Effect of the D178N Mutation and the Codon 129 Polymorphism on the Metabolism of the Prion Protein*

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Prion diseases are thought to be caused by the conversion of the normal, or cellular, prion protein (PrP\text{C}) into an abnormal protease-resistant conformation (PrP\text{R}). There are three familial forms of human prion disease, Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker syndrome, and fatal familial insomn Men (FFI) (Parchi and Gambetti, 1995). Despite their phenotypic differences, FFI and one familial type of CJD (CJD\text{D178N}) are both linked to a single mutation of PrP with conformational changes resulting in the substitution of asparagine for aspartic acid (D178N) (Goldfarb et al., 1992). They differ, however, at the PrP gene level, since a natural polymorphic site encoding either methionine or valine (Goldfarb, Petersen et al., 1992). Codon 129 of the mutant allele specifies the disease phenotype associated with the D178N mutation: the 129M,178D polymorphism is linked to FFI, the 129V,178N haplotype to CJD\text{D178N} (Goldfarb, Petersen et al., 1992). A variety of data clearly demonstrate the importance of the mutation in the pathogenesis of FFI and CJD\text{D178N} and other inherited prion diseases. They provide no information, however, on the precise metabolic events that lead to the disease. In this study we used transfected human neuroblastoma cells to examine the effect of the PrP D178N mutation associated with either the 129M or 129V codon on the synthesis and metabolism of the PrP\text{N}.

EXPERIMENTAL PROCEDURES

Cloning and Production of Celi Lines—M-17 human neuroblastoma cells (Ross et al., 1983; provided by Dr. J. Biedler) were transfected with the episomal vector pCAP8 (Harbor et al., 1988) containing prion protein coding sequence (27\,µg/10-cm plate) using the cationic lipid DOTAP (Boehringer Mannheim, 46\,µg/10-cm plate) in serum-free Opti-MEM (Life Technologies, Inc.). The inserted prion protein coding sequence, under the control of the cytomegalovirus promoter, was either normal (178D) or mutant (D178N) with either a Met or Val at codon 129 (129M or 129V), a natural polymorphic site in the PrP gene, and the 129M,D178N haplotype is linked to FFI, the 129V,D178N haplotype to CJD\text{D178N} (Goldfarb, Petersen et al., 1992). A variety of data clearly demonstrate the importance of the mutation in the pathogenesis of FFI and CJD\text{D178N} and other inherited prion diseases. They provide no information, however, on the precise metabolic events that lead to the disease. In this study we used transfected human neuroblastoma cells to examine the effect of the PrP D178N mutation associated with either the 129M or 129V codon on the synthesis and metabolism of the PrP\text{N}.

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Mutations underlying an increasing number of inherited diseases are being discovered. The task now is to define the individual steps through which the mutated protein causes a specific disease, in some cases by becoming a pathogen, often after a symptom-free interval of several decades (Hamilton et al., 1992).

The prion protein (designated PrP\text{C}) has been implicated in a variety of human and animal diseases referred to as prion diseases, spongiform encephalopathies, or transmissible spongiform encephalopathy (Prusiner and DeArmond, 1994). These results offer new insight into the effect of the D178N mutation on the metabolism of the prion protein.

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The inherited prion diseases associated with PRNP mutations fall into three major groups: Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker syndrome, and the recently discovered fatal familial insomnia (FFI) (Parchi and Gambetti, 1995).

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‡ The abbreviations used are: CJD, Creutzfeldt-Jakob disease; FFI, fatal familial insomnia; PI-PLC, phosphatidylinositol-specific phospholipase C; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; BFA, brefeldin A; GPI, glycosylphosphatidylinositol; ER, endoplasmic reticulum.

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octapeptide repeat deletion in the mutant allele and from an age-matched control.

Western Blot Analysis—Cells were washed once with serum-free Opti-MEM and then incubated in serum-free Opti-MEM with PI-PLC (59 ng/ml, provided by T. Rosenberry, Case Western Reserve University) for 30 min at 37°C. The media was removed and centrifuged at 290 × g for 5 min to remove any cells. Cells were washed once with PBS (phosphate-buffered saline) and lysed off the plates in ice-cold cell lysis buffer (100 mM NaCl, 10 mM EDTA, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 10 mM Tris, pH 7.4, 1 mM PMSF (phenylmethylsulfonyl fluoride)). Nuclei and large debris were removed by centrifugation at 200 × g (4°C) for 10 min. The supernatants were then precipitated with 4 volumes of MeOH (−20°C for 2 h). The resultant precipitates, suspended in 50 mM Tris-HCl (pH 7.5) containing 0.5% Nonidet P-40 and 1 mM PMSF, were digested in a 20-μl reaction for 1 h with 0.1 U of endoglycosidase H (1000 units, New England Biolabs, in 50 mM Tris-HCl, pH 7.4) for 60 min at 37°C. The glycosylated proteinase K (Boehringer Mannheim) or 20-μl cell equivalents were digested in a 20-μl reaction for 1 h with 0.5, 1, 5, or 10 μg/mL proteinase K. The supernatants were precipitated with 4 volumes of methanol, resuspended in sample buffer, resolved on 14% SDS-PAGE, blotted onto Immobilon P, and detected with the 3F4 antibody.

