Effect of the E200K Mutation on Prion Protein Metabolism

Comparative Study of a Cell Model and Human Brain

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The hallmark of prion diseases is the cerebral accumulation of a conformationally altered isoform (PrP^{Sc}) of a normal cellular protein, the prion protein (PrP^C). In the inherited form, mutations in the prion protein gene are thought to cause the disease by altering the metabolism of the mutant PrP (PrP^M) engendering its conversion into PrP^{Sc}. We used a cell model to study biosynthesis and processing of PrP^M carrying the glutamic acid to lysine substitution at residue 200 (E200K), which is linked to the most common inherited human prion disease. PrP^M contained an aberrant glycan at residue 197 and generated an increased quantity of truncated fragments. In addition, PrP^M showed impaired transport of the unglycosylated isoform to the cell surface. Similar changes were found in the PrP isolated from brains of patients affected by the E200K variant of Creutzfeldt-Jakob disease. Although the cellular PrP^M displayed some characteristics of PrP^{Sc}, the PrP^{Sc} found in the E200K brains was quantitatively and qualitatively different. We propose that the E200K mutation cause the same metabolic changes of PrP^M in the cell model and in the brain. However, in the brain, PrP^M undergoes additional modifications, by an age-dependent mechanism that leads to the formation of PrP^{Sc} and the development of the disease. (*Am J Pathol* 2000, 157:613–622)

Transmissible spongiform encephalopathies or prion diseases are a group of neurodegenerative diseases that affect both humans and animals and are caused by changes in an ubiquitous and highly conserved protein, called prion protein (PrP).¹ In humans, the normal or cellular prion protein (PrP^C)¹ comprises 209 amino acids, a disulfide bridge between residues 179 to 214,^{2–5} a glycosylphosphatidyl inositol anchor,^{6,7} and two sites of nonobligatory N-linked glycosylation at amino acids 181

and 197.^{8,9} The central event in the pathogenesis of all prion diseases is a conformational change in which the α -helical content of PrP^C is diminished and the amount of β -sheets increases, resulting in the formation of an abnormal PrP^C isoform, called PrP scrapie or PrP^{Sc}. PrP^{Sc} is resistant to proteases, insoluble in nonionic detergents, and is not released from the cell surface by phosphatidylinositol-specific phospholipase C (PI-PLC).^{10–12}

Prion diseases comprise a sporadic, idiopathic form and forms that are genetically determined or transmitted by an infectious mechanism. Genetic prion diseases are linked to mutations in the gene encoding PrP^C, *PRNP*, and are inherited as three major autosomal dominant phenotypes: familial Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler Scheinker disease, and fatal familial insomnia.¹³ The most common of the human *PRNP* mutations occurs at codon 200 and results in the substitution of glutamic acid with lysine (E200K) in PrP.¹⁴ The E200K mutation is linked to a disease phenotype that resembles that of the typical sporadic CJD, the most common human prion disease.¹⁵ Although the presence of the *PRNP* E200K mutation increases the probability of developing CJD from 1:1 million, the prevalence of the sporadic form, to more than 1:1.1, the penetrance of the E200K mutation,¹⁵ the carriers of the mutation remain asymptomatic for several decades.¹⁵ Therefore, the changes caused by the E200K mutation in the mutant PrP (PrP^M) make the conversion of PrP^M into PrP^{Sc} almost inevitable, but the disease becomes clinically detectable only after a long incubation time. These findings raise important questions concerning the nature and, above all, the timing of the mutation-related changes that promote the conversion of PrP^M into PrP^{Sc} and the beginning of the disease. In a series of studies, it has been proposed that *PRNP* mutations *per se* cause the PrP^M to transform in a PrP^{Sc}-like isoform soon after its synthesis, suggesting that the long incubation time of the disease results from a slow rate of accumulation of this iso-

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form.¹⁶⁻¹⁸ However, this issue remains controversial.¹⁵⁻¹⁹

In the present study, we investigated the effects of the *PRNP* E200K mutation on the metabolism of PrP^M in human neuroblastoma cells and we demonstrated several abnormal features of PrP^M, such as an abnormal glycosylation, an increased formation of truncated fragments, and a partial insolubility and increased resistance to digestion with proteinase K (PK). Then, we looked for these abnormal features in the PrP extracted from brains of patients affected by the E200K subtype of familial CJD. Our results demonstrate that several posttranslational changes are produced by the E200K mutation and are shared by the cell model and the E200K CJD-affected brains. However, basic characteristics of the PrP^{Sc} present in the E200K brains are not reproduced by the cell model, suggesting that although the E200K mutation renders PrP^M susceptible to conversion into PrP^{Sc}, the conversion requires additional modifications of the protein to occur.

