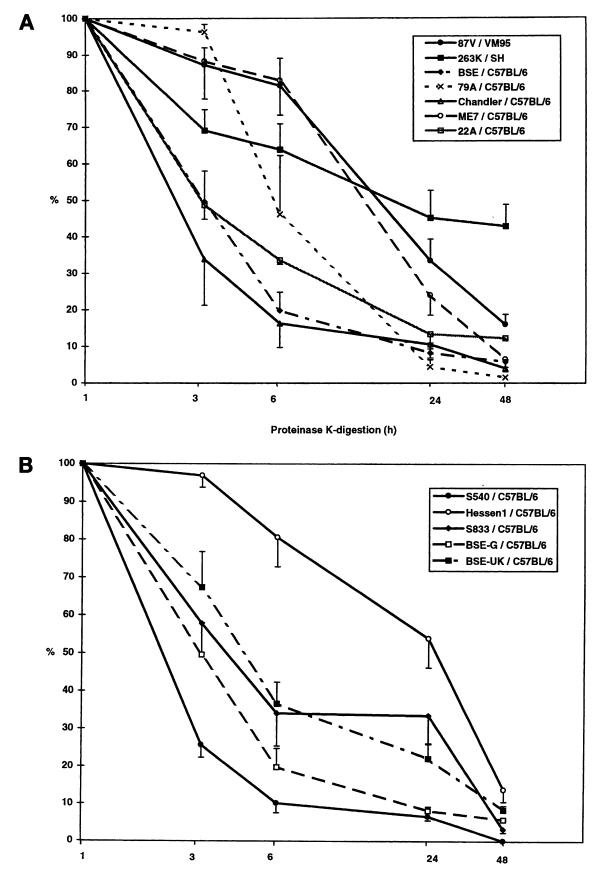[a]Isolated in a C57BL/6 mouse and subpassaged there.
[b]Isolated in a C57BL/6 mouse and subpassaged in VM95 mice.

dues (the exact number depends on the species and strain of agent) of the 211–221 residues encompassing the amino acid backbone of post-translationally processed PrP^{Sc}, leaving behind a resistant core fragment with a molecular mass of 27–30 kD, if in diglycosylated form. In contrast to PrP^{C}, PrP^{Sc} contains a high β-sheet content (14–16).

A variety of different prion strains have been discovered that can be distinguished according to differences in their transmissibility, incubation time, induced clinical symptoms, brain lesions, and resistance to inactivation (17–24). Western blot analysis has revealed that nonglycosylated, proteinase K–treated PrP^{Sc} of the scrapie strain 87V and of the transmissible mink encephalopathy (TME) strain "Hyper" exhibit a 1–2 kD lower molecular mass than PrP^{Sc} accumulated during the infection of mice or hamsters with other scrapie or TME strains (25–26). Three different molecular masses of nonglycosylated CJD-derived PrP^{Sc} have been reported (27), whereby PrP^{Sc} derived from the new variant CJD (nvCJD) clusters into the category of low molecular mass (27). A low molecular mass was also reported for BSE-derived PrP^{Sc}. However, no data are as yet

available on the molecular masses of mouse-passaged scrapie-derived PrP^{Sc} prions obtained from diseased individuals of the same host species.

Comparative analysis of glycoform ratios (nonglycosylated, monoglycosylated, and diglycosylated fraction of PrP^{Sc}) is another useful means for discriminating TSE strains (so-called glycotyping). In conventional cases of CJD, GSS, and kuru (27), as well as in mice infected with the scrapie strains Chandler and 79A, the monoglycosylated fraction is the major compound of PrP^{Sc} (28,29). In contrast, infections with the nvCJD, BSE, and scrapie strains 87V and 263K lead to the aberrant deposition of diglycosylated PrP^{Sc} (27–29).

Molecular analysis of the deposited PrP^{Sc} has thus provided important criteria for the discrimination of prion strains. It must be noted, however, that PrP^{Sc} produced in the course of infection by certain strains or isolates (e.g., BSE and 87V) are hardly distinguishable at all by the glycotyping technique. In the current study, we introduce further criteria for the characterization of strains or isolates. We were able to demon-

A

Legend:
- 87V / VM95
- 263K / SH
- BSE / C57BL/6
- 79A / C57BL/6
- Chandler / C57BL/6
- ME7 / C57BL/6
- 22A / C57BL/6

%

Proteinase K-digestion (h)

B

Legend:
- S540 / C57BL/6
- Hessen1 / C57BL/6
- S833 / C57BL/6
- BSE-G / C57BL/6
- BSE-UK / C57BL/6

%

Proteinase K-digestion (h)

strate that PrP^{Sc} resists the long-term exposure to proteinase K and followed this up by comparing the molecular mass of proteinase K–treated PrP^{Sc} of mouse-passaged BSE and scrapie strains, as well as by quantitatively analyzing the distribution of PrP^{Sc} deposits in different sites in the brain. The data presented here confirm the existence and distinctiveness of prion strains and underline the fact that BSE isolates can be distinguished from experimental and field scrapie strains or isolates by physicochemical analysis.