Brain tissue was homogenized in 9 volumes of 0.32 M sucrose, 20 mM Tris, pH 7.5, containing 2 μg/mL PMSF, 1 μg/mL leupeptin, 1 μg/mL pepstatin. The supernatant was clarified by centrifugation at 10,000 × g for 10 min, and recentrifuged at 100,000 × g for 1 h to obtain a membrane fraction. The total homogenate and the membrane fraction, before and after deglycosylation, were resolved on 12% SDS-polyacrylamide gels, transferred to Immobilon P, and probed with an antiserum to the carboxyl-terminal residues 220–231 (Chen et al., 1995), or anti-N, an antiserum to the amino-terminal residues 23–40.

Pulse-Chase PI-PLC—Cells were grown to 95% confluence on 10-cm plates. Cells were washed once with Opti-MEM and then incubated in Opti-MEM ± PI-PLC (59 ng/ml) for 2 h at 4°C. Media was then collected and cells were lysed for immunoprecipitation.

Pulse-Chase PI-PLC in the Presence of Tunicamycin—Cells were grown to 95% confluence on 10-cm plates. Cells were washed once with deficient MEM and then incubated for 1 h in deficient MEM ± 2 μg/mL tunicamycin (Boehringer Mannheim). 2 μg/mL was empirically determined to be the lowest concentration that would effectively block glycosylation while not affecting protein synthesis.2 A 1-h pulse with 0.5 mCi of Trans35S-label in deficient MEM ± tunicamycin (2 μg/mL) was followed by a 0 or 4-h chase in Opti-MEM lacking tunicamycin. Following the chase, cells were washed once with Opti-MEM and then incubated in Opti-MEM ± PI-PLC (59 ng/ml) for 2 h at 4°C. Media was then collected and cells were lysed for immunoprecipitation.

Pulse-Chase PI-PLC in the Presence of Brefeldin A (BFA)—Cells were grown to 90% confluence on 60-mm plates. Cells were washed once with deficient MEM and then incubated in deficient MEM for 30 min (37°C). A 30-min pulse with 0.175 mCi of Trans35S-label in deficient MEM with 1 μg/mL BFA (Epicentre Tech) was followed by a 0, 30-min, 60-min, 90-min, 1-h, or 2-h chase in Opti-MEM with 1 μg/mL BFA. 1 μg/mL was empirically determined to be the lowest concentration that would inhibit transport beyond the Golgi.3 Cells were washed in phosphate-buffered saline and lysed for immunoprecipitation.

Cells were washed once with phosphate-buffered saline and lysed for immunoprecipitation. Cells were washed once with phosphate-buffered saline and then lysed in 1 ml of 0.5% Nonidet P-40 phosphate-buffered saline supplemented with 1 mM PMSF. After incubating on ice for 10 min, lysates were centrifuged at 2100 × g for 10 min (4°C) to remove nuclei and large debris. 250 μl of the cell lysates were immunoprecipitated in a total volume of 1 ml. The prion protein was immunoprecipitated in 1% bovine serum albumin, 0.1% N-laurylsarcosine buffer (2% SDS, 10% glycerol, 5% β-mercaptoethanol) for 4 h at 4°C. Immunoprecipitation was carried out with one of the following antibodies: 3F4, anti-C, an antiserum to the carboxyl-terminal residues 220–231 (Chen et al., 1995), or anti-N, an antiserum to the amino-terminal residues 23–40. Protein-antibody complexes were bound to Protein A-agarose beads (Boehringer Mannheim) by rocking at 4°C for 3 h. To wash, beads were pulse spun at 690 × g (4°C), the supernatant was aspirated, and the beads were resuspended in 0.5 ml of ice-cold wash buffer (150 mM NaCl, 10 mM Tris, pH 7.8, 0.1% N-laurylsarcosine with 0.1 mM PMSF). Beads were washed this way six times and then resuspended in sample buffer (2% SDS, 10% glycerol, 5% β-mercaptoethanol) and boiled to release the bound proteins.

PNGase F and Endoglycosidase H Treatment—Immunoprecipitated protein was removed from the beads and precipitated in 4 volumes of methanol to remove sample buffer. Protein pellets were resuspended in denaturing buffer (0.5% SDS, 1% β-mercaptoethanol) and boiled for 10 min. Samples were deglycosylated with PNGase F (1000 units, New England Biolabs, in 50 μM sodium citrate, pH 7.5) or endoglycosidase H (1000 units, New England Biolabs, in 50 μM sodium citrate, pH 5.5) for 60 min at 37°C. Proteins were again precipitated with methanol and the resulting pellet was boiled in sample buffer and separated on 16% SDS acrylamide gels.

