

## Differential Epitope-Mapping of the Two Forms of the Prion Protein: Alterations at the C-Terminus

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Most of the diagnostic methods for transmissible spongiform encephalopathies are based on the detection of the abnormal protease-resistant conformers of the prion protein (PrP<sup>Sc</sup>), when the normal protease-sensitive conformers (PrP<sup>C</sup>) are also present. A selective immune detection of PrP<sup>Sc</sup> with high sensitivity is hampered by the absence of sufficient knowledge about the structure of PrP<sup>Sc</sup>. An economical and rapid mapping approach is used here for the identification of epitopes that are exposed differently on PrP<sup>Sc</sup> and PrP<sup>C</sup>. This approach revealed remarkable differences in the conformation/accessibility of the two long helical segments between the two prion forms. This result is not in harmony with recent models of PrP<sup>Sc</sup>.

**Key Words** : Prion, Epitope, Conformation specific antibody

### Introduction

Transmissible spongiform encephalopathies (TSE-s) are fatal, infectious, neurodegenerative disorders in humans and other mammals.<sup>1-3</sup> The central event in the disease is the transformation of a normal cell-surface glycoprotein (PrP<sup>C</sup>), the prion protein, to an abnormal, protease-resistant, increased  $\beta$ -sheet-containing form (PrP<sup>Sc</sup>). The assertion that the PrP<sup>C</sup>-to-PrP<sup>Sc</sup> transition is purely conformational is based on the absence of an alteration either in the disulfide bond pattern<sup>4</sup> or in other systematic covalent modifications<sup>5</sup> in the two forms of the protein. The disease can be transmitted to humans, both from animals through the food chain and from humans by medical intervention.<sup>6</sup> Therefore, there is a demand to determine the presence of a prion agent in tissues or blood of prion-affected animals destined for the human food chain before the appearance of any clinical symptoms. This also applies to the detection of prions in humans, who may participate in tissue and blood donation programs.<sup>7,8</sup>

Although there is now considerable evidence that prion diseases arise by the conformational conversion of PrP<sup>C</sup> into PrP<sup>Sc</sup>, neither the exact nature of the infectious agent nor the identity of the species that is toxic to the cells is known.<sup>7,9,10</sup> Most of the diagnostic methods are based on the detection of the surrogate marker, PrP<sup>Sc</sup>, which is deposited in the brain, spleen, and lymphatic tissues of diseased individuals and animals during the progression of TSE.<sup>7</sup> These methods, which result in a *definitive* diagnosis of TSE infection, are based on invasive brain biopsy or postmortem

testing. In order to establish a diagnosis that employs minimally invasive body-fluid samples (*e.g.*, blood), the sensitivity of the detection of PrP<sup>Sc</sup> has to be increased significantly. Detection methods that are based on the amplification of PrP<sup>Sc</sup> are continuously being improved.<sup>11-17</sup> Another promising approach is the generation of more sensitive antibodies (Ab-s) that are selective to either PrP<sup>Sc</sup> or PrP<sup>C</sup>. Thus, the Proteinase K (PK) digestion step, which is used for distinguishing PrP<sup>Sc</sup> from PrP<sup>C</sup> in most detection methods, could be eliminated.<sup>17-23</sup> There are few reports of Ab-s that can selectively recognize PrP<sup>Sc</sup> without reacting with the normal, cellular form of the protein.<sup>18,20,22-25</sup> However, there are no reports of these antibodies being successfully exploited for the development of a sensitive diagnostic procedure to identify the pathologic isoform of PrP.

