Novel Differences between Two Human Prion Strains Revealed by Two-dimensional Gel Electrophoresis*

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The phenotype of human sporadic prion diseases is affected by patient genotype at codon 129 of the prion protein (PrP) gene, the site of a common methionine/valine polymorphism, and by the type of the scrapie PrP (PrPSc), which likely reflects the prion strain. However, two distinct disease phenotypes, identified as sporadic Creutzfeldt-Jakob disease (M/M2 sCJD) and sporadic fatal insomnia (sFI), share methionine homozygosity at codon 129 and PrPSc type 2. One-dimensional gel electrophoresis and immunoblotting reveal no difference between the M/M2 sCJD and sFI species of PrPSc in gel mobility and glycoform ratio. In contrast, the two-dimensional immunoblot demonstrates that in M/M2 sCJD the full-length PrPSc form is overrepresented and carries glycans that are different from those present in the PrPSc of sFI. Because the altered glycans are detectable only in the PrPSc and not in the normal or cellular PrP (PrPC), they are likely to result from preferential conversion to PrPSc of rare PrPC glycoforms. This is the first evidence that a qualitative difference in glycans contributes to prion diversity.

The normal or cellular prion protein (PrPC)1 in humans is a cell-surface, glycosylphosphatidylinositol-anchored glycoprotein that has 209 residues and two potential N-glycosylation sites (1–3). According to the degree of glycosylation, three full-length PrPC glycoforms can be distinguished: the diglycosylated, the monoglycosylated, and the unglycosylated form, which migrate as 33–42 kDa, 28–33 kDa, and 28–30 kDa glycoproteins in SDS-PAGE, respectively (1, 4–6). In addition, human PrPC brain preparations also comprise three N-terminally truncated species, which are generated following cycling through the plasma membrane (7).

It is widely accepted that the change in conformation of PrPC is the central event in the pathogenesis of prion diseases (2, 3, 8). PrPC, which has predominantly an α-helical conformation and is sensitive to proteolytic enzymes such as proteinase K (PK), is converted into a form commonly identified as scrapie PrP (PrPSc) that is rich in β-sheet and is PK-resistant and infectious (9–11). However, an important issue that remains to be clarified is the molecular events that lead to the formation of distinct isoforms of PrPSc called prion strains.

Prion strains are defined, stable forms of PrPSc with characteristic physical-chemical features that, upon inoculation in animals, cause disease with a unique incubation time as well as type and distribution of the lesions (2, 3). Prion strains are thought to reflect different conformations that PrPSc may adopt (5, 12, 13). Both the genotype of the host and the prion strains determine the disease phenotype (2, 3).

Prion strains also exist in human prion diseases, which include sporadic and genetic forms as well as forms acquired by infection (13–15). The sporadic form is the most common and comprises five distinct subtypes of Creutzfeldt-Jakob disease (sCJD) and the sporadic form of fatal insomnia (sFI) (8, 16). The mechanism of the phenotypic diversity in sporadic prion diseases is unclear but is believed to be determined by the genotype at codon 129, the site of a common Met/Val polymorphism (17, 18) and the PrPSc type (18). Two major types of PrPSc are recognized, which differ in the size of the PrPSc fragment generated by PK-digestion (5). In PrPSc type 1, the unglycosylated fragment migrates at 21 kDa; in type 2 it migrates at 19 kDa (5). The different sizes are likely due to conformational differences between PrPSc species resulting in the exposure of different PK cleavage sites. PrPSc types 1 and 2 have been transmitted to transgenic mice and are considered to represent human prion strains (13–15).

The combination of the codon 129 genotype with the PrPSc type correlates fairly well with the phenotype in four of the six distinct phenotypes in sporadic prion disease (16). However, affected subjects that are Met homozygous at codon 129 and have PrPSc type 2 (M/M2) present either one of two quite different disease phenotypes (16, 19). The sFI phenotype is characterized by insomnia and severe thalamic atrophy with little involvement of the cerebral cortex. In contrast, the M/M2 sCJD phenotype is clinically similar to CJD and is characterized by severe spongiosis of the cerebral cortex but with little involvement of the thalamus (16, 19). One-dimensional immunoblotting and preliminary microsequencing of the PK-resistant PrPSc fragment (26) reveal no difference between the two PrPSc species.

