A sensitive filter retention assay for the detection of PrP^{Sc} and the screening of anti-prion compounds

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Abstract **A hallmark of prion diseases is the accumulation of an abnormally folded prion protein, denoted PrP^{Sc}. Here we describe a new and highly sensitive method for the detection of PrP^{Sc} in brain and other tissue samples that utilizes both PrP^{Sc} diagnostic criteria in combination; protease resistance and aggregation. Upon filtration of tissue extracts derived from scrapie- or bovine spongiform encephalopathy-infected animals, PrP^{Sc} is retained and detected on the membranes. Laborious steps such as SDS–PAGE and Western blotting are avoided with concomitant gain in sensitivity and reliability. The new procedure also proved useful in a screen for anti-prion compounds in a scrapie-infected cell culture model. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

Key words: Anti-prion compound; Bovine spongiform encephalopathy; Creutzfeldt–Jakob disease; Prion

1. Introduction

The fundamental event in prion diseases such as Creutzfeldt–Jakob disease (CJD) in humans, bovine spongiform encephalopathy (BSE) in cattle and scrapie in sheep is the conversion of the cellular prion protein, PrP^C, into a pathogenic isoform, PrP^{Sc} [1–3]. Accumulation of PrP^{Sc} in the brain of prion-infected animals correlates with the rise in the titer of infectious prions and is used as a diagnostic marker for prion diseases. In light of the threat of an interspecies transmission of BSE to humans, a large number of domestic animals must be tested for the presence of PrP^{Sc} in the brain or other suitable material [4–6]. In the absence of covalent modifications that would allow a distinction between PrP^{Sc} and PrP^C, PrP^{Sc} is routinely detected in proteinase K (PK)-treated homogenates by Western blotting or enzyme-linked immunosorbent assay (ELISA) utilizing the fact that PrP^{Sc}, but not PrP^C, is partially protease resistant. Notably, these currently available assays do not take advantage of the fact that PrP^{Sc} forms aggregates. Indeed, formation of detergent-resistant PrP aggregates is a general biochemical property of PrP^{Sc}, even for rare prion strains where PrP^{Sc} is sensitive to proteolytic digestion [7].

As shown previously, detection of amyloid aggregates in neurodegenerative diseases caused by polyglutamine proteins is possible by a simple filter retention assay [8]. Here we developed a similar technique for the reliable and sensitive detection of PrP^{Sc} in tissue homogenates or cell lysates using a slot blot device and nitrocellulose (NC) or cellulose acetate (CA) membranes. With adaptation, this method will allow the rapid high-throughput testing of large numbers of tissue samples for PrP^{Sc} and the screening of compound libraries for substances with anti-prion activity.

2. Materials and methods

2.1. Preparation of protein extracts

2.1.1. Mouse brain. Whole brains derived from CD1 mice or from clinically ill CD1 mice infected with RML prions were homogenized at room temperature in 10 volumes of PBS (phosphate-buffered saline) containing 0.25% trypsin by successive passages through 16-, 18- and 20-gauge needles. Trypsin digestion was stopped by the addition of trypsin inhibitor. The homogenate was then centrifuged (800 rpm, 5 min) to remove debris and connective tissue (see Fig. 1, step A).

When PK digestion of membrane-bound PrP was performed (see below) the brain homogenate was diluted to 1% (w/v) with PBS-containing Triton X-100 and deoxycholate (DOC, 0.5% each), incubated on ice for 10 min and cleared by a low-speed spin (1500 rpm, 10 min) to obtain a post-nuclear supernatant (PNS). The PNS was diluted with sarkosyl buffer (0.5% in PBS) and analyzed by the filter retention assay. Alternatively, the PNS was subjected to a high-speed centrifugation (13 000 rpm, 20 min) and the pellet resuspended in sarkosyl buffer prior to analysis by the filter retention assay (see Fig. 1, step B1).

2.1.2. Bovine brain. Bovine brain stem tissue of a control and a BSE-diagnosed animal was homogenized in 10 volumes Triton X-100/ DOC buffer. PNSs were prepared and filtered through NC membranes. Detection of PrP was carried out as described for the mouse brain extracts (see below). For Western blotting analysis, the homogenates were digested with PK (100 μg/ml, 1 h at 37°C) or mock-treated, the samples boiled in SDS sample buffer separated by SDS–PAGE, followed by electrotransfer to NC and Western blotting [13].