SDS-PAGE, Fluorography, and PhosphorImager Analysis—Proteins were separated on 16% acrylamide gels (37:5:1 acrylamide:bis-acrylamide). Gels were fixed in methanol:acetic acid:H2O (40:10:50) for 15 min, dehydrated in dimethyl sulfoxide for 1 h and radioactivity was enhanced by rocking the gels in PPO(dimethyl sulfoxide)22%) for 90 min. Gels were then dried and exposed to film (Kodak XAR-5). Densitometric analysis of the fluorograms was performed using a PDI 42000e scanner. Some gels were also analyzed using a Molecular Dynamics PhosphorImager. Microsoft Excel 5.0 was used for statistical analysis.

RESULTS

Expression and Localization of the PrP—Human PrPc is a 209-amino-acid glycoprotein anchored to the cell membrane by a glycosylphosphatidylinositol (GPI) moiety (Prusiner and DeArmond, 1994). As is the case for other membrane glycoproteins, most of the PrPc molecules receive N-linked oligosaccharide chains, a disulfide bond, and the GPI anchor while being translocated into the lumen of the endoplasmic reticulum (ER) (Vidugiriene and Menon, 1994). During this processing PrPc is also folded. The oligosaccharide chains are trimmed in the ER and additional modifications take place in the Golgi apparatus. Human PrPc has two sites for N-glycosylation corresponding to residues 181 and 197 (Locht et al., 1986; Robakis et al., 1986). Three major forms of mature PrPc are found which differ in their degree of glycosylation and are referred to as glycoforms. They include one unglycosylated form and two glycosylated

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1 S. L. Richardson and R. B. Petersen, unpublished data.
2 C. B. Urig and R. B. Petersen, unpublished data.
Metabolism of Normal and Mutated Prion Protein

We used human neuroblastoma cells transfected with DNA constructs expressing the human prion protein sequence to determine the effect of the D178N mutation on the metabolism of PrP (Fig. 1). The constructs are based on an episomal vector, so hygromycin-resistant bulk cultures, rather than cloned cell lines, were used. The advantage of this system is that the expression level is not influenced by the site of integration facilitating direct comparison in metabolic studies of the PrP expressed by different constructs. Initially, we compared the cell surface forms of PrP and PrP in transfected cells following cleavage of the GPI anchor with the enzyme PI-PLC (Fig. 2A).

The PrP released from transfected control cells shows three distinct glycoforms which are similar to those previously described in non-transfected mouse neuroblastoma cells (Fig. 2A; Caughey et al., 1988; Scott et al., 1988). Since our cells overexpress PrP, we compared, in a separate experiment, the ratio of PI-PLC released glycoforms with those of a non-transfected human cell, M17 BE2C a clonal line from the same tumor, to ensure that we were not observing a processing bias toward less glycosylated forms due to an overload of the glycosylation machinery. In addition, the under-representation of unglycosylated PrP in the normal cell lysates exhibited the same immunoprecipitable bands in comparison to the samples from the mutant cells which accounts for 8.3 ± 3.6% of the PI-PLC released PrP (the loading of the samples from the mutant cells was adjusted by a factor of 3 so that they would be comparable to the control cells) and 2) a selective decrease in the forms that migrate most rapidly in gels. Denaturing and reducing PI-PLC (Fig. 2B), the samples in panel A were treated with the enzyme peptideN-glycosidase F to remove the sugars and analyzed as above. A single band that comigrates with the unglycosylated form and a small amount of a 20-kDa product are observed in the normal cell lines.

After treatment of the PI-PLC released PrP with PNGase F, which cleaves N-linked oligosaccharides, the PrP migrates as a single band (Fig. 2B) which has the same mobility as the most rapidly migrating form in the untreated preparations. This finding confirms that the three PrP forms differ in N-glycosylation and that the most under-represented PrP glycoform at the cell surface is the unglycosylated form.

To confirm that the unglycosylated PrP was missing from the cell surface, and not merely resistant to PI-PLC, we surface labeled cells with biotin that were untreated or previously treated with PI-PLC. As shown in Fig. 3, cells expressing PrP showed a marked reduction in surface labeling after treatment with PI-PLC indicating that mutant PrP is not resistant to PI-PLC. Thus, the under-representation of unglycosylated PrP released from the surface of the cells by PI-PLC (Fig. 2) is not a result of an inability to cleave the GPI anchor of PrP.

Metabolism of the PrP—The under-representation of PrP on the surface of the mutant cells could be the result of rapid turnover or unsuccessful transport. In addition, the under-representation of the unglycosylated PrP may result from its selective degradation or increased glycosylation. We investigated synthesis and glycosylation of PrP using a pulse-chase paradigm. After the chase, the cells were treated with PI-PLC to remove the GPI-anchored protein from the cell surface. The cell lysate (intracellular PrP) and the medium (cell surface PrP) were immunoprecipitated and analyzed by SDS-PAGE.

