Immunological characterization of abnormal prion protein from atypical scrapie cases in sheep using a panel of monoclonal antibodies

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After the implementation of an active surveillance programme for scrapie in sheep in the EU, the number of diagnosed classical scrapie cases rose sharply and a novel kind of so-called atypical scrapie case was discovered. These atypical scrapie cases display unusual features concerning the distribution of the abnormal prion protein (PrPSc) in the brain, a distinct electrophoretic profile of PrPSc and an inconsistent reaction pattern in the currently used rapid tests. In this report, PrPSc of two German atypical sheep scrapie cases was characterized by epitope mapping using a panel of 18 monoclonal antibodies that were directed against epitopes located throughout the prion protein. This analysis suggests that PrPSc derived from atypical scrapie cases and treated with proteinase K is largely composed of an 11 kDa fragment (previously referred to as the 12 kDa band) and of polymeric fragments thereof. The 11 kDa band corresponds to a prion protein fragment spanning approximately aa 90–153 and may therefore represent a novel PrPSc type.

INTRODUCTION

Transmissible spongiform encephalopathies (TSEs) are a group of fatal, neurodegenerative diseases that include scrapie in sheep and goats, as well as bovine spongiform encephalopathy (BSE) in cattle and Creutzfeldt–Jakob disease in humans. TSEs are associated with the accumulation of an abnormal isoform (PrPSc) of the host-encoded prion protein, PrPC. In contrast to PrPC, PrPSc shows a partial resistance to digestion with proteinase K (PK).

The implementation of an obligatory active surveillance programme for scrapie in 2002 resulted in an increase of diagnosed classical scrapie cases in all EU member states. Moreover, atypical scrapie cases were newly discovered that were similar, if not identical, to the so-called Nor98 cases that were found previously in Norway (Benestad et al., 2003). Atypical scrapie cases have been reported in Germany, France, Portugal, Ireland, Sweden, Belgium and elsewhere (Buschmann et al., 2004a; Orge et al., 2004; Onnasch et al., 2004; Gavier-Widen et al., 2004; De Bosschere et al., 2004).

In Germany, approximately 45 % of all currently diagnosed scrapie cases and 84 % of the affected flocks represent atypical scrapie. The atypical phenotype is characterized by the absence of or a faint and mostly granular deposition of PrPSc at the level of the obex. On the other hand, in the majority of cases, massive accumulations of PrPSc are detectable in the cerebellum and cerebrum. The PrPSc electrophoretic profile by Western blot is different from that of classical scrapie and includes an additional fragment with a molecular mass of approximately 12 kDa (Benestad et al., 2003; Buschmann et al., 2004a).

The abnormal PrPSc derived from atypical scrapie cases displays a lower resistance to PK than does PrPSc from classical scrapie. Furthermore, these scrapie cases are diagnosed only by one (Bio-Rad TeSeE, formerly Bio-Rad Platelia) of the four currently used rapid tests (Buschmann et al., 2004a). Although atypical scrapie cases are frequently found only in single animals of an affected flock, atypical scrapie cases are indeed infectious when inoculated into transgenic mice (Le Dur et al., 2005; G. Lühken, A. Buschmann, H. Brandt, M. Eiden, M. H. Groschup & G. Erhardt, unpublished data). Notably, such atypical scrapie cases were often found in sheep carrying PrP genotypes that were believed to convey scrapie resistance (Buschmann et al., 2004b). It has been reported recently that polymorphisms at codons 141 and 154 in the ovine prion protein gene may be correlated with atypical scrapie cases (Moum et al., 2005).

Here, we report differences in the immunoreactivity of PrPSc derived from atypical scrapie, from BSE and from classical scrapie after PK digestion. These differences were found
when a panel of monoclonal antibodies (mAbs) directed against a broad range of prion protein epitopes were used for PrP\textsuperscript{Sc} detection by Western blot.