2.1.3. Cultured cells. N2a and scrapie-infected mouse neuroblastoma (ScN2a) cells were grown in modified Eagle's medium supplemented with antibiotics (1 U/ml penicillin G and 1 mg/ml streptomycin) and 10% fetal calf serum. N2a cells are an immortalized neuroblastoma cell line (ATCC No. CCl 131). ScN2a cells were established by infecting N2a cells with an enriched preparation of prions isolated from the brains of scrapie-ill mice infected with RML prions [10]. For treatment with DOSPA ScN2a, cells cultivated for 4 days were incubated for 16 h in DOSPA as described [14]. Trypsin treatment of the cells (0.25% final) was carried out on the cell culture dish maintained on ice. After complete detachment of the cells, digestion was terminated by the addition of a 10-fold excess of soybean trypsin inhibitor (Gibco BRL). The cells were collected by a brief centrifugation, washed twice with PBS-containing trypsin inhibitor and then protein lysates were prepared as described for brain samples. An equivalent of 10^6 cells was used for the filter retention assay.

2.2. PK treatment

2.2.1. In homogenates. 10% homogenates were first incubated with DNase I (Roche) (1 h, 37°C) to eliminate high-molecular weight DNA. Subsequently, PK (Roche) (100 μg/ml) was added, the lysates

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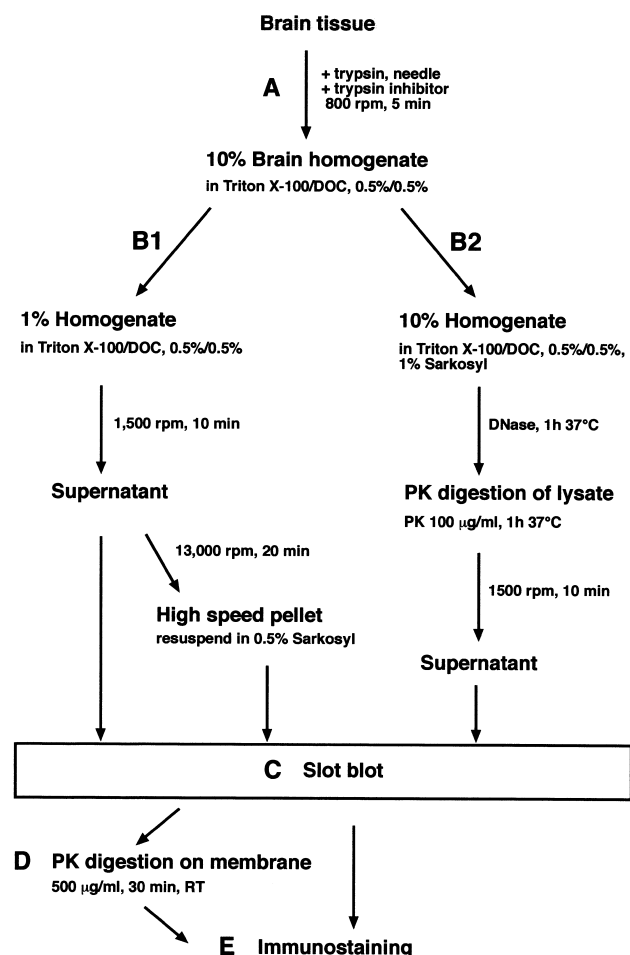*E-mail address:* tatzelt@biochem.mpg.de (J. Tatzelt).

