Prion diseases are characterized by the conversion of the normal cellular prion protein (PrPC), a glycoprotein that is anchored to the cell membrane by a glycosylphosphatidylinositol moiety, into an isoform that is protease-resistant (PrP\textsuperscript{res}) and pathogenic. In inherited prion diseases, mutations in the prion protein (PrPM) engender the conversion of PrPM into PrP\textsuperscript{res}. We developed a cell model of Gerstmann-Sträussler-Scheinker disease, a neurodegenerative condition characterized by PrPM, containing amyloid deposits and neuronal loss, by expressing the Gerstmann-Sträussler-Scheinker haplotype Q217R-129V in human neuroblastoma cells. By comparison to PrPC, this genotype results in the following alterations of PrPM: 1) expression of an aberrant form lacking the glycosylphosphatidylinositol anchor, 2) increased aggregation and protease resistance, and 3) impaired transport to the cell surface. Most of these alterations are temperature-sensitive, indicating that they are due to misfolding of PrPM.

Prion diseases include inherited and sporadic forms as well as forms that are acquired by infection. They are commonly associated with three major phenotypes referred to as: 1) Creutzfeldt-Jakob disease (CJD), 2) fatal familial insomnia (FFI), and 3) Gerstmann-Sträussler-Scheinker disease (GSS). All prion diseases are believed to share the same basic pathogenic mechanism that involves the conversion of a normal protein called cellular prion protein (PrPC) into a form that is partially resistant to proteases (PrP\textsuperscript{res}) and infectious (2). PrP\textsuperscript{res}, a glycoprotein encoded by a gene (PRNP) located on human chromosome 20 (3), undergoes the non- obligatory addition of one or two N-linked oligosaccharide chains leading to the expression of three glycoforms containing none, one, or two glycans (4–6) and the linking of a glycosylphosphatidylinositol (GPI) anchor at the C terminus (7, 8). PrP\textsuperscript{res} is transported to the plasma membrane through the secretory pathway and is then reinternalized and cleaved in the endocytic compartment to generate N-terminal and C-terminal fragments, the latter being returned to the cell surface (9, 10). The conversion of PrP\textsuperscript{res} to PrP\textsuperscript{res} entails the switch from a predominantly α-helical to a β-sheet conformation resulting in a form that, beside being protease-resistant and infectious, is insoluble in non-ionic detergents, has the propensity to aggregate, and can polymerize into amyloid fibrils (2).

In sporadic prion diseases, PrP\textsuperscript{res} is thought to form initially as the result of a spontaneous stochastic event, and the conversion process would then be maintained autocatalytically by the endogenous PrP\textsuperscript{res}. In inherited prion diseases, it has been hypothesized that the mutant PrP (PrPM) spontaneously converts into PrP\textsuperscript{res} (11).

A distinctive feature of inherited prion diseases is the phenotypic heterogeneity (1). One determinant of this heterogeneity is the PRNP codon 129, the site of a common methionine (129M)/valine (129V) polymorphism (12). Therefore, the PRNP determinant of the phenotype in inherited prion diseases is the haplotype established by the pathogenic mutation and codon 129. The Q217R-129V haplotype, which segregates with GSS, is associated with a phenotype characterized by prominent PrP deposits, often with the characteristics of amyloid, which contain PrP\textsuperscript{res} forms of all sizes but especially N-terminal and C-terminal truncated forms (13).

To understand the individual steps involved in the pathogenesis of inherited prion diseases, we have generated several transfected cell models using a human neuroblastoma cell system to characterize PrP\textsuperscript{res}. In this study we report on the metabolism of the PrP\textsuperscript{res} expressed in cells transfected with the Q217R-129V PRNP construct. We observed complex changes in the biogenesis of PrP\textsuperscript{res} which include the following: 1) expression of aberrant PrP\textsuperscript{res} forms, some of which lack the GPI anchor and are retained in the endoplasmic reticulum (ER)-cis Golgi compartment; 2) presence of aggregated forms that exhibit decreased protease sensitivity in intracellular compartments and fail to reach the plasma membrane; and 3) underrepresentation of PrP\textsuperscript{res} at the cell surface. Remarkably, these changes largely revert at reduced temperature, suggesting that abnormal folding of PrP\textsuperscript{res} plays a major role in the pathogenesis of this GSS variant.

**EXPERIMENTAL PROCEDURES**

**Materials, Cell Culture Conditions, and Production of Transfected Cell Lines**—The human neuroblastoma cell line M17 was obtained from Dr. J. Biedler (Memorial Sloan-Kettering Cancer Center, New York); Opti-MEM, fetal bovine serum, penicillin-streptomycin, methionine and cysteine-free DMEM, and Lipofectin were from Life Technologies, Inc.; hygromycin B was from Calbiochem; sulfosuccinimidobiotin (Sulfo-NHS-Biotin) and streptavidin-agaroase were from Pierce; Tran\textsuperscript{35S} was from ICN (Costa Mesa, CA); N-glycosidase-F and endoglycosidase-H were from Boehringer Mannheim; protein A-Sepharose was from Pharmacia Biotech Inc. Recombinant Bacillus thuringiensis phos-
Cellular Processing of Q217R Mutant Prion Protein

phatidylinositol-specific phospholipase-C (PI-PLC) was purified according to Deeg et al. (14) or donated by T. Rosenberry (Case Western Reserve University).