Materials and Methods

Propagation of BSE and Scrapie Strains

C57BL/6 mice were intracerebrally inoculated (20 μl of 1% mouse brain homogenate) with cloned mouse scrapie strains ME7, 22A, 79A, and 87V (kindly provided by M. Bruce, BBSRC & MRC Neuropathogenesis Unit, Edinburgh) and with the Chandler strain (kindly provided by C. Weissmann, University of Zürich). Similarly, VM95 mice (a gift from M. Bruce, BBSRC & MRC Neuropathogenesis Unit, Edinburgh) were inoculated with strains 87V and 22A. The Chandler strain was passaged in outbred white Swiss (CD1) mice and transgenic Tg20 mice [(29a) obtained from C. Weissmann] that overexpressed PrP^C. The scrapie strain 263K (obtained from H. Diringer, Robert Koch Institute, Berlin) was used to infect Syrian hamsters. The Hessen1 isolate was derived from a field case of scrapie in a 4-year-old Suffolk ram (30) and was isolated and subpassaged in C57BL/6 mice. Isolates S833 and S540 from the brain of Suffolk sheep that had been naturally infected by scrapie were transmitted to C57BL/6 mice by primary passage. BSE strains isolated from cattle that had developed BSE in the United Kingdom (BSE-UK; courtesy of R. Bradley, Weybridge) and in Germany (BSE-G) were subpassaged in C57BL/6 and VM95 mice. To prevent mixing the strains by cross-contamination, all instruments for inoculation were porous-load autoclaved at 136°C and 3 bars for 4 hr prior to use and the animals were kept in isolation in facilities set up for this purpose for the respective scrapie strain. At the clinical stage of the disease, the animals were killed and their brains removed and stored at −20°C. Conservation of strain-specific characteristics were monitored by brain-lesion profile-scoring performed on indicator mice (data not shown). The measurement of the incubation period was calculated as the interval between inoculation and the well-defined appearance of typical symptoms of scrapie disease.

Preparation of Brain Homogenates and Purification of Prion Proteins (SAFs)

For each strain or isolate, separate brain homogenates from individual mice were examined separately and the pools contained brain tissues of several animals. Brain tissues were homogenized in phosphate-buffered saline (PBS) containing 0.5% NP-40 and 0.5% sodium deoxycholate (27) and were sonicated for 20–30 sec. Debris was removed by centrifugation at 2000 rpm for 5 min and supernatants [10% (w/v) homogenates] were stored at −20°C.

PrP^{Sc} was purified using a modified protocol as described previously (8). In summary, brain tissue was homogenized in brain lysis buffer (10 mM sodium phosphate containing 10% *N*-lauryl-sarcosinate, 3 mM phenylmethylsulfonylfluoride (PMSF), and 3 mM *N*-ethylmaleimide). Cell detritus was pelleted at 20,000 rpm for 5 min and in a further centrifugation step fibrils were pelleted at 100,000 rpm for 30 min.

Fig. 1. Long-term proteinase K sensitivity of PrP^{Sc} derived from different scrapie and BSE strains (A) or field isolates (B). To investigate the sensitivity of scrapie and BSE strains, isolates to proteinase K prion rods were purified from mouse brain homogenate pools (a minimum of four mice per trial) by differential centrifugation. Fractions containing PrP^{Sc} were standardized according to their protein concentrations and signal intensities. Aliquots were subsequently exposed to 50 μg/ml (final concentration) proteinase K for 1, 3, 6, 24, or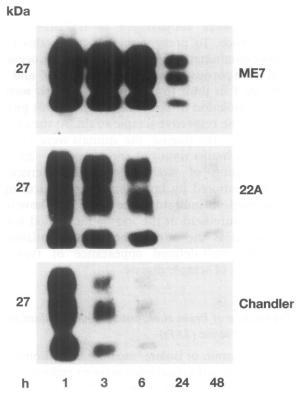 48 hr at 37°C. Residual PrP^{Sc} was then visualized by immunoblot and total band signals quantified by the photoimager technique. Residual PrP^{Sc} antigen signals were calculated as arithmetic means from a minimum of two extraction and digestion experiments per strain. Each sample taken was analyzed on at least four different immunoblots. Combined PrP^{Sc} signals (non-, mono- and diglycosylated bands) obtained after 1 hr of proteolysis were set at 100% to minimize artefacts because of the possible initial contamination of fibrils with other proteins. Standard error values are indicated.