In this study, we used two-dimensional gel electrophoresis followed by immunoblotting as a novel approach to explore differences among prion strains. We demonstrate for the first time major differences between the PrPSc associated with sFI and that present in M/M2 sCJD.

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‡ The abbreviations used are: PrP, cellular (normal) prion protein; PAGE, polyacrylamide gel electrophoresis; PK, proteinase K; PrPSc, scrapie PrP; sCJD, sporadic Creutzfeldt-Jakob disease; sFI, sporadic form of fatal insomnia; M/M, Met homozygous at codon 129; M/M2, Met homozygous at codon 129 and having PrPSc type 2; PNgase F, peptide-N-glycosidase F; mAb, monoclonal antibody.
Experimental Procedures

Tissues—Brain tissue was obtained from the frontal cortex of four autopsy-confirmed cases with sporadic prion disease that were M/M at codon 129 of the PrP gene (PRNP) and had the PrPSc type 2 and of two genotype-matched cases free of prion disease used as controls (5, 20). The four prion disease cases were categorized as sFI (n = 2) or M/M2 sCJD (n = 2) based on their histopathological and PrP immunohistochemical features (16). In one case each of sFI and M/M2 sCJD, tissue was also obtained from the hippocampus (CA1) and the thalamus (mediodorsal nucleus). Frontal cortex obtained at biopsy was also examined in one case of M/M2 sCJD.

Homogenization and Fractionation—Brain homogenates were prepared in 10 volumes of lysis buffer (10 mM Tris, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 5 mM EDTA, pH 8.0) and 1 mM phenylmethylsulfonyl fluoride, centrifuged at 12,000 × g for 10 min, and the supernatants were stored at −80 °C. To obtain detergent-soluble and detergent-insoluble fractions, tissues were homogenized in 10 volumes of sucrose buffer (0.25 mM sucrose, 10 mM Tris, 5 mM EDTA, pH 8.0) and centrifuged at 6000 rpm for 10 min, and the supernatants were centrifuged again at 60,000 × g for 1 h at 4 °C and stored. The pellets were resuspended in 10 volumes of TNE buffer (20 mM Tris, 150 mM NaCl, 2 mM EDTA, pH 8.0) with 2% Sarkosyl, centrifuged at 60,000 × g for another 1 h at 4 °C, and stored.

Proteinase K and PNGase F Treatments—For proteinase K treatment, brain homogenates without phenylmethylsulfonyl fluoride were incubated at 37 °C for 1 h in the presence of 50 μg/ml PK (Roche Molecular Biochemicals). The digestion was stopped with 3 mM phenylmethylsulfonyl fluoride. Before PNGase F treatment, PK-treated and untreated brain homogenates were mixed with 0.1 volumes of 10× denaturing buffer (20 mM Tris, 150 mM NaCl, 2 mM EDTA, 10% 2-mercaptoethanol, 5% SDS, pH 7.5) and heated for 10 min at 95 °C. PNGase F (New England Biolabs Inc.) digestion was carried out at 37 °C for 1 h with the addition of 0.1% of 10% Nonidet P-40.

Immunoprecipitation—mAb 8H4 to the C terminus and mAb 8B4 to the N terminus of PrPSc were coupled to Sepharose 4B immunoaffinity columns (21). Immunoprecipitation was carried out at 4 °C overnight by equilibrating 20 μl of immunoaffinity beads with a predetermined amount of brain preparation. The bead-bound PrP was eluted and processed for two-dimensional electrophoresis.

One- and Two-dimensional Electrophoresis (PAGE) and Immunoblotting—Brain homogenate was mixed with PAGE loading buffer (160 mM Tris, 4% SDS, 4% 2-mercaptoethanol, 50% glycerol, 0.04% bromphenol blue, pH 6.8) and heated for 10 min at 95 °C before loading onto a 15% SDS-PAGE gel (Bio-Rad). Two-dimensional PAGE was performed as described by the supplier with minor modifications using Mini-Protean II two-dimensional cell and Mini-Protean II tube module (Bio-Rad). Equal amounts of PrP were loaded onto the tube gel, subjected to 500 V for 10 min and 750 V for 5 h. The extruded gel tubes were equilibrated in gel equilibration buffer (50 mM Tris, 6% urea, 30% glycerol, 2% SDS, pH 6.8) for 15 min and loaded onto the two-dimensional gel (15% SDS-PAGE). The pH gradients of two-dimensional gels were determined from duplicate gels loaded with two-dimensional gel marker and detected with Coomassie Blue. PAGE slabs were electrotransferred onto nitrocellulose membranes and immunoblotted (21). PrP was detected using mAb 8H4 in combination with horseradish peroxidase-conjugated goat anti-mouse IgG Fc antibody and visualized with chemiluminescence (Roche Molecular Biochemicals).