Cell cultures were maintained at 37 °C in Opti-MEM supplemented with 5% fetal calf serum and penicillin/streptomycin, in a humidified atmosphere containing 5% CO2. Opti-MEM was supplemented with 500 μg/ml Penicillin. Human neuroblastoma cells (M17) expressing normal (Q217–129V) or mutant (Q217R-129V) PrP were generated as described (15). Transfected cells were maintained in selective medium containing hygromycin. Experiments were performed at different times post-transfection on bulk-selected cells. For all experiments, cells were replated overnight and used at 90–95% confluency. The conditions for the antibodies were used: 3F4, rabbit anti-immunohistologic peptide corresponding to human PrP residues 23–40 (B. Gheti, Indiana University); 3F4, a monoclonal antibody that recognizes an epitope on human PrP residues 109–112 (R. Kascak, New York State Institute for Basic Research in Developmental Disabilities; Ref. 16); anti-C, rabbit immune serum to synthetic human PrP residues 220–231 (A. Helenium, Yale University); anti-A, rabbit immune serum to synthetic human PrP residues 211–220 (University of California, San Diego), and anti-cathepsin-D (R. A. Nixon, Harvard University).

SDS-PAGE and Western Blotting—In a typical experiment, 9 × 10⁶ cells were used for each condition. An equal amount of total protein was used from cells expressing either normal or mutant PrP. Protein concentrations were determined with bichinchonic acid according to the manufacturer (Fierce), using bovine serum albumin as a standard. To detect PI-PLC-digested PrP in cell culture extracts, cells were rinsed, warmed, and lysed in a buffer containing 0.5% Nonidet P-40, 0.5% deoxycholate, and 10 mM EDTA in Tris-buffered saline (TBS, Tris 20 mM, NaCl 150 mM, pH 7.4), containing 10 μg/ml each of leupeptin, antipain, pepstatin, and 1 mM phenylmethylsulfonyl fluoride (PMSF). Cell debris was cleared by centrifugation at 290 × g, and the protein in the supernatant was precipitated with 5 volumes of cold methanol at −20 °C. Cellular proteins were fractionated by SDS-PAGE and electrophoretically transferred to Immobilon-P (Millipore) for 2.5 h at 70 V at 4 °C. Membranes containing transferred proteins were blocked in TBS containing 10% non-fat dry milk and 0.1% Tween 20 for 1 h at 37 °C and probed with anti-PrP antibodies (anti-N diluted 1:4000, 3F4 diluted 1:50,000, or anti-C, rabbit immune serum to synthetic human PrP residues 220–231 (B. Gheti, Indiana University); 3F4, a monoclonal antibody that recognizes an epitope on human PrP residues 109–112 (R. Kascak, New York State Institute for Basic Research in Developmental Disabilities; Ref. 16); anti-C, rabbit immune serum to synthetic human PrP residues 220–231 (A. Helenium, Yale University); anti-A, rabbit immune serum to synthetic human PrP residues 211–220 (University of California, San Diego), and anti-cathepsin-D (R. A. Nixon, Harvard University).

SDS-PAGE and Western Blotting—In a typical experiment, 9 × 10⁶ cells were plated overnight in 10-cm tissue culture dishes were used for each time point. Cells were washed with methionine-cysteine-free Dulbecco’s modified Eagle’s medium and pre-incubated in the same medium for 1 h at 37 or 24 °C. Cellular proteins were metabolically labeled with 0.166 mCi/ml Tran³⁵S-label (ICN) in labeling medium (methionine-cysteine-free Dulbecco’s modified Eagle’s medium with 5% dialyzed serum) for 2 or 30 min at 37 or 24 °C, as indicated. The cells were washed and chased in serum-free Opti-MEM with 1 mM cold methionine and cysteine for indicated times. Where indicated, media was added 30 min before chase and the cells were re-incubated at 37 °C. At the end of the chase, the medium was collected, and the cells were washed with cold PBS followed by lysis in 1% Nonidet P-40, 0.5% deoxycholate, PBS, pH 7.4, containing 10 μg/ml each of leupeptin, antipain, pepstatin, and 1 mM PMSF. Clarified cell lysate and medium samples were rocked overnight with anti-PrP antibodies in the presence of 1% bovine serum albumin and 0.1% N-laurylsarcosine. Protein-antibody complexes were collected with 40 μl of protein A-Sepharose (Pharmacia) and washed four times with 0.5 ml of wash buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.8, 0.1% N-laurylsarcosine, and 0.1 mM PMSF). Bound protein was eluted by boiling in sample buffer (2% SDS, 10% glycerol, 5% β-mercaptoethanol) and analyzed by SDS-PAGE fluorography. PrP bands were quantitated by PhosphorImager analysis (Molecular Dynamics).