Ab-s with high sensitivity that recognized selectively PrP<sup>C</sup> but not PrP<sup>Sc</sup> could be equally valuable for PrP<sup>Sc</sup> detection using a two-step procedure. In the first step, the samples are depleted of PrP<sup>C</sup> by the action of the PrP<sup>C</sup>-specific antibody. Subsequently, in the second step, the remaining PrP<sup>Sc</sup> in the samples is detected with any anti-prion antibody that can react with PrP. The difficulties in generating such selective Ab-s include the absence of sufficient knowledge of the structure of PrP<sup>Sc</sup>, which would make it possible to target immunogen sequences that are exposed only in one isoform. Additional problems are the extensive sequence identity between mammalian PrP-s and the fact that PrP<sup>C</sup> is on the surface of T-cells that are among the probable reasons for the poor immunogenicity of the prion protein.<sup>7</sup> Here we present the results of a differential epitope-mapping approach for the rapid identification of epitopes that are exposed only in one isoform by generating *polyclonal* chicken (IgY) anti-prion Ab-s derived from immunization with short peptides. The immunization approach that is generally followed in the field uses long polypeptides of PrP containing  $\beta$ -sheet conformations characteristic to PrP<sup>Sc</sup>.<sup>26-29</sup>

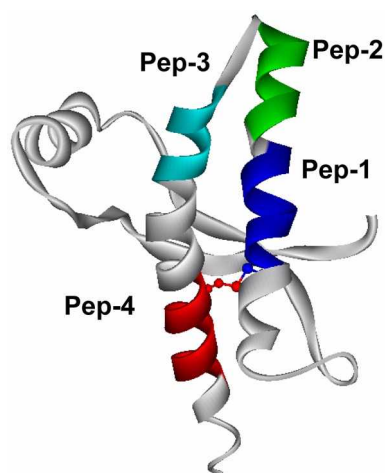
**Abbreviations**: Ab-s, sensitive antibodies; EIA, Enzyme Immunoassay; ELISA, Enzyme Linked Immunosorbent assay; IgY, *polyclonal* chicken antibody; PK, Proteinase K; PrP, prion; PrP<sup>C</sup>, normal protease-sensitive conformers; PrP<sup>Sc</sup>, abnormal protease-resistant conformers of the prion protein; TMB, 3,3',5,5'-tetramethylbenzidine; TSE, Transmissible spongiform encephalopathies.

However, the short 7 or 8-mer peptide fragments of PrP, which are used in our approach, are more appropriate for mapping the accessibility of PrP segments on the prion forms. The idea of using chicken is based on the fact that the sequence of the chicken prion protein has only ~40% sequence identity with mammalian counterparts.<sup>27</sup> Additionally, the generation of polyclonal Ab-s is preferred as it is more rapid and economical for this rapid screening purposes than is the generation of monoclonal Ab-s. The epitopes that are identified by this mapping approach can facilitate the targeted generation of monoclonal Ab-s in knock-out mice and in chickens.<sup>27-30</sup>

## Results and Discussion

Four peptides representing segments of the two long helices (H2 and H3) of PrP<sup>C</sup> (Fig. 1) where we hypothesized that the two forms of the prion protein might be different, were selected. Polyclonal chicken (IgY) anti-prion Ab-s were generated against these segments as described in the Material and Methods section. The purified IgY Ab-s (Ab147, Ab153, Ab157 and Ab151) recognize both the unfolded reduced and the folded disulfide intact forms of the recombinant prion protein spanning residues 23-231 or 121-231 (data not shown). In order to determine whether the IgY Ab-s were specific to epitopes accessible in PrP<sup>Sc</sup>, an indirect sandwich ELISA was performed. The IgY Ab-s were immobilized on a microtiter plate, and were incubated overnight with normal and TSE-infected hamster brain (strain 263 K) homogenates (with or without PK digestion) as well as with recombinant PrP and PBS, for positive and negative controls, respectively. A SPI-bio Enzyme Immunoassay (EIA) for prion protein (SPI-bio, Massy Cedex, France) with antibody-coated plates that recognize the DYEDRYREN residues 142-160 (per package insert) was utilized<sup>19</sup> for a comparison with the IgY coated Ab-s.

