Proteolytic processing and glycosylation influence formation of porcine prion protein complexes

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High level of heterogeneity seems to be a ubiquitous feature of mammalian PrPs (prion proteins) and may be relevant to the pathogenesis of prion diseases. In the present study, we describe the heterogeneity of PrP^C (cellular form of PrP) from porcine brain. It was disclosed and characterized by a combination of one-dimensional PAGE and two-dimensional PAGE analyses with enzymic deglycosylation and copper-affinity experiments. We found that the identified two main populations of porcine PrP^C consist of diglycosylated forms and correspond to the full-length (molecular mass 32–36 kDa) and proteolytically modified protein (molecular mass 25–30 kDa), known as C1. The two populations were fully separated during Cu²⁺-loaded immobilized metal affinity chromatography, indicating different affinity for copper ions. The more basic forms, migrating as species of higher molecular mass, exhibited stronger affinity for copper ions, whereas those with more acidic pI and of lower molecular mass were low-affinity Cu²⁺-binding molecules and thus could represent

ent N-terminally truncated PrP^C. Size-exclusion chromatography revealed that most of the PrP^C molecules in porcine brain extracts exist in the form of high-molecular-mass complexes (probably with other proteins). The heterogeneity of porcine PrP^C, resulting from proteolytic modification and glycosylation, influences its ability to assemble into these complexes. N-truncated molecules dominate over full-length PrP^C in fractions of molecular mass over the range 65–130 kDa, whereas the full-length species are the major forms of PrP^C present in the monomeric fraction and in complexes above 130 kDa. Two-dimensional PAGE analysis indicated that the complexed PrP^C differs in the composition of pI forms from the monomers.

Key words: two-dimensional PAGE, porcine prion protein, copper binding, glycosylation, prion heterogeneity, proteolytic processing.

INTRODUCTION

The cellular form of PrP (prion protein), PrP^C, is a key molecule implicated in the pathogenesis of TSEs (transmissible spongiform encephalopathies) [1]. It is postulated that a disease-associated form of this protein named PrP^{Sc} (scrapie form of PrP) is a conformer generated from PrP^C in a conversion process of unknown molecular mechanism. PrP^C is a glycoprotein expressed mainly in the neuronal tissue and attached to the cell membrane through a GPI (glycosylphosphatidylinositol) anchor [2]. However, transmembrane isoforms with either C- or N-terminus located in the endoplasmic reticulum lumen have also been described. Mammalian PrPs contain two potential N-glycosylation sites and appear as bi-, uni- and unglycosylated forms; however, the latter two are relatively rare cell-surface components [3–5]. Preparations of PrP from hamster and human brain exhibit a high degree of heterogeneity [6–8], which is a result of structural variability of attached N-glycans as well as GPI moieties. The complexity of PrP population is also enhanced by proteolytic modifications occurring *in vivo* [9–11]. The two mapped cleavage sites lie at 110–112 and 80–100 residues (human PrP^C, see Figure 1) and processing of PrP by metalloproteases leads to the generation of N-truncated forms referred to as C1 and C2 respectively. C1 is the major product of normal PrP^C metabolism, whereas C2 dominates in TSE brains. The consequence of the N-truncation is a loss of an octameric repeat region (octarepeats), shown to comprise the main Cu²⁺-binding site responsible for superoxide dismutase activity of PrP^C [12–15]. The role of the post-translational modifications of PrP is not known. However, it is speculated that they may

be important for targeting to a particular region of the cell or brain [16] and regulating protein–protein interactions, thus influencing the as yet undefined physiological function of PrP^C. It has been demonstrated that in bovine brain extracts PrP^C may form dimers, whereas unglycosylated recombinant protein is monomeric [17,18]. It was postulated that N-glycosylation affects the conversion of PrP^C into PrP^{Sc}, the event believed to be crucial for the pathogenesis of TSE, since PrP^{Sc} was produced more rapidly in cells with inhibited glycosylation [19]. Moreover, the so-called prion strains can be characterized by the specific banding pattern of PrP^{Sc} on one-dimensional PAGE, reflecting post-translational modifications, and this pattern seems to be transmitted from the donor organism into newly generated PrP^{Sc} in the recipient [20,21]. Since the structural stability of a protein conformation may be affected by glycosylation, it has been postulated that the strain diversity may be, at least in part, a result of this modification [22]. Thus the heterogeneity of PrP may play a significant role in the pathogenesis of TSE. It is not known whether the post-translational modifications have an impact on the interactions of PrP^C with its ligands identified so far, such as dystroglycan, tubulin, laminin, laminin receptor/precursor, glial fibrillary acidic protein, adaptor protein Grb2, synapsin Ib, stress-inducible protein 1, heat-shock protein 60 etc. ([23–25], reviewed in [26]).