Metabolic Labeling and Immunoprecipitation—In a typical pulse-chase experiment, ~9.6 × 10⁶ cells plated overnight in 10-cm tissue culture dishes were used for each time point. Cells were washed with methionine-cysteine-free Dulbecco’s modified Eagle’s medium and pre-incubated in the same medium for 1 h at 37 or 24 °C. Cellular proteins were metabolically labeled with 0.166 mCi/ml Tran³⁵S-label (ICN) in labeling medium (methionine-cysteine-free Dulbecco’s modified Eagle’s medium with 5% dialyzed serum) for 2 or 30 min at 37 or 24 °C, as indicated. The cells were washed and chased in serum-free Opti-MEM with 1 mM cold methionine and cysteine for indicated times. Where indicated, media was added 30 min before chase and the cells were re-incubated at 37 °C. At the end of the chase, the medium was collected, and the cells were washed with cold PBS followed by lysis in 1% Nonidet P-40, 0.5% deoxycholate, PBS, pH 7.4, containing 10 μg/ml each of leupeptin, antipain, pepstatin, and 1 mM PMSF. Clarified cell lysate and medium samples were rocked overnight with anti-PrP antibodies in the presence of 1% bovine serum albumin and 0.1% N-laurylsarcosine. Protein-antibody complexes were collected with 40 μl of protein A-Sepharose (Pharmacia) and washed four times with 0.5 ml of wash buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.8, 0.1% N-laurylsarcosine, and 0.1 mM PMSF). Bound protein was eluted by boiling in sample buffer (2% SDS, 10% glycerol, 5% β-mercaptoethanol) and analyzed by SDS-PAGE fluorography. PrP bands were quantitated by PhosphorImager analysis (Molecular Dynamics).

Assay of Detergent Insolubility and Proteinase K Resistance—Cells were lysed at 4 °C in 1 ml of Western blot lysis buffer (TBS, Tris 20 mM, pH 7.4, NaCl 150 mM, EDTA 10 mM, Nonidet P-40 0.5% and deoxycholate 0.5%) containing protease inhibitors and clarified by centrifugation. The supernatant was ultracentrifuged at 100,000 × g in a Beckman SW50.1 rotor for 1 h at 4 °C. The high-speed supernatant was collected and the pellet fraction was redissolved in 1 ml lysis buffer. Proteins in the supernatant and pellet fraction were precipitated with cold methanol at −20 °C for 2 h, dissolved in sample buffer, and immunoblotted as above. For proteinase K treatment, cells were lysed as above in the absence of protease inhibitors, and clarified lysates were treated with 3.3 μg/ml proteinase K for 5 or 10 min. The reaction was stopped by the addition of 2 mM PMSF, and the proteins were metabolically labeled and immunoblotted as above.

Cell Surface Biotinylation—Subconfluent cell cultures were washed with PBS (containing 0.1 mM CaCl₂, 1 mM MgCl₂) and biotinylated at 4 °C with 1 mg/ml cold sulfo-NHS-biotin (dissolved in PBS) for 10–15 min, as described (19). Excess biotin was quenched with 50 mM glycine in PBS. The cells were washed with PBS, lysed as above, and clarified by centrifugation for 10 min. PrP in the supernatant was immunoprecipitated as described above, fractionated by SDS-PAGE, and transferred to Immobilon-P. Biotinylated PrP molecules were revealed by blotting with streptavidin/horseradish peroxidase enhanced chemiluminescence (Amersham Corp.). Alternatively, biotinylated cellular proteins were retrieved by incubating the cell lysate at 4 °C with streptavidin-agarose for 16–18 h. Non-biotinylated cellular proteins in the supernatant were methanol-precipitated and dissolved in sample buffer. Biotinylated proteins were recovered after washing the beads several times with increasing stringency and eluting bound proteins by boiling in sample buffer (20). Intracellular and biotinylated samples were fractionated by SDS-PAGE, and the amount of PrP in each was revealed by immunoblotting with 3F4 or anti-C antibody.

Peptide Mapping—N- and C termini of the 32-kDa form were evaluated by peptide mapping using endoproteases Asp-N and Lys-C. Cells were radiolabeled in the presence of tunicamycin to achieve a clear separation between the 27-kDa normal and 30-kDa aberrant form on SDS-PAGE. After immunoprecipitation and SDS-PAGE, the bands were visualized by autoradiography, excised, and soaked in elution buffer (Tris 20 mM, pH 8.0, SDS 1%, dithiothreitol 2 mM) for 1 h at 37 °C. The gel pieces were homogenized and incubated at 60 °C for 2 h,
followed by treatment with 5 mM iodoacetic acid for 1 h at 4 °C. The gel debris was removed by centrifugation of the homogenate in a Micropure filter (0.22 mm) inserted into a Microcon 10 unit (Amicon) at 10,000 g for 20 min. The protein sample was digested overnight at 37 °C with Asp-N or Lys-C (Boehringer Mannheim) in 0.1% SDS, 50 mM sodium phosphate, pH 8.0, as described (17). The resulting peptides were separated on Tris-Tricine SDS-PAGE (21) and visualized by fluorography.