Brain tissue

A | + trypsin, needle
+ trypsin inhibitor
800 rpm, 5 min

10% Brain homogenate
in Triton X-100/DOC, 0.5%/0.5%

B1 / \ **B2**

1% Homogenate
in Triton X-100/DOC, 0.5%/0.5%

10% Homogenate
in Triton X-100/DOC, 0.5%/0.5%,
1% Sarkosyl

1,500 rpm, 10 min   DNase, 1h 37°C

Supernatant   **PK digestion of lysate**
PK 100 μg/ml, 1h 37°C

13,000 rpm, 20 min   1500 rpm, 10 min

High speed pellet
resuspend in 0.5% Sarkosyl

Supernatant

C   Slot blot

D   PK digestion on membrane
500 μg/ml, 30 min, RT

E   Immunostaining

Fig. 1. Flow chart of the filter retention assay describing alternative procedures for the detection of PrP^{Sc} in brain homogenates (see text and Section 2 for details). In route B1, treatment with PK is performed on the membranes (D), whereas in B2, incubation with PK occurs in the lysates.

incubated for 1 h at 37°C, and proteolysis terminated by the addition of Pefabloc (Roche). After a low-speed spin, the resultant supernatant was analyzed for PrP^{Sc} (see Fig. 1, step B2).

2.2.2. On filter. Membranes loaded with protein were submerged in PK buffer (500 μg/ml in PBS) for 30 min at room temperature. The reaction was stopped by extensive washing (three times in PBST buffer (PBS, 0.1% Tween)) (see Fig. 1, step D).

2.3. Filter retention and immunodetection

A commercially available slot blot device (Amersham Pharmacia Biotech, PR 648 Slot Blot Manifold) was used for the filtration of protein homogenates through NC (0.45 μm pore size) or CA (0.2 μm pore size) membranes (Schleicher and Schuell, Dassel, Germany). All homogenates were applied in duplicate and in a stepwise 10-fold dilution with sarkosyl buffer as the diluent. After the samples were filtered through the membrane, each slot was washed with 500 μl sarkosyl buffer (see Fig. 1, step C). For immunodetection, the membranes were incubated with 3 M guanidinium–HCl for 10 min, washed three times in PBST, and PrP was detected by the anti-PrP antibody A7 using the enhanced chemoluminescence (ECL) detection system (Amersham Pharmacia Biotech) (see Fig. 1, step E).

3. Results

3.1. A filter retention assay to detect PrP^{Sc} in brain homogenates

A filter retention assay was developed to monitor the pres-

ence of detergent-insoluble PrP^{Sc} aggregates in homogenates of scrapie- or BSE-infected brains and in lysates of scrapie-infected cultured cells. Alternative strategies were employed for the preparation of protein extracts from scrapie-infected mouse brains and the selective proteolysis of PrP^{C}. These variations of the procedure are outlined in the flow diagram of Fig. 1. Briefly, whole mouse brains were homogenized in Triton X-100/DOC detergent buffer by successive passages through 16-, 18- and 20-gauge needles (Fig. 1, step A). Addition of trypsin during homogenization facilitated tissue dissociation and degraded cell surface PrP^{C}. After addition of trypsin inhibitor, cell debris and connective tissue were removed by centrifugation. The resulting supernatant was processed either with or without PK treatment. Homogenate B1 (no PK treatment) was subjected to low-speed centrifugation to prepare a PNS. After addition of sarkosyl, this PNS was used directly for analysis by filtration. Alternatively, PrP^{Sc} aggregates were first pelleted from the PNS and then resuspended in sarkosyl buffer. Homogenate B2 (with PK treatment) was incubated with DNase to eliminate high-molecular weight DNA prior to incubation with PK. Filtration through CA or NC membranes was performed with a commercially available slot blot device (step C). To minimize sample handling, we also explored the possibility to degrade PrP^{C} directly on the filter membrane after filtration of the homogenates (step D). PrP^{Sc} was detected by immunostaining after a brief incu-
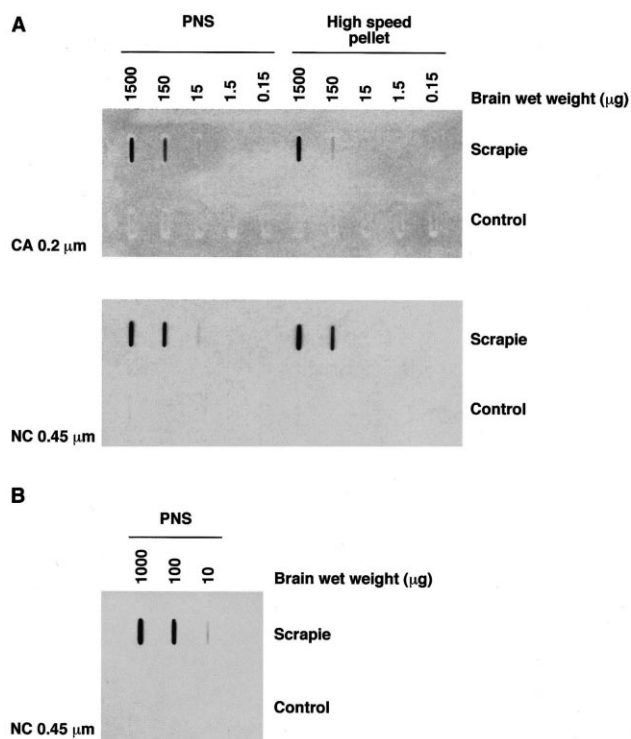
Fig. 2. Selective filter retention of PrP^{Sc} from mouse brain homogenates. A: 10% brain homogenates were processed as described to obtain a PNS or a high-speed pellet fraction in sarkosyl buffer (Fig. 1, step B1). Serial dilutions were prepared in sarkosyl buffer and applied onto CA or NC filters. Amounts of wet weight of brain tissue analyzed are indicated in μg. B: PNS from scrapie-infected brain was diluted with nine volumes of PNS from control brain. The amounts indicated refer to the wet weight of scrapie-infected brain tissue present in the sample. Immunodetection was carried out after PK digestion on the membrane (Fig. 1, step D) with the anti-PrP antibody A7 and the ECL detection system.