**METHODS**

**Animals and diagnostic confirmation.** Two German atypical scrapie cases, S39/02 (genotype AHQ/ARR) and S40/04 (genotype ARQ/ARQ), were used for epitope mapping. One German classical scrapie case, S33/02 (genotype ARQ/ARQ), and a German BSE isolate, R128/04, served as controls. PrP\textsuperscript{Sc} of an undigested brain homogenate from a scrapie-negative sheep served as internal control for antibody binding on each Western blot. All described German TSE cases were confirmed by either scrapie-associated fibril (SAF) immunoblotting or immunohistochemistry, or by both methods according to the OIE manual (Anonymous, 2004; Gretzschel et al., 2005).

**Tissue treatment, SDS-PAGE and Western blotting.** From all sheep or cattle samples, 10% (w/v) brain homogenates were prepared in 0.42 mL sucrose solution containing 0.5% deoxycholic acid sodium salt and 0.5% (w/v) Nonidet P40 by using a Ribolysor (Hybaid). Precipitation with phosphotungstic acid (PTA) was carried out according to the protocol established by Wadsworth et al. (2001) with some modifications (Gretzschel et al., 2005). After PTA precipitation, samples were loaded on 16% Tris/polyacrylamide gels. Electrophoresis was carried out by using the Bio-Rad Mini Protein II system with a running buffer containing 25 mM Tris, 190 mM glycine and 3 mM SDS. If not mentioned otherwise, sample preparation, SDS-PAGE and Western blotting were carried out as described previously (Gretzschel et al., 2005) using a panel of 18 mAbs (Table 1). All chemicals were derived from Sigma unless otherwise noted. The molecular mass markers Precision Plus Protein Standard Dual Colour and Precision Plus Protein Standard Unstained (both from Bio-Rad) were used, with fragment sizes of 10, 15, 20, 25, 37, 50, 75, 100, 150 and 250 kDa. Whilst the dual-colour standard is mostly recommended for molecular mass determination on gels and for the monitoring of transmission efficiency, the unstained marker is recommended for molecular mass determination on gels and Western blots.

**PrP-specific antibodies.** The following mAbs were used (Table 1): L42 and P4 (R-Biopharm), FH11 and BG4 (TSE Resource Centre, Compton, UK), F89/160.1.5 and F99/97.6.1 (VMRD Inc.), ICSM18 and ICSM35 (DC-Gen), 6H4 (Prionics AG), 12B2, 9A2, 94B4, SAF32, SAF34, 8G8 and 12F10 [J. Grassi, Commissariat à l’Energie Atomique (CEA), Gif-sur-Yvette, France], R145 [Community Reference Laboratory, Veterinary Laboratories Agency (VLA), Weybridge, UK] and 3B5 (W. Bodemer, German Centre for Primates, Göttingen, Germany).

Seventeen of these antibodies were mouse mAbs, which were subsequently incubated with a secondary antibody (alkaline phosphatase-conjugated anti-mouse immunoglobulin; Dianova) at 0.15 µg ml\textsuperscript{-1}. Only mAb R145 was a rat mAb, which was incubated with an alkaline phosphatase-conjugated anti-rat immunoglobulin (Dianova) at 0.15 µg ml\textsuperscript{-1}. All antibodies were tested with undigested, as well as with PK-digested, brain homogenates. Before precipitation, PK digestion was performed for 1 h at 55°C, using a final concentration of 50 µg ml\textsuperscript{-1}.

**Pepscan analysis.** To detect the linear epitope specificities of mAbs L42, 12B2, 9A2, 6H4, 8G8, 94B4, 12F10, F89/160.1.5, F99/97.6.1, BG4 and FH11, Pepscan analyses of antibodies were performed in an ELISA-like procedure on solid-phase synthetic peptides bound to plastic surfaces, as described by Geyser et al. (1984) and Thuring et al. (2004). Complete sets of overlapping 15mer peptides were synthesized based on the amino acid sequence of the ovine PrP (GenBank accession no. AJ000739). A site was considered positive (i.e. antigenic) when the A$_{405}$ values of two or more consecutive peptides were at least three times the background. The background was calculated as the mean A$_{405}$ value measured for 20 consecutive peptides with low levels of reaction, where the standard deviation (SD) was 20% of the mean A$_{405}$ value. A linear epitope (or epitope core) was defined as the sequence of amino acids common to all antigenic peptides in a site.