RESULTS

Characterization of Total Cell-associated and Plasma Membrane-anchored PrPC and PrPM at Steady State—Immunoblots of total cell-associated PrP probed with 3F4 show that the major full-length forms (H, I, and U) are common to PrPC and PrPM (lanes 1 and 3) and that H and I comigrate with U after PNGase treatment (lanes 2 and 4). PrPM contains an additional 32-kDa band (32, lane 3) that migrates at 30 kDa after deglycosylation (30, lane 4). A faint band of 20 kDa (20) is also detected in both PrPC and PrPM preparations (see text). Total cell-associated PrP probed with the anti-C antibody reveals additional minor bands that consist of N-terminally truncated I and U forms of PrPC (I^T and U^T, of 22 and 18 kDa, respectively) (lane 1), and PrPM contains only ill-defined bands in the 25–22-kDa range (22–25 bracket, lane 3). After PNGase, all truncated PrP forms migrate at 18 kDa (U^T, lane 2), whereas truncated PrPM forms contain additional peptides of 17 and 14 kDa (17 and 14, lane 4). C, the immunoblots of surface PrPC cleaved by PI-PLC demonstrate the presence of full-length and truncated forms (lanes 1 and 3), while surface PrPM is comprised predominantly of the H form (lanes 2 and 4); the 32-kDa form is not detected. D, indirect immunofluorescent staining of cell surface PrP confirms the underrepresentation of PrPM compared with PrPC (panels 3 versus 1). Following PI-PLC treatment, the decrease of surface PrPM and PrPC is comparable (panels 4 versus 2).

Fig. 1. Total cell-associated and PI-PLC-released PrPC^C and PrPM^M at steady state. A, immunoblots of total cell-associated PrP probed with 3F4 show that the major full-length forms (H, I, and U) are common to PrPC^C and PrPM^M (lanes 1 and 3) and that H and I comigrate with U after PNGase treatment (lanes 2 and 4). PrPM^M contains an additional 32-kDa band (32, lane 3) that migrates at 30 kDa after deglycosylation (30, lane 4). A faint band of 20 kDa (20) is also detected in both PrPC^C and PrPM^M preparations (see text). B, total cell-associated PrP probed with the anti-C antibody reveals additional minor bands that consist of N-terminally truncated I and U forms of PrPC^C (I^T and U^T, of 22 and 18 kDa, respectively) (lane 1), and PrPM^M contains only ill-defined bands in the 25–22-kDa range (22–25 bracket, lane 3). After PNGase, all truncated PrP forms migrate at 18 kDa (U^T, lane 2), whereas truncated PrPM forms contain additional peptides of 17 and 14 kDa (17 and 14, lane 4). C, the immunoblots of surface PrPC^C cleaved by PI-PLC demonstrate the presence of full-length and truncated forms (lanes 1 and 3), while surface PrPM^M is comprised predominantly of the H form (lanes 2 and 4); the 32-kDa form is not detected. D, indirect immunofluorescent staining of cell surface PrP confirms the underrepresentation of PrPM^M compared with PrPC^C (panels 3 versus 1). Following PI-PLC treatment, the decrease of surface PrPM^M and PrPC^C is comparable (panels 4 versus 2).

followed by treatment with 5 mM iodoacetic acid for 1 h at 4 °C. The gel debris was removed by centrifugation of the homogenate in a Micropure filter (0.22 mm) inserted into a Microcon 10 unit (Amicon) at 10,000 × g for 20 min. The protein sample was digested overnight at 37 °C with Asp-N or Lys-C (Boehringer Mannheim) in 0.1% SDS, 50 mM sodium phosphate, pH 8.0, as described (17). The resulting peptides were separated on Tris-Tricine SDS-PAGE (21) and visualized by fluorography.

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prominent following radiolabeling (see below). The N-terminal truncated H form is not detectable probably because it is obscured by the full-length forms. However, it is likely to comigrate at 18 kDa with the truncated I and U forms following PNGase-F treatment since the 18-kDa band markedly increases following deglycosylation (Fig. 1B, lane 2).

Immunobots to detect PrPM with the 3F4 antibody show a prominent band of 32 kDa in addition to the three glycoforms present in PrPC (Fig. 1A, lane 3) (see below for detailed description). The pattern after deglycosylation is similar to that of PrPC, except that the deglycosylated product of 32-kDa form migrates at 30 kDa (Fig. 1A, lane 4).

Immunobots with the anti-C antibody demonstrate that PrPM differs from PrPC in three ways as follows: 1) the I truncated form is replaced by ill defined bands of 25–22 kDa (Fig. 1B, lane 3); 2) deglycosylation reveals additional truncated forms at approximately 17 and 14 kDa (Fig. 1B, lane 4); and 3) the 32-kDa PrPM form is not detected by the anti-C antibody.

Quantitative analyses reveal that the total amounts of cell-associated PrPM and PrPC are not significantly different (total PrPM 92 ± 7% of total PrPC; n = 8), but the percentage distribution of the various glycoforms differs and shows greater than 50% reduction of the PrPC U form. The II, I, and U glycoforms account for 47 ± 9, 33 ± 9, and 7 ± 5%, respectively, of the total PrPM and are significantly different (p < 0.006) from the corresponding values of PrPC that are 64 ± 5; 20 ± 3, and 15 ± 2%, respectively. The 32-kDa form accounts for 11 ± 3% of the total PrPM.

Cell surface PrPC was released with phosphatidylinositol-specific phospholipase C (PI-PLC), an enzyme that specifically cleaves GPI anchors. Immunobots of the released protein reacted with the 3F4 or anti-C antibodies reveal the same forms as in total cell extracts, i.e., all three full-length and truncated PrPC forms (Fig. 1C, lanes 1 and 3). The amount of cell surface PrPM released by PI-PLC is 45 ± 21% (p < 0.003; n = 4) less than that of PrPC, and the I and U truncated forms and the 32-kDa form are not detected (Fig. 1C, lanes 2 and 4).