Experimental Procedures

Antibodies

The following antibodies were used: anti-N, a rabbit antiserum to a synthetic peptide corresponding to human PrP residues 23 to 40 (B. Ghetti, Indiana University, Indianapolis, IN); 3F4, a monoclonal antibody that recognizes human PrP residues 109 to 112;²⁰ anti-C, a rabbit antiserum to synthetic human PrP residues 220 to 231;²¹ and 8H4 a monoclonal antibody whose epitope is located within the 145 to 220 sequence.²²

Cell Lines

The human neuroblastoma cell line M-17 BE(2)C (kindly provided by B. Spengler and J. Biedler, Memorial Sloan-Kettering Cancer Center, New York, NY), which does not express PrP,²³ was transfected with the episomal vector *Cep4B* containing the human PrP coding sequence under the control of the cytomegalovirus promoter and the hygromycin B resistance gene for selection. The PrP coding sequence, obtained from genomic human DNA, was cloned into the bacterial plasmid pVZ1 and oligonucleotide-directed mutagenesis was used to create the mutant PrP coding sequence (Bio-Rad Muta-Gene phagemid *in vitro* mutagenesis kit; Bio-Rad, Richmond, CA).²³ The following cell lines were used: control/129M or C, expressing normal PrP, bearing a methionine at codon 129, or mutant at codon 200 with either methionine (E200K/129M) or valine (E200K/129V) at codon 129. Moreover, cell lines with PrP mutated at codon 181 or 199, either combined or not with the E200K mutation (N181Q/129M; N181Q/129M/E200K; T199A/129M; T199A/129M/E200K), were constructed. Transfected cells were grown as bulk-selected, hygromycin-resistant cultures.²³ Multiple independent transfections were used to avoid selection bias. For each experiment cells were detached with trypsin, counted, and an

identical number of cells were seeded onto 10-cm plates and grown overnight to ~95% confluence.

Patients and Tissues

Four patients carrying the E200K mutation were studied. All patients were clinically affected and died after a duration of symptoms ranging from 4 to 18 months. Tissue was obtained at autopsy in three patients and from a biopsy in the fourth.

Frozen tissue from the cerebral cortex and cerebellum was used for the biochemical studies. A semiquantitative evaluation of spongiosis, neuronal loss, and gliosis was carried out in the same brain regions sampled for the biochemical studies.²⁴ The histopathology was rated as follows: a, minimal where only minimal gliosis was present; b, intermediate where spongiosis and gliosis were mild to moderate; and c, severe where the spongiosis and astrogliosis were moderate to severe and neuronal loss was visually detectable.

Preparation of Samples

Whole Cell Proteins

Cells were washed three times with cold phosphate-buffered saline and lysed in ice-cold lysis buffer (100 mmol/L NaCl, 10 mmol/L ethylenediaminetetraacetic acid, 0.5% Nonidet P-40, 0.5% Na-deoxycholate, 10 mmol/L Tris, pH 7.4, 1 mmol/L phenylmethyl sulfonyl fluoride, and 10 mg/ml each of leupeptin, antipain, pepstatin). Nuclei and large debris were removed by centrifugation at 690 × *g* for 10 minutes at 4°C. The supernatant was precipitated with 4 volumes of methanol at -20°C overnight.

Surface Proteins (Released by PI-PLC)

Cells were washed twice and then incubated in serum-free Opti-MEM (Life Technologies, Inc., Grand Island, NY) containing 59 ng/ml PI-PLC¹⁴ for 30 minutes at 37°C. The medium was removed, centrifuged at 290 × *g* at 4°C for 10 minutes and methanol precipitated.

Brain Tissue

Gray matter brain samples were obtained from frozen brains of E200K-affected patients and age-related controls.²⁴ From each brain sample ~100 mg of tissue was homogenized in 9 volumes of lysis buffer and aliquots equivalent to 0.3 mg of wet tissue were used for PK digestion.²⁴ All tissue preparations were carried out at 4°C.

Western Blots

Protein samples (brain tissue equivalent to 0.3 mg of wet tissue or lysate from ~25,000 cells, double quantity for surface PrP) were resuspended in sample buffer (6% sodium dodecyl sulfate [SDS], 5% β-mercaptoethanol, 4

mmol/L ethylenediaminetetraacetic acid, 20% glycerol, 125 mmol/L Tris, pH 6.8) and boiled for 10 minutes before loading. Protein samples were separated in 12, 14, or 16% SDS-polyacrylamide gel (37.5:1 acrylamide: bis-acrylamide) or in 10% Tris-16.5% Tricine gels.²⁵ Proteins were transferred to Immobilon P (Millipore Corp., Bedford, MA) for 2 hours at 60 V, blocked with 10% nonfat milk in Tris-buffered saline, pH 7.5, and probed with the appropriate antibody. The immunoreactivity was visualized by enhanced chemiluminescence (ECL; Amersham, Arlington Heights, IL) on Kodak X-Omat film (Eastman Kodak, Rochester, NY) and quantified using a computer-assisted densitometric scanner.²⁴ Data analysis was performed using Excel 5 (Microsoft).