kDa

Fig. 2. **Immunoblot depicting the difference in long-term stability of PrP^{Sc} fibrils during proteinase K digestion.** Scrapie strains ME7, 22A, and Chandler were propagated in C57BL/6 mice. Fibrils were incubated with 50 μg/ml (final concentration) proteinase K for 1, 3, 6, 24, and 48 hr. Proteinase K activity of samples taken at each time point was halted by the addition of PMSF (final concentration, 5 mM) followed by heating at 95°C for 15 min. Samples were run on 16% SDS-PAGE gels and immunoblotted using the polyclonal anti-peptide antibody Ra5/7 to mouse PrP (residues 95–110).

Pellets were resolubilized in 16 mM Tris buffer (pH 7.4) and incubated at 37°C. After 30 min, potassium iodide buffer (15% potassium iodide, 60 mM sodium thiosulfate 5-hydrochloride, 10 mM Tris buffer, pH 7.4) was added and samples were incubated for another 30 min at 37°C. After adding potassium iodide buffer (10%) to each sample, fibrillar structures were pelleted at 100,000 rpm for 30 min, supernatants were removed, and pellets were dried.

Treatment with Proteinase K and PNGaseF

To digest PrP^{Sc} of homogenates or SAFs, proteinase K (50 μg/ml) was added to the sample and incubation was carried out for 1 hr (37°C), unless otherwise indicated. Digestion was terminated by adding 5 mM PMSF (Boehringer Mannheim,

Mannheim, Germany) and heating the samples at 95°C for 15 min. For deglycosylation, the proteinase K–treated samples were digested with 10 mU/ml PNGaseF (Boehringer, Mannheim) for 6 hr at 37°C. The reaction was terminated by heating to 95°C for 15 min and the samples were stored at −20°C.

Immunoblot Analysis

Electrophoretic separation of proteins by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) on a mini-slab-gel apparatus (Mini-protein II dual slab cell, Biorad, Munich) was performed with 16% or 20% polyacrylamide gels as previously described (31). Before loading onto the gels, the samples were suspended in SDS-loading buffer and heated to 95°C for 5 min. Proteins were electroblotted onto Immobilon-P membranes (Millipore, Bedford, MA) using a semi-dry blotting system (Biorad). Membranes were blocked in PBS containing 0.05% TWEEN 20 (PBST), 5% non-fat milk powder, and 5% fetal calf serum (FCS) and were incubated with a mouse-specific polyclonal anti-peptide rabbit antibody Ra5/7 at 1:2000 dilution in blocking buffer to detect mouse-passaged PrP^{Sc}. This antibody was raised to the amino terminus of mouse PrP^{Sc} (amino acids 95–110: THNQWNKPSKPKTNMK). To detect hamster-passaged PrP^{Sc}, the monoclonal antibody 3F4 was used at a dilution of 1:5000 in PBST. Horseradish peroxidase–conjugated affinity-purified goat anti-rabbit immunoglobulin G (IgG) or goat anti-mouse IgG (Dianova, Hamburg, Germany) served as the detection antibody. The membranes were developed using a chemiluminescence enhancement kit (ECL, Amersham, Little Chalfont, UK) and the protein bands were visualized on high-performance luminescence detection films (Hyperfilm ECL, Amersham).

Quantitative and Statistical Analysis

In order to glycotype the PrP strains or isolates, the banding intensities of the non-, mono-, and diglycosylated PrP isoforms were determined in the linear range of light emission, which was verified for each strain or isolate by analyzing serially diluted samples after digestion of PrP^{Sc} with proteinase K for 1 hr at 37°C, as previously described (29). The signal bands were scanned on a chemiluminescence photoimager (Fujifilm, Düsseldorf, Germany) with the CSC program. For quantification, the banding intensities of the non-,
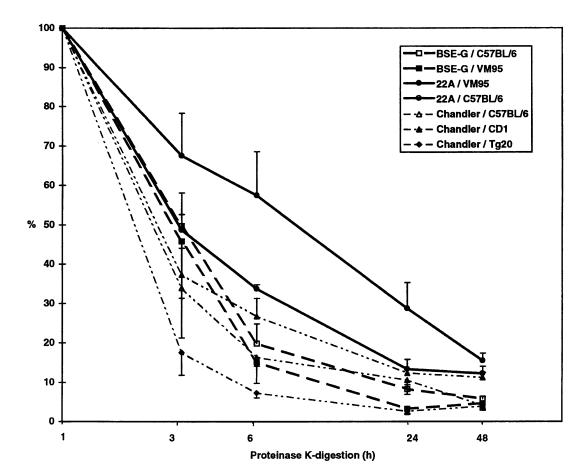
Fig. 3. Analysis of long-term stability of proteinase K of strains 22A, Chandler, and BSE-G propagated in different mouse lines. PrP^{Sc} was purified from pooled mouse brain homogenates (mouse lines Tg20, C57BL/6, CD-1, and VM95) and exposed to proteinase K for 1, 3, 6, 24, or 48 hr at 37°C prior to immunoblot analysis. Combined PrP^{Sc} signals were recorded using the photoimager technique and arithmetic means of at least four runs per sample were calculated. Standard error values are indicated.