Results

On one-dimensional immunoblots, all untreated PrP preparations from control, sFI, and M/M2 sCJD, and sFI brains display the typical pattern of human brain PrP, although control brains contain only PrPSc whereas PrP from M/M2 sCJD and sFI comprises both PrPC and PrPSc (and presumably other abnormal PrP forms). Proteinase K digestion was performed with PK-treated and untreated brain homogenates to obtain detergent-insoluble and detergent-soluble PrP preparations (P). The addition of 0.1 volume of 10% Nonidet P-40 directed to the N-terminal region of PrP. Equal amounts of PrP were loaded onto the tube gel, subjected to 500 V for 10 min and 750 V for 5 h. The extruded gel tubes were equilibrated in gel equilibration buffer (50 mM Tris, 6% urea, 30% glycerol, 2% SDS, pH 6.8) for 15 min and loaded onto the two-dimensional gel (15% SDS-PAGE). The pH gradients of two-dimensional gels were determined from duplicate gels loaded with two-dimensional gel marker and detected with Coomassie Blue. PAGE slabs were electrotransferred onto nitrocellulose membranes and immunoblotted (21). PrP was detected using mAb 8H4 in combination with horseradish peroxidase-conjugated goat anti-mouse IgG Fc antibody and visualized with chemiluminescence (Roche Molecular Biochemicals).

On two-dimensional immunoblots, five major sets of PrPSc forms are detected in controls (Fig. 1B). The uppermost set of 35–42 kDa, which includes between 13 and 20 individual spots spanning pI values that range from pH 4.5 to 8.0, presumably corresponds to the full-length diglycosylated form. The second, third, and fourth sets of ~35, 29, 35, and 24–28 kDa, contain 6–13 spots and have a pI range between pH 4.5 to pH 6.0. They are likely to represent mostly glycosylated forms of the N-terminal-truncated PrPSc species and, to a lesser extent, the full-length monoglycosylated and unglycosylated species. The fifth set of ~18 kDa includes three spots and probably contains the unglycosylated truncated form. A similar pattern is seen for the total PrP from the cases of sFI (Fig. 1D). In contrast, the pattern of M/M2 sCJD comprises two additional sets of spots of ~36 and ~31 kDa, which span the entire pI range, from pH 4.5 to 8.0, and match in pI range the 35–42 kDa set containing the full-length diglycosylated form (Fig. 1F). The other four PrP sets are similar to those of controls and sFI. All these patterns were reproducible.

We separated the detergent-soluble PrP fractions that contain mostly PrPSc from the detergent-insoluble fractions that are highly enriched in PrPSc (and, presumably, other abnormal PrP isoforms) in control, sFI, and M/M2 sCJD (Fig. 2) (22). Similar profiles are obtained in the two-dimensional immunoblots of the soluble PrP from the control, sFI, and M/M2 sCJD brains as well as of the insoluble PrP obtained from the sFI brains (Fig. 2, A–E). In contrast, the insoluble PrP from M/M2 sCJD displays the same two ~36 and ~31 kDa additional sets of spots observed in total PrP preparations (Figs. 2F and 1F).