At the end of the labeling period (time 0; Fig. 4A), all cell lysates exhibit the same immunoprecipitable bands in comparable amounts including three distinct bands and a slowly migrating smear. The three discrete bands are a high mobility band corresponding to the unglycosylated PrP and two bands with slightly lower gel mobility. The latter two bands are sensitive to digestion with endoglycosidase H (endo H), indicating that they correspond to the PrP forms glycosylated with the high mannose core and that they are still located in the ER or the Golgi complex (data not shown; Caughey et al. (1989)). After a 30-min chase, as a result of modifications to the sugar chains, these two forms become endo H resistant (data not shown). The slowly migrating smear is the PrP on which the carbohydrates have been highly modified (Caughey et al., 1989). At 2 h, only mature forms of PrP are detected (Fig. 4A). At the 2-h time point PrP is under-represented in the cells expressing either the 129M,D178N or the 129V,D178N. The average amount of unglycosylated PrP from both mutant cell

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**Fig. 1.** Schematic representation of the prion protein and the cell lines used in this study. A, linear representation of the human prion protein indicating pertinent features: signal sequence, amino-terminal epitope, 3F4 epitope, the polymorphic codon 129 (methionine or valine), codon 178 site of the aspartic acid to asparagine mutation, glycosylation sites at residues 181 and 197, carboxyl-terminal cleavage site for GPI anchor addition, carboxyl-terminal epitope, and termination site. B, the cell types listed with the genotype of the transfected constructs.

**Fig. 2.** Less modified forms of PrPM, the unglycosylated (U) and intermediate (I) forms are under-represented on the surface of cells. A, transfected cells were treated with PI-PLC and the released protein was concentrated by precipitation. The protein was then resolved on SDS-polyacrylamide gels, blotted onto Immobilon P, and reacted with the monoclonal antibody 3F4. The amount of mutant protein loaded was three times that of the normal. The antibody reaction was detected using horseradish peroxidase-conjugated sheep anti-mouse IgG followed by a chemiluminescent substrate. M, mature glycosylated PrP; I, an intermediate migrating form of PrP; U, unglycosylated PrP. B, the samples in panel A were treated with the enzyme peptideN-glycosidase F to remove the sugars and analyzed as above. A single band that comigrates with the unglycosylated form and a small amount of a 20-kDa product are observed in the normal cell lines.
types accounts for 1.9 ± 2.3% of the total PrPM while the unglycosylated PrP accounts for 8.2 ± 2.6% in control cells (n = 6; p < 0.003; Fig. 4C). Although there is also an apparent under-representation of the intermediate form, as noted above, the species migrating at this position change with time making quantitation difficult. Similar results are obtained using antibodies directed against the amino (anti-N) and carboxyl (anti-C) termini, except that anti-N recognizes an additional band at 20 kDa which is an unglycosylated, carboxyl-terminal truncated form of PrP and anti-C detects a truncated form of the unglycosylated PrP, not recognized by 3F4, that migrates at approximately 18 kDa (data not shown; Chen et al. (1995)).

The unglycosylated PrP is a significant fraction of the total PrPM at the zero time point indicating that this form is normally produced by all cell types (18.0 ± 2.2% versus 23.3 ± 2.2% in control cells; n = 8). At the zero time point, the labeled PrP is still in the ER-Golgi complex region as shown by the barely detectable quantity of PrP that is PI-PLC-released from the cell surface (Fig. 4B). The decrease of the intracellular unglycosylated PrP occurs at the 30-min and 2-h time points when the glycosylated PrPM forms are still intracellular or are being inserted into the plasma membrane. When analyzed separately, the unglycosylated cell surface PrPM is undetectable after 2 h in the 129M mutant cells (n = 4), but accounts for 0.6 ± 0.4% of total PrP in 129V mutant cells (n = 4; Fig. 4D).

In contrast, the unglycosylated form accounts for 5.3 ± 0.6% in 129M and 129V control cells (n = 4; p < 0.008 (129 M) and p < 0.0008 (129V) two tailed t-test; Fig. 4D). These findings, and the previous finding that PrPM is under-represented at the cell surface in the steady state, indicates that PrPM is inefficiently transported through the secretory pathway. This is especially evident for the less modified glycoforms.

The absence of the truncated, unglycosylated 20-kDa form on the surface of the mutant cells also supports this conclusion (Fig. 4B).

In addition to the surface and intracellular forms of PrP detailed above, we detected a small amount of PrP (<5% of the total) in the chase medium without addition of PI-PLC (data not shown). Epitope mapping indicates that the secreted PrP corresponds to the previously described PrP form lacking the last four carboxyl-terminal residues and the GPI anchor (Stahl et al., 1993). The secreted PrP is under-represented in the media from the mutant cells relative to the normal cells, indicating that the low amount of PrPM at the surface of the mutant cells is not the result of increased secretion.