The underrepresentation of PrPM at the cell surface after PI-PLC cleavage may be due to its resistance to PI-PLC because of aggregation or aberrant association with the plasma membrane (22, 23). Therefore, we further examined cell surface expression of PrPC and PrPM by biotinylation and immunofluorescence.

PrPC and PrPM present at the cell surface were biotinylated and either 1) immunoprecipitated, fractionated by SDS-PAGE, and detected by blotting with streptavidin/horseradish peroxidase or 2) retrieved with streptavidin-agarose following cell lysis, fractionated, and detected by immunoblotting. The results obtained with either of these procedures are similar to those obtained with PI-PLC cleavage and show a reduction of the total surface PrPM of 48 ± 23% (p < 0.006; n = 4) (data not shown).

The PrPC and PrPM present on the plasma membrane were further investigated by quantitative immunofluorescence using 3F4 (Fig. 1D). Compared with PrPC, the fluorescence intensity of PrPM preparations is reduced on average by 62 ± 5% (p < 0.0004; n = 8), further confirming the underrepresentation of PrPM (Fig. 1D, panel 1 versus 2). Treatment with PI-PLC results in a 57 ± 8% decrease of the immunofluorescence associated with PrPC and 77 ± 5% of that associated with PrPM (Fig. 1D, panel 3 versus 4).

Together, these findings show that although comparable amounts of total PrPM and PrPC are present in the intracellular compartments, PrPM is underrepresented at the plasma membrane, consistent with its impaired transport to that cell locale. In addition, PrPM is processed aberrantly, producing at least two additional fragments of 14 and 17 kDa.

Therefore, metabolic studies were carried out to investigate the synthesis, processing, turnover, and transport of PrPM employing a pulse-chase paradigm.

Metabolism and Transport of PrPC and PrPM—Immunoprecipitation of newly synthesized PrP with 3F4 after a 2-min labeling with Tran35S-label reveals three bands that are common to both PrPC and PrPM. The two upper bands represent the precursors of the H and I forms (H+, I+; Fig. 2A, lanes 1 and 5, upper panel) and exhibit gel mobilities different from those of the mature forms because they contain immature glycans. The lowest band comigrates with the U form (Fig. 2A, lanes 1 and 5, upper panel) (6, 15). PrPM preparations show two additional forms migrating at 32 and 24 kDa (Fig. 2A, lane 5, upper and lower panels, respectively). After a 40-min chase period, modification of the high mannose core glycans yields the mature H and I glycoforms that were observed in the immunoblots (Fig. 2A, lanes 4 and 8, upper panel). An additional 20-kDa form is detected in both PrPC and PrPM preparations. Thus, the synthesis and early kinetics of the H, I, and U forms are comparable in both PrPC and PrPM cells. However, when chased for longer times, the turnovers of PrPC and PrPM are markedly different (Fig. 2B). Following a 30-min labeling and chase periods from 1 to 6 h, “intracellular” (i.e., PI-PLC-treated) PrPC and PrPM progressively decrease in quantity, although PrPM decreases more rapidly, becoming less than half the amount of PrPC after a 6-h chase (Fig. 2B, lanes 1–5 versus 6–10). The 32-kDa form is relatively stable for 2 h and decreases thereafter (Figs. 2B, lanes 6–10). The chase period, this form accounts for 21–45% of the cell-associated PrPM, whereas the 24-kDa peptide, which is less represented, becomes undetectable after a 2-h chase (Fig. 2B, lanes 6–10). Immunoprecipitation of the intracellular PrPM with the anti-C antibody indicates that the truncated forms are more prominent in PrPM than in the PrPC preparations after 2 or 4 h of chase (Fig. 2C, lanes 3–5 versus 8–10).

The PrP released from the plasma membrane by PI-PLC, and immunoprecipitated with the 3F4 antibody, peaks at the 2-h chase time point in both PrPC and PrPM cells (Fig. 2D, lanes 3 and 8). The amount of PrPM released is approximately 60% less than that of PrPC (17 ± 5% versus 42 ± 7% of the total cell-associated PrPM and PrPC, respectively; p < 0.002; n = 3) (Fig. 2D, lanes 7–10 versus 2–5), and only the H form of PrP is detected at the surface of the mutant cells (Fig. 2D, lanes 7–10), whereas all three full-length glycoforms are well represented in the control cells (Fig. 2D, lanes 2–5). Immunoprecipitation of the PI-PLC-released PrPC with the anti-C antibody reveals a delay in the appearance of the I- and U-truncated forms with respect to the full-length forms (Fig. 2E, 3–5) consistent with the notion that these peptides result from the cleavage of full-length PrP (9, 24). In contrast, in PrPM cells, the ill-defined 25–22 kDa (probably corresponding to PrPC I-truncated form) and the U-truncated forms are not transported to the surface in significant quantities (Fig. 2C, lanes 8–10 versus Fig. 2E lanes 8–10).