To investigate further the nature of the two additional sets of spots observed in M/M2 sCJD, the detergent-insoluble PrP was affinity-purified in control, sFI, and M/M2 sCJD with mAb 8B4, directed to the N-terminal region of PrP. Equal amounts of purified PrP carrying an intact N terminus were processed for two-dimensional blotting and immunoreacted with mAb 8H4 directed to the PrP C terminus (Fig. 3). Again, control and sFI preparations form similar patterns characterized by a single set of spots, whereas M/M2 sCJD two-dimensional blots contain the two additional populations of PrP (Fig. 3). This experiment clearly shows that the variant insoluble PrP pattern associated with M/M2 sCJD is, for the most part, generated by
the full-length form of the insoluble PrP, which carries at least two additional sets of different glycans. Consistent with this conclusion, the removal of the N-linked glycans by PNGase F treatment greatly reduces the difference in insoluble PrP pattern that M/M2 sCJD displays when compared with sFI and control (Fig. 4). These preparations show four major spots migrating at 18–21 kDa, which likely represent N-truncated PrP fragments (Fig. 4). However, the M/M2 sCJD preparations also contain a larger spot of 27–29 kDa and pI between pH 7 and 8, which corresponds to the PrP full-length form and is much weaker in sFI and control. These findings argue that the different PrP pattern of M/M2 sCJD results not only from a larger variety of glycans but also from the presence of a larger quantity of full-length PrP.

To examine PrPSc, equal amounts of PK-treated total PrP from sFI or M/M2 sCJD were processed for one- and two-dimensional immunoblotting. In one-dimensional immunoblots, PrPSc distributes in three bands, which migrate at 30–35, 27–28, and ~19 kDa and are indistinguishable in the sFI and M/M2 sCJD preparations (Fig. 5, A and C). In contrast, although sFI and M/M2 sCJD two-dimensional immunoblots share many spots, at least 14 of them are present only in the M/M2 sCJD preparations (Fig. 5D). However, after deglycosylation all differences in PrPSc pattern have vanished. Both blots contain a similar set of 5–6 spots that migrate at ~19 kDa as the PK- and PNGase F-treated PrPSc in two-dimensional immunoblots. Therefore, in PrPSc preparations the distinctive two-dimensional immunoblot pattern of M/M2 sCJD is generated mostly by the presence of more diverse glycans.

**DISCUSSION**

Analysis of PrPSc by one-dimensional gel electrophoresis and immunoblotting has shown that PrPSc associated with distinct animal and human prion diseases may differ in gel mobility and in the ratio of the glycoforms, providing the first insight into the molecular basis of prion diversity (4–6, 12, 13). However, one-dimensional immunoblotting has obvious limitations because of its relatively low capacity to separate different proteins, especially glycoproteins, which may differ only in the type of their glycans.

When we used two-dimensional immunoblotting to compare the insoluble PrP and the PrPSc associated with the M/M2 sCJD phenotypes with that of sFI, we observed that in M/M2 sCJD the PrP pattern is characterized by two distinctive features: 1) the presence of three rather than one set of glycans linked to the full-length PrP, and 2) a relatively larger quantity of the full-length PrP. The single set of glycosylated, full-length PrP present in sFI and control is distributed over a wide pI range but displays a fairly uniform molecular mass of 38–43 kDa, indicating that this set carries glycans that are heterogeneous with respect to the charges, but have the mass expected for the presence of two glycans. In M/M2 sCJD, the two additional sets of spots have lower molecular masses. The nature of these glycans needs to be analyzed, but the upper two sets are consistent with the PrP di- and monoglycosylated forms whereas the lower set might contain simpler glycans chains. In two-dimensional preparations of a PK-resistant PrPSc fragment generated by the PK treatment, the M/M2 sCJD patterns differ from that of sFI in the presence of several spots, which span a wide pI range and occupy the upper half of the blot.
cently observed in the United Kingdom and believed to have been acquired from bovine spongiform encephalopathy (BSE), PrPSc has a glycoform ratio similar to that observed in the PrPSc of BSE and to that of mice infected with PrPSc derived from variant CJD and BSE (6, 23). These findings suggest that the glycoform ratio reflecting the glycosylation site occupancy is reproduced upon inoculation to receptors. However, it is unclear whether preferential glycan site occupancy is consistently reproduced upon transmission to receptive animals (13). A comparative study of experimental scrapie in Syrian hamsters has shown that PrPC and PrPSc contain the same set of at least 52 glycan species but in different relative amounts (24). These changes could be due to a preferential conversion to PrPSc of PrPβ that contains certain kinds of sugars (24). Alternatively, the glycosylation machinery may be impaired in certain cell populations as the result of the neurodegeneration associated with the disease process (24). This, in turn, results in the underrepresentation of some glycans, which, therefore, are less available for conversion.