In the present study, we show that most of the PrP molecules extracted from porcine brain were in the form of complexes of molecular mass higher than those described for PrP^C dimer. Moreover, the high degree of porcine PrP^C heterogeneity, originating from proteolytic processing and glycosylation, affects its ability to participate in these complexes. The high similarity (> 91%)

Abbreviations used: 2-ME, 2-mercaptoethanol; Cu²⁺-IMAC, Cu²⁺-loaded immobilized metal affinity chromatography; DTT, dithiothreitol; GPI, glycosylphosphatidylinositol; IEF, isoelectric focusing; LB, lower band; mAb, monoclonal antibody; PNGase F, peptide N-glycosidase F; PrP, prion protein; PrP^C, cellular form of PrP; PrP^{Sc}, scrapie form of PrP; TSE, transmissible spongiform encephalopathy; UB, upper band.

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Figure 1 Alignment of porcine versus human PrP sequences (NCBI accession numbers P49927 and P04156 respectively; Clustal W [39])

Marked are regions and residues known to be modified in human PrP: N- and C-terminal signal peptides, lacking in mature protein (faint font), proteolytic cleavage sites (✂), GPI attachment site and two glycosylated asparagine residues. The octarepeat domain is presented as a grey rectangle and the epitope for antibody 6H4 is underlined. Residue numbering is shown for porcine PrP. Note that the identity between the mature proteins reaches 91% (indicated by *).

between porcine and human PrP sequences and a similar pattern of heterogeneity make porcine PrP^C a very useful model for studying the biochemistry of the human protein with a complement of post-translational modifications not available with recombinant PrP.

MATERIALS AND METHODS

Brain extracts

Immediately after pigs were killed at a local slaughterhouse, brain tissues were removed from their skulls, collected and transported on ice. All preparative steps were performed on ice or at 4°C. Brains from ten animals were thoroughly homogenized in (1 ml/g of tissue) buffer containing 1 mM ATP, 2 mM EGTA, 0.1 mM EDTA, 2 mM DTT (dithiothreitol), 33% (w/v) glycerol and 100 mM PIPES, pH 6.9. The homogenate was centrifuged for 15 min at 32 000 g and the obtained pellets were stored in aliquots at -20°C. Extraction of the pellets was performed for 30 min in 9 ml of buffer E [150 mM NaCl, 1% Igepal, 0.5% Tween 20, 0.1 mM DTT, 2 mM PMSF, 10 µg/ml pepstatin A (Sigma, St. Louis, MO, U.S.A.), 15 mM Tris/HCl, pH 7.5] for 1 g of pellet. During extraction, the material was gently homogenized in a Potter homogenizer. The extract was then centrifuged at 34 500 g for 20 min and the resultant supernatant was saved.

Size-exclusion chromatography

The extract described above was subjected to fractionation with ammonium sulphate at 25% of saturation and centrifuged at 34 500 g for 20 min. The supernatant was brought to 35% of ammonium sulphate saturation. Centrifugation (as above) resulted in a 'floating pellet' that was resuspended in column buffer (150 or 500 mM NaCl, 0.1% Igepal, 0.05% Tween 20, 0.5 mM DTT,

0.5 mM PMSF and 15 mM Tris/HCl, pH 7.5) and dialysed overnight. Subsequently, the preparation was clarified by centrifugation at 34 500 g for 20 min, diluted to obtain 8 mg/ml of total protein and applied to a Sephadex G-100 column (150 cm × 0.8 cm) equilibrated with the column buffer. The flow rate was 5 ml/h. The presence of PrP^C in collected fractions was monitored by dot-blot analysis. Calibration of the column was performed using protein molecular-mass standards, which were 21 kDa rabbit skeletal-muscle myosin light chain 1, 65 kDa BSA (Sigma) and 130 kDa rabbit skeletal-muscle myosin papain subfragment 1.