The apparent discrepancy between the immunoblot data showing comparable PrPC and PrPM pools and pulse-chase data showing that intracellular PrPM is unstable and transported only in small amounts to the cell surface, even after overnight chase times (data not shown), suggests that PrPM aggregates and is retained in an intracellular compartment. If present as an aggregate, the mutant protein may not be efficiently immunoprecipitated by our procedure. This would account for the observed decrease of PrPM in the pulse-chase experiments described above. Therefore, we studied the site of
intracellular retention and determined whether the bulk of intracellular PrPM is aggregated.

Solubility and Protease Sensitivity of Intracellular and Surface PrPM—Solubility of total cell-associated PrPM and PrPC was examined following treatment with non-ionic detergents and ultracentrifugation to separate aggregated from soluble proteins. Supernatant and pellet fractions were recovered and analyzed by blotting (Fig. 3, A and B). Although PrPC is almost entirely detergent-soluble, 50–63% (n = 6) of total cell-associated PrPM, including a portion of the 32-kDa form, is detergent-insoluble (Fig. 3, lane 3 versus 6). The insolubility is inversely related to the degree of glycosylation, the unglycosylated form being entirely insoluble. In contrast, both PI-PLC cleaved (not shown) and surface biotinylated PrPM are detergent-soluble (Fig. 3B, lane 3 versus 6). With the exception of the 32-kDa form, all detergent-insoluble PrPM is Endo-H-resistant (Fig. 3C), indicating that aggregation of the non-32-kDa fraction takes place in a compartment distal to the cis-Golgi (25).

To evaluate if aggregated PrPM could be immunoprecipitated efficiently, total cell extract, soluble, and insoluble fractions of PrPC and PrPM were subjected to immunoprecipitation, and the resulting supernatant was immunoblotted for any remaining PrP (Fig. 3D). All PrPC and almost all of the PrPM-soluble (S2) fraction are immunoprecipitated by our procedure (Fig. 3D, lanes 1–3 and 5), whereas a substantial amount of insoluble PrPM remains in the supernatant after the immunoprecipitation (Fig. 3D, lane 6). Thus, the decrease in cell-associated PrPM observed in pulse-chase experiments is primarily due to aggregation. Since the aggregated PrPM was not solubilized even by boiling in SDS (22), the kinetics of aggregation could not be determined.

The presence of proteinase K-resistant PrPM was assessed by treating PrPC and PrPM cell lysates with various concentrations of PK (0.5–10 μg/ml) for different times (0–30 min) at 37 °C. Treatment with 3.3 μg/ml PK for 5 min revealed that approximately 7% of the total PrPM is resistant, whereas PrPC is completely digested (Fig. 3E, lanes 2 and 3 versus 5 and 6).
**Fig. 3. Detergent insolubility and protease resistance of PrPM.** A, total cell-associated PrP\(^{C}\) and PrP\(^{M}\) was solubilized in detergent, and after pelleting the nuclei and cell debris (S\(_1\)), fractionated by high speed centrifugation to yield soluble (S\(_2\)) and insoluble (P\(_2\)) fractions. These were analyzed by SDS-PAGE and immunoblotting with 3F4. Although PrP\(^{C}\) is completely soluble (lanes 1 and 3), PrP\(^{M}\) is largely recovered in the detergent-insoluble fraction (lanes 2 and 3). B, biotinylated cell surface PrP\(^{M}\) is fully soluble (lanes 5 and 6) as observed for biotinylated PrP\(^{C}\) (lanes 2 and 3) (biotinylated surface proteins were retrieved with streptavidin-agrose and immunoblotted with 3F4. S\(_1\), low speed detergent-soluble lysate; S\(_2\), and P\(_2\), high speed detergent-soluble (S\(_2\)) and -insoluble (P\(_2\)) fractions. C, Endo-H treatment of the insoluble fraction (P\(_2\)) shows that all full-length PrP\(^{M}\) forms are Endo-H-resistant except the 32 kDa that migrates at 30 kDa after Endo-H treatment (lane 1 versus 2). D, soluble (S\(_1\)) and insoluble (P\(_2\)) fractions of PrP\(^{C}\) and PrP\(^{M}\) were immunoprecipitated with 3F4, and the resultant supernatant was immunoblotted with the same antibody. In contrast to PrP\(^{C}\) (lanes 1–3), a significant amount of PrP\(^{M}\) (lanes 4 and 6), most of which is insoluble, fails to immunoprecipitate and is recovered in the supernatant; the soluble PrP\(^{M}\) (S\(_1\)) is immunoprecipitated efficiently, except for the 32-kDa form (lane 5). E, a small amount of PrP\(^{M}\) is resistant to PK treatment (3.3 \(\mu\)g/ml) for 5 min (lane 5); in contrast, PrP\(^{C}\) is completely digested (lane 2). The PK-resistant PrP\(^{M}\) shows the expected faster mobility of the resistant C-terminal fragments and contains all the major (H\(^{res}\), I\(^{res}\), U\(^{res}\)) and, possibly, the 32-kDa (39\(^{res}\)) forms (lanes 5 and 6).

All full-length PrP\(^{M}\) forms, and possibly the 32-kDa forms, are represented in the PK-resistant fraction. All the forms show a decrease in size after PK digestion as in prion diseases (Fig. 3E, lane 4 versus 5).