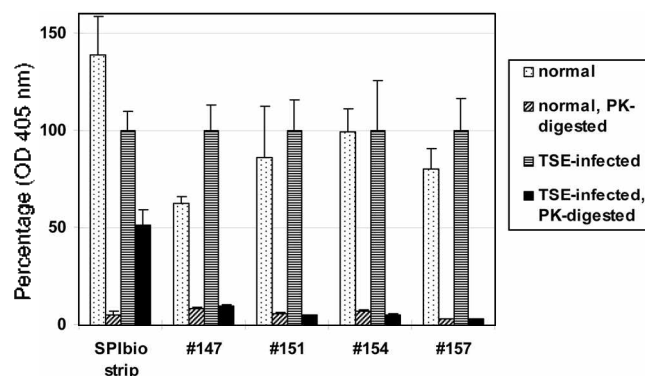
Figure 2 shows the results of these experiments. In order to facilitate a comparison of the antibodies, the measured



**Figure 1.** NMR structure<sup>39</sup> of the human PrP<sup>C</sup> residues 121-231. The peptide segments of PrP<sup>C</sup> used for generating the IgY Ab-s are: residues 179-186 (Pep-1), 189-195 (Pep-2), 199-205 (Pep-3) and 214-221 (Pep-4). The disulfide bond between the helices is indicated.

values are expressed as the percentages of the values that are found with the TSE-infected brain homogenate without PK digestion. It was necessary because the immobilized Ab-s have different sensitivity toward the various protease-resistant and protease-sensitive isoforms that are present in normal and TSE-infected hamster brain homogenates. All four IgY Ab-s as well as the SPI-bio kit capturing Ab detected prion protein forms in both normal and TSE-infected hamster brain homogenates. However, the IgY Ab-s have higher affinity toward isoforms in TSE-infected versus normal hamster brain homogenate, relative to the SPI-bio capturing Ab. After PK digestion, PrP<sup>Sc</sup> is clearly detected with the SPI-bio capturing Ab. By contrast, PK digestion decreased the signals in TSE-infected samples with the IgY Ab-s to near background levels that are represented by the respective signals for the normal brain homogenate that contains no PrP<sup>Sc</sup>. Thus, the epitopes of the four Ab-s are exposed on protease-sensitive forms of the prion protein (*i.e.*, the cellular and the unfolded proteins), but they are little or not available on the PK-digested protease-resistant core of PrP. These results were reproduced in independent experiments repeating both the Ab-immobilizations and PK-digestion steps.

These data have important structural implications for the conformational conversion of the prion proteins. In a recent  $\beta$ -helical model of the PK resistant core of PrP<sup>Sc</sup>, (PrP27-30) the two long helices, H2 and H3, of the cellular form of PrP are proposed to be preserved and the epitopes on H2 and H3 suggested to be exposed equally for antibody binding on both forms.<sup>31</sup> The four IgY in our study that recognize the protease-sensitive isoforms of PrP in brain homogenates were generated against short peptides of helices two and three (Fig. 1). Although these segments cover more than half



**Figure 2.** Sandwich ELISA experiments. Normal and scrapie infected hamster brain homogenates with and without PK digestion were tested using the IgY anti-prion polyclonal Ab-s as capture Ab-s as well as the SPI-bio kit capture Ab as a reference. Ab147, Ab153, Ab157 and Ab151 were generated against Pep-1, Pep-2, Pep-3 and Pep-4, respectively. Values are expressed as the percentages of the respective scrapie samples. An acetylcholinesterase-conjugated Fab' from the SPI-bio kit was used as the secondary Ab. Dotted bar: normal hamster brain homogenates, bar with slanted lines: PK-digested normal hamster brain homogenates, bar with horizontal lines: scrapie-infected hamster brain homogenates, solid bar: PK-digested scrapie-infected hamster brain homogenates. Error bars show sample ranges.