**Genotyping.** PrP genotypes of the German TSE sheep were determined as described previously (Lübben et al., 2004).

**RESULTS**

The immunoblot patterns of PrP\textsuperscript{Sc} derived from classical and atypical scrapie cases were similar to those reported in the literature, i.e. the different glycoforms of classical PrP\textsuperscript{Sc} had molecular masses of approximately 36, 33 and 29 kDa that shifted down to 28, 21 and 17.5 kDa following PK treatment. In contrast to this, PrP\textsuperscript{Sc} derived from atypical scrapie cases displayed one major, distinct band of a low molecular mass and three to five additional bands ranging from 15 to 30 kDa in size. Repeated measurements of the size of this low-molecular-mass PrP\textsuperscript{Sc} band, previously described as the 12 kDa band in the literature (Benestad et al., 2003), consistently gave it a lower molecular size in our hands. The size of this band was therefore determined by using a molecular size marker with 5 kDa increments from 10 to 25 kDa (Fig. 1). This measurement reproducibly gave a molecular size of about 11 kDa, so this band is referred to as the ‘11 kDa fragment’ in this report.

Eighteen antibodies were used for immunochemical characterization of PrP\textsuperscript{Sc} of classical and atypical scrapie cases before and after digestion with PK. These antibodies were divided into five groups according to the PrP epitopes that they detect (Table 1).

Two mAbs, FH11 (Fig. 2a, b; Table 2) and BG4, which recognize epitopes at the N terminus of the prion protein (aa 54–58), detected the undigested PrP of atypical and classical scrapie cases, as well as that from the BSE isolate. However, the 11 kDa fragment of the atypical scrapie cases was detected only faintly, if at all. After PK digestion, these antibodies failed to recognize all TSE isolates.

mAb 3B5 (group 2; Fig. 2c, d; Table 2) recognizes aa 51–90 of the prion protein. Prior to PK digestion, mAb 3B5 detected classical scrapie and the upper fragments of atypical scrapie and also the BSE isolate, but again, the 11 kDa fragment of the atypical scrapie cases was detected very weakly or not at all. After PK digestion, this antibody recognized only classical scrapie, but failed to recognize atypical scrapie or the BSE isolate.

Five mAbs of group 3 (12B2, P4, 8G8, SAF32, SAF34), which were raised against an epitope close to the PK-cleavage site, epitope-mapped to the region from aa 59 to 107 of the prion protein. Without PK digestion, they detected classical
scrapie, as well as atypical scrapie with the 11 kDa fragment and also the BSE isolate. After PK digestion, these antibodies failed to recognize the BSE isolate, but clearly detected the classical and the atypical scrapie cases, including the 11 kDa fragment. Examples of the results obtained with mAb 12B2 are shown in Fig. 2(e, f) and Table 2.