Transport and Stability of the PrP—To better characterize the effect of glycosylation on the transport of the PrPM, we labeled cells in the presence of the antibiotic tunicamycin which blocks the synthesis of the high mannose core preventing glycosylation of newly synthesized proteins in the ER (Elbien, 1987). We analyzed the cell lysate and media from PI-PLC-treated cells as in the pulse-chase experiment described above, with or without addition of tunicamycin. In contrast with the untreated controls, the cell lysates from normal and mutant tunicamycin-treated cells show only a readily detectable PrP band at the zero time point (Fig. 5A) which, as expected, migrates with the unglycosylated form of the PrP. After a 4-h
chase period, the intracellular PrP, although decreased by over 90% (93 ± 2.3%; n = 6), is still present in the tunicamycin-treated cells expressing PrPC (Fig. 5A). In contrast, after the 4-h chase, the PrPM is barely detectable in the tunicamycin-treated mutant cells although the amount of PrPM in the tunicamycin-treated control and mutant cells is comparable immediately after labeling (Fig. 5A). At the cell surface, PrPC is readily detectable in the tunicamycin-treated control cells at the zero time point and increases 3.5-fold during the 4-h chase (Fig. 5, B and C). In contrast, PrPM is present at one-half the amount of PrPC at the surface of the tunicamycin-treated mutant cells at time 0 and is essentially unchanged over the 4-h chase (Fig. 5C).

The finding that when glycosylation is prevented PrPM barely reaches the cell surface and is undetectable inside the cell shortly after its synthesis lends additional support to the notion that the unglycosylated form of the D178N PrPM is degraded in an intracellular compartment and that PrPM, but not PrPC, requires glycosylation to facilitate transport to the cell surface.

Cellular Compartment of PrP Degradation—We used brefeldin A (BFA), which blocks the transport of glycosylated proteins from the ER through the Golgi complex (Lippincott-Schwartz et al., 1991), to determine whether PrPM is degraded in an intracellular compartment and that PrPM, but not PrPC, requires glycosylation to facilitate transport to the cell surface.

Fig. 6. Unglycosylated 129M PrPM is less stable than 129V PrPM in cells treated with brefeldin A. A, cells were preincubated in media lacking methionine and cysteine and labeled for 30 min in media containing Trans35S-label and 1 μg/ml brefeldin A. Cells were chased for the times indicated above the lanes, cell homogenates were prepared and immunoprecipitated with monodonal antibody 3F4. The immunoprecipitated samples were resolved on SDS-polyacrylamide gels and visualized by fluorography. The fastest migrating band is the unglycosylated PrP, the two upper bands are glycosylated with the high mannose core which is modified to endoglycosidase H resistance over the course of the 2-h chase. G, glycosylated; U, unglycosylated. B, graphical representation of the decrease in the unglycosylated PrP as a percent of total PrP after a 2-h chase in brefeldin A-treated cells (*, p < 0.003).
Metabolism of Normal and Mutated Prion Protein

The central event in the pathogenesis of the prion diseases is thought to be a change in the conformation of PrP<sup>C</sup> (Prusiner and DeArmond, 1994) that renders the PrP<sup>C</sup> protease resistant (PrP<sup>res</sup>) (Hope et al., 1986; Caughey and Raymond, 1991; Pan et al., 1993; Safar et al., 1993). The abnormally conformed PrP<sup>res</sup> is believed to act as a template for the conversion of newly synthesized PrP<sup>C</sup> into PrP<sup>res</sup>. This mechanism is thought to be shared by all forms of prion diseases: sporadic, transmitted, and inherited. In the sporadic form, the change in conformation of PrP<sup>C</sup> would be the consequence of either a somatic mutation or of a stochastic event involving the direct conformational modification of a PrP<sup>C</sup> molecule. In the transmitted forms, the conversion would be triggered by the exogenous PrP<sup>res</sup>. In the inherited prion diseases the pathogenic mutation presumably predisposes the PrP<sup>M</sup> to spontaneous conversion into PrP<sup>res</sup>, however, this conversion occurs as a function of age even though mutant protein is produced throughout the life of the individual.

The study of the metabolism of the PrP<sup>M</sup> in cells transfected with constructs homologous to the two PRNP haplotypes linked to FFI and CJD<sup>D178</sup> was undertaken to assess the metabolic differences, if any, between the two forms of PrP<sup>M</sup> and PrP<sup>C</sup>. Studying cell models of FFI and CJD<sup>D178</sup> is of special interest since FFI and CJD<sup>D178</sup> share the same D178N mutation in PRNP, but have two different phenotypes providing a striking example of phenotypic heterogeneity (Goldfarb, Petersen et al., 1992). Since the only heterogeneity in the PRNP coding sequence between these two diseases is at codon 129 of the mutant allele, the phenotypic differences are likely to be due to the amino acid present at position 129 of PrP<sup>M</sup> which in all FFI subjects examined to date is methionine and in all CJD<sup>D178</sup> subjects is valine (Goldfarb, Petersen et al., 1992; Gambetti et al., 1995). These differences extend to the PrP<sup>res</sup> associated with the two diseases (Monari et al., 1994). The ratio of the three glycoforms and the size of the PrP<sup>res</sup> fragment generated by protease K digestion are different. In FFI the unglycosylated form is under-represented and the PrPres fragment generated by protease K treatment is smaller than in CJD<sup>D178</sup> (Monari et al., 1994). The difference in size of the PrP<sup>res</sup> fragments, which is due to different sites of degradation by the protease K in the two PrP<sup>res</sup> forms, is consistent with the hypothesis that the PrP<sup>res</sup> present in FFI and CJD<sup>D178</sup> have distinct conformations. The simplest explanation for these findings, based on a large body of experimental data, is that the presence of methionine or valine at position 129 of the PrP<sup>M</sup> results in PrP<sup>res</sup> that differ in the ratios of the glycoforms and in conformation. In turn, these differences determine two distinct disease phenotypes (Monari et al., 1994; Gambetti et al., 1995).