Immobilized Cu²⁺ affinity chromatography

The resin was prepared by 1 h incubation of equal volumes of Chelating Sepharose FF (Amersham Biosciences, Piscataway, NJ, U.S.A.) slurry with 0.2 M CuCl₂ followed by extensive washing with deionized water and equilibration in dialysis buffer (150 mM NaCl, 0.1% Igepal, 0.05% Tween 20, 0.5 mM DTT, 0.5 mM PMSF and 10 mM sodium phosphate buffer, pH 7.0). The copper-loaded resin was packed into a Spin Cup Column (Pierce, Rockford, IL, U.S.A.). The brain extracts were fractionated with ammonium sulphate (at 25 and 35% of saturation) and the obtained 'floating pellet' was dialysed overnight against the buffer described above. The clarified preparations were then loaded on the resin and after 15 min incubation on ice the resin was centrifuged at 2000 g for 1 min. The resin was washed with one volume (settled gel) of the dialysis buffer and subsequently with three volumes of this buffer. The method of elution of proteins from the resin was the same as the washing method using the dialysis buffer containing 15, 30, 150, 500 and 850 mM imidazole (pH 7.0) or 50 mM EDTA. Each first wash was collected and precipitated with acetone for the preparation of one- and two-dimensional PAGE samples.

Enzymic deglycosylation

The brain extracts were dialysed overnight against a buffer containing 0.2 mM EDTA, 15 mM NaCl, 1 % (v/v) 2-ME (2-mercaptoethanol), 0.1 % Igepal, 0.5 mM PMSF and 10 mM Tris/HCl, pH 8.5. After clarification, 10 μ l of denaturing buffer (5 % SDS, 15 % 2-ME) was added to 90 μ l of the preparation and the mixture was boiled for 10 min. Subsequently, 12.5 μ l of 0.5 M sodium phosphate buffer (pH 7.5), 12.5 μ l of 10 % Igepal and 5 μ l of PNGase F (peptide N-glycosidase F) from *Flavobacterium meningosepticum* (New England Biolabs, Beverly, MA, U.S.A.) or water were added. The reaction was performed for 1 h at 37 °C and for 21 h at 25 °C.

Electrophoretic separations

One-dimensional PAGE (SDS/PAGE) was performed on 5 % stacking gel or 12 or 15 % (PNGase F-treated samples) separating gels by the method of Laemmli [27]. Before two-dimensional PAGE, the preparations were precipitated with three volumes of cold acetone and left at -20 °C overnight. The precipitates were pelleted and suspended in GLB buffer [9 M urea, 2.4 % (w/v) Ampholine (pH 3.5–9.5; Amersham Biosciences), 2 % (w/v) Triton X-100, 1 % 2-ME and 0.04 % Bromophenol Blue]. Subsequently, the suspension was diluted twice with 2 % Triton X-100. Before IEF (isoelectric focusing), the samples were clarified by 1 min centrifugation at 14 000 g. The tube gels for IEF were composed of 4 % (w/v) acrylamide, 2 % Triton X-100, 9.2 M urea, 2 % Ampholine (pH 3.5–9.5) and were prerun for 15 min at 150 V. The electrofocusing was performed in 20 mM NaOH as catholyte and 10 mM H₃PO₄ as anolyte for 30 min at 500 V and for 2.5 h at 750 V with cooling of the electrophoretic apparatus. The tube gels were subsequently equilibrated in 5 % 2-ME, 2.3 % SDS, 10 % glycerol and 62.5 mM Tris/HCl (pH 6.8) for 15 min and subjected to SDS/PAGE on 12 or 15 % (PNGase F-treated samples) slab gels. The pH gradient formed during IEF was determined for tube gels electrofocused simultaneously with the gels loaded with samples. These empty gels were cut into 5 mm slices and incubated separately for at least 1 h in 10 mM KCl. Subsequently, pH was measured for each of the slices.