mono- and diglycosylated PrP isoforms were determined with the TINA2.0 program and calculated as described (32). For experiments determining long-term proteinase K resistance, signal intensities of digested pooled PrP^{Sc} (1 hr) were defined as 100% for each strain, respectively, and the percentages of long-term hydrolyses were calculated. Standard errors were calculated from the means of different gel runs. For the calculation of PrP^{Sc} deposits in different brain regions, identical amounts of each homogenate and of each individual animal were loaded onto the gels. Protein intensities of the investigated areas in an individual animal were determined and the deposits were calculated as percentages of the total signals. After separate SDS-PAGE and immunoblotting, the deposits were analyzed by calculating the means of each individual. The standard errors were assessed from the means of the animals for each PrP banding pattern

to analyze the differences among individuals. To exclude variations in the separations in different SDS-PAGE runs, each brain homogenate was electrophoresed, immunoblotted, and scanned at least four times. Relative PrP^{Sc} amounts of different scrapie and BSE strains or isolates were determined by photoimager recording of immunoblot signals' combined values of all three glycoforms. Standard error values were calculated from means of the gels runs of the individual animals tested.

Results

In the current study, six phenotypically well-defined rodent scrapie strains (87V, 79A, Chandler, 22A, ME7, 263K) as well as five mouse-passaged field BSE (BSE-G and BSE-UK) and scrapie (Hessen1, S833, and S540) isolates were
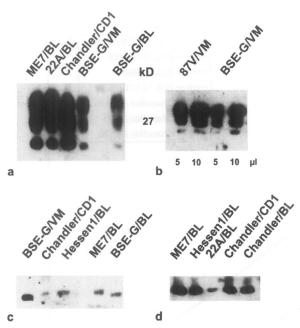
Fig. 4. Molecular mass of proteinase K–treated PrP^{Sc} elicited by different scrapie and BSE strains or isolates. Brain homogenates (10%) from C57BL/6 (BL), VM95 (VM), and CD-1 mice infected with different mouse-adapted BSE isolates and scrapie strains were treated with 50 μg/ml proteinase K for 1 hr at 37°C and run on 20% (a) or 16% SDS-PAGE (b–d) gels. PrP^{Sc} bands were revealed by immunoblotting using a rabbit anti-peptide antibody to mouse PrP sequences (aa 95–110). In c and d, PrP^{Sc} was deglycosylated by PNGase F treatment in addition to the proteinase K cleavage.

propagated in at least one out of four different conventional or transgenic mouse lines or in Syrian hamsters (Table 1). All strains and isolates led to the typical characteristics of scrapie disease after unique incubation times in the animals, which formed one basis for their discrimination. PrP^{Sc} molecules accumulated in the neuronal systems were subsequently analyzed with respect to their glycosylation patterns after proteinase K digestion.

Long-Term Resistance of PrP^{Sc} to Proteinase K

Abnormal PrP (PrP^{Sc}) was purified from brain tissue samples of the infected mice and hamsters by standard procedures established for the preparation of scrapie-associated fibrils. Fibrils were subsequently exposed for prolonged times (1, 3, 6, 24, and 48 hr) to proteinase K (50 μg/ml) to determine their relative proteolytic stability. Non-degraded PrP^{Sc} compounds were visualized as immunoblots using an antiserum to the amino terminus of the proteinase K–resistant PrP^{Sc} and