The metabolism of PrP<sup>C</sup> as observed in the human cell line expressing the normal PRNP is in accord with the data previously reported for the mouse neuroblastoma N2A cells (Caughey et al., 1989; Tarabooulos et al., 1992; Harris et al., 1993). In our transfected neuroblastoma cells, the ratio of the three glycoforms is essentially the same as the ratio found in non-transfected cells (data not shown). We also observed an ~20-kDa band in the cells transfected with the normal construct which corresponds to one of the N-terminally truncated form described previously (Harris et al., 1993; Chen et al., 1994).

![PNGase](image)

**Fig. 7.** Uglycosylated PrP<sup>M</sup> is under-represented in the brain of a subject affected by FFI. Membrane fractions obtained from the occipital cortex of a control subjects (lane 1) and one FFI subject heterozygous for a deletion of one of the octarepeats within PRNP codons 76 and 91 (lane 2). The samples were immunoblotted and stained with an antibody that recognizes the amino-terminal region of PrP. As expected the unglycosylated form migrates as two uneven bands in the FFI subjects because of the deletion, whereas in the control the unglycosylated form migrates as a single band. The samples were treated with the enzyme PNGase F to remove the N-linked sugars and analyzed as above (control, lane 3; FFI, lane 4). In the FFI subject the two bands, which now contain all the original glycoforms, are comparable indicating that the unglycosylated form is selectively decreased. (U, unglycosylated; M, mature).

point to the 2-h time point, whereas the percent decrease of PrP<sup>C</sup> is 27.9 ± 6.5% over the 2-h chase (n = 3, p < 0.05; two-tailed t-test). In the cells expressing 129V,D178N PrP<sup>M</sup>, the unglycosylated form is more stable than in the cells expressing 129M,D178N as it decreases by only 17.5 ± 1.1% during the 2-h chase. Thus, the degradation of the unglycosylated PrP<sup>M</sup> is likely to occur in a cellular compartment beyond the Golgi, probably the endosomal-lysosomal system.

D178N Mutant Cells Lack PrP<sup>res</sup>—Cell homogenates from normal and mutant cells were tested for the presence of protease K-resistant PrP. Enzyme titrations from 0.5 to 10 μg/ml in 1-h incubations at 37°C as well as a time course of protease digestion using 5 μg/ml protease K for 5, 10, 15, and 20 min failed to demonstrate any protease K-resistant PrP. Concentrations of protease K 10–200 times greater than this are typically used to demonstrate PrP<sup>res</sup> in brain homogenates from affected individuals or cell extracts from scrapie-infected cells (Monari et al., 1994; Neary et al., 1991).

PrP<sup>M</sup> Is Under-represented in the Brain—To determine whether the unglycosylated PrP<sup>M</sup> is decreased in the brain of subjects with FFI, the ratio of the PrP glycoforms was examined on Western blots of brain microsomal fractions obtained from a normal brain and a brain of an FFI affected subject. The FFI subject had, in addition to the D178N mutation, a deletion of one octapeptide repeat within codon 76 and 91 of the mutant allele. The presence of the deletion does not alter the characteristics of the FFI disease phenotype or of the PrP<sup>res</sup> when compared to FFI subjects without deletion (Parchi et al., 1995). Histologically normal brain regions lacking PrP<sup>res</sup> were used to avoid the potential effect of the lesions and of the conversion into PrP<sup>res</sup> on the amount of PrP<sup>M</sup>. Immunoblots of normal human gray matter tissue stained with an anti-N serum show three major bands corresponding to three different glycoforms which migrate as a single band after deglycosylation (Fig. 7). Densitometric analyses revealed that the unglycosylated PrP<sup>M</sup> is present at one-third of the amount of the unglycosylated PrP<sup>C</sup> expressed by the normal allele (1.033 ± 0.02) (Fig. 7, lanes 1 and 2). However, after deglycosylation, the product of the two alleles is comparable (1.086 ± 0.09), indicating that the decrease of the unglycosylated form is selective (Fig. 7, lanes 3 and 4).