Immunoblotting

For dot-blot analysis, 2 μ l of sample was spotted on a nitrocellulose membrane (Amersham Biosciences). The antigen was detected by means of a primary antibody, mouse monoclonal antibody 6H4 (Prionics AG, Schlieren, Switzerland) diluted at 1:5000 and secondary antibody, rabbit anti-mouse IgG conjugated with horseradish peroxidase (Sigma) diluted at 1:80 000. Colorimetric detection was performed using 3,3',5,5'-tetramethylbenzidine (Sigma).

For Western blot, gels after electrophoresis were subjected to electrotransfer on to nitrocellulose and immunodetection was performed as described for dot-blot except that a higher concentration of the secondary antibody (1:40 000) was used. Enhanced chemiluminescent detection was performed by incubation of the blot with SuperSignal West Femto substrate (Pierce) followed by exposition against X-ray film. Dot-blots and X-ray films were analysed densitometrically using the Image Quant software (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

RESULTS

Electrophoretic characterization of porcine brain PrP^C

To characterize PrP^C extracted from whole porcine brain, we performed a set of one- and two-dimensional electrophoretic separations

Figure 2 Immunoblots of porcine PrP^C separated by one-dimensional (left panel and left lane in the right panel) and two-dimensional PAGE (right panel)

The inset and the left lane in the right panel show overexposed film. A representative Western blot for four independent preparations is presented. Molecular masses are indicated in kDa.

followed by immunodetection with mAb (monoclonal antibody) 6H4. This antibody had been raised against the sequence DYEDRYRE (residues 144–152 in human PrP), which is also conserved in the porcine PrP (residues 148–156) (Figure 1) [28]. Thus mAb 6H4 allows the detection of full-length PrP as well as its N-terminally truncated forms, C1 and C2 [9,10].

On one-dimensional immunoblot, porcine PrP^C appears as two major sets of bands (Figure 2). The UBs (upper bands) correspond to molecular masses from 32 to 36 kDa, whereas the LBs (lower bands) migrate as 25–30 kDa molecules. In extracts from whole brain, PrP^C migrating as UB is present at a higher concentration compared with the fraction migrating as LB. The mAb 6H4 detects also three minor bands of approx. 57, 20 and 17 kDa (Figure 2, overexposed film). The first one may represent the already described covalent PrP dimer [29,30], and the low-molecular-mass species presumably correspond to truncated forms.

Only two-dimensional electrophoresis reveals a very high degree of heterogeneity of PrP extracted from porcine brain. After two-dimensional PAGE, porcine PrP^C is separated into at least 52 forms spanning the pI values from 7.6 to 4.8 (Figure 2). Each of the bands observed on one-dimensional PAGE, except the 17 kDa species, is composed of multiple spots. The UB comprises 33 spots corresponding to PrP^C forms with pI spanning over 7.6 to 6.0. However, in this population the less acidic forms are better represented. The bands migrating as 25–30 kDa are composed of approx. 12 spots of acidic pI (6.3–4.8). Also the 20–24 kDa species, resolved into six charge forms, display exclusively acidic pI (6.3–5.1). The barely detectable 17 kDa band migrates as a single spot of pI 6.3. Despite the observed high degree of complexity of the PrP^C preparation from porcine brain, a simplified pattern appears, in which the PrP molecules of higher molecular mass are of less acidic pI, whereas those migrating as lower molecular mass forms are apparently more acidic. The ratio of the intensity of UB to LB observed on one-dimensional PAGE is almost inverted after two-dimensional PAGE. This may suggest that some PrP forms are missing on IEF and predominantly UB comprises molecules of pI values beyond the pH range of the gel.

Removal of PrP^C N-linked glycans

In an attempt to identify the major PrP^C species, migrating as UB and LB, the extracts from porcine brain were subjected to enzymic

Figure 3 Electrophoretic characterization of enzymically deglycosylated porcine PrP^C

The left panel shows one-dimensional immunoblot obtained for PNGase F-treated and -untreated material. Two-dimensional separation of deglycosylated PrP^C is presented on the right panel of the Figure. Molecular masses are indicated in kDa.

deglycosylation. The preparations were treated with PNGase F, an enzyme that specifically removes asparagine-linked glycans. This enzyme converted porcine PrP^C into species migrating as approx. 26 and 17 kDa molecules, with the UB slightly dominating (Figure 3). An additional minor band of approx. 20 kDa could also be detected. The 20 and 17 kDa species were not observed on immunoblots of untreated PrP^C (on not-overexposed films, see Figure 2). Presumably, the 26 kDa band is formed from the UB and 17 kDa band from the LB.