**Intracellular Localization of PrP—Employing double immunofluorescence with antibodies directed to PrP and to specific cellular organelles, we observed that the majority of intracellular PrP\(^{C}\) is co-localized with anti-o-mannosidase II, a marker of the medial Golgi (Fig. 4A, panels 1–3).** The distribution of intracellular PrP\(^{M}\) is more widespread; part co-localizes with the Golgi apparatus, just like PrP\(^{C}\) (Fig. 4A, panels 4–6), and part maps with calnexin, an ER marker (Fig. 4B, panel 1 versus 2), and with cathepsin D, a marker of the endosomal-lysosomal compartment (Fig. 4B, panel 3 versus 4).

**Further Characterization of the 32-kDa and the Other Aberrant PrP\(^{M}\) Forms—**The detection of the 32-kDa form, even after a 2-min pulse (Fig. 2A), and its Endo-H sensitivity, even after a sustained chase (Fig. 3C, lanes 1 and 2; and data not shown), suggest that it remains in the ER-cis Golgi region (25). Moreover, the 32-kDa form migrates at 30 kDa on SDS gels following deglycosylation (~3 kDa larger than unglycosylated PrP) and immunoreacts with the N-terminal antibody to PrP\(^{C}\) and immunoreacts with the N-terminal antibody to PrP\(^{M}\) residues 23–40 (Fig. 1A, lanes 3 and 4 and data not shown) suggesting that it maintains the 22-residue C-terminal GPI signal peptide and does not acquire the GPI anchor (26). To explore this possibility, Q217R and wild-type-transfected cells were labeled either with \[^{35}\text{S}\]methionine and -cysteine (trans\[^{35}\text{S}\]S-label) (Fig. 5, lanes 1 and 2) or \[^{3}H\]ethanolamine (Fig. 5, lanes 3 and 4), which labels the GPI anchor. In contrast to the H, I, and U forms of PrP\(^{C}\) and PrP\(^{M}\), the 32-kDa form is not detected after \[^{3}H\]ethanolamine labeling, confirming the absence of the GPI anchor (Fig. 5, lane 2 versus 4). The persistence of the C-terminal GPI signal peptide is supported by preliminary data obtained from peptide mapping (data not shown) and may account for the lack of immunoreactivity of the 32-kDa form with the anti-C antibody.

The 24-kDa as well as the 17- and 14-kDa forms seen after deglycosylation are the other aberrant forms that are only detected in the PrP\(^{M}\) cells (Figs. 2A and 1B, respectively). Like the 32-kDa form, the 24-kDa form is sensitive to Endo-H (data not shown) and probably resides in the ER-cis Golgi (25). It reacts with 3F4, but not with antibodies to the N (not shown) or C termini (Fig. 2C). The 17- and the 14-kDa forms are recognized only by the anti-C antibody and are detected only after deglycosylation (Fig. 1B, lane 4).

**Reversibility of Changes at 24 °C—Evidence that the aggregation of the mutant protein is associated with an aberrant conformation was obtained when cells were analyzed at 24 °C, a temperature known to promote folding of proteins (27, 28).** Pulse-chase experiments carried out at this temperature show that synthesis and turnover of intracellular PrP\(^{M}\) is similar to that of PrP\(^{C}\) for up to 6 h of chase (Figs. 6A, lanes 1–5 versus 6–10) and different from that at 37 °C (Fig. 2, B and D). However, the aberrant 32- and 24-kDa forms remain Endo-H-sensitive (data not shown). Moreover, the PrP\(^{M}\) cleaved from the plasma membrane with Pl-PLC also contains the I and U forms that are undetectable at 37 °C (Fig. 6B, lanes 8–10 versus Fig. 2D, lanes 8–10). Quantitative analysis of total PrP
ing a 24-h exposure to 24 °C, PrPM co-localizes predominantly of glycans.

A lower temperature promotes correct folding even in the absence the role of glycans in protein folding and also indicate that

3F4, FITC, and anti-cathepsin D, RITC superimposed.

2 panels 2

PrPM—

the secretory pathway.

A, panels 1–3

Intracellular localization of PrPC and PrPM determined by double immunofluorescence. A, PrPc co-localizes with the Golgi apparatus which appears well formed (panels 1–3). In contrast, PrPM has a more widespread distribution (panels 4–6). Panels 1 and 4, 3F4, FITC; panels 2 and 5, anti-α-mannosidase (Anti-Mann.) II, RITC; panels 3 and 6, color superimposition. B, unlike PrPc (panels 1 and 3), PrPM (panels 2 and 4) partially co-localizes with the ER (panel 2 versus 1) and the endosomal-lysosomal compartments (panel 4 versus 3). Panels 1 and 2, 3F4, FITC, and anti-calnexin, RITC superimposed; panels 3 and 4, 3F4, FITC, and anti-cathepsin D, RITC superimposed.

shows that the turnover of PrPc and PrPM is comparable at 24 °C, whereas at 37 °C PrPM turns over more rapidly than PrPc (Fig. 6, C and D).

Immunoblots of cells exposed to 24 °C show that over 60% less PrPM sediments in the detergent-insoluble form than at 37 °C (Fig. 6E, lane 6; versus Fig. 3A, lane 6); the H- and I-glycosylated forms become more readily soluble than the unglycosylated U form, whereas the 32-kDa form becomes entirely soluble (Fig. 6E, lanes 5 and 6). These findings reinforce the role of glycans in protein folding and also indicate that lower temperature promotes correct folding even in the absence of glycans.