**DISCUSSION**
The PrPM is relatively stable in this compartment suggesting that the unglycosylated form of PrPM is digested in the endosomal-lysosomal system. Our data also support the hypothesis that N-linked oligosaccharides aid in the folding process and provide structural stability to proteins (Helenius, 1994; et al., 1994).

Taken together these data indicate that the D178N PrPM is unstable and this instability is partially corrected by N-glycosylation. Thus, while the glycosylated forms are synthesized and transported to the cell surface in a fairly normal fashion, although in reduced amounts, the unglycosylated form remains unstable and is broken down before it reaches the cell surface. In contrast to the recent reports by Lehmann and Harris (1995, 1996) on the metabolism of mouse homologues of several pathogenic human mutations in Chinese hamster ovary cells, the PrP is expressed in our cells was released from the cell surface by PI-PLC suggesting a normal association with the cell membrane. In addition, the PrP produced in our cells was fully digested by proteinase K. Whether the discrepancies between these results are the consequence of homologous versus heterogeneous systems or the sequence differences between the human and mouse prion protein remains to be determined.

We examined the PrP from the brain of a FFI subject with the D178N mutation and one octapeptide repeat deletion in the mutant allele in an area of the brain lacking PrPres (Bosque et al., 1992; Parchi et al., 1995). Because of the 8-residue difference, the PrP and PrPC can be separated by gel electrophoresis. The unglycosylated PrP was present at one-third of the amount of the unglycosylated PrPC. These findings are consistent with the conclusion that the unglycosylated form of the 129M, D178N PrP is also unstable in the brain cells of FFI patients and that the neuroblastoma cells transfected with the 129M, D178N construct recapitulate the early metabolic events of the PrP occurring in brain cells of the FFI patients.

The reduced stability of the D178N PrP may be based on the spatial proximity of residues 178 and 129. Secondary structural predictions generated by the alignment of PrP sequences place these two residues in close proximity of each other in the hydrophobic core (Huang et al., 1994; Nguyen et al., 1995). If this model of PrP secondary structure is correct, methionine and valine at position 129 would be strategically located to influence the conformation of the D178N PrP (Huang et al., 1994). The brefeldin A experiment suggests that a subtle but significant difference exists in the processing of PrP in 129M and 129V cells. The minor differences do not immediately shed light on the different phenotypes observed in FFI and CJD.127.

Within the more complex cellular environment provided by the intact brain, and the longer time frame needed to develop the disease, the subtle differences we observe between the 129M and 129V PrP proteins may be sufficient to result in two distinct diseases.

Finally, although the present study provides no direct data as to the mechanism by which PrP is converted into PrPres in FFI and CJD, it suggests that in the FFI brain the ratios of PrP to PrPC and PrP glycoforms are comparable. As expected from the studies by Caughey et al. (1990), the prion protein must reach the cell surface for it to be converted to PrPres explaining the observation that in FFI very little unglycosylated PrP is detected (Monari et al., 1994). Therefore, the under-representation of the unglycosylated PrP in FFI appears to result solely from the degradation of the unglycosylated form before it reaches the cell surface.

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REFERENCES

Bosque, P. J., Vnencak-Jones, C. L., Johnson, M. D., Whitlock, J. A., and McLean, M. J. (1992) Neurology 42, 1864–1870

Caughey, B., and Raymond, G. J. (1991) J. Biol. Chem. 266, 18217–18223

Caughey, B., Race, R. E., Vogel, M., Buchmeier, M. J., and Chesebro, B. (1988) Proc. Natl. Acad. Sci. U.S.A. 85, 4657–4661

Caughey, B., Race, R. E., Ernst, D., Buchmeier, M. J., and Chesebro, B. (1989) J. Virol. 63, 175–181

Caughey, B., Neary, K., Buller, R., Ernst, D., Perry, L. L., Chesebro, B., and Race, R. (1990) J. Virol. 64, 1093–1101

Chen, S. G., Teplow, D. B., Parchi, P., Teller, J. K., Gambetti, P., and Autilio-Gambetti, L. (1995) J. Biol. Chem. 270, 19173–19180

Elbein, A. D. (1987) Annu. Rev. Biochem. 56, 497–534

Fra, A., and Sitia, R. (1993) in Subcellular Biochemistry (Norgese, N., and Harris, J. R., eds) Vol. 21, Plenum Press, New York

Hambor, J. E., Hauer, C. A., Shu, H. K., Groger, R. K., Kaplan, D. R., and Harris, D. A., Huber, M. T., van Dijken, P., Shyng, S. L., Chait, B. T., and Wang, R. (1993) Biochem. 32, 806–808

Hambor, J. E., Hauer, C. A., Shu, H. K., Groger, R. K., Kaplan, D. R., and Harris, D. A. (1995) Brain Pathol. 5, 43–51