Pulse Chase

Cells were washed and pre-incubated for 30 minutes at 37°C with methionine-deficient MEM (def MEM; ICN Bio-medicals, Irvine, CA). A pulse with 0.5 mCi ³⁵S-translabel (ICN) in 3 ml of def MEM was followed by washing with Opti-MEM and incubation at 37°C in the same media for the different chase points. When indicated PI-PLC treatment was performed by incubating the cells in Opti-MEM + PI-PLC for the last 30 minutes of chase at 37°C. Medium was collected and cells were lysed at different time points.

Pulse Chase with Inhibitors

Plated cells were pre-incubated for 30 minutes with the inhibitor, then pulsed and chased as above in the presence of the drug. For each inhibitor the lowest effective concentration was empirically determined. Inhibitors' concentrations were as follows: 2 mg/ml tunicamycin (Boehringer Mannheim, Mannheim, Germany), 2 mmol/L dithiothreitol, 50 mmol/L Swainsonine (Oxford Glyco-system).

Immunoprecipitation

Medium, PI-PLC-released proteins, and cell lysates were prepared as described above. The postcentrifugation supernatant was immunoprecipitated with the appropriate antibody in 1% bovine serum albumin, 0.1% *N*-lauryl sarcosine, 0.1 mmol/L phenylmethyl sulfonyl fluoride by rocking at 4°C overnight. Protein-antibody complexes were bound to protein A Sepharose beads. The beads were washed 6 times in 1 ml of ice-cold wash buffer (150 mmol/L NaCl, 10 mmol/L Tris, pH 7.8, 0.1% *N*-lauryl sarcosine with 0.1 mmol/L phenylmethyl sulfonyl fluoride), resuspended in sample buffer, and boiled to release the bound proteins. After protein separation by SDS-polyacrylamide gel electrophoresis, the gels were fixed by soaking in methanol:acetic-acid:water (40:10:50) for 15 minutes, dehydrated in dimethylsulphoxide for 1 hour, and enhanced by rocking the gels in 2,5-diphenyloxazole/dimethylsulphoxide (22%) for 90 minutes, followed by precipitation of the 2,5-diphenyloxazole with water.

Gels were dried, exposed to film, and analyzed as for Western blots (see above).

Endoproteinase Lys-C Digestion

³⁵S-labeled PrP was extracted from SDS gels, denatured in 6 mol/L guanidine hydrochloride in 50 mmol/L Tris-HCl, pH 8, reduced with 2 mmol/L dithiothreitol, carboxy-methylated with 6 mmol/L Na-iodoacetate, and precipitated with 10 volumes of ethanol at -20°C. The pellet was resuspended in 0.01% SDS, 1 mmol/L ethylenediaminetetraacetic acid, 25 mmol/L Tris-HCl, pH 8.5, and digested overnight at 37°C.²¹

PNGase-F, Endoglycosidase-H Digestion

Proteins were precipitated in 4 volumes of methanol, resuspended in denaturing buffer (0.5% SDS, 1% β -mercaptoethanol), boiled for 10 minutes, and treated with PNGase-F (New England Biolabs, Beverly, MA) in 1% Nonidet P-40, 50 mmol/L sodium-citrate, pH 7.5, or with endoglycosidase-H (New England Biolabs) in 50 mmol/L sodium-citrate, pH 5.5, overnight at 37°C.

Detergent Solubility Test

To determine detergent solubility the tissues were lysed in 9 volumes of lysis buffer and spun at 690 $\times g$ for 10 minutes at 4°C. The supernatants were centrifuged at 100,000 $\times g$ for 1 hour to obtain the detergent-soluble -S₂- (supernatant) and detergent-insoluble -P₂- (pellet) fractions. Both fractions were methanol-precipitated and resuspended in the same volume of buffer.

PK Digestion

Brain homogenates were digested with 100 μ g/ml PK for 1 hour at 37°C.²⁴ Cells lysates were digested with 3.3 or 5 μ g/ml of PK (Boehringer-Mannheim) for 10 minutes at 37°C. The reaction was terminated by the addition of phenylmethyl sulfonyl fluoride to a final concentration of 3 mmol/L.

Results

PrP^M and PrP^C after Metabolic Labeling

After a 3-minute pulse and immunoprecipitation with the 3F4 antibody, both PrP^M and PrP^C migrated as three well-defined bands (Figure 1A, lanes 1 and 2). The upper two bands have been shown to be the precursors (PH, PI) of the diglycosylated or high (H) and of the monoglycosylated or intermediate (I) mature PrP forms, whereas the lowest band contains the unglycosylated (U) form.^{23,26} No difference was detected between PrP^M and PrP^C preparations. Therefore, early stages of PrP synthesis and posttranslational modification seem to be primarily unaffected by the mutation. However, at increasing chase times, during which in PrP^C the H and I precursors