In M/M2 sCJD, only insoluble PrP and PrPSc form the variant two-dimensional pattern, whereas the pattern of the soluble PrP is similar to that of PrPSc from controls. Therefore, the most economical explanation for these findings is that the variant PrPSc present in M/M2 sCJD is the result of preferential conversion and the accumulation of full-length glycoforms that are normally underrepresented, rather than being secondary to an impairment of the cell glycosylation machinery. The finding that prolonged exposure of the two-dimensional immunoblots from controls reduces somewhat the pattern differences with M/M2 sCJD supports this conclusion (data not shown).

Glycans affect many characteristics of the glycosylated protein, including stability, protein-to-protein interactions, packaging, and orientation at the cell surface (24, 25). Therefore, it is likely that the conformation and other properties of the glycosylated, full-length PrPSc present in M/M2 sCJD are different from that of the corresponding PrPSc species present in sFI. This, in turn, would determine the different phenotype.

If the only conformational differences between the sFI and M/M2 sCJD PrPSc species were those determined by the variant glycans, this work, to our knowledge, would be the first observation that two PrPSc species associated with distinct disease phenotypes differ only in the glycans they carry. Transmission experiments are needed to test this hypothesis.

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FIG. 5. One- and two-dimensional immunoblots of PK-resistant PrPSc before and after PNgase F treatment. PK-resistant brain lysates from sFI (A, B, E, and F) and M/M2 sCJD (C, D, G, and H) were treated (E–H) or untreated (A–D) with PNgase F and probed for one-dimensional (A, C, E, and G) and two-dimensional (B, D, F, and H) immunoblots. Asterisks are M/M2 sCJD PrPSc spots not detected in sFI. After deglycosylation, PK-treated PrPSc from sFI and M/M2 sCJD show no difference in two-dimensional pattern.

These spots are likely to represent the PK-truncated forms of the full-length, glycosylated PrPSc, which form the 35–37 and 30–33 kDa sets of spots in the M/M2 sCJD-insoluble preparations not treated with PK.

The amount of PrPSc present in the cerebral cortex from M/M2 sCJD is 5–10-fold larger than that in sFI and might result in the presence of PrPSc forms that generate the variant patterns in M/M2 sCJD but are undetectable in sFI. Furthermore, the different PrP pattern might result from the vast difference in histological lesions between sFI and M/M2 sCJD. We investigated these two possibilities but observed no difference in pattern regardless of whether the two-dimensional immunoblots were loaded with total tissue homogenate (containing different amounts of PrP) or with equal protein amounts of immunopurified total PrP or of PrPSc. The pattern was also unchanged when tissue was obtained from the hippocampal formation that has minimal histopathology in both diseases or from the thalamus that is more severely affected in sFI than in M/M2 sCJD. Finally, the PrP pattern remained unchanged regardless of whether we used brain tissue obtained from an autopsy (in which a different rate of postmortem autolysis might play a role in the PrP pattern formation because there may be delays of 8–18 h before the tissue is harvested) or whether the brain tissue was obtained from a biopsy, which has minimal autolysis. Combined, these findings indicate that the different two-dimensional gel patterns formed by the insoluble PrP and by the PrPSc in sFI and M/M2 sCJD are likely to be due to the intrinsic differences of PrPSc rather than being secondary to the different quantities of PrPSc, the different histopathology, or postmortem autolysis.

The role of the glycans in prion strain determination is controversial. In variant CJD, the human prion disease results from the acquisition of a conformational advantage of the cellular prion protein (PrPC) to the scrapie prion protein (PrPSc). This conversion is driven by neuronal degeneration and membrane perturbations, leading to the formation of PrPSc species that are then prionogenic. The role of glycans in this process is debated, with some studies suggesting that glycans play a role in the conversion of PrPC to PrPSc and others arguing that glycans are not essential for conversion.

Glycans are known to influence the conformation and stability of proteins, and their role in prion disease is still under investigation. The glycosylation pattern of PrPSc has been shown to vary between different prion strains, and this variation may contribute to the differences in disease phenotype. However, the exact nature of the glycan contribution to prion strain determination is not fully understood, and further research is needed to elucidate the role of glycans in this process.
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