Table 1. Characteristics of mAbs used for Western blotting

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Linear epitope on ov PrP*</th>
<th>Immunogen</th>
<th>Species tested and recognized†</th>
<th>Antibody concentration</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FH11</td>
<td>54–58</td>
<td>rec ov PrP</td>
<td>Sheep, cattle, hamster, human</td>
<td>1000 1 : 1000</td>
<td>Jeffrey et al. (2001); present paper</td>
</tr>
<tr>
<td>BG4</td>
<td>54–57</td>
<td>rec bo PrP</td>
<td>Sheep, cattle, hamster, human</td>
<td>1000 1 : 1000</td>
<td>Jeffrey et al. (2001); Martin et al. (2005); present paper</td>
</tr>
<tr>
<td>3B5</td>
<td>51–90</td>
<td>rec hu PrP</td>
<td>Sheep, cattle, mouse</td>
<td>– 1 : 20</td>
<td>Bodemer (1999)</td>
</tr>
<tr>
<td>SAF32</td>
<td>59–89</td>
<td>ha SAF</td>
<td>Sheep, cattle, mouse, hamster, human</td>
<td>– 1 : 1000</td>
<td>Féraudet et al. (2005)</td>
</tr>
<tr>
<td>SAF34</td>
<td>59–89</td>
<td>ha SAF</td>
<td>Sheep, cattle, mouse, hamster, human</td>
<td>– 1 : 1000</td>
<td>Féraudet et al. (2005); Moudjou et al. (2001)</td>
</tr>
<tr>
<td>12B2</td>
<td>93–97</td>
<td>bo PrP peptide</td>
<td>Sheep, cattle, mouse, human, deer</td>
<td>1700 1 : 666</td>
<td>Jeffrey et al. (2006); Yull et al. (2006)</td>
</tr>
<tr>
<td>P4</td>
<td>93–99</td>
<td>ov PrP peptide</td>
<td>Cattle, sheep</td>
<td>1000 1 : 2500</td>
<td>Hardt et al. (2000); Harmeyer et al. (1998); Thuring et al. (2004)</td>
</tr>
<tr>
<td>8G8</td>
<td>100–107</td>
<td>rec hu PrP</td>
<td>Sheep, cattle, mouse, hamster, human</td>
<td>– 1 : 1000</td>
<td>Féraudet et al. (2005); Krasemann et al. (1996, 1999); present paper</td>
</tr>
<tr>
<td>ICSM35</td>
<td>93–102</td>
<td>hu PrP</td>
<td>Sheep, cattle, mouse, hamster, human</td>
<td>3000 1 : 2857</td>
<td>Beringue et al. (2003); White et al. (2003)</td>
</tr>
<tr>
<td>F89</td>
<td>140–147</td>
<td>bo PrP peptide</td>
<td>Sheep, cattle, deer, human</td>
<td>1000 1 : 333</td>
<td>O'Rourke et al. (1998, 2000); present paper</td>
</tr>
<tr>
<td>L42</td>
<td>148–153</td>
<td>ov PrP peptide</td>
<td>Sheep, cattle, mink, human</td>
<td>1000 1 : 2500</td>
<td>Hardt et al. (2000); Harmeyer et al. (1998); present paper</td>
</tr>
<tr>
<td>12F10</td>
<td>146–155</td>
<td>rec hu PrP</td>
<td>Sheep, cattle, human</td>
<td>– 1 : 1000</td>
<td>Krasemann et al. (1996, 1999); Féraudet et al. (2005); present paper</td>
</tr>
<tr>
<td>ICSM18</td>
<td>146–156</td>
<td>hu PrP</td>
<td>Sheep, cattle, mouse, hamster, human</td>
<td>3600 1 : 3333</td>
<td>Beringue et al. (2003); White et al. (2003)</td>
</tr>
<tr>
<td>6H4</td>
<td>148–157</td>
<td>rec hu PrP</td>
<td>Sheep, cattle, mouse, hamster, human</td>
<td>– 1 : 10000</td>
<td>Korth et al. (1997); present paper</td>
</tr>
<tr>
<td>94B4</td>
<td>190–197</td>
<td>rec bo and hu PrP</td>
<td>Cattle, sheep</td>
<td>650 1 : 1666</td>
<td>Thuring et al. (2004); present paper</td>
</tr>
<tr>
<td>F99</td>
<td>221–224</td>
<td>ov PrP peptide</td>
<td>Sheep, cattle, deer</td>
<td>1000 1 : 333</td>
<td>O'Rourke et al. (2000); Spraker et al. (2002); present paper</td>
</tr>
</tbody>
</table>

*This represents at least part of the epitope.
†This column is not exhaustive.