Figure 1. Processing of PrP^C and PrP^M. **A:** After a 3-minute pulse the cells were chased from 0 to 60 minutes and the lysates were immunoprecipitated using the 3F4 antibody. Note the difference in the highly modified glycans (H), between PrP^M and PrP^C which is not visible in their corresponding precursors (PH, PI; lanes 1 and 2). **B:** After a 30-minute pulse the cells were chased for 4 hours. Lanes 1 and 3, whole cell PrP; lanes 2 and 4, surface PrP removed by PI-PLC. Immunoprecipitation with the 3F4 antibody. **C:** Truncated PrP forms present in the intracellular compartment and at the cell surface. The cells were labeled for 30 minutes then chased for 3 hours. The cell lysate was immunoprecipitated with the 3F4 antibody to clear the full-length PrP, then with the anti-C antibody to detect the N-terminally truncated fragments and their glycoforms. Lanes 1 and 2, whole cell; lanes 3 and 4, cell surface as in (B). 18^I, monoglycosylated isoform of the 18-kd fragment; 18^H, diglycosylated isoform of the 18-kd fragment. The position of the full-length U isoform is indicated (C, control; M, mutant).

undergo processing of the glycans and attain the mature migration pattern, the PrP^M H form differed in gel mobility from HPrP^C (Figure 1A, lanes 5 and 6). HPrP^M migrated as an ill-defined smear of 31 to 40 kd, as opposed to HPrP^C that migrated at 33 to 42 kd, suggesting that the maturation of the glycans is abnormal in PrP^M (Figure 1A).

PrP^M and PrP^C at the cell surface were analyzed by surface biotinylation as well as by cleavage of the glycosylphosphatidyl inositol anchor with the enzyme PI-PLC.^{6,27} Biotinylation showed that UPrP^M is selectively decreased by 50% compared to UPrP^C ($5 \pm 0.2\%$ of the total PrP versus $10 \pm 2.1\%$, $P < 0.01$, $n = 3$) (data not shown). Similarly, UPrP^M and possibly IPrP^M were selectively released in lesser amounts by PI-PLC (Figure 1B). The UPrP^M released after a 4-hour chase accounted for $1 \pm 0.2\%$ of the total UPrP^M form at the 0 chase time, whereas UPrP^C accounted for $4 \pm 0.6\%$ ($P < 0.01$, $n = 3$).

The three full-length PrP glycoforms are known to have N-terminal-truncated forms that are generated by cleavage at residues 111 to 112 after re-internalization from the plasma membrane.^{28,29} We examined these forms by sequential double-immunoprecipitation using the 3F4 antibody to eliminate the full-length forms followed by recovery of the N-terminally truncated fragments by using the anti-C antibody. The truncated PrP^M and PrP^C glycoforms were visualized as 25- to 30-kd and 28- to 33-kd bands for the H forms and as 20- to 23-kd and 20- to 25-kd bands for the I forms, respectively, whereas the U form migrated at 18 kd in both preparations (Figure 1C). The truncated HPrP^M showed a faster migration than the truncated HPrP^C, as was found in the corresponding full-length forms. In addition, the unglycosylated 18-kd PrP^M peptide was preferentially underrepresented (Figure 1C). Additional PrP fragments were seen after immunoprecipitation (see below). 3F4 revealed a 20-kd fragment, whereas the anti-C antibody detected 20-kd and 12-kd fragments. The 12-kd fragment became detectable in the intracellular compartment only after a 3-hour chase and was more abundant in the E200K cell preparations than in the controls (Figure 1C). In conclusion, analyses of PrP^M by metabolic labeling show three major and consistent changes: 1) presence of abnormal glycans in

the H form; 2) underrepresentation of the U form at the cell surface in both the full-length and truncated forms; and 3) increase in quantity of the PrP^M fragments. We examined these three changes in more detail.

Abnormal Glycosylation of the PrP^M H Form

After removal of the glycans with PNGase-F, PrP^M and PrP^C display similar gel mobility, confirming that the glycans are the cause of the difference between the two H forms (Figure 2A). We then examined whether the change affects the glycans at one or both glycosylation sites. First, we treated the PrP preparations with the endoprotease Lys-C, which generates a fragment containing only the 181-glycosylation site, and found no difference between PrP^M and PrP^C (Figure 2B). Second, we used N181Q and T199A glycosylation knock-out mutants with or without the E200K substitution, and demonstrated a difference in mobility between the PrP^M and PrP^C I forms only in the 181-glycan knock-out (N181Q) mutant (Figure 2C). Therefore, only the glycan attached to residue 197 is aberrant.

We then examined the glycan processing to identify the step at which the change occurred (Figure 2D).³⁰ The blockade of the α 3-6 mannosidase II by Swainsonine, which prevents the removal of α 3-6 mannose residues after the addition of the first *N*-acetylglucosamine in the medial region of the Golgi apparatus, was the first step at which the two H forms differed (Figure 2, E and D; step 3). Thus in PrP^M, the 197 glycan begins to differ from the corresponding PrP^C glycan at the step in which normally one *N*-acetylglucosamine molecule is added (Figure 2D, step 3).