residual antigen amounts quantified using enhanced chemiluminescence and photoimager techniques. In this set of experiments, banding signal intensities of PrP^{Sc} after digestion with proteinase K for 1 hr were defined as 100% for each strain or isolate. PrP^C was proteolytically degraded entirely in this process. To minimize artefacts, all experiments were performed in duplicate and each sample was run on SDS-PAGE gels, immunoblotted, and analyzed in quadruplicate. To exclude specific effects arising from the mouse line involved, all BSE and scrapie strains (except 87V and 263K) used in this experiment were propagated in C57BL/6 mice. Most interestingly, PrP^{Sc} did not show a uniform resistance to proteinase K treatment (Figs. 1A, B, 2). PrP^{Sc} antigenicity of scrapie strain Chandler in C57BL/6 mice was degraded by as much as 84% after only 6 hr of exposure, whereas PrP^{Sc} of strain 87V was reduced by only 19% under identical conditions. Strain ME7 exhibited a similar proteinase K resistance to 87V with a reduction of PrP^{Sc} amounting to as little as 17% (Fig. 1A). The strains 22A and 263K had intermediate proteinase K resistance. PrP^{Sc} of 22A was decreased by 66% and PrP^{Sc} of 263K was reduced by 36%. However, an initially low proteinase K degradation rate did not automatically indicate that PrP^{Sc} resisted proteolytic cleavage to the same relative degree for extended times. After 48 hr of proteinase K exposure, the degree of PrP^{Sc} degradation of strains 87V, ME7, Chandler, 22A, and 263K was 86%, 94%, 99%, 88%, and 57%, respectively (Figs. 1A, 2). Thus, PrP^{Sc} of strain 263K was highly stable to extended proteinase K exposure (even beyond 72 hr of exposure; data not shown).

PrP^{Sc} from field isolates of scrapie, retrieved, and passaged in C57BL/6 mice was as disparate in long-term proteinase K stability as that from the defined BSE and scrapie strains (Fig. 1B). While over 90% of PrP^{Sc} from isolate S540 was diminished after 6 hr of incubation, the Hessen1 PrP^{Sc} isolate was almost entirely proteinase K–resistant. Scrapie isolate S833 along with the BSE isolates showed intermediate susceptibility (34% reduction after 6 hr of exposure). PrP^{Sc} of BSE-G and BSE-UK was degraded by 80% and 63%, respectively, after 6 hr of exposure.

In a second set of experiments, the influence of the different mouse lines used for propagation of TSE agents was analyzed. C57BL/6, CD1, and Tg20 carry the mouse prion allele Prnp^a in homozygous form, while VM95 mice harbor the Prnp^b allele. No effect of the mouse line used for
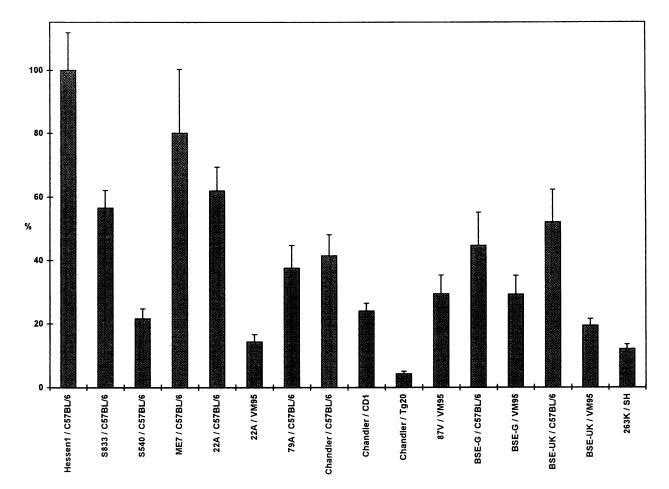
Fig. 5. Relative amounts of PrP^{Sc} in different scrapie and BSE strains or isolates. Proteins of pooled brain homogenates (10%) were separated by SDS-PAGE gel runs (16%) after proteinase K digestion and immunoblotted using a rabbit anti-peptide serum to mouse PrP sequences. For quantification of the signals, protein bands were scanned on a chemiluminescence photoimager. Reciprocal values from homogenates of different mouse lines infected with the appropriate scrapie and BSE strains or isolates to give identical signal intensities are depicted. Overall percentages were calculated as arithmetical means ± standard error of the means (SEM) of individual mice. The following numbers of mice and SDS-PAGE were included into each calculation: C57BL/6 mice were infected with Hessen1 (homogenates of 5 mice divided up into 31 gels), S833 (4 mice/16 gels), S540 (4 mice/26 gels), ME7 (5 mice/27 gels), 22A (5 mice/17 gels), 79A (6 mice/23 gels), Chandler (6 mice/39 gels), BSE-G (5 mice/30 gels), and BSE-UK (5 mice/24 gels). VM95 mice were inoculated with 87V (6 mice/32 gels), 22A (4 mice/16 gels), BSE-G (4 mice/22 gels) and BSE-UK (4 mice/21 gels). The Chandler strain was used to infect both CD-1 (8 mice/43 gels) and Tg20 mice (4 mice/16 gels). Syrian hamsters (SH) were inoculated with 263K (4 hamsters/16 gels).

propagation was observed for the BSE-G isolate or for the scrapie strain Chandler. However, a slight, yet statistically significant difference in long-term proteinase K resistance was found in PrP^{Sc} of scrapie strain 22A propagated in VM95 or C57BL/6 mice. This was all the more remarkable, as all PrP^{Sc} displayed strain-specific glycotyping patterns independent of the mouse type in which they were tested (data not shown). Thus, depending on the prion strain, the mouse line used as PrP^{Sc} source may have an effect,

albeit minor, on its long-term proteinase K stability (Fig. 3).