bation of the membranes in 3 M guanidinium–HCl to expose PrP epitopes [9].

Fig. 2A shows a comparison of the PrP^{Sc} retention properties of CA (0.2 μm pore size) and NC (0.45 μm pore size) membranes loaded with PNS or resuspended high-speed pellets from scrapie-infected and control brains (see Fig. 1, step B1). PK digestion was performed after filtration directly on the membranes (see Fig. 1D, step D). For both membranes the assay was highly specific for PrP^{Sc} in scrapie-infected mice as no signal above background was detected with control homogenates from non-infected animals. Analysis of serial dilutions of the various preparations indicated that with both membranes a clear signal was obtained with an amount of homogenate corresponding to 150 μg of wet weight of brain tissue. Sensitivity and the signal to background ratio were better for the NC membrane. To demonstrate that the sensitivity of the filter retention assay is unaffected by the total amount of tissue analyzed, homogenates of scrapie brain were serially diluted with control homogenate, leaving the total amount of protein constant. Again, the PrP^{Sc} present in 100 μg of scrapie brain homogenate, now analyzed in the presence of 900 μg of control homogenate, was clearly detected by the filter retention assay (Fig. 2B).

Homogenates treated with PK before filtration (see Fig. 1, step B2) were used to compare the sensitivity of the filter retention assay with the Western blotting procedure that is frequently used for the routine detection of PrP^{Sc} (Fig. 3A,B). After PK digestion, equivalent amounts of homogenates were either filtered through a NC membrane (Fig. 3A) or analyzed by SDS–PAGE and electrotransfered to the same type of NC membrane (Fig. 3B). Immunodetection of PrP on both membranes was done in parallel. Similar to the results

obtained with PK treatment after filtration, an amount of PrP^{Sc} derived from 150 μg brain tissue was clearly detected in the filter retention assay and PrP^{Sc} from 15 μg of tissue was still weakly detectable (Fig. 3A, left panel). The assay gave an essentially linear response between 15 μg and 15 mg of scrapie brain tissue. In contrast, the analysis of PrP^{Sc} by Western blotting was at least 10-fold less sensitive (Fig. 3B, right panel).

In summary, the filter retention assay detects PrP^{Sc} in brain homogenates of mice with experimental scrapie specifically and with high sensitivity. The laborious steps of SDS–PAGE and immunoblotting can be omitted. PK treatment of individual samples is circumvented, thus further minimizing sample handling and avoiding a potential source of variability.

3.2. Detection of PrP^{Sc} in the brain of preclinical BSE-infected cattle

We tested whether the filter retention assay for PrP^{Sc} can also be used to detect BSE-infected cattle. Bovine brain stem tissue from a diagnosed, preclinical animal was obtained from Dr. M. Groschup (Federal Research Center for Virus Diseases of Animals, Tübingen, Germany). The most rapid procedure for sample preparation outlined in Fig. 1 was chosen for this analysis. Control and BSE-infected brain tissue was directly homogenized in Triton X-100/DOC buffer. PNSs were prepared and serial dilutions thereof were filtered through a NC membrane. Prior to immunodetection with the A7 anti-PrP antiserum, membrane-bound proteins were subjected to limited PK digestion. PrP^{Sc} was specifically detected in samples derived from 100 μg of BSE brain tissue (Fig. 4A).

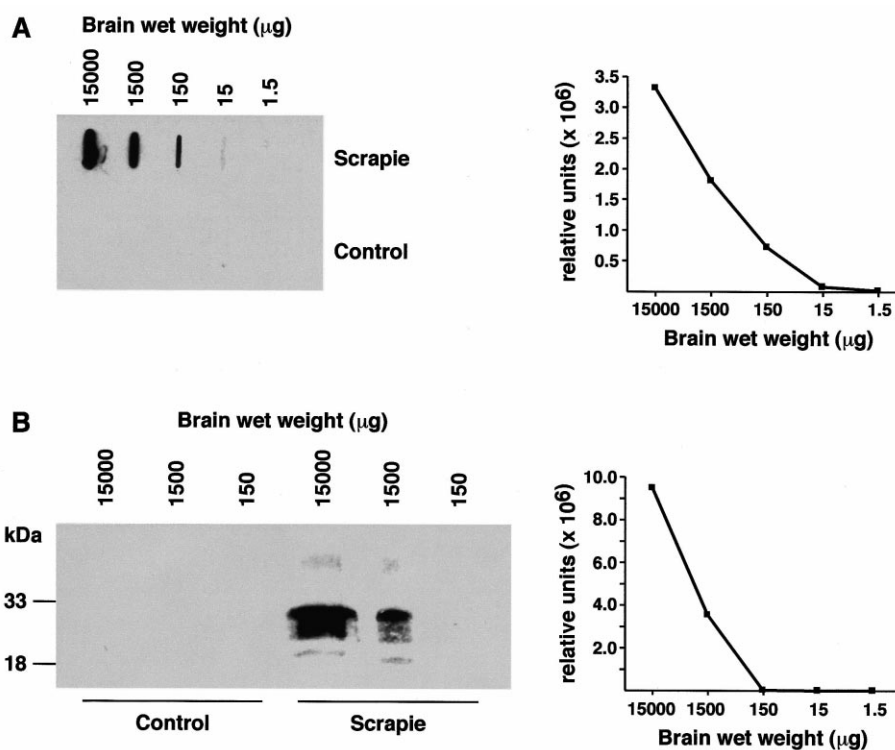To compare the sensitivity of the filter retention assay with

Fig. 3. Detection of PrP^{Sc} in mouse brain by filter retention assay and by SDS–PAGE/Western blotting. Brain homogenates were subjected to PK treatment (Fig. 1, step B2) and serial dilutions analyzed in parallel by the filter retention assay (A) and by SDS–PAGE and Western blotting (B). PrP was detected as described in Fig. 2. Results were quantified by phosphorimager analysis (right panels).

a Western blotting approach, 10% brain homogenates were digested with PK (100 µg/ml, 1 h at 37°C), separated with SDS–PAGE, and transferred to NC membranes. Immunodetection was done in parallel with the same type of NC filter used in the filter assay approach. The BSE-infected sample was clearly identified, however, with an at-least 10-fold reduced sensitivity compared to the filter retention assay (Fig. 4B). Thus, the filter retention assay is capable of detecting PrP^{Sc} in brain stem tissue of cattle with preclinical BSE.

3.3. A screening method for anti-prion compounds

ScN2a cells offer the possibility to study the propagation of PrP^{Sc} in cell culture [10–12]. We successfully used this cell culture model to identify compounds that interfere with the accumulation of PK-resistant and -infectious PrP^{Sc} [13–15]. Notably, the lipopolyamine DOSPA at nanomolar concentrations induced degradation of pre-existing PrP^{Sc} aggregates in live cells within 16 h [14]. In order to explore the possible use of the filter retention assay as a screening method for anti-prion compounds, we adapted the sample preparation employed for brain tissue (see Fig. 1). Total cell lysates with and without PK digestion, PNSs and high-speed pellets, were prepared from an equal number of cells (Fig. 5A). A positive signal for PrP was only obtained with lysates prepared from ScN2a cells, indicative of the presence of PrP^{Sc}.

Next, ScN2a cells that had accumulated substantial amounts of PrP^{Sc} were incubated for 16 h with increasing nanomolar concentrations of DOSPA (Fig. 5B). As for brain homogenates, low-speed supernatants and resuspended high-

Fig. 5. A cell culture model for the screening of anti-prion compounds. A: Detection of PrP^{Sc} from ScN2a cells. Protein lysates from ScN2a and non-infected N2a cells were prepared, and PrP was analyzed by filter retention assay. B: DOSPA induces degradation of PrP^{Sc}. ScN2a cells cultivated for 4 days were incubated in DOSPA (5 or 10 nM) or mock-treated (DOSPA, 0 nM) for an additional 16 h. PNS and high-speed pellets were prepared and PrP analyzed by filter retention assay. Immunodetection was carried out as described in Fig. 2.
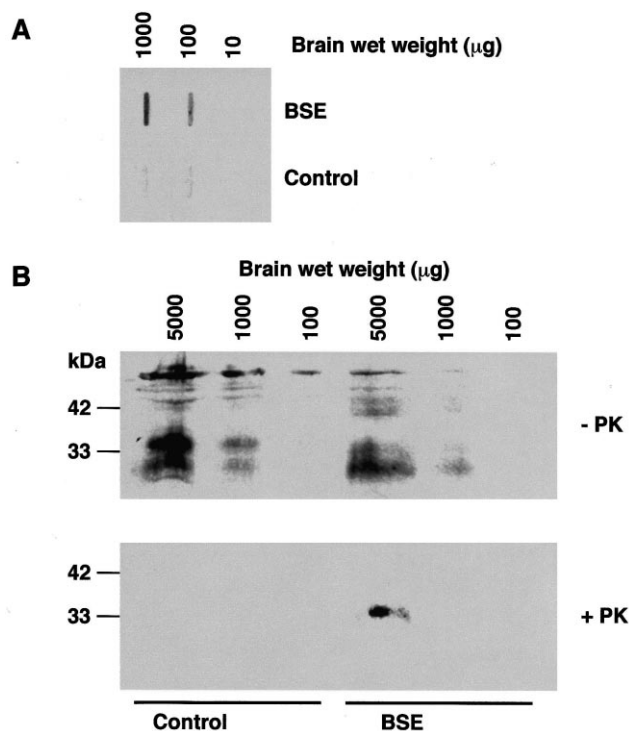
speed pellets of cell lysates were analyzed by the filter retention assay. With both methods a concentration-dependent decrease in the amounts of detectable PrP^{Sc} was observed, demonstrating the suitability of the filter retention assay as a screening method for anti-prion compounds.

4. Discussion

The advent of new variant CJD suggests that prion diseases can be transmitted from animals to humans [5,6,16–18]. To eliminate scrapie-infected material from food and pharmaceutical products, a large number of domestic animals must be tested for the presence of infectious prions. Currently available assays for the detection of PrP^{Sc} in brain samples utilize the protease resistance of PrP^{Sc} relative to PrP^{C} as the single diagnostic criterion for PrP^{Sc}. Brain homogenates are incubated with PK individually, a potential source of variability and false-positive test results, and the remaining PrP protein is detected immunologically by ELISA or by laborious SDS–PAGE and Western blotting procedures. To avoid these problems, we have developed a simple filter retention assay for PrP^{Sc} that measures two diagnostic biochemical properties of PrP^{Sc} in combination, protease resistance and the presence of a detergent-insoluble aggregated state. PK treatment can be performed conveniently and under standardized conditions directly on the filter membrane for many samples in parallel.

Providing various options for sample preparation, the filter retention assay is adaptable to different tissues and also to liquor samples. Its main advantage is the concentration of PrP^{Sc} from large sample volumes on a small filter area, resulting in highly sensitive detection with very little or no background signal from normal PrP^{C}. The PrP^{Sc} present in as little

Fig. 4. Detection of PrP^{Sc} in brain extracts of BSE-infected cattle. A: Serial dilutions of PNS were filtered onto NC membranes (0.45 µm pore size) and membrane-bound proteins subjected to limited PK digestion. B: 10% brain homogenates were mock-treated (-PK) or incubated with PK (+PK), boiled in SDS sample buffer, separated by SDS–PAGE and electrotransferred onto NC filters (0.45 µm). Immunodetection of membrane-bound PrP was carried out with anti-PrP antiserum A7.

as 100 μg of wet weight of infected mouse or bovine brain can be detected reliably. In contrast, upon SDS–PAGE and Western blotting, the PrP signal spreads over a mass range from 27 to 30 kDa. This and the limited efficiency of electrotransfer may explain why in a side-by-side comparison, the performance of SDS–PAGE and Western blotting was inferior to that of the filter retention assay.

Another application of the filter retention assay is the screening of compound libraries for anti-prion agents. Proof of principle was provided by the demonstration of the concentration-dependent effect of the lipopolyamine DOSPA in causing degradation of PrP^{Sc} in a cell culture model of ScN2a cells [14]. We are currently adapting the cell culture assay to a 96-well format to establish a high-throughput screen for anti-prion compounds.

References

[1] Collinge, J. (2001) Annu. Rev. Neurosci. 24, 519–550.
[2] Weissmann, C. et al. (1996) Cold Spring Harbor Symp. Quant. Biol. 61, 511–522.
[3] Prusiner, S.B., Scott, M.R., DeArmond, S.J. and Cohen, F.E. (1998) Cell 93, 337–348.
[4] Aguzzi, A., Montrasio, F. and Kaeser, P.S. (2001) Nat. Rev. Mol. Cell. Biol. 2, 118–126.
[5] Lasmezas, C.I. et al. (2001) Proc. Natl. Acad. Sci. USA 98, 4142–4147.
[6] Scott, M.R., Will, R., Ironside, J., Nguyen, H.O., Tremblay, P., DeArmond, S.J. and Prusiner, S.B. (1999) Proc. Natl. Acad. Sci. USA 96, 15137–15142.
[7] Scott, M.R., Groth, D., Tatzelt, J., Torchia, M., Tremblay, P., DeArmond, S.J. and Prusiner, S.B. (1997) J. Virol. 71, 9032–9044.
[8] Wanker, E.E., Scherzinger, E., Heiser, V., Sittler, A., Eickhoff, H. and Lehrach, H. (1999) Methods Enzymol. 309, 375–386.
[9] Taraboulos, A., Jendroska, K., Serban, D., Yang, S.L., DeArmond, S.J. and Prusiner, S.B. (1992) Proc. Natl. Acad. Sci. USA 89, 7620–7624.
[10] Butler, D.A., Scott, M.R.D., Bockman, J.M., Borchelt, D.R., Taraboulos, A., Hsiao, K.K., Kingsbury, D.T. and Prusiner, S.B. (1988) J. Virol. 62, 1558–1564.
[11] Caughey, B. and Raymond, G.J. (1991) J. Biol. Chem. 266, 18217–18223.
[12] Borchelt, D.R., Taraboulos, A. and Prusiner, S.B. (1992) J. Biol. Chem. 267, 16188–16199.
[13] Tatzelt, J., Prusiner, S.B. and Welch, W.J. (1996) EMBO J. 15, 6363–6373.
[14] Winklhofer, K.F. and Tatzelt, J. (2000) Biol. Chem. 381, 463–469.
[15] Gilch, S. et al. (2001) EMBO J., in press.
[16] Hill, A.F., Desbruslais, M., Joiner, S., Sidle, K.C., Gowland, I., Collinge, J., Doey, L.J. and Lantos, P. (1997) Nature 389, 448–450.
[17] Collinge, J., Sidle, K.C., Meads, J., Ironside, J. and Hill, A.F. (1996) Nature 383, 685–690.
[18] Bruce, M.E. et al. (1997) Nature 389, 498–501.