Underrepresentation of the U Form of PrP^M at the Cell Surface

After treatment with tunicamycin, which prevents glycosylation, all of the PrP produced by the cell is in the U form.³¹ After a 2-hour chase, we observed that, of the total PrP produced at time 0, the amount of PrP^C that is

Figure 2. Characterization of aberrant PrP^M glycosylation. **A:** PrP from total cell lysate was treated with endoglycosidase-H or PNGase-F and immunoreacted with the 3F4 antibody. **B:** ³⁵S-methionine-labeled PrP^C and PrP^M were cut with the enzyme endoproteinase Lys-C to generate an ~20-kd fragment containing only the 181 glycosylation site. Note that this fragment co-migrates in the control and the mutant. **C:** Cell lines expressing the normal or mutant PrP, in which the 181 or 197 glycosylation sites were knocked out. Only PrP^M carrying the 197 glycan shows a glycan that is different (*) from the corresponding control. **D:** Diagram of the N-glycosylation process. ○, Glucose molecule; ●, mannose molecule; and □, N-acetylglucosamine molecule. **E:** Cells were treated with Swainsonine to inhibit α3-6 mannosidase. The cell lysates were immunoprecipitated with the 3F4 antibody. A lower band is detected in the mutant (@) which is not present in PrP^C preparations.

left exceeded that of PrP^M by 32% intracellularly and by 46% at the cell surface (data not shown), indicating that the preferential decrease of UPrP^M at the cell surface is not because of hyperglycosylation. Other mechanisms that may account for this decrease are the preferential degradation of the UPrP^M or its preferential aggregation, with consequent epitope masking and inefficient immunoprecipitation. In the pulse-chase experiments, the UPrP^M obtained with immunoprecipitation with the 3F4 antibody is reduced compared to UPrP^C (Figure 1, B and C). In contrast, by immunoblotting the supernatant after immunoprecipitation, we detected a higher amount of residual PrP^M, especially of the U form, compared to PrP^C (Figure 3). Because comparable amounts of PrP were detected in immunoblots of the cell lysate (Figures 5C and 6D), a finding that suggests that the antibody does

not have a different affinity for PrP^M or PrP^C, it seems that aggregation is the immediate cause of the UPrP^M being underrepresented in the immunoprecipitate.³² However, the aggregated UPrP^M is not increased compared to UPrP^C in immunoblots of total cell lysates, hence the UPrP^M does not accumulate in intracellular compartments (Figures 5C and 6D).

Increased Production of Truncated Fragments

Immunoblot analysis confirmed the presence of 20-kd and 12-kd PrP fragments, in addition to the 18-kd peptide (Figure 4). The 20-kd band, present in both mutant and control cell lines, was formed by two PrP fragments similar in length but truncated at different sites. The first corresponded to the N-terminal 20-kd band that was previously described.²³ This peptide, which reacts with the anti-N antibody, and, therefore, lacks the C-terminus, was equally represented in the mutant and control cell lysates at 0 chase time (Figure 2). In contrast, the second 20-kd fragment, recognized by the anti-C, 8H4, and 3F4 antibodies, but not by the anti-N antibody, was overrepresented in PrP^M preparations compared to PrP^C (10.1 ± 4 versus 4.5 ± 2.2 , $P < 0.01$, $n = 4$) (Figure 4). This C-terminal fragment displayed a glycoform ratio similar to that of full-length surface PrP (Figure 1C) and appeared after a 1-hour chase, suggesting that it is generated after re-internalization. Immunoblotting with the anti-C antibody confirmed the increased amount of the 12-kd fragment in the mutant compared to the control cells (Figure 4).

Figure 3. The U-form aggregates soon after synthesis. Cell lysates were immunoprecipitated with the 3F4 antibody and the residual supernatant was blotted and detected with the 3F4 antibody.

Figure 4. Increased quantity of truncated fragments. The 20-kD fragments and the full-length U form were immunostained with the anti-N, anti-C, 8H4, and 3F4 antibody. Note the similar quantity in mutant and control preparations and the lack of glycoforms of the 20-kD N-terminal fragment, whereas the 20-kD C-terminal fragment was more prominent in the mutant preparations. The cell lysate was digested with PNGase-F before staining with the anti-C antibody to allow better detection of the bands. Only the anti-C antibody detected the 12-kD fragment. *, Indicates the 20-kD fragments.

Insolubility and Protease Resistance of PrP^M

E200K PrP^M has been reported to have some of the properties of the PrP^{Sc}, namely insolubility in nonionic detergents as well as partial resistance to PI-PLC and protease treatments.^{16–18} Thus, we assessed these properties in our PrP^M preparations.

PrP^M was recovered in significantly higher amounts than PrP^C in the detergent insoluble fraction, P₂, ($22 \pm 7\%$ of total PrP^M versus $8 \pm 2\%$ of total PrP^C, $P < 0.01$, $n = 4$) (Figure 5A). All PrP^M glycoforms were represented in the aggregated fraction, but the U form was relatively overrepresented, accounting for ~40% of the total aggregated PrP^M (Figure 5A). Except for the 18-kD fragment, all fragments were more highly represented in the mutant cells. This feature was especially pronounced in the 12-kD PrP^M fragment, of which ~20% was insoluble, whereas no insoluble 12-kD fragment was present in control cells (Figure 5B).

PI-PLC was significantly less effective in cleaving the anchor in the E200K than in the control cells ($56\% \pm 6$ of PrP^M cleaved versus $71\% \pm 7$ of PrP^C, $P < 0.01$, $n = 3$) (data not shown).

The sensitivity to proteases was examined, as in previous studies,¹⁹ by treating the cell lysates with $3.3 \mu\text{g/ml}$ of PK at 37°C for 10 minutes (Figure 5C). Several fragments were observed. After immunoreaction with the 3F4 antibody, a fragment corresponding to the 20-kD C-terminal peptide described above was the main isoform present. This fragment is close, in size, to the so-called PrP_{27–30} generated after digestion with PK (50 to $100 \mu\text{g/ml}$) of affected brains, and was significantly more abundant in PrP^M than PrP^C preparations ($25 \pm 4.5\%$ versus $2.8 \pm 2.5\%$, $n = 3$, $P < 0.001$) (Figure 5C). Immunoblotting with the anti-C antibody showed the

presence of the 12-kD fragment described above only in PrP^M preparations. Moreover, the 8H4, as well as the anti-C antibody (data not shown for the last), showed in both PrP^M and PrP^C preparations substantial amounts of an 18-kD fragment, which is known to be generated by a cleavage at residues 111/112 (Figure 5, B and C).²⁹ To test whether the increased PrP^M resistance to PK digestion was simply the consequence of the increase in the aggregated form, preparations from the E200K and control cells were normalized for the content of the detergent insoluble PrP, and digested with PK (Figure 5D). Although under this condition, a higher PK concentration was required for PrP^C to be digested, the resistance of PrP^M to digestion remained significantly higher. Therefore, the PrP^M resistance to PK digestion is not simply because of PrP^M-increased aggregation, but it is likely to be because of newly acquired properties of the mutant protein.

PrP Properties in E200K CJD Brains

To assess the relevance of the alterations observed in the cell model to the corresponding human disease, we compared the cellular PrP^M with the total PrP and with PrP^{Sc} extracted from brains of patients affected by the E200K subtype of CJD. Although the direct comparison between the two systems is limited by the heterozygosity of the E200K mutation, which results in the presence of both PrP^M and PrP^C in the brain samples, we observed several similarities (Table 1).

The full-length UPrP from the E200K brains appeared as a smear compared to the sharp band of slightly higher mobility present in control brain preparations (Figure 6A). This finding is likely to result from both heterogeneity in

Figure 5. Characterization of PrP^M aggregation and PK resistance. **A:** Total cell lysates were centrifuged in nonionic detergents. The supernatant (S₂) and the pellet (P₂) were resuspended in equal volumes and stained with the 3F4 antibody. **B:** First two lanes: same treatment as in (A) to demonstrate the insolubility of the 12-kd fragment. Last two lanes: PK digestion. Staining with the anti-C antibody. **C:** Whole cell lysates were digested for 10 minutes with 3.3 mg/ml of PK. The blot was stained with the 3F4 or 8H4 antibody. The 8H4 recognizes the C-terminal PrP fragments in addition to the 20-kd fragment, which however, is not visible in this figure because is covered by the more abundant monoglycosylated isoform of the 18-kd fragment. **D:** PrP^M and PrP^C preparations normalized to contain equal amounts of insoluble PrP^C and PrP^M were digested with two concentrations of PK for 10 minutes.

gel mobility and reduction in quantity of the U forms in the E200K brain. Indeed, the U form in the total brain homogenate from an area with minimal pathology was decreased by ~40% compared to the control brain (Figure 6A). In the cell model, PrP^M shows a slightly slower gel migration of the PrP^M U form (UPrP^M) compared to the control (Figures 5D or 6D). Therefore, the brain findings concerning UPrP are consistent with the results of the cell studies, which show that UPrP^M has reduced gel mobility and, especially at the cell surface, is lower in amount than UPrP^C.

The 20-kd C-terminal fragment that was increased in the mutant cells was also increased in the affected brains (Figure 6B), where it corresponded to the so-called

“PrP27–30” fragment that is also formed *in vivo*.²⁹ In addition, the 12-kd peptide that was increased in the mutant cells, accounted for ~13% of the total PrP in the E200K CJD brains whereas it was not detected in control brains. The presence of these truncated fragments in preparations from brain biopsies excluded the possibility that they are simply postmortem artifacts (Figure 6B).

It has been shown that in CJD and other human prion diseases, on treatment with PK, PrP^{Sc} generates either one of two major fragments, which have a relative molecular mass (*Mr*) of 21 kd and 19 kd (Figure 6C) and have been designated type 1 and type 2, respectively.^{33–35} Although the CJD patients carrying the more common E200K-129M haplotype examined in this study form

Table 1. Diagram Summarizing the PrP Changes Occurring in the Cells and in the Human Brain

	Cellular PrP ^M	Brain-derived PrP ^M	PrP ^{Sc}
Aberrant glycosylation	+	+	+
Underrepresentation of the U form	+	+	+
Increased quantity of truncated fragments	+	??	+
Detergent insolubility	Partial	??	Complete
PK resistance	Up to 3.3 µg/ml for 10 minutes	??	Up to 100 µg/ml for 24 hours
Fragments after PK digestion	1) ~20 kd in both the E200K-129M and E200K-129V 2) 18 kd well-represented	??	1) ~21 kd in the E200K-129M, ~19 kd in the E200K-129V 2) no 18 kd

*Data not available due to the presence of PrP^{Sc}.

Figure 6. PrP in affected brains. **A:** Immunoblot of total PrP from a control or E200K-affected brain stained with the 3F4 antibody. **B:** Whole brain lysate from an E200K-affected patient (lane 1), a control (lane 2), an E200K brain biopsy of an affected patient (lane 3), and after digestion of sample 1 with PK (lane 4); all samples were probed with the anti-C antibody. **C:** PK-digested samples from affected brains were blotted and stained with the 3F4 antibody. Type 1 PrP^{res} from sporadic CJD (T.1), E200K CJD, and type 2 PrP^{res} from sporadic CJD (T.2) are shown. Note the reduction in the quantity of the E200K U form and the faster migration of all type 2 PrP^{res} forms compared to type 1 PrP^{res}. **D:** PrP from control, E200K-129M and E200K-129V cell lines, before (first three lanes) and after (last three lanes) limited PK digestion, were blotted and immunoreacted with the 3F4 antibody.

PrP^{Sc} type 1, those carrying the rare E200K-129V haplo-type form PrP^{Sc} type 2.³⁶ Therefore, we generated a mutant cell line carrying the E200K mutation coupled with valine rather than methionine at codon 129 (E200K-129V). The PrP^M expressed by both the E200K cell lines was similarly resistant to PK, and in both cell lines it generated a PK-resistant PrP^M fragment that co-migrated at ~20 kd (Figure 6D). Therefore, the E200K cell models do not reproduce the PrP^{Sc} dualism found in the corresponding human diseases (Figure 6C).

Discussion

The neuroblastoma cell model carrying the E200K mutation in *PRNP* demonstrates the presence of several posttranslational changes of PrP^M that are related to the mutation. These changes, which include aberrant glycosylation, underrepresentation, and abnormal gel migration of UPrP^M, as well as increased quantity of truncated PrP^M forms, were validated by demonstrating the presence of comparable mutation-related changes in the brains of patients affected by the corresponding human disease. As previously observed in cell models carrying this or other *PRNP* mutations,^{16–18} E200K PrP^M also displayed increased aggregation and resistance to PI-PLC and PK treatments. All of these changes are likely to play different roles in the conversion of PrP^M into PrP^{Sc}, the central event in the pathogenesis of inherited prion diseases.

Amino acid substitutions in flanking or adjacent regions are known to influence both the efficiency and type of glycosylation.^{37,38} However, this study shows that the E200K mutation does not affect glycosylation efficiency but rather selectively interferes with the modifications of the glycan chain at residue 197 which results in an enhanced gel migration of the highly glycosylated PrP^M form. The mutation-related glycan change is first detected at the stage of *N*-acetylglucosamine addition, which takes place in the medial Golgi. A possible explanation for the higher gel mobility of the PrP^M 197 glycan is that at this stage an increased number of glycans receive a bisecting *N*-acetylglucosamine molecule that cannot be extended further and consequently migrates faster on gel. The HPrP^{Sc} present in the affected E200K

brains and the HPrP^M recovered from fibroblasts of affected patients also show an increase in gel mobility that is comparable to what is observed in the cell model.³⁹ These findings argue that in the corresponding human disease only the 197 PrP^M glycan is changed in a manner similar to that of the cell model.

Although glycans are known to contribute to protein stability, it is unlikely that the abnormal glycan attached to residue 197 significantly increases the susceptibility of PrP^M to convert into PrP^{Sc}. In contrast, this may be because of the other changes associated with the E200K mutation, such as the increased aggregation and resistance to PI-PLC and PK treatments, which may all be related to the misfolding and destabilizing effect of the mutation on PrP^M. A reasonable cascade of events, which, according to our data, applies to both the cell model and the brain, is that the increased instability and aggregation make PrP^M more dependent on the presence of the glycans to remain soluble and reach the cell surface (Table 1). This mechanism easily explains the underrepresentation, especially at the cell surface, of the PrP^M U form, which is the least soluble and most likely to be degraded before reaching its destination. The underrepresentation of UPrP^M in the cell model is a feature shared by the Q217R and the D178N mutations²³ and is common to the E200K and D178N familial variants of CJD and to Fatal Familial Insomnia.^{21,24} The present study strongly argues that the underrepresentation of the U form in the E200K PrP^{Sc} results from the effect of the E200K mutation on PrP^M before, not after, the conversion of PrP^M into PrP^{Sc} occurs.