Molecular Mass of PrP^{Sc} after Proteinase K Cleavage

Proteinase K–treated brain homogenates of mice infected with strains BSE, 87V, 22A, Hessen1, Chandler, and 79A were run on high-resolution SDS-PAGE gels and PrP^{Sc} bands were visualized by immunoblotting. As expected, the molecular mass of nonglycosylated PrP^{Sc} in almost all

strains averaged 21 kD (Fig. 4). However, as previously described (25), PrP^{Sc} of strain 87V was cleaved by proteinase K to a 1–2 kD smaller fragment (molecular mass approximately 19 kD). As already demonstrated by the comparison of PrP^{Sc} produced in different species, the same characteristic could now be observed for both mouse-passaged BSE isolates: nonglycosylated proteinase K–degraded PrP^{Sc} molecules from mouse-passaged BSE isolates exhibited a lower molecular mass than those from the other scrapie strains. This difference was independent of mouse line (C57BL/6 or VM95 mice) used for propagation of BSE PrP^{Sc}.

Relative Amounts of PrP^{Sc} Deposits

Relative amounts of PrP^{Sc} deposits induced by the infection of mice with different prion strains or isolates were determined by photoimager recording of immunoblot signals (combined values of all three glycoforms). The most abundant PrP^{Sc} accumulation was found in C57BL/6 mice infected with the scrapie isolate Hessen1, which was thus set at 100% (Fig. 5). Similarly, pronounced accumulation levels were also observed in C57BL/6 mice infected with the mouse-adapted strain ME7 (Fig. 5). Intermediate PrP^{Sc} accumulation levels were found in C57BL/6 mice infected by 22A, 79A, Chandler, BSE-G, and BSE-UK. Interestingly, the levels of PrP^{Sc} produced were dependent of the respective mouse line used. PrP^{Sc} accumulation levels of all strains were lower in VM95 mice. Moreover, the neuronal overexpression of mouse PrP^{C} in transgenic Tg20 mice (a factor of 10) did not give rise to elevated PrP^{Sc} amounts after inoculation with Chandler strain. Rather, PrP^{Sc} levels were eight times higher in C57BL/6 and five times higher in CD1 than in Tg20 mice.

Distribution of PrP^{Sc} Deposits

Consistent differences among the TSE strains were also observed with regard to the localization of PrP^{Sc} deposits in the brains of scrapie- and BSE-diseased mice (Fig. 6). In VM95 mice infected with either scrapie strain 87V or with BSE, PrP^{Sc} was accumulated largely in the brain stem. Interestingly, both BSE isolates exhibited the same deposition pattern in VM95 mice. In contrast, infection with strain 22A led to major PrP^{Sc} deposits in the cerebellum. Scrapie isolates Hessen1 and S833 differed in PrP^{Sc} distribution in the brain. Whereas PrP^{Sc} of Hessen1 accumu-
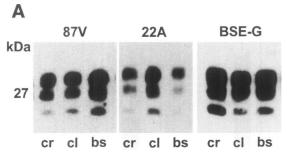

Fig. 6. Distribution of PrP^{Sc} deposits in different brain areas. Relative amounts of PrP^{Sc} deposits in the cerebrum (cr), cerebellum (cl), and brain stem (bs) were determined by immunoblotting (A) and quantification through the photoimager technique (B). Relative percentages of cerebrum, cerebellum, and brain stem were calculated as arithmetic means ± standard error of the means of individual mouse homogenates. VM95 mice were inoculated with 87V (6 mice/24 gels); BSE-UK (4 mice/16 gels); BSE-G (4 mice/24 gels); and 22A (4 mice/19 gels). C57BL/6 mice were inoculated with 22A (4 mice/18 gels); Chandler (5 mice/31 gels); 79A (3 mice/12 gels); BSE-G (3 mice/26 gels); Hessen1 (4 mice/19 gels); and S833 (4 mice/16 gels). Outbred CD-1 mice were inoculated with Chandler (6 mice/34 gels). Tg20 mice were inoculated with the Chandler strain (4 mice/16 gels) and Syrian hamsters (SH) were inoculated with 263K (4 hamsters/16 gels).

lated largely in the brain stem, major PrP^{Sc} deposits of S833 were detected in the cerebellum, as with strain 22A.