Immunofluorescence microscopy further reveals that following a 24-h exposure to 24 °C, PrPM co-localizes predominantly with the Golgi apparatus as does PrPc (Fig. 6F; compare with Fig. 4A, panels 1–3).

DISCUSSION

The complex changes in the biogenesis of PrPαM induced by the Q217R-129V genotype show that a PRNP point mutation affects the metabolism of human PrPαM in significant ways as was previously shown for the D178N mutation (15). This information is relevant to the pathogenesis of human GSS. In addition, the present findings have implications regarding GPI anchor addition, folding, and storage of abnormal proteins in intracellular compartments, and the “quality control” system in the secretory pathway.

Effects of the Q217R-129V Genotype on the Biogenesis of PrPαM—Although synthesis and maturation of full-length PrPαM are similar to those of PrPc, aberrant PrPαM forms are detected soon thereafter, and the the full-length PrPαM forms undergo surprising posttranslational changes.

The aberrant PrPαM forms include the 32- and 24-kDa forms and minor fragments of 17 and 14 kDa. The 32-kDa form is likely to lack the GPI anchor and to retain the 22-residue C-terminal anchoring code (8, 29). Incomplete anchor addition to PrPM cannot be merely due to PrPM overexpression since the CEP4β expression system yields comparable synthesis of PrPc which is fully anchored (Fig. 2A) (15). In addition, the 32-kDa form appears to be retained in the ER since it remains Endo-H-sensitive. The origin and characteristics of the other aberrant PrPαM forms is unclear. On the basis of the immunoreactivity and Endo-H sensitivity, the 24-kDa peptide is either an internal or an N-terminally truncated fragment which, like the 32-kDa form, resides in the ER. The 17- and 14-kDa peptides must be generated by the aberrant cleavage of a glycosylated form.

The Q217R mutation also destabilizes the full-length PrPαM forms, the majority of which aggregate, whereas a small amount may undergo degradation. Since PrPαM is not transported efficiently to the plasma membrane and the tendency to aggregate is inversely related to the extent of glycosylation, the subcellular distribution of PrPαM and its glycoforms are different from that of PrPc; the relative amount of PrPαM associated with the cell membrane at steady state is 45% less than the amount of PrPc (and is represented largely by the highly glycosylated form, although the total intracellular pools of PrPc are comparable, and all the glycoforms are represented in the PrPαM pool (Fig. 1C versus A and B). The underrepresentation of the full-length I and U PrPαM forms at the cell surface, in turn, may change the way truncated forms are generated.

Aggregated PrPαM accumulates in intracellular compartments. Although the 32-kDa form appears to aggregate in the ER-cis Golgi, other PrPαM forms aggregate in more distal intracellular compartments, judging by their resistance to Endo-H and their co-localization with markers of the endosomal-lysosomal system.

An important observation of this study is that the amount of aggregated PrPαM is significantly reduced following the culture of mutant cells at 24 °C. This finding suggests that aggregation of PrPαM is the result of altered folding in the ER (27). A similar temperature effect has been observed on a mutant cystic fibrosis transmembrane conductance regulator with deletion of phenylalanine (CFTRAF508) (28). This finding has therapeutic implications since substances like glycerol have been shown to mimic the effect of low temperature on CFTRAF508 (30) and hence may facilitate proper folding of other mutated proteins such as PrP.

The finding that PrPαM has increased resistance to protease K (PK) digestion strengthens the validity of the present cell sys-
tem as a model of the human prion disease. The PK-resistant fragments generated by the exogenous protease in the Q217R mutant cells, as in the PrP\textsuperscript{Rsc} present in prion diseases, lacks the N terminus (data not shown). Our data show that the PK-resistant PrP\textsuperscript{M} contains the same forms in a similar ratio to the PrP\textsuperscript{M} forms present in the aggregated fraction (Fig. 3E versus Fig. 3A and D). This is consistent with a precursor-product relationship between aggregated (but PK-sensitive) and PK-resistant PrP\textsuperscript{M} forms as indicated (23). Thus, the present data support the notion that in inherited prion diseases, as in other prion diseases, the formation of the PK-resistant PrP\textsuperscript{M} is preceded by the formation of the aggregated but protease-sensitive PrP\textsuperscript{M}.

Collectively these findings indicate that the primary effect of the Q217R-129V genotype is a perturbation of the PrP\textsuperscript{M} conformation. This, in turn, leads to the expression of a variety of PrP\textsuperscript{M} forms, only some of which can be transported while the others aggregate and result in an altered subcellular distribution of PrP\textsuperscript{M}.

**The Q217R Cell Model and the Q217R Human Prion Disease**—The presence of a 7-kDa internal peptide spanning from residues 81/82 to residues 145/146 has been demonstrated in
Cellular Processing of Q217R Mutant Prion Protein

Amyloid deposits from brains of affected subjects carrying the Q217R-129V haplotype (13). In addition, brain extracts contained the common PK-resistant PrP of 27–30 kDa, a fragment of 8–13 kDa, and unidentified lower molecular weight fragments, the latter probably being the result of sequential proteolytic cleavage (13).

The present data suggest that during the pathogenesis of the disease, the above changes are preceded by the formation of detergent-insoluble and, in part, PK-resistant aggregