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Characterization of proteinase K-resistant N- and C-terminally truncated PrP in Nor98 atypical scrapie

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An increasing number of scrapie cases with atypical characteristics, designated Nor98, have recently been recognized. Here, the proteinase K (PK)-resistant prion protein (PrP) fragments from two Swedish cases of Nor98 atypical scrapie have been characterized. The prominent, fast-migrating band in the distinct Nor98 Western immunoblot electrophoretic profile was determined to be of 7 kDa in size and was accordingly designated Nor98-PrP7. The antigenic composition of Nor98-PrP7, as assayed by a panel of anti-PrP antibodies, revealed that this fragment comprised a mid-region of PrP from around aa 85 to 148. N- and C-terminally truncated fragments spanning the mid-region of PrP have only been observed in the genetic prion disorder Gerstmann–Sträussler–Scheinker disease. It is shown here that the long-term PK resistance of Nor98-PrP7 is reduced compared with that of PrP^{res} in classical scrapie. Enzymic deglycosylation did not change the distinct electrophoretic profile of Nor98-PrP7. A previously unidentified, PK-resistant, C-terminal PrP fragment of around 24 kDa was detected and its PK resistance was investigated. After deglycosylation, this fragment migrated as a 14 kDa polypeptide and was designated PrP-CTF14. Antigenic determination and the size of 14 kDa suggested a fragment spanning approximately aa 120–233. The existence of two PK-resistant PrP fragments, Nor98-PrP7 and PrP-CTF14, that share an overlapping region suggests that at least two distinct PrP conformers with different PK-resistant cores are present in brain extracts from Nor98-affected sheep. The structural gene of PrP in three Nor98-affected sheep was analysed, but no mutations were found that could be correlated to the aberrant PK-resistant profile observed.

INTRODUCTION

Scrapie in sheep and goats belongs to the prion diseases or transmissible spongiform encephalopathies (TSEs), together with Creutzfeldt–Jakob disease (CJD) in man, bovine spongiform encephalopathy (BSE) in cattle and chronic wasting disease in cervids (Prusiner, 1998; Sigurdson & Miller, 2003). The cellular prion protein (PrP^{C}) is a glycoprotein with a function that is not fully understood and is, in the diseased animal, present in a partially protease-resistant, alternatively folded isoform (PrP^{Sc}). PrP^{Sc} can impose its aberrant conformation onto PrP^{C}, thereby amplifying the aberrant prion protein. The infectious prion is composed largely, if not entirely, of PrP^{Sc} and is the transmissible agent of TSEs (Prusiner, 1998). Scrapie has been recognized in the sheep population for more than two centuries (Stockman, 1913). Recently, a number of atypical cases of scrapie were diagnosed in sheep in Norway and subsequently designated Nor98 (Benestad *et al.*, 2003). Similar atypical scrapie cases were also reported from other parts of Europe (De Bosschere *et al.*, 2004; Onnasch *et al.*, 2004), including Sweden (Gavier-Widen *et al.*, 2004). Nor98 cases differ from classical scrapie and BSE in several features, including the pattern of PrP^{Sc} deposits and the neuroanatomical distribution of the histopathological lesions in the brain (Benestad *et al.*, 2003). Other uncommon features are observed in the clinical presentation, epidemiology and frequency of genotypes of sheep affected (Benestad *et al.*, 2003; Moum *et al.*, 2005). The differentiation between classical scrapie and Nor98 is based on these features and substantiated by the observation of the Nor98 Western blot electrophoretic profile, typified by a fast-migrating, proteinase K (PK)-resistant material (Benestad *et al.*, 2003). PK-treated brain extracts from Nor98 cases demonstrate PrP of full-length size, as well as a smaller fragment migrating with an electrophoretic mobility consistent with an estimated size of around 12 kDa. The smallest

fragment seen in classical scrapie extracts is a C-terminal, PK-resistant peptide of around 19–21 kDa (Hayashi *et al.*, 2005; Hope *et al.*, 1999; Stahl *et al.*, 1993). In CJD, two patterns of PK-resistant fragments are seen, type 1 and type 2, with unglycosylated forms of 21 and 19 kDa, respectively (Monari *et al.*, 1994; Parchi *et al.*, 1996, 1997, 2000). However, brain extracts from subjects affected by certain types of Gerstmann–Sträussler–Scheinker disease (GSS) contain PrP^{res} fragments of around 7–11 kDa, carrying ragged N and C termini (Ghetti *et al.*, 1996; Parchi *et al.*, 1998; Piccardo *et al.*, 1995, 1996, 2001; Tagliavini *et al.*, 1991, 2001). GSS is a prion disease with a genetic background with various mutations in the prion protein (reviewed by Young *et al.*, 1999). The corresponding GSS fragment of 7 kDa synthesized *in vitro* could be assembled into amyloid fibrils (Salmona *et al.*, 2003; Tagliavini *et al.*, 2001).

This study was undertaken to characterize the Nor98 PrP^{res} fragmental pattern by immunoblot mapping and to investigate its resistance to PK in relation to classical scrapie. The prion protein from different cases of Nor98 was analysed in order to find out whether a genetic background to Nor98 exists.

METHODS

Reagents. Peptide : *N*-glycosidase F (PNGase F) was from New England BioLabs. PK and protein molecular mass markers (MagicMark and SeeBlue Plus2) were from Invitrogen. Iodoacetamide (IAA), sodium deoxycholate and PMSF were from Sigma-Aldrich. Dithiothreitol (DTT) was from USB Corporation. Nonidet P40 (NP40) was from Fluka AG.

Case tissues and DNA sequencing. Clinical tissue samples from three Swedish Nor98-affected sheep were used in this study. The three codons associated with scrapie susceptibility and resistance (codons 136, 154 and 171) are shown in parentheses. Case 1 was from the southern county of Skåne (136A/A 154R/H 171R/Q), case 2 from the Baltic island of Gotland (136A/A 154R/R 171Q/Q) and case 3 from Västra Götaland (136A/A 154R/R 171R/Q) [cases 1, 2 and 4, respectively, in the paper by Gavier-Widen *et al.* (2004)]. Nor98 cases 1 and 2 and a Swedish scrapie case from 1986 (Elvander *et al.*, 1988) were used in Western immunoblot analyses. The Western immunoblotting results on Nor98 brain homogenates from the two cases reported in this study were the same, irrespective of genotype, and were each replicated two to five times. For sequencing of the prion protein gene (PRNP) open reading frame (ORF) in Nor98-affected sheep, material from cases 1, 2 and 3 was used. Brain-tissue samples from the obex region of Nor98 and scrapie cases were kindly provided by the National Veterinary Institute, Uppsala, Sweden. Ovine brain tissue from a healthy animal was provided by Dr M. A. Tranulis, Norwegian School of Veterinary Science, Oslo, Norway. DNA was extracted from brain tissue and analysed for the presence of known or new coding mutations in the PRNP ORF. The complete PRNP ORF was amplified by PCR using two oligonucleotide primers, 5′-GCTGACACCCTCTTTATTTTGC-3′ (forward) and 5′-GCAAGAAATGAGACACCACCAC-3′ (reverse). The PCR products were sequenced on both DNA strands by using a MegaBACE 1000 (Amersham Biosciences). Chromatograms were analysed with DNAStar SeqMan version 5.01 (DNAStar Inc.).

Antibodies. The ovine PrP numbering scheme is applied throughout, except where specifically stated otherwise. mAb P4 (R-Biopharm AG) recognizes amino acid residues 93–99 (Harmeyer *et al.*, 1998; Thuring *et al.*, 2004). mAb 6H4 (Prionics AG) recognizes aa 147–155 (Korth *et al.*, 1997). mAbs 8B4 and 8F9 (kindly donated by Dr M.-S. Sy, Case Western Reserve University, Cleveland, OH, USA) recognize aa 40–47 (Li *et al.*, 2000) and aa 228–234 (Kang *et al.*, 2003), respectively. mAb L42 (R-Biopharm AG) is raised against aa 145–163 (Vorberg *et al.*, 1999). mAb 2A11 [kindly donated by Dr J. M. Torres, Centro de Investigación en Sanidad Animal (CISA-INIA), Valdeolmos, Spain] recognizes aa 163–171 (Brun *et al.*, 2004). mAb 34C9 (Prionics AG) recognizes aa 141–145. mAb F89 (VMRD Inc.) was kindly donated by Dr M. A. Tranulis, Norwegian School of Veterinary Science, Oslo, Norway, and recognizes aa 142–145 (O'Rourke *et al.*, 1998; Van Everbroeck *et al.*, 1999). The rabbit polyclonal antisera R505 and R521 (kindly donated by Dr J. P. M. Langeveld, Lelystad, The Netherlands), are raised against aa 100–111 and 94–105 (Garssen *et al.*, 2000; van Keulen *et al.*, 1995), respectively.

Western immunoblot analysis of brain extracts. Homogenates were prepared as 10 % (w/v) brain tissue in lysis buffer [PBS, 0·5 % (v/v) NP40, 0·5 % (w/v) sodium deoxycholate] by using a PlusOne Sample grinding kit (Amersham Biosciences) according to the manufacturer's protocol. Following homogenization, extracts were centrifuged at 500 ***g*** in a microcentrifuge for 5 min at 25 °C. Supernatants were collected and treated with PK at 100 μg ml^{-1} for 1 h at 37 °C or for the indicated time in the PK time-course experiments. The digestion was terminated by addition of 2 mM PMSF. Proteins were then denatured and incubated in the absence or presence of PNGase F according to the manufacturer's protocol (New England Biolabs). After PK and PNGase F treatment, proteins were precipitated with trichloroacetic acid (TCA) by using a PlusOne SDS-PAGE Clean-Up kit (Amersham Biosciences) according to the manufacturer's protocol. The resulting protein pellet was dissolved in NuPAGE LDS sample buffer (Invitrogen) supplemented with 0·1 M DTT, and heated for 10 min at 100 °C. We found it prudent to test all antibodies on normal, scrapie and Nor98 sheep-brain homogenates, which were treated with and without alkylation, respectively (Yuan *et al.*, 2005). Of the antibodies used in this study, only the reactivity of mAb 6H4 was affected notably by alkylation. The signal detected from alkylated normal, scrapie and Nor98 samples was decreased compared with non-alkylated samples when 6H4 was used as detection antibody. In light of this, all samples probed with mAb 6H4 were not alkylated. After boiling and reduction, samples were incubated in the presence or absence of 1 % (w/v) of the alkylating agent IAA for 15 min at room temperature to prevent reformation of disulphide bonds. Samples were then centrifuged at 9000 ***g*** for 5 min at 25 °C in a microcentrifuge, loaded onto 12 % NuPAGE Bis-Tris gels (Invitrogen) and electrophoresed in MES buffer (Invitrogen). MES electrophoresis buffer provides an optimum separation of proteins in the 1–20 kDa size range (according to the manufacturer's instructions) and was therefore chosen for studies of the Nor98 small fragment. After electrophoresis, proteins were electrotransferred onto PVDF membranes (Hybond-P; Amersham Biosciences), which were then blocked for non-specific binding with 5 % (w/v) non-fat milk in PBS with 0·05 % Tween 20 (PBST) for 1 h at room temperature. When necessary, the blocked PVDF membranes were cut into strips and probed individually with antibody of choice. The membranes were incubated with primary antibody for 1 h at room temperature. After washing with PBST, the membranes were incubated for 1 h at room temperature with peroxidase-conjugated rabbit anti-mouse immunoglobulin antibodies or peroxidase-conjugated swine anti-rabbit immunoglobulin antibodies (Dako A/S), depending on the origin species of the primary antibody. The membranes were washed again in PBST and bands were visualized on X-ray film (Hyperfilm; Amersham Biosciences) by enhanced chemiluminescence (ECL; Amersham Biosciences). All antibodies were diluted in 1 % (w/v) non-fat milk in PBST.

Quantification of PrP fragments. Western immunoblots were scanned and the relative amounts of the bands of interest were obtained by computerized integration of peaks representing the bands using ImageJ software version 1.34m (US National Institutes of Health, Bethesda, MA, USA; http://rsb.info.nih.gov/ij/). Scanning was done on exposures within the linear range of the photographic film.

RESULTS

Distinct PK-resistant PrP fragments in Nor98-affected sheep-brain extracts

In Western immunoblotting, the Nor98 PK-resistant PrP has been characterized by an abnormal glycoprotein profile, contrasting with the triplet pattern in classical scrapie comprising the typical di-, mono- and unglycosylated PrP bands (Somerville & Ritchie, 1990). The glycoprotein profiles of classical scrapie and Nor98 display a clear difference in the banding pattern after PK treatment (Fig. 1a). The most notable difference in Nor98 samples compared with the scrapie pattern is the appearance of a fast-migrating band, determined here to be around 7 kDa in size and accordingly designated Nor98-PrP7 (Fig. 2a). This band has previously been reported to be around 12 kDa (Benestad *et al.*, 2003). However, by using a gel-electrophoresis buffer system that provides an optimum separation of proteins in the 1–20 kDa size range combined with the appropriate molecular markers, we could determine that its migration corresponded to a size of around 7 kDa. Traces of P4-immunoreactive material at the position of Nor98-PrP7 could be seen even in the untreated brain extract, suggesting that a fragment corresponding to Nor98-PrP7 is a metabolic product in the Nor98 disease (Fig. 1b). The mAb P4 and the polyclonal antipeptide sera R521 and R505 detected, in addition to the prominent Nor98-PrP7 band, signals of varying intensity from three PK-resistant PrP fragments, migrating to higher apparent molecular masses of 33, 28 and 15 kDa (Fig. 1a; Table 1). In contrast, when Western immunoblots of Nor98 brain homogenates were probed with mAb 6H4, only one PK-resistant fragment of around 24 kDa was detected and none of the fragments visualized by mAb P4 were seen (Fig. 1b). As a control of the reactivity of mAb 6H4, a scrapie brain extract and an extract from healthy sheep brain were analysed by Western immunoblotting (Fig. 1d, lanes 1–7).

The characteristic Nor98-PrP7 fragment is not affected by deglycosylation

Deglycosylation of the scrapie extract after PK treatment results in a fragment of around 20 kDa, corresponding to an N-terminally truncated, unglycosylated fragment (Chen *et al.*, 1995; Hayashi *et al.*, 2005; Stahl *et al.*, 1993; Yadavalli *et al.*, 2004). The Nor98-PrP7 did not contain any sugars, as its migration was unchanged by PNGase F treatment (Fig. 1c). Neither the 15 kDa nor the 28 kDa fragment shifted in electrophoretic mobility after deglycosylation, suggesting that these fragments are also non-glycosylated. However, the slowest-migrating band of 33 kDa contained carbohydrates, as it disappeared after deglycosylation, probably migrating to the 28 kDa position (Fig. 1c). As a control for the mAb P4, an extract from normal sheep brain was analysed by Western immunoblotting (Fig. 1d, lanes 8–11).

Nor98-PrP7 is a both N- and C-terminally truncated fragment spanning the mid-region of PrP

By using various anti-PrP antibodies that recognize distinct epitopes located in the N and C termini and mid-region of PrP in Western immunoblotting, we determined which epitopes were present in Nor98-PrP7. The fragment reacted with antibodies P4, R521, R505 and L42, whose epitopes span from aa 93 to 163. No reactivity was obtained with the extreme N-terminal mAb 8B4 (Table 1). mAb 6H4 (epitope, aa 147–155) did not detect Nor98-PrP7 (Fig. 2a) and thus the C terminus of the 7 kDa fragment does not show a 6H4-reactive epitope. However, mAb L42, which was raised towards a peptide encompassing aa 145–163 and partially overlaps the epitope of mAb 6H4, detected Nor98-PrP7 (Fig. 2a). This places the C terminus of the fragment in the region of aa 145–155. Its apparent size of 7 kDa and the reactivity towards mAbs P4 and L42 suggest that the small fragment is a result of PK truncation in both the N- and the C-terminal parts of PrP. N- and C-terminally truncated fragments of similar sizes spanning the mid-region of PrP have previously only been observed in certain subjects with the human genetic prion disorder GSS (Young *et al.*, 1999). In addition to the Nor98-PrP7 band, mAb P4 and the polyclonal antipeptide sera R521 and R505 also detected minor populations of three PK-resistant PrP species of varying intensity, migrating to around 33, 28 and 15 kDa (Figs 1a, b, 2a; Table 1). In spite of their sizes, these populations of PK-resistant material failed to react with several mAbs in Western immunoblotting (Table 1). The expected triplet pattern is seen when the scrapie brain extract was immunoblotted with mAb P4 (Fig. 2b). Both mAbs L42 and 6H4 showed a similar pattern, but with a proportionally weaker reactivity towards the unglycosylated band (Fig. 2b). As a further control of the reactivity of mAb L42, Western immunoblots on brain extracts from Nor98, scrapie and healthy sheep with and without PK and PNGase F treatment were performed (Fig. 2c)

Identification and characterization of a PK-resistant C-terminal PrP fragment in Nor98, PrP-CTF14

In addition to the Nor98-PrP7 fragment, mAb L42 detected a PK-resistant fragment of around 24 kDa (Fig. 2a, lane 3). The same fragment of molecular mass 24 kDa was detected by antibodies with epitopes C-terminal to the epitope of L42, such as 6H4 (Fig. 2a), 2A11 (Fig. 3c) and the extreme C-terminal mAb 8F9 (Table 1). However, neither the extreme N-terminal mAb 8B4 nor antibodies P4, R505 and R521, directed towards the mid-region of PrP, detected this fragment (Figs 1b, 2a; Table 1). The fact that the C-terminal

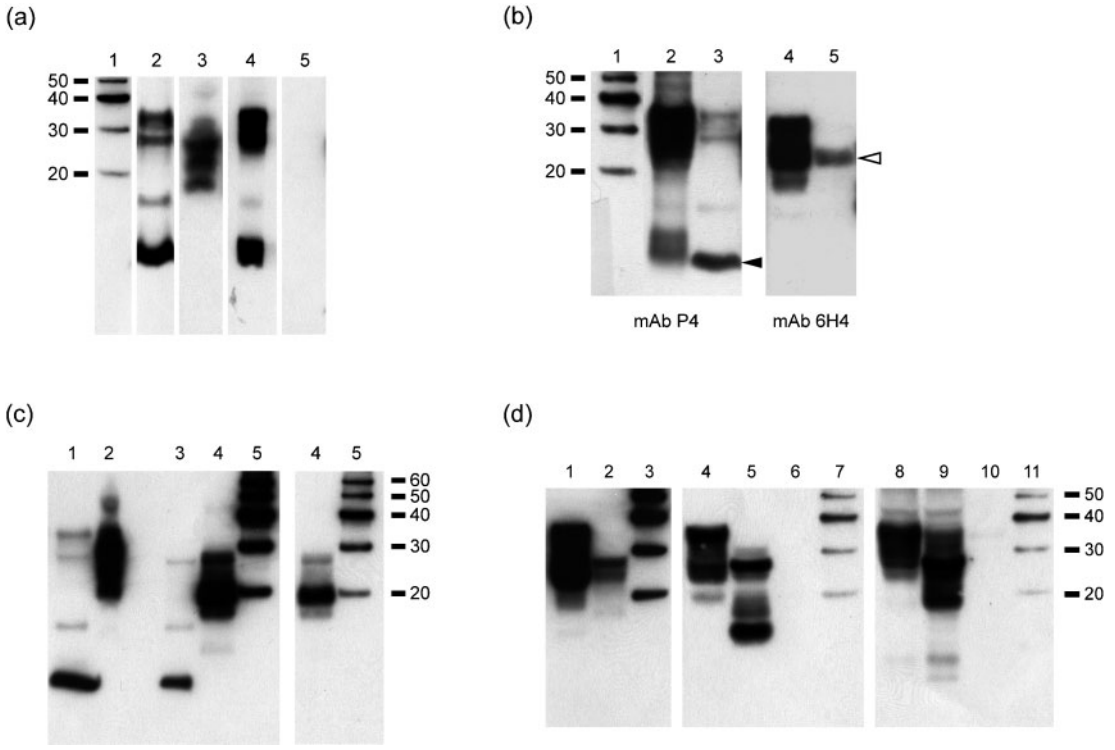

Fig. 1. Western immunoblot characterization of PrP^{res} in Nor98 by mAbs P4 and 6H4. (a) Brain homogenates were treated with PK (see Methods). Lanes: 1, molecular mass marker (MagicMark; Invitrogen); 2, Nor98 case 1; 3, sheep scrapie; 4, Nor98 case 2; 5, normal sheep. Membranes were probed with mAb P4. (b) Nor98 brain homogenate (case 1) was treated without PK (lanes 2 and 4) and with PK (lanes 3 and 5) and the filters were probed with mAb P4 (lanes 2 and 3) and mAb 6H4 (lanes 4 and 5) (see Methods). Filled arrowhead, Nor98-PrP7 detected by mAb P4; open arrowhead, 24 kDa fragment detected by mAb 6H4. Lane 1, molecular mass marker (MagicMark). (c) PNGase F treatment of PrP^{res} in Nor98. Brain homogenates were treated first with PK and then incubated without (lanes 1–2) or with (lanes 3–4) PNGase F (see Methods). Lanes 1 and 3, Nor98 case 2; lanes 2 and 4, sheep scrapie. Membranes were probed with mAb P4. A shorter exposure of lanes 4–5 is shown in a separate panel to the right. Lane 5, molecular mass marker (MagicMark). (d) Controls for antibody specificity. Brain homogenates were incubated without PK (lanes 1, 4 and 8), with PK (lanes 2, 6 and 10) or with PNGase F (lanes 5 and 9). Lanes 1–2, sheep scrapie; lanes 4–6 and 8–10, normal sheep. Membranes were probed with mAb 6H4 (lanes 1–7) and mAb P4 (lanes 8–11). Lanes 3, 7 and 11, molecular mass marker (MagicMark). Apparent molecular masses based on migration of protein standards are indicated (kDa).

mAbs, as well as L42, reacted with a 24 kDa, PK-resistant PrP fragment from Nor98 extracts has not been reported before. Its identity towards the C-terminal part of PrP is further substantiated by its sensitivity to deglycosylation (Fig. 2a). PNGase F treatment shifted the band from around 24 to 14 kDa and the fragment was therefore denoted PrP-CTF14 (C-terminal fragment 14 kDa). Its size of around 14 kDa, the absence of the epitopes for 8B4, P4, R505 and R521 and reactivity with the extreme C-terminal mAb 8F9 estimated its N terminus to be in the region of aa 120. The reactivity towards mAb 8F9 (Table 1) suggested that this fragment extends to the glycophosphatidylinositol anchor-attachment site at the C terminus, i.e. a fragment similar to the previously characterized C1, but PK-resistant (Chen *et al.*, 1995; Zhao *et al.*, 2006). The two mAbs 34C9 and F89, with epitopes located N-terminally adjacent to the epitope of mAb L42, reacted only with the 24 kDa fragment and not with Nor98-PrP7. In spite of the extensive denaturation performed both before the deglycosylation and the electrophoresis (with or without alkylation of the denaturated sample before electrophoresis), the epitopes situated on Nor98-PrP7 were not accessible by mAbs 34C9 or F89 in Western immunoblotting. When the same membranes were reprobed with mAb P4, Nor98-PrP7 was detected (not shown). Both fragments encompass the amino acid sequences corresponding to the epitopes for mAbs 34C9 and F89.

Reactivity of various antibodies towards PK-treated Nor98 brain extracts

In Table 1, a summary of the reactivity of various antibodies towards PK-treated Nor98 and scrapie extracts is shown. mAb 8B4 reacted with neither Nor98 nor scrapie, as the PK treatment degrades its epitope. The antibodies with epitopes located in the mid-region of PrP reacted with the prominent N- and C-terminally truncated fragment Nor98-PrP7 in

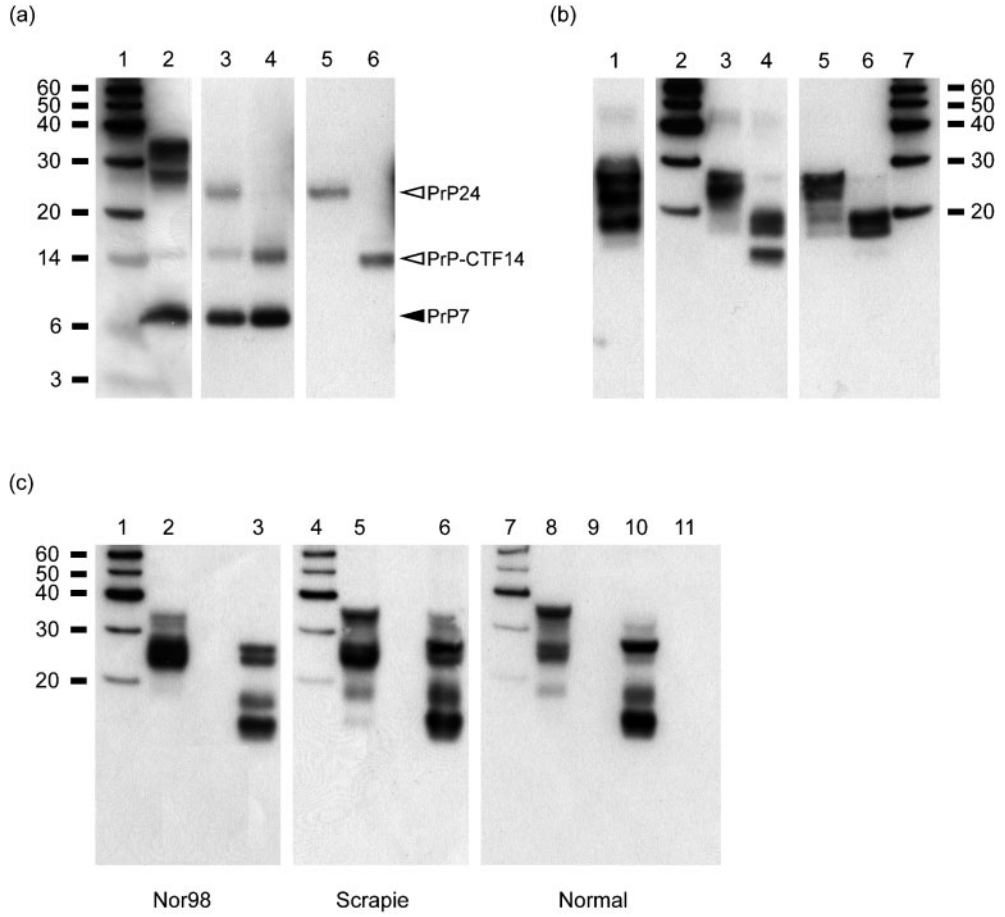

Fig. 2. Further characterization and determination of the apparent molecular masses of Nor98-PrP7 and a novel PK-resistant fragment of ovine PrP in Nor98 extracts, PrP-CTF14. (a) Nor98 brain homogenates (case 2) were treated with PK and then incubated in the absence (lanes 2–3 and 5) or presence (lanes 4 and 6) of PNGase F. Membranes were probed with mAb P4 (lanes 1–2), mAb L42 (lanes 3–4) and mAb 6H4 (lanes 5–6). Lane 1, molecular mass marker (a mixture of SeeBlue Plus2 and MagicMark). Indicated to the right are the positions of Nor98-PrP24, Nor98 PrP-CTF14 and Nor98-PrP7. (b) Scrapie brain homogenates were treated with PK and then incubated in the absence (lanes 1, 3 and 5) or presence (lanes 4 and 6) of PNGase F. Membranes were probed with mAb P4 (lane 1), mAb L42 (lanes 3–4) and mAb 6H4 (lanes 5–6). Lanes 2 and 7, molecular mass marker (MagicMark). (c) Nor98 case 1 (lanes 2–3), scrapie (lanes 5–6) and normal sheep (lanes 8–11) brain homogenates were incubated without (lanes 2–3, 5–6, 8 and 10) or with (lanes 9 and 11) PK and then incubated in the absence (lanes 2, 5 and 8–9) or presence (lanes 3, 6 and 10–11) of PNGase F. Membranes were probed with mAb L42. Incubation mixtures were analysed by Western immunoblotting. Apparent molecular masses based on migration of protein standards are indicated (kDa).

Nor98 extracts. In scrapie, the typical, three-banded glycopattern $PrP^{27–30}$ was seen. Deglycosylation did not affect Nor98-PrP7 but, in scrapie, the expected 20 kDa, deglycosylated fragment appeared. Antibodies with epitopes located in the C-terminal region of PrP did not detect Nor98-PrP7 but, instead, a 24 kDa band (when deglycosylated migrating to 14 kDa, PrP-CTF14) could be detected. In scrapie, the classical glycoprofile was again seen. mAb L42 detected both the prominent Nor98-PrP7 derived from the middle part of PrP and the more C-terminal fragment PrP-CTF14. In scrapie extracts, L42 reacted mainly with the two glycoforms of PrP, $PrP^{25–30}$ (Fig. 2b). The estimated termini of the fragments are for Nor98-PrP7 around aa 85 and 150 and for PrP-CTF14 around aa 120 and 233 (Fig. 4). As the PK-resistant fragments have partly overlapping sequence, at least two conformers of PrP with different PK-resistant cores must be present in the Nor98 brain extract. Fig. 4 presents a schematic representation of the positions of PK-resistant fragments found in Nor98 and scrapie brain extracts.

Nor98-PrP7 and PrP-CTF14 have a reduced PK resistance compared with $PrP^{27–30}$ in classical scrapie

The strain concept of scrapie is partially reflected in a different susceptibility of different PrP molecules to PK.

Table 1. PK-resistant PrP fragments detected in Western immunoblot analysis of Nor98

Material*	Antibodies							
	8B4		P4, R521, R505		L42		34C9, F89, 6H4, 2A11, 8F9	
	−PNGase F	+PNGase F	−PNGase F	+PNGase F	−PNGase F	+PNGase F	−PNGase F	+PNGase F
Nor98	–	ND†	33 kDa 28 kDa 15 kDa 7 kDa§	28 kDa 15 kDa 7 kDa§	24 kDa‡ 7 kDa§	14 kDa‡ 7 kDa§	24 kDa‡	14 kDa‡
Scrapie	–	ND	20–30 kDa	20 kDa‖	20–30 kDa	20 kDa‖	20–30 kDa	19–20 kDa‖

*Following PK treatment, brain homogenates were incubated in the absence or presence of PNGase F as indicated.
†ND, Not done.
‡PrP-CTF14.
§Nor98-PrP7.
‖Deglycosylated $PrP^{27–30}$, encompassing aa 85/89/94–233 (Stahl *et al.*, 1993; Hayashi *et al.*, 2005).

This is probably due to a strain-specific conformation of PrP (Prusiner, 1998). Nor98-PrP7 showed a reduced PK resistance compared with that of classical scrapie (Fig. 3a). After 180 and 360 min, around 66 and 31 %, respectively, of the Nor98-PrP7 remained, compared with the amount at 60 min (Fig. 3b). The 24 kDa fragment (migrating at 14 kDa after deglycosylation, PrP-CTF14) showed reduced PK resistance when compared with classical scrapie, similar to Nor98-PrP7 (Fig. 3c). After 360 min incubation with PK, around 14 and 30 % remained of the mAb 6H4- and mAb 2A11-immunoreactive PrP-CTF14 fragment, respectively (Fig. 3d). The scrapie PrP^{res} showed only minor further degradation, even after 360 min incubation with PK. The difference in PK resistance and the different banding profile suggest different conformations of the scrapie and Nor98 prions.

Sequence determination of the ORF of the prion protein gene from Swedish cases of Nor98

Western blot analyses of sporadic CJD (sCJD), GSS and familial fatal insomnia cases have, in addition to the normal banding, shown additional bands of lower molecular masses (Montagna *et al.*, 2003; Prusiner, 1998; Young *et al.*, 1999; Zou *et al.*, 2003). In the majority of these cases, a mutation in the PRNP ORF has been attributed to explain the pattern (Young *et al.*, 1999). The PRNP ORF sequence from the two cases analysed here and a third Swedish case sequenced by us did not contain new variants, mutations or the previously reported polymorphisms in sheep (Goldmann *et al.*, 2005). Sequence studies of the prion protein from cases of Nor98 in Norway showed an association with phenylalanine at codon 141 (20 of 38 cases) (Moum *et al.*, 2005). The six Swedish Nor98 cases found to date (including the cases analysed here) all show homozygosity for leucine at codon 141 (this study; M. Isaksson & L. H. M. Renström, unpublished data). It is worth noting that the Western immunoblotting results from Nor98 brain homogenates reported in this study were the same in the two cases, irrespective of genotype (A_{136}/A_{136} R_{154}/H_{154} R_{171}/Q_{171} in case 1 and $A_{136}R_{154}Q_{171}/A_{136}R_{154}Q_{171}$ in case 2). The genotype distribution among the six Swedish Nor98 cases resembles the pattern found among Nor98 cases in Norway, in that $A_{136}R_{154}Q_{171}$ and/or $A_{136}H_{154}Q_{171}$ is often present, whereas $V_{136}R_{154}Q_{171}$ is missing. One case carried the $A_{136}R_{154}R_{171}/A_{136}R_{154}H_{171}$ genotype.

DISCUSSION

Recently, an atypical form of scrapie in sheep, named Nor98, has been recognized in several countries in Europe. When compared with classical scrapie, Nor98 cases are characterized by a fragmental pattern containing a low-molecular-mass form of PK-resistant PrP that was previously estimated to be around 12 kDa in size (Benestad *et al.*, 2003; Gavier-Widen *et al.*, 2004). By using optimal conditions for SDS-PAGE analysis in a defined separation system combined with appropriate molecular markers, we determined the molecular mass to be around 7 kDa. This fragment, Nor98-PrP7, is detected with antibodies directed towards the mid-region of PrP. However, mAbs directed to the C-terminal region of PrP revealed a previously unidentified PrP fragment of around 24 kDa. After deglycosylation, this fragment shifted to around 14 kDa, demonstrating that it contains *N*-linked sugars. It was accordingly designated PrP-CTF14 (C-terminal fragment 14 kDa).

The PK-resistant Nor98-PrP7 and PrP-CTF14 differ not only in migration from scrapie $PrP^{27–30}$, but also in protease sensitivity. These differences may be explained by different conformations of PrP^{Sc} in Nor98 and scrapie. The migration of Nor98-PrP7 is not affected by enzymic deglycosylation, suggesting that it corresponds to a central region of PrP that does not contain the glycosylation sites at N_{184} and N_{200}. A PrP fragment of similar size is the major component of the GSS amyloid. In GSS A117V and F198S, antibodies to the mid-region detected both 7 and 14 kDa bands that, similar

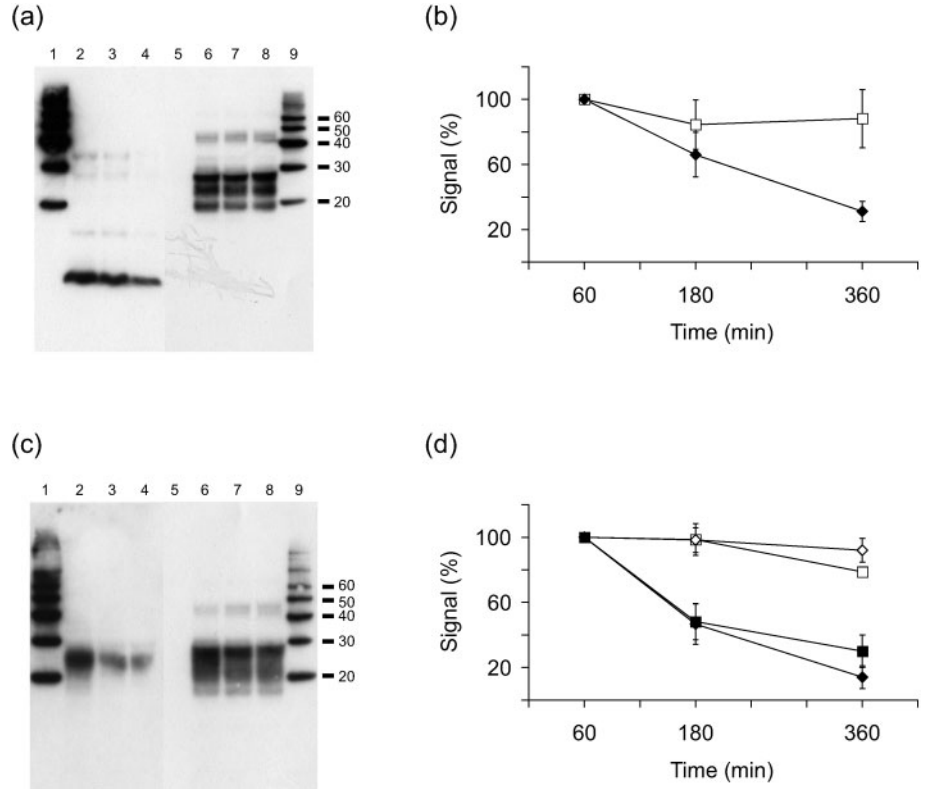

Fig. 3. Time-course studies by Western immunoblotting of PK resistance of PrP fragments in Nor98 brain extracts compared with that of classical sheep scrapie. (a) Time-course study of the PK resistance of Nor98-PrP7. Membrane was probed with mAb P4. (b) Quantification of the signals of the PK-resistant fragments Nor98-PrP7 (◆) and scrapie $PrP^{27–30}$ (□) as detected by mAb P4. (c) Time-course study of the PK resistance of PrP-CTF14. Membrane was probed with mAb 2A11. (d) Quantification of the signals of the PK-resistant fragments Nor98 PrP-CTF-14 (filled symbols) and scrapie $PrP^{27–30}$ (open symbols) as detected by mAbs 2A11 (■, □) and 6H4 (◆, ◇), respectively. Brain homogenates [in (a) and (c)] were treated with 100 μg PK ml^{-1} for indicated times. Nor98 case 2 (lanes 2–4) was incubated for 60, 180 and 360 min, respectively; normal sheep (lane 5) was incubated for 60 min; sheep scrapie (lanes 6–8) was incubated for 60, 180 and 360 min, respectively. Lanes 1 and 9, molecular mass marker (MagicMark). Apparent molecular masses based on migration of protein standards are indicated (kDa). Left part (lanes 1–4) and right part (lanes 5–9) of the immunoblot membrane were exposed for (a) 5 and 1 min, respectively, and for (c) 20 and 5 min, respectively. The signal detected at 60 min incubation with PK has been set to 100 % [in (b) and (d)]. Data points and bars represent the mean ± SD based on two experiments.

to Nor98-PrP7, were also not shifted upon deglycosylation (Piccardo *et al.*, 2001).

Nor98-PrP7 is detected with antibodies P4, R505 and R521, whose epitopes span aa 93–111, and with L42, which is raised against a peptide spanning aa 145–163. It has been shown that the tyrosine at position 148 is crucial for binding of mAb L42 to PrP (Vorberg *et al.*, 1999), which would suggest that the C terminus of Nor98-PrP7 is C-terminal to this position. The region of PrP that mAb L42 is raised against partially overlaps the epitope of mAb 6H4 (aa 147–155), but 6H4 does not recognize the Nor98-PrP7 fragment, which suggests that the 6H4 epitope is partially degraded. This places the C terminus of the fragment in the region of aa 148–155. Furthermore, mAb 6H4 has been reported (Korth *et al.*, 1997) to react well with mouse and hamster PrP, which carry a tryptophan residue instead of tyrosine at position 148, thus indicating that the emphasis of the epitope of 6H4 is somewhat biased towards the C-terminal part of aa 147–155 or that a structural element is necessary. Antibodies with epitopes located closer to the C terminus of PrP than 6H4 fail to react with Nor98-PrP7. Taken together, these data strongly support the hypothesis that the C terminus of Nor98-PrP7 is located in the region of aa 148–155. With a size of 7 kDa and an inferred C terminus in the region of aa 148–155, the Nor98-PrP7 fragment is suggested to have an N terminus around aa 85 (Fig. 4), similar to that of scrapie $PrP^{27–30}$. The exact PK-cleavage points at the N and C termini of Nor98-PrP7 and PrP-CTF14 await confirmation by mass spectrometry and/or amino acid sequencing. Due to a limited amount of clinical material, it has to date not been possible to perform these analyses. The GSS

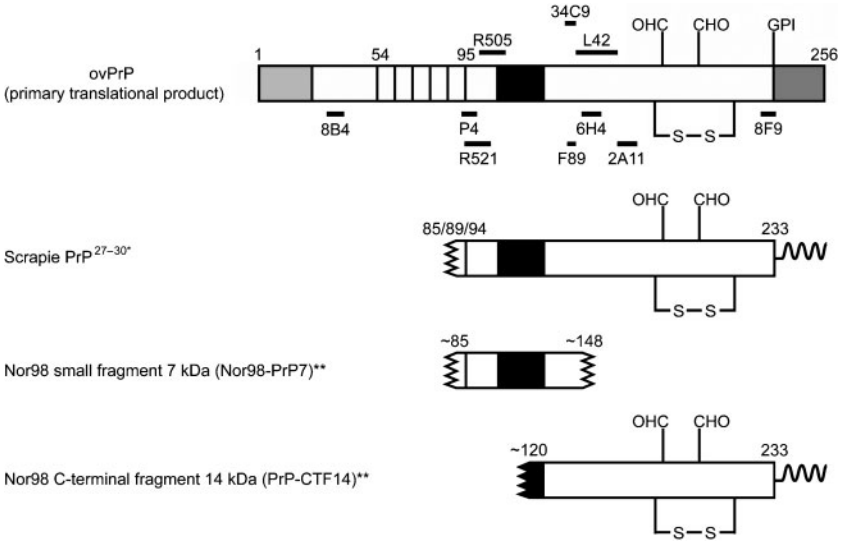

Fig. 4. Schematic representation of ovPrP and PK-resistant PrP fragments observed in ovine prion diseases. The epitopes recognized by the anti-PrP antibodies used in this study are indicated on the ovPrP primary translational product. *Scrapie $PrP^{27–30}$ encompasses aa 85/89/94–233. It is generated by PK cleavage between residues 85 and 94 of ovine PrP^{Sc}, producing a highly ragged N terminus (Hayashi *et al.*, 2005; Parchi *et al.*, 2000; Stahl *et al.*, 1993). **Nor98-PrP7 and PrP-CTF14 were identified in this study. Putative regions for PK-cleavage sites, based on reactivity with anti-PrP antibodies and apparent molecular masses in Western immunoblotting, are indicated.

PrP7-8 fragments have been shown to span aa 77/94–149/156 (ovine numbering) (Tagliavini *et al.*, 2001). This fragment and the Nor98-PrP7 fragment mapped here cover a region that, when using a corresponding synthetic peptide, easily formed amyloid fibrils that were partially resistant to proteolytic digestion (Salmona *et al.*, 2003; Tagliavini *et al.*, 2001).

The mapping with mAbs directed to the C-terminal region of PrP revealed the PrP fragment designated PrP-CTF14. The size and the reactivity with C-terminal, but not N-terminal, antibodies suggested that PrP-CTF14 spans from around aa 120 to the extreme C terminus. A similarly sized fragment can be found in healthy humans and cattle. Human and bovine PrP^{C} is cleaved endogenously at residues K_{110}/H_{111}/M_{112} (human numbering) and K_{121}/H_{122} (bovine numbering), respectively (Chen *et al.*, 1995; Zhao *et al.*, 2006), into an N-terminally truncated, C-terminally intact C1 fragment with a size of 15 kDa (after deglycosylation); however, this fragment has complete sensitivity to PK treatment. The identification of PK-resistant PrP fragments similar to PrP-CTF14 has previously only been described in certain subjects with sCJD (Zou *et al.*, 2003).

PrP-CTF14 is likely to originate from a subpopulation of PrP^{Sc} distinct from that generating Nor98-PrP7. These fragments have overlapping regions and mAb L42 detects both the Nor98-PrP7 and the PrP-CTF14 fragments, indicating that its epitope is in the overlapping part of the fragments. N-terminal to the epitope of mAb L42 and also in the overlapping part of the two fragments are located the epitopes for mAbs 34C9 and F89. However, only PrP-CTF14 is detected by these mAbs. The C-terminal part of Nor98-PrP7 could be too compact to expose the epitopes for mAbs 34C9 and F89 except at the extreme C-terminal end, where the epitope of mAb L42 was found to be accessible. Antisera towards the mid-region of PrP detected, in addition to the dominant band of Nor98-PrP7, minor populations migrating to estimated sizes of 15, 28 and 33 kDa. Their sizes suggested the presence of the epitopes for the C-terminal antibodies used; however, these fragments were not detected by these antibodies. How or whether these bands relate to Nor98-PrP7, as oligomers or whether they have other N and C termini, remains to be investigated.

It was recently suggested that PrP could maintain certain tertiary structure in PVDF membranes, although the sample had been treated with boiling, denaturants and reducing and/or alkylating agents before electrophoresis (Yuan *et al.*, 2005). In a recent report, Nelson *et al.* (2005) showed that the core structure of amyloid fibrils can be packed so closely that even water molecules are excluded. A closely packed amyloid of this sort would also be consistent with the observation that certain antibody epitopes are concealed in the PVDF membrane (Yuan *et al.*, 2005). In view of this, it is therefore possible that certain epitopes on PK-resistant PrP fragments in Nor98 brain homogenates could be inaccessible, due to structural constraints. The presence of N- and C-terminally truncated, PK-resistant PrP fragments with anomalous reactivity with anti-PrP antibodies in brain homogenates from Nor98 cases suggests alternative PrP conformations different from those of both normal PrP^{C} and classical scrapie PrP^{Sc}.

In GSS, one of the alleles encoding the prion protein is mutated and only the mutated protein is found in the PK-resistant amyloid (Tagliavini *et al.*, 1994). Our analysis of the Swedish Nor98-affected sheep showed no mutations in the structural gene of PrP that could be correlated to the aberrant PK-resistant profile observed. Moum *et al.* (2005) recently sequenced the ORF of PRNP in 38 Norwegian cases of Nor98 and found an association with polymorphisms at codons 141 and 154, but no mutations that could be correlated directly to the aberrant electrophoretic profile of Nor98. In this aspect, all six cases found in Sweden were homozygous for leucine at position 141, and the genotype

distribution at codons 136, 154 and 171 in the six cases resembled the pattern found among Nor98 cases in Norway in that ARQ and/or AHQ is often present, whereas VRQ is missing. However, the Western immunoblotting results from Nor98 brain homogenates reported in this study were the same in the two cases, even though they carried different genotypes. It will be interesting to analyse the presence of Nor98-PrP7 and PrP-CTF14 in atypical scrapie-affected sheep from different geographical origins in Europe and also with distinct genotypes to find out whether amino acid differences in PrP influence the size of the PK-resistant fragments.

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