of the two helices, none of these four antibodies recognizes PrP27-30. By contrast, the more N-terminal control SPI-Bio antibodies, which are known to recognize PrP27-30, do recognize it in the very same samples tested. It is presently not clear which epitope is shielded by the sugar moieties and which becomes cryptic in the conformational transition/aggregation process. Nevertheless, our results revealed remarkable differences in the conformation/accessibility of these two segments between the two prion forms contrary to presumptions on which the present  $\beta$ -helical model of PrP27-30 was based.<sup>31</sup> Particularly, Peretz *et al.* demonstrated that the very C-terminal 6 amino acids of PrP are likely exposed on both PrP forms.<sup>32</sup>

There are two reports, where different accessibility of helices 2 and 3 of PrP between the two protein forms have been reported. Horiuchi and co-workers<sup>33</sup> described the generation of a new panel of diverse monoclonal Ab-s. Epitope-mapping revealed that some of these Ab-s recognized segments of the two long helices of PrP. Surprisingly, none of the Ab-s that are generated in this work recognized PrPSc, although their epitopes correspond more than one third of the sequences of PrP between residues 56 and 229.<sup>33</sup> In a more recent study, Serbec and co-workers<sup>34</sup> reported the generation of monoclonal Ab-s against 13 mer peptides of helices 2 and 3. Some of these Ab-s recognized selectively PrPSc without recognizing PrPC or the recombinant protein. Although the authors also considered that their binding to PrPSc was not PrP-specific,<sup>35</sup> this question was not experimentally addressed.<sup>35</sup>

Finally, it is worth to mention that this result is in accord with recent studies on amyloid fibrils of the recombinant prion protein where the two long helices of PrP were shown to undergo a conformational transition to  $\beta$ -structures.<sup>36-38</sup> Bocharova *et al.* observed that  $\beta$ -sheet-rich conformation was located at proteinase K-resistant core region, PrP27-30, where two long helices were located, using annealing method by refolding the recombinant PrP in presence of Triton X-100 after brief exposure to 80 °C to  $\beta$ -structures.<sup>36</sup> Moreover, MS analysis of H/D backbone amide exchange, and site-directed spin labeling EPR spectroscopy were employed to probe the  $\beta$ -structures by Cobb *et al.* and Lu *et al.*, respectively.<sup>37,38</sup> They also found that the  $\beta$ -sheet core of PrP amyloid corresponded to the C-terminal region from residue 169, including  $\alpha$ -helix 2, 3, and the loop between these two helices. Hence, these new observations including our current antibody results strongly suggested the C-terminal region of  $\alpha$ -helix 2 and 3 would through conformational changes from PrPC to the pathogenic PrPSc, at least synthetic PrPSc-like aggregates.<sup>38</sup>

## Materials and Methods

**Materials.** Eggcellent IgY purification kit, maleimide-activated BSA, 1-Stop TMB (3,3',5,5'-tetramethylbenzidine) peroxidase solution, BupH<sup>TM</sup> Carbonate-Bicarbonate buffer, and peroxidase-conjugated rabbit Anti-chicken IgY antibody were obtained from Pierce Biotechnology, Inc. SPI-bio

Enzyme Immunoassay (EIA) for prion protein was purchased from SPI-bio Inc. (Massy Cedex, France). Proteinase K (PK), ready-made Phosphate buffered saline (PBS) solution, Tris buffered saline solution (final 0.1% v/v), and protease cocktail were purchased from Sigma. Brain homogenate of TSE-infected (263 K strain) Syrian golden hamster (tittered  $2.0 \times 10^9$  in animal bioassay) and normal control samples were obtained from Baltimore Research and Education Foundation (BREF, University of Maryland). Ac-cvntikq-NH<sub>2</sub> (for Ab147), Ac-(c)-vttttkg-NH<sub>2</sub> (for Ab153), Ac-(c)-tetdvkm-NH<sub>2</sub> (for Ab157) and Ac-cvtqyqke-NH<sub>2</sub> (for Ab151), corresponding to residues 179-186, 189-195, 199-205 and 214-221 of the prion protein, respectively, were synthesized at the W.M. Keck Foundation Biotechnology Resource Laboratory at Yale University. CNBr-activated Sepharose was purchased from GE Healthcare Inc. For ELISA Immulon 4 HBX microtiter plates from Thermo Labsystems (Helsinki, Finland) and Block Ace from Serotech Inc. were used. Recombinant hamster PrP protein was purchased from Alicon AG (Switzerland).