After limited digestion with PK, the cellular PrP^M generates a C-terminal fragment, which is similar in size to the most common PK resistant fragment of PrP^{Sc}. The PK resistance of the cellular fragment is at least two orders of magnitude lower than that of PrP^{Sc}. However it is significantly increased in the mutant protein compared to PrP^C. It has been proposed that PrP^M expressed in cell and animal models has the essential properties of PrP^{Sc} and that the lower level of PK resistance is because of the shorter time available for PrP^M conversion and accumulation in these models.^{16–18} The present study does not support this conclusion. We confirm that the C-terminal

region of E200K PrP^M has an increased resistance to PK digestion. Furthermore, by correcting for the amount of the aggregated form, we show, for the first time, that the increased PK resistance is not simply because of the higher aggregation of PrP^M, but is likely to result from an intrinsic change in the structure of PrP^M. However, the present data also show that the PK-resistant PrP^M expressed in the cell model is qualitatively different from PrP^{Sc} (Table 1). In most human prion diseases, including virtually all of the sporadic and familial variants of CJD, PK treatment generates either one of two major protease-resistant fragments of PrP^{Sc}: a fragment of ~21 kd called type 1 and one of 19 kd called type 2.^{33,34} The difference in Mr of the two PrP^{Sc} fragments is because of the different site of PK cleavage which are most commonly located at residue 82 for PrP^{Sc} type 1 and 97 for PrP^{Sc} type 2, respectively.⁴⁰ The different cleavage site, in turn, is likely to result from the different conformation of the two PrP^{Sc} isoforms, or from PrP^{Sc} binding to different ligands. In inherited prion diseases, the presence of either of the two PrP^{Sc} is determined primarily by the 129 codon coupled with the mutation on *PRNP*.^{33,34} Thus, in addition to the most common familial CJD in which the E200K mutation is coupled to the codon 129 expressing methionine and PrP^{Sc} type 1 is present in brain, there also is a E200K-129 valine familial CJD associated with PrP^{Sc} type 2.³⁶ When we modeled these two diseases in cells, the E200K-129 methionine and E200K-129 valine cell lines failed to form the PK-resistant PrP^M fragments of 21 kd and 19 kd, respectively, but both lines formed only a PK-resistant PrP^M isoform of ~20 kd. These findings strongly argue that whereas in the human disease PrP^M destabilized by the mutation is eventually refolded into a specific PrP^{Sc} isoform, in the cell model PrP^M fails to reach this stage. Therefore, although in the cell model the PrP^M reproduces the changes associated with the E200K mutation and makes PrP^M susceptible to convert into the PrP^{Sc} form, the cellular PrP^M does not undergo this conversion and remains different from PrP^{Sc}.

An unexpected finding of this study is the formation, after limited PK digestion, not only of the 20-kd fragment that is increased in the PrP^M, but also of a much greater amount of an 18-kd C-terminal fragment in both PrP^M and PrP^C. The weak PK resistance of the 20-kd fragment in the PrP^M preparations and of the 18-kd fragment in both PrP^M and PrP^C may be interpreted in view of recent nuclear magnetic resonance data of recombinant PrP. The structural data have demonstrated that PrP comprised a highly ordered region encompassing the C-terminus of PrP^C approximately from residue 113, whereas the remaining N-terminal region is primarily unstructured.^{41–43} Thus, we propose that the E200K mutation (and other *PRNP* mutations having a similar effect) cause the tertiary structure to extend toward the N-terminal region to include the unstructured 112 to 90 segment. These findings point to a conformational alteration of the region between 90 to 112 of PrP^M, as the underlying factor in the pathogenic process. In the E200K affected brains, as well as in other prion diseases, this extended C-terminal region is likely to be the site of major conformational changes during the conversion of PrP^C to

PrP^{Sc}.^{1,44} Furthermore, because this region includes the cleavage sites for the generation of the 20-kd and 18-kd fragments, the altered structure of PrP^M results in the incorrect cleavage of the mutant protein during the recycling. This would explain the increased formation of the 20-kd fragment in the mutant cells. In scrapie-infected cells, a C-terminal PrP^C fragment equivalent to the 20 kd has been shown to be directly converted into the PrP^{Sc} conformer.⁴⁵ Moreover the 20-kd fragment might also be inherently pathogenic because it retains the 106 to 126 region that has been shown to be toxic *in vitro*,⁴⁶ while it is cleaved to generate the 18-kd fragment.²⁹

In conclusion, our data show that multiple alterations in PrP^M are driven by the E200K mutation. Although some of these alterations, such as the assembly of abnormal glycans in the site flanking the mutation, are probably not critical for the pathogenic process, others, like the structural changes in the N-terminal region and the generation of potentially harmful fragments, are more likely to play a role in PrP^M susceptibility to conversion into PrP^{Sc}. However other events possibly related to aging and not occurring in cell models are probably required for PrP^{Sc} formation and the clinical onset of the disease. The identification of these events and of their timing is needed to elucidate the pathogenetic mechanism and establish a preventive treatment in inherited prion diseases.

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