Discussion

There is a growing need to establish criteria for distinguishing TSE agents. Variations among scrapie, BSE, and CJD strains and isolates were detected by the glycotyping technique (27–29), but this technique does not discriminate every individual strain. In the present work, we have selected a number of biochemical and biological markers of PrP^{Sc}, which have not yet been studied in detail for their application in BSE and scrapie strains and isolates; as it turned out, these markers are useful in distinguishing prion strains more precisely.

We have analyzed the long-term proteinase K resistance, molecular mass of PrP^{Sc}, and topology and quantity of the PrP^{Sc} deposits. Our results demonstrate that PrP^{Sc} proteins of different BSE and scrapie strains consistently display differences in their long-term proteinase K resistance. For example, PrP^{Sc} of 87V and ME7 were relatively stable to proteinase K hydrolysis for up
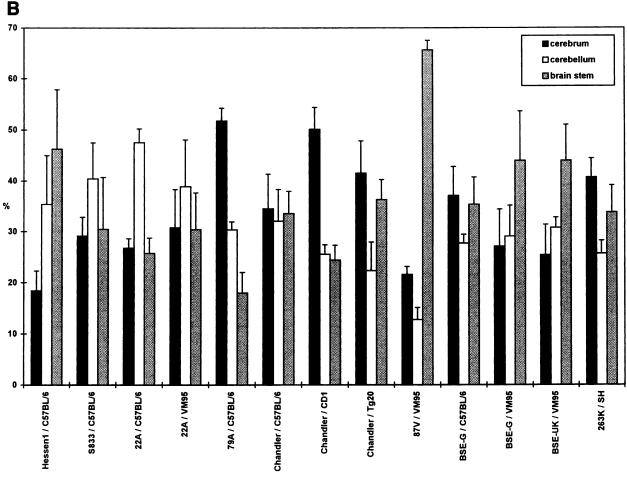
Fig. 6.   Continued

to 24 hr of digestion, whereas PrP^{Sc} of the Chandler strain was almost completely destroyed after as little as 6 hr of exposure. PrP^{Sc} of scrapie strains 22A and 263K displayed intermediate resistance. Interestingly, field scrapie isolates in mice were not as diverse as experimental scrapie strains. While the Hessen1 isolate PrP^{Sc} was almost totally proteinase K–resistant, PrP^{Sc} of isolates S833 and S540 exhibited intermediate and high sensitivity to this enzyme. In comparison to most other scrapie strains, PrP^{Sc} of the BSE isolates exhibited intermediate resistance to proteinase K digestion. Scrapie strain 87V and strains of BSE exhibited a very similar pattern of signal intensities of the non-, mono- and diglycosylated PrP^{Sc} isoform (29) but after long-term proteinase K treatment, both strains could be clearly distinguished, BSE PrP^{Sc} being markedly less stable.

Our results are not unprecedented; strain-specific differences in the proteinase K stability of

PrP^{Sc} compounds have already been observed. Kascsak et al. (25) exposed fibril preparations from scrapie strains 263K, 139A, and ME7 to proteinase K and separated the proteins on SDS-PAGE gels. In these experiments, the three typical bands were weaker in varying degrees, indicating that PrP^{Sc} of strain 139A was less resistant to proteinase K digestion than PrP^{Sc} of scrapie 263K and ME7. Later, similar differences were observed for transmissible mink encephalopathy strains Hyper and Drowsy, the latter being less resistant to proteinase K treatment (26).

The species and particular breed used for the generation of PrP^{Sc} may have an influence on its molecular characteristics. We compared PrP^{Sc} generated in a variety of different mouse lines carrying prion alleles Prnp^a or Prnp^b. These encode mouse PrP differing by polymorphisms at amino acids 108 (Leu/Phe) and 189 (Thr/Val) (33). According to our results, the effect of these polymorphisms on the long-term proteinase K

resistance of PrP^{Sc} is not entirely clear. While no differences were found in long-term proteinase K resistance of PrP^{Sc} from the BSE-G isolate propagated in homozygous Prnp^a C57BL/6 mice and Prnp^b VM95 mice, PrP^{Sc}s of scrapie strain 22A propagated in these two different mouse lines were distinct from another. It has been reported that SAF proteins from scrapie strains ME7 and 87V isolated from different mouse lines differed in sensitivity to proteinase K (34), a result which contradicts that of another study that found no differences between strains 139A and ME7 (35).