The region from aa 93 to 163 was covered by five antibodies of group 4 (L42, ICSM35, 9A2, F89/160.1.5). They detected PrPSc derived from classical scrapie, atypical scrapie including the 11 kDa fragment and the BSE isolate, both before and after PK digestion. Results obtained from L42 are shown (Fig. 2g, h; Table 2).
Six mAbs of group 5, which epitope-mapped between aa 146 and 224 of the prion protein (ICSM18, 94B4, 6H4, 12F10, R145 and F99/97.6.1), recognized PrP\textsuperscript{Sc} of classical scrapie, of the BSE isolate and the upper fragments of atypical scrapie before PK digestion. However, after PK digestion, the atypical scrapie cases were not detected by these antibodies, as shown for antibody ICSM18 (Fig. 2i, j; Table 2).

**DISCUSSION**

In contrast to PrP\textsuperscript{C}, PrP\textsuperscript{Sc} derived from classical as well as from atypical scrapie cases exhibits a remarkable partial resistance to digestion with PK. This is due to the increased \(\beta\)-sheet content in the PrP core following conversion from the normal \(\alpha\)-helical form. As a consequence, only the unstructured, flexible N terminus can be degraded proteolytically. According to our data, PrP\textsuperscript{Sc} derived from atypical scrapie cases differs substantially from that of classical scrapie cases. The N-terminal PK-cleavage site seems to be a
few amino acids further towards the C terminus of the protein, still being closer to that determined for classical scrapie than to that determined for BSE. Furthermore, our results indicate clearly that the 11 kDa band (which was formerly designated the 12 kDa fragment) observed in PK-treated atypical scrapie PrPSc constitutes only a minor fragment of the prion protein. Incidently, to form this fragment, PrPSc must be trimmed proteolytically from the N terminus as well as the C terminus, so that only a core peptide spanning aa 90–153 (an estimate based on the mAb-binding sites) is retained. Moreover, based on the distinct mAb-binding patterns and respective molecular sizes, we propose that the upper bands in immunoblotted PrPSc derived from atypical scrapie cases may represent multimers of the 11 kDa band or of fragments thereof.

These results were obtained by immunochemical characterization of atypical scrapie PrPSc before PK digestion, but fail to detect the cleaved N terminus after PK digestion. However, the 11 kDa fragment of atypical scrapie cases was not detected by these antibodies even before PK digestion. We therefore conclude that this flexible, N-terminal epitope of the prion protein is not present in the 11 kDa fragment of atypical scrapie cases (Fig. 3).

The exact N-terminal PK-cleavage site of PrPSc depends on the particular prion strain and on the applied reaction conditions. For discrimination between BSE and scrapie, the difference in the strain-specific N-terminal PK-cleavage sites, which vary from aa 81 to 89 for scrapie and aa 96 to 97 for BSE (Hayashi et al., 2005), is targeted. Thus, mAbs raised against an epitope between positions 75 and 89 of the prion protein detect scrapie-derived PrPSc very well, but fail to recognize bovine or ovine BSE.

mAb 3B5, representing group 2 of the applied antibodies, binds close to the PK-cleavage site and can therefore be used for the discrimination between BSE and scrapie (Groschup et al., 2000), like antibodies from group 3 (Baron & Biacabe, 2001; Stack et al., 2002). However, although mAb 3B5 has a reaction pattern similar to that of these antibodies with BSE and classical scrapie PrPSc, it fails to detect PrPSc derived from atypical scrapie cases after strong PK digestion. Furthermore, mAb 3B5 detects the 11 kDa fragment of atypical scrapie cases before PK digestion either very weakly