Goldfarb, L. G., Petersen, R. B., Tabaton, M., Brown, P., LeBlanc, A. C., Montagna, P., Cortelli, P., Jullien, J., Vital, C., Pendlebury, W. W., Haltia, M., Willis, P. R., Hauw, J. J., McKeever, P. E., Monari, L., Schrank, B., Swergold, G. D., Autilio-Gambetti, L., Gajdusek, C., Lugaresi, E., and Gambetti, P. (1992) Science 258, 806–808

Hamilton, J. A., Steinrauf, L. K., Liepnieks, J., Benson, M. D., Holmgren, G., Sandgren, O., and Stoen, L. (1992) Biodm. Biophys. Acta 1139, 9–16

Harris, D. A., Huber, M. T., van Dijken, P., Shyng, S. L., Chait, B. T., and Wang, R. (1993) Biochem. 32, 1009–1016

Helenius, A. (1994) Mol. Biol. Cell 5, 253–265

Henskoff, S., and Eghtedarzadeh, M. (1987) Genetics 117, 711–725

Hope, J., Morton, J. L. D., Farquhar, C. F., Multhaup, G., Beyreuther, K., and Kimmemo, R. H. (1986) EMBO J. 5, 2591–2597

Huang, Z., Gabriel, J. M., Baldwin, M. A., Fletterick, R. J., Prusiner, S. B., and Lin, H., Huang, Z., Fletterick, R. J., Cohen, F. E., and Prusiner, S. B. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 10962–10966

Kornfeld, R., and Kornfeld, S. (1985) Annu. Rev. Biochem. 54, 631–664

Kretzschmar, H. A., Stowing, L. E., Westaway, D., Stubbilbine, W. H., Prusiner, S. B., and DeArmond, S. J. (1986) DNA (N.Y.) 5, 315–324

Lehmann, S., and Harris, D. (1995) J. Biol. Chem. 270, 24589–24597

Lehmann, S., and Harris, D. (1996) J. Biol. Chem. 271, 1633–1637

Lippincott-Schwartz, J., Yuan, L., Tipper, C., Amherdt, M., Orli, L., and Klausner, R. D. (1991) Cell 67, 601–616

Locht, C., Chesebro, B., Race, R., and Keith, J. M. (1986) Proc. Natl. Acad. Sci. U.S.A. 83, 6372–6376

Monari, L., Chen, S. C., Brown, P., Parchi, P., Petersen, R. B., Mikol, J., Gray, F., Cortelli, P., Montagna, P., Ghetti, B., Goldfarb, L. G., Gajdusek, D. C., Lugaresi, E., Gambetti, P., and Autilio-Gambetti, L. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 2839–2842

Neary, K., Caughey, B., Ernst, D., Race, R. E., and Chesebro, B. (1991) J. Virol. 65, 1031–1034

Nguyen, J., Baldwin, M. A., Cohen, F. E., and Prusiner, S. B. (1995) Biochemistry 34, 4186–4192

Pan, K.-M., Stahl, N., and Prusiner, S. B. (1992) Protein Sci. 1, 1343–1352

Pan, K.-M., Baldwin, M., Nguyen, J., Gasset, M., Serban, A., Groth, D., Methhorne, I., Huang, Z., Fletterick, R. J., Cohen, F. E., and Prusiner, S. B. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 10962–10966

Parchi, P., and Gambetti, P. (1995) Curr. Opin. Neurobiol. 5, 259–263

Parchi, P., Castellani, R., Cortelli, P., Montagna, P., Chen, S. G., Petersen, R. B., Manetto, V., Vnencak-Jones, C. L., McLean, M. J., Sheller, J. R., Lugaresi, E., Autilio-Gambetti, L., and Gambetti, P. (1995) Ann. Neurol. 38, 21–29

Prusiner, S. B., and DeArmond, S. J. (1994) Annu. Rev. Neurosci. 17, 311–339

Robakis, N. K., Sawh, P. R., Waife, G. C., Rubenstein, R., Carp, R. I., and Innis, M. A. (1986) Proc. Natl. Acad. Sci. U.S.A. 83, 6377–6381

Ross, R. A., Spenger, B. A., and Biedler, J. L. (1983) J. Biol. Chem. 258, 374–377

Safar, J., Rollar, P. P., Gajdusek, D. C., and Gibbs, C. J., Jr. (1993) J. Biol. Chem. 268, 20276–20284

Sampath, D., Varkey, A., and Freeze, H. H. (1992) J. Biol. Chem. 267, 4440–4455

Scott, M. R. D., Butler, D. A., Bredesen, D. E., Wahlch, M., Hsiao, K., and Prusiner, S. B. (1988) Protein Eng. 2, 69–76

Stahl, N., Baldwin, M. A., Teplow, D. B., Hood, L., Gilson, B. W., Burlingame, A. L., and Prusiner, S. B. (1993) Biochemistry 32, 1991–2002

Taraboulos, A., Raeder, A. J., Borchelt, D. R., Serban, D., and Prusiner, S. B. (1992) Mol. Biol. Cell 3, 851–863

Vidugiriene, J., and Menon, A. K. (1994) J. Cell Biol. 127, 333–341