The close linkage between PrP^{Sc} and the infectious agent itself is indisputable. It would seem that the strain-specific characteristics of prion diseases are brought about, if not encoded, by differences in the PrP^{Sc} conformations. Evidence for different conformations of several PrP^{Sc} strains has been demonstrated recently through use of a highly sensitive conformation-dependent immunoassay. Several hamster-passaged PrP strains differed in their conformation and each strain exhibited a distinct pattern (36). The underlying mechanisms for these strain-specific PrP^{Sc} conformations are still unclear—either these differences are solely due to variations in structure and conformation of PrP^{Sc} or to post-translational modifications of PrP.

Another unique characteristic of PrP^{Sc} from BSE isolates independent of the mouse line used is that proteinase K–treated PrP^{Sc} possesses a 1–2 kD lower molecular mass compared to similarly treated PrP^{Sc} of other experimental scrapie strains. The same characteristic was revealed for scrapie strain 87V PrP^{Sc}. However, none of the three German field isolates of scrapie included in this study induced the accumulation of proteinase K–treated PrP^{Sc} with such low molecular mass. Similar differences in the molecular mass of prion proteins were found for scrapie, as well as for BSE and human diseases such as CJD and FFI (27,34,37). A conformational change of strain-specific PrP^{Sc} could be one reason for differences in molecular mass, by additional N-terminal amino acids to proteinase K digestion. In addition to mouse lesion profile-scoring (38) and PrP^{Sc} glycotyping, this characteristic of BSE-derived PrP^{Sc} might therefore provide us with the first distinctive parameter for future discrimination of BSE and scrapie infections in small ruminants.

Total PrP^{Sc} amounts were determined in proteinase K–digested homogenates of mouse brain tissues. High levels were found in C57BL/6

mouse brains infected with the scrapie isolate Hessen1 and strain ME7, respectively, and the lowest levels were detected in transgenic Tg20 mice infected with Chandler. The formed PrP^{Sc} amounts differed in scrapie strains as well as in the mouse line used. In this way, we were able to recognize an influence according to the mouse line used. VM95 mice expressed less PrP^{Sc} than C57BL/6 mice independent of the strain.

We compared the levels of PrP^{Sc} accumulation in the cerebrum, cerebellum, and brain stem of diseased mice. Substantial differences in the distribution of the PrP^{Sc} deposits in these brain areas were found depending on the nature of the strain or isolate. By this criterion, scrapie strain 87V could be distinguished from all other prion strains, including BSE isolates. While strain 87V PrP^{Sc} is deposited most abundantly in the brain stem, BSE infection leads to a rather even range of PrP^{Sc} deposits in the brain stem, cerebellum, and cerebrum. PrP^{Sc} of strain 22A is deposited primarily in the cerebellum of the mice. These results are in agreement with histoblot results on strain 22A mouse brains, with predominant PrP^{Sc} staining in the cortex, granular cell layer of the cerebellum, hippocampus, and hypothalamus. Most PrP^{Sc} of 87V accumulated in the thalamus, locus ceruleus, and raphe nuclei of the brain stem; very few accumulated in the neocortex, hippocampus, and hypothalamus (39–40).

The inoculation of field agents of scrapie into C57BL/6 mice resulted in clearly different patterns. Each isolate had a specific incubation time, a particular amount of formed PrP^{Sc}, a characteristic distribution of PrP^{Sc} deposits in brain areas, and a characteristic resistance to proteinase K digestion. Scrapie isolate S833 exhibited properties similar to those of strain 22A with regard to proteinase K resistance and PrP^{Sc} deposition in brain. This finding suggests that 22A is a representative field strain of scrapie. Moreover, PrP^{Sc} glycotyping profiles, molecular mass, and/or long-term PK resistance results obtained indicated that the three German field isolates of scrapie can be distinguished from the BSE isolates.

In summary, these studies have demonstrated that the PrP^{Sc} elicited by distinct prion strains and isolates passaged in in- and outbred mouse lines has different but unique, strain-specific properties. Glycotyping of the non-, mono- and diglycosylated PrP^{Sc} isoforms, as well as analysis of the molecular mass of the protein bands, was not sufficient for providing a clear discrimination of some strains, such as scrapie

strain 87V and the BSE isolates. Through differences in long-term resistance to proteinase K digestion, we were able to pinpoint a characteristic that helped us distinguish between these two strains/isolates. Analysis of the deposits and physicochemical characterization of PrP^{Sc} in BSE and scrapie strains/isolates thus yield important information for the possible discrimination between strains. Furthermore, it seems that PrP^{Sc} strains differ in their structural conformation; this can result in different proteinase K sensitivities, varying PrP^{Sc} accumulations, deposition in different brain areas, and individual glycoform patterns.

Acknowledgements

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