After two-dimensional PAGE (Figure 3), deglycosylated porcine PrP^C was separated into a much lower number of charge forms than the untreated preparation (Figure 2). This indicates that glycosylation is the major modification responsible for the observed PrP heterogeneity. As it is shown in two-dimensional immunoblot (Figure 3), the 26 kDa species comprise several basic forms (pI of approx. 7.6), not detected for LB (Figure 2). The band of approx. 17 kDa is separated into three spots within pI values of 6.1–5.7. mAb 6H4 also detected a minor form of approx. 18 kDa and pI 6.2. In the range of molecular mass from approx. 20 to 25 kDa, migration was barely detectable with multiple forms spanning pI values of the 26 and 17 kDa species. Again, the ratio of 26 and 17 kDa deglycosylated PrP^C, observed on one-dimensional PAGE, is inverted on a two-dimensional PAGE (Figure 3), which may imply that some of the forms composing the species of higher molecular mass have a pI (probably more basic) that does not allow focusing during IEF with the ampholytes used.

Copper-binding properties of PrP^C species

It is known that all mammalian PrP^Cs are copper-binding proteins. To characterize the affinity of porcine PrP^C for Cu²⁺ ions and further to identify the PrP species, we performed fractionation of brain extracts on copper-loaded resin. During IMAC (immobilized metal affinity chromatography), porcine PrP^C is retained on the resin and eluted at two different concentrations of imidazole (Figure 4A), leading to full separation of the UB and LB. The PrP^C species migrating as the approx. 27 kDa band (LB) are eluted from the resin at 15 mM imidazole, whereas the molecules composing the UB display a much higher affinity for copper and elute only at 500 mM imidazole.

The origin of the two differentially eluting forms is confirmed on two-dimensional PAGE (Figures 4B and 4C). The fraction eluted at the low imidazole concentration is composed of 27 kDa forms spanning acidic pI values (5.9–4.8), seen in Figure 2. This fraction also includes acidic species of approx. 20–24 kDa and the 17 kDa form. The fraction of PrP^C displaying high affinity for the Cu²⁺-loaded resin has a two-dimensional PAGE pattern characteristic of the UB (compare Figures 2 and 4C).

Figure 4 Separation of porcine PrP^C species by Cu²⁺ affinity chromatography

(A) The one-dimensional immunoblot shows sample applied on the IMAC resin (S), flowthrough fraction (U), fractions eluted with a step gradient of imidazole concentration and EDTA. Two-dimensional immunoblots for the fractions eluted from Cu²⁺-loaded resin by 15 mM (B) and 500 mM (C) imidazole. Insets show overexposed films. Molecular masses are indicated in kDa.

Porcine PrP^C forms complexes of high molecular mass

Taking into consideration the ability of bovine PrP to dimerize [17], we decided to perform experiments aimed at the characterization of oligomeric properties of porcine PrP^C. Extracts from whole porcine brain were subjected to fractionation with ammonium sulphate. The precipitate enriched in PrP^C, forming a 'floating pellet' on centrifugation, was solubilized and applied on a size-exclusion chromatography column. PrP^C eluted from the column at volumes corresponding to molecular mass ranging from approx. 30 to 200 kDa (Figure 5). Surprisingly, in 150 mM salt, only approx. 10% of porcine PrP migrate as monomers and another 12% over the range up to approx. 70 kDa possibly corresponds to

Figure 5 Size-exclusion chromatography of porcine PrP^C

Elution profiles of PrP subjected to molecular filtration in a column buffer containing either 150 or 500 mM NaCl are presented in the Figure. The relative concentrations of PrP^C (expressed in arbitrary units) in eluted fractions were determined by the dot-blot analysis. Arrows indicate molecular mass (in kDa) calculated from calibration curves corresponding to appropriate elution volumes. The presented chromatograms were highly repeatable and the mean for four independent experiments is shown.

PrP^C dimers. Approximately 60% of PrP^C was fractionated as complexes of molecular mass above 97 kDa. Most of the PrP molecules were engaged in 97–180 kDa assemblies. The complexes were not destabilized by high salt concentration (500 mM NaCl) and the equilibrium was even slightly shifted towards the high-molecular-mass species (Figure 5).

The heterogeneity of porcine PrP^C influences its participation in complexes

It is commonly assumed that proteolytic modification as well as glycosylation may affect the interaction between protein molecules. We examined whether the above-described heterogeneity in molecular mass and charge of porcine PrP^C influences its ability to form the complexes analysed by size-exclusion chromatography. One-dimensional immunoblotting (Figure 6A, see also one-dimensional separations shown in Figure 7A) of obtained gel filtration fractions indicates apparent differences in the composition of PrP forms. The PrP^C molecules migrating as UB and LB display different tendencies to participate in complexes of a particular size. Densitometric analysis (Figure 6B) of the blots revealed that UB species greatly dominate over LB or are even the sole species in the monomeric fraction of porcine PrP^C. Surprisingly, the UBs also dominate in the very first fractions eluted from the column (complexes of ~200 kDa), whereas in the fractions corresponding to molecules over the range 65–130 kDa the ratio between the two PrP species is apparently inverted. To explain the observed domination of UB over LB in complexes of high molecular mass as well as in monomeric PrP, we subjected the fractions corresponding to particles of molecular masses of approx. 180, 77 and 40 kDa to two-dimensional PAGE. Comparison of two-dimensional immunoblots obtained for these fractions (Figure 7A) indicates differences in the pattern of charge forms contributing to the UB. The ratio of intensity among the individual spots separated during IEF differs most notably between the fraction of the high-molecular-mass complexes and the

Figure 6 One-dimensional PAGE analysis of PrP^C fractionated by size-exclusion chromatography

(A) Immunoblot obtained after one-dimensional electrophoresis. Insets show overexposed films. Molecular masses are indicated in kDa. (B) Densitometric analysis of the immunoblot presented in (A). Shown are the means $\pm$ S.D., $n=3$. Molecular masses indicated by arrows were calculated from calibration curves.

fraction of monomers (see densitometric analysis shown in Figure 7B). Generally, in the last of the compared fractions, approx. 35 kDa population of PrP^C molecules contain a higher proportion of the more acidic charge forms than the first two fractions. Moreover, there is an additional charge form with a slightly higher molecular mass (marked no. 5 in Figure 7A) present in the monomeric fraction. Its presence exclusively in this fraction was confirmed by one-dimensional IEF (results not shown). These results may suggest that the charge forms of pI values from 7.6 to 7.4 (nos. 1–4 in Figure 7A), migrating as the UB, have a higher tendency to form high-molecular-mass complexes compared with the forms of pI 7.3–6.8 and 6.3 (nos. 5–8 and 11) comprising the same band on one-dimensional PAGE. Moreover, the slightly acidic species of UB (nos. 10 and 11, pI 6.5 and 6.3 respectively) are enriched in the fraction corresponding to 77 kDa complexes. The forms carrying negative charge (pI 6.1–4.8), migrating as LB, are present predominantly in the intermediate (77 kDa) complexes (Figure 7A).

DISCUSSION

One of the intriguing features of PrP, reported previously for human PrP^C by means of two-dimensional PAGE [7], is its high degree of heterogeneity resulting from post-translational modifications. In the present study, we show that it is also a feature of porcine PrP^C. The gene encoding porcine PrP has been sequenced [28] and the deduced amino acid sequence exhibits very high

Figure 7 Two-dimensional PAGE analysis of PrP^{Sc} fractions separated by size-exclusion chromatography

(A) Immunoblots of PrP^{Sc} fractions corresponding to the molecular masses of 40, 77 and 180 kDa obtained after two-dimensional electrophoresis. Molecular masses are indicated in kDa. (B) Densitometric analysis of PrP^{Sc} forms composing the UB from the fractions presented in (A).

similarity to the human protein (Figure 1). Moreover, experimental transmission of bovine spongiform encephalopathy to the pig succeeded [31]. However, the porcine protein has not been examined before.

In the present study, two-dimensional PAGE allowed us the separation of porcine brain PrP^{Sc} into as high number of forms (~52 spots) as had been described for human PrP [7]. The population of PrP molecules is heterogeneous with respect to charge and molecular mass. It is commonly assumed that a variety of

charge forms are predominantly accounted for by the differences in the amount of sialic acid contained in the attached *N*-glycans and GPI [6,32]. A detailed analysis performed by Rudd et al. [6] revealed that Syrian hamster PrP can carry various *N*-glycans containing 0–5 moieties of sialic acid. Enzymic deglycosylation experiments (Figure 3) clearly show that *N*-glycosylation is the major post-translational modification responsible for introducing heterogeneity also into PrP^{Sc} from porcine brain. Separation of deglycosylated PrP^{Sc} into a few charge forms may result from compositional variability of GPI and, for C1, alternative cleavage sites around His-111 (human PrP, see Figure 1). Since the N-terminal sequence carries a strong positive charge, the normal proteolytic processing of this region is responsible not only for the observed molecular mass heterogeneity but also for charge diversity of PrP^{Sc}. Some heterogeneity of molecular mass is also introduced by differences in the number and size of *N*-glycans attached.

Two-dimensional PAGE patterns obtained for the human [7] and porcine proteins exhibit some similarities. However, because of the differences in electrophoretic conditions applied in those experiments, a direct comparison is not possible. The work referring to human PrP concludes that this protein exists predominantly as full-length diglycosylated and N-terminally truncated (C1 fragment) diglycosylated molecules. Thus most of the human PrP^{Sc} molecules were found to be in the fully glycosylated form. Again, the C2 fragment was shown to be a rare species in brains of healthy organisms [9,10]. We decided to identify the major species of porcine brain PrP^{Sc} and check if the above characteristics are also true for the porcine protein. On the basis of the very high level of similarity to human PrP (Figure 1), we assumed that the porcine protein may undergo similar post-translational modifications to those described for the human protein. The population of porcine PrP^{Sc} is resolved during one-dimensional PAGE into two major species migrating as molecules of molecular mass 25–30 and 32–36 kDa (Figure 2), denoted for simplicity as LB and UB respectively. Two-dimensional electrophoresis indicated that LB is composed solely of acidic forms, whereas UB migrates as forms of pI within the range 7.6–6.0. A similar pattern was described for the two dominating species of human PrP^{Sc} [7]. This may be explained in terms of the basic character of full-length PrP sequence (pI values of 9.39 for the human and 9.45 for the porcine protein sequence) and acidic pI calculated for the C1 fragment (pI values of 5.75 for human and 5.88 for porcine protein sequence) lacking the highly basic sequence of approx. 9 kDa. Since *N*-glycosylation may introduce additional negative charges into the PrP molecule, the pI values calculated for amino acid sequences may be lowered after this modification. To verify if indeed UB represents full-length molecules and LB is composed solely of C1, we subjected the preparations from porcine brain to enzymic deglycosylation by PNGase F. The 9 kDa difference in molecular mass between the bands obtained after PNGase F treatment may correspond to the N-terminal sequence, indicating that the 17 kDa band represents C1, whereas the 26 kDa band is formed by full-length molecules. Moreover, two-dimensional PAGE analysis revealed that the *in vitro* deglycosylated 26 kDa species is solely composed of basic forms not detected for LB in extracts not treated with PNGase F (compare two-dimensional immunoblots in Figures 2 and 3). It should be remembered that unglycosylated full-length PrP is a strongly basic molecule with a pI value beyond the range of ampholytes applied by us, thus would not be detected on two-dimensional immunoblots but still would contribute to the LB on one-dimensional PAGE. To exclude the possibility that LB also comprises unglycosylated full-length molecules, we characterized the affinity of the two species for copper ions. Since the C1 fragment lacks the N-terminal sequence

spanning octarepeats (postulated to be the major copper binding region in the molecule) [12] as well as His-96 comprising together with His-111 the high affinity site [33,34], it should exhibit a lower affinity for Cu²⁺ than intact PrP^C. Indeed, we observed a complete separation of the LB from the UB on copper affinity chromatography (Figure 4A), where the PrP^C species of molecular mass approx. 27 kDa exhibited weak Cu²⁺-binding properties, whereas the approx. 34 kDa species exhibited strong Cu²⁺-binding properties. The two fractions separated during Cu²⁺-IMAC were composed of acidic charge forms and more basic forms described for LB and UB respectively (compare two-dimensional immunoblots in Figures 2, 4B and 4C). Such a two-step elution of PrP^C from copper-loaded resin has already been reported for the protein from hamster brain [35] and may indicate the presence of two Cu²⁺-binding sites, the N-terminal sequence and residues located within the C1 fragment. The identified copper-binding motif in the C-terminal part of the molecule overlaps His-187 [36,37]. Our results correlate with the finding that the peptide 177–230 contains a weak binding site [38]. Taken together, the results presented above allowed us to identify the two major porcine brain PrP^C species as the diglycosylated C1 and the diglycosylated full-length protein.

A promising approach, that may lead to an understanding of the physiological function(s) of PrP^C and the molecular mechanism of its pathogenic conversion into PrP^{Sc}, is to study PrP^C oligomerization and interactions with other proteins. It was previously demonstrated that bovine PrP^C molecules are in a dimer–monomer equilibrium [17]. Interestingly, in the present study, at the physiological ionic strength (150 mM NaCl), more than 70 % of porcine PrP is fractionated during size-exclusion chromatography in the form of complexes of molecular mass significantly exceeding that calculated for the PrP dimer (Figure 5). However, there are substantial differences in the preparative procedure applied by us and in the mentioned work, which may explain the discrepancy in the results. Meyer et al. [17] performed thermal denaturation of brain extracts as a purification step preceding the analysis of oligomeric properties of PrP^C. Such an approach may have led to the removal of proteins that would remain associated with PrP during the mild conditions of preparation used in our study. Hence, we assumed that the molecular assemblies of PrP^C observed by us are complexes with other proteins rather than oligomers of PrP^C. We believe that these complexes are *in vivo* formed raft constituents.

Our work and previous reports [6,7] present mammalian PrP^C as a highly heterogeneous protein; however, the physiological function of this feature remains unknown. The question raised in the present study was whether the heterogeneity of PrP^C, resulting from glycosylation and limited proteolysis, affects the tendency of the porcine protein to assemble into complexes described in this work. We found that the proteolytic processing exerts the most significant effect on PrP^C fractionation during size-exclusion chromatography (Figure 6). Since exclusively intact molecules, retaining the N-terminal region, are present in the fraction corresponding to monomeric protein, it is possible that this unstructured segment of PrP^C hinders some protein–protein interactions. On the other hand, full-length PrP^C dominates over C1 also in the fraction of complexes with the highest molecular mass. An explanation of such a fractionation might be that the N-terminal sequence can constitute a docking site for some of the PrP interactors. Among the already identified ones are tubulin and laminin receptor/precursor [24,25]. The simplest explanation of the domination of full-length PrP^C in the monomeric fraction as well as in the fraction of high-molecular-mass complexes is a different composition of glyco-forms in these fractions. A comparison of two-dimensional electrophoretic separations of the mentioned

fractions revealed some differences in the composition of charge forms. The observed differences are rather quantitative (with the exception of the additional charge form 5 present only in the monomeric fraction, see Figure 7A) and suggest that the more basic forms are somewhat more readily incorporated into high-molecular-mass complexes. It is known that *N*-glycans as well as GPI introduce a negative charge to the PrP thereby influencing ionic interactions with other molecules. However, this is not the case in our studies, where PrP^C complexes are stable even at a high ionic strength (0.5 M NaCl). This may suggest that the described complexes are stabilized by hydrophobic interactions.

In conclusion, our findings demonstrated that porcine brain PrP^C exists as a highly heterogeneous population of mostly fully glycosylated molecules. The heterogeneity is introduced into the protein by post-translational modifications: glycosylation and proteolytic processing. Most of the PrP^C molecules form hetero-complexes not described to date for the cellular form of PrP. Glycosylation and N-terminal truncation affect the ability of the PrP to assemble into complexes. Further studies are required to determine the composition of these complexes, which may lead to identification of physiological interactors of PrP^C and elucidation of the role of PrP in the cell.

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