<table>
<thead>
<tr>
<th>Group</th>
<th>mAb</th>
<th>Linear epitope on PrP</th>
<th>–PK</th>
<th>+ PK</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Classical scrapie S33/02 Atypical scrapie S40/04 BSE R128/04 Scrapie-negative sheep</td>
<td>Classical scrapie S39/02 Atypical scrapie S40/04 BSE R128/04</td>
</tr>
<tr>
<td>1</td>
<td>FH11</td>
<td>54–57</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>2</td>
<td>BG4</td>
<td>54–58</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>3</td>
<td>3B5</td>
<td>51–90</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>4</td>
<td>SAF32</td>
<td>59–89</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>5</td>
<td>12B2</td>
<td>93–97</td>
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<td>12F10</td>
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<td>++</td>
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<td></td>
<td>ICSM18</td>
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<td>++</td>
<td>++</td>
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<td>6</td>
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<td>148–157</td>
<td>++</td>
<td>++</td>
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<td>9B4</td>
<td>190–197</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>R145</td>
<td>221–234</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
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<td>F99</td>
<td>221–224</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Table 2. Classification of antibodies according to the binding affinity towards PrPSc derived from atypical scrapie cases in Western blot.

−, No signal in Western blot; ±, weak signal in Western blot; +, slight signal in Western blot; ++, moderate signal in Western blot; ++++, strong signal in Western blot; ND, not done.
PK-cleavage sites of PrP<sub>Sc</sub> from BSE and classical scrapie are pie, atypical scrapie and BSE before and after PK digestion.

Antibody epitopes of PrP<sub>Sc</sub> derived from classical scrapie cases, but fail to detect BSE-derived PrP<sub>Sc</sub> after PK digestion. Therefore, the PK-cleavage site of atypical scrapie cases seems to be equidistant from or even closer to the PK-cleavage site of PrP<sub>Sc</sub> than to the PK-cleavage site of BSE. The 11 kDa fragment of atypical scrapie cases is detected easily by the antibodies of group 3 before and after PK digestion, although mAbs SAF 32 and SAF34 seem to bind somewhat more weakly than mAbs 12B2, P4 and 8G8. Thus, this epitope is present in atypical scrapie cases before and after PK digestion (Fig. 3).

Antibodies of group 4 detect classical scrapie, atypical scrapie including the 11 kDa fragment and the BSE isolate equally well before and after PK digestion. Therefore, this region represents the PK-resistant core not only of PrP<sub>Sc</sub> derived from classical scrapie and BSE, but also of PrP<sub>Sc</sub> derived from atypical scrapie cases (Fig. 3).

Antibodies of group 5 fail to detect the 11 kDa fragment of atypical scrapie cases even before PK digestion and do not recognize the upper fragments or the 11 kDa fragment of atypical scrapie cases after PK digestion. Therefore, this 11 kDa band seems not to extend beyond approximately aa 153 towards the C terminus of the protein. The proteolysis of the C terminus also appears to take place in vivo, as this band is also not detectable in non-PK-treated preparations. However, the origin of the upper bands of undigested PrP<sub>Sc</sub> derived from atypical scrapie cases remains unclear, because their signals may be superimposed by the signals of non- or only partially cleaved PrP<sub>C</sub> or PrP<sub>Sc</sub> moieties.

Our findings are reminiscent of the major PrP<sub>Sc</sub> peptide fragment found in amyloid fibrils extracted from familial Gerstmann–Sträussler–Scheinker (GSS) syndrome victims (Salmona et al., 2003; Tagliavini et al., 2001). This major amyloid component spans the residues of approximately aa 82–146 of human PrP and has an approximate size of 7 kDa. Synthetic peptides of this amino acid sequence easily form amyloidotic aggregates that are partially resistant to protease digestion and assemble to form fibrils with a parallel cross-β structure. It is conceivable that the 11 kDa fragment observed in atypical scrapie originates – similarly to the GSS fragment – from full-length PrP<sub>Sc</sub>, which is deposited in the extracellular compartment and partially degraded by proteases, and then further digested by tissue endopeptidases.

Taken together, we propose that PTA-precipitated and PK-treated PrP<sub>Sc</sub> derived from atypical scrapie is largely composed of a novel and unique fraction of the prion protein. The infectious nature of this fraction and its potential molecular and cellular pathomechanism remain to be established.

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