**Preparation of peptide antigen.** Two milligram of peptides, Ac-cvntikq-NH<sub>2</sub> (for Ab147), Ac-(c)-vttttkg-NH<sub>2</sub> (for Ab153), Ac-(c)-tetdvkm-NH<sub>2</sub> (for Ab157) and Ac-cvtqyqke-NH<sub>2</sub> (for Ab151), were conjugated to maleimide-activated BSA (6 mg) in PBS at 37 °C during a four-hour incubation. [(c) indicates an extra cysteine residue added for facilitating the immobilization of the peptides.] The unreacted maleimide groups were blocked by incubating the solution with mercaptoethanol (5 mM) at room temperature for 30 min. Excess peptide and mercaptoethanol were dialyzed at 4 °C overnight. The peptide-BSA antigens were concentrated by an Amicon concentrator (10,000 m.w. cut-off).

**Generation and purification of IgY antibody.** Polyclonal chicken (IgY) anti-prion Ab-s were generated at QED Bioscience Inc. The peptide-BSA antigens combined with complete Freund's adjuvant were injected intradermally into chickens. At days 8, 15, 22 and 50, boost injections were administered, chicken eggs were collected, and the yolks isolated. IgY antibodies were purified from the yolks using the Eggcellent IgY purification kit following the manufacturer's protocol (Pierce Biotechnology, Inc.) and further purified on the respective peptide affinity columns.

**Immobilization of antibodies onto ELISA plates.** The antibodies were immobilized to microtiter plates in 100  $\mu$ L coating buffer (BupH<sup>TM</sup> Carbonate-Bicarbonate buffer, Pierce Biotechnology, Inc) at 5  $\mu$ g/mL IgY concentration overnight at 4 °C in a dark moisture chamber. The plates were washed with 300  $\mu$ L of PBS three times then-incubated with 300  $\mu$ L 10% Blockace (Serotec) for 1 hour at room temperature in a dark moisture chamber. The plates were washed again with 300  $\mu$ L of PBS three times and dried.

**Sample preparation and ELISA.** 50  $\mu$ L of hamster brain homogenate (10% in PBS) (provided by Dr. Rohwer) was treated with or without PK (50  $\mu$ g/mL, for 30 min) at 37 °C in 1% Triton X-100. PK digestion was stopped by adding protease inhibitor (10 mM) and heating for 10 min at 100 °C. The sample was then diluted in TBST to a final concen-

tration of 1%.

ELISA procedures were carried out on the IgY-immobilized plates, following the procedure described below, using a commercially available assay (SPI-Bio). The ELISA plate was rinsed 5 times with wash buffer (300  $\mu$ L/well). 100  $\mu$ L PBS was dispensed to control wells for assessing the non-specific binding. Recombinant PrP was used as positive control. Samples (100  $\mu$ L) were dispensed to the appropriate wells and the plates were incubated for 1 hour at 37 °C under continuous shaking on an orbital shaker (60 rpm). After 10 min cooling, the plate was washed 4 times with the wash buffer. 100  $\mu$ L of Anti-PrP acetylcholinesterase-Fab' conjugate (SPI-Bio) was applied to detect the bound PrP proteins by incubating for 1 hour at 37 °C with slow shaking on an orbital shaker. After 10 min cooling, the plate was washed four times with the wash buffer (300  $\mu$ L/well). Ellman's reagent solution (200  $\mu$ L) was then added to develop signal. After 30 min incubation in the dark the reaction was read at 405 nm using a plate-spectrophotometer. The experiments were performed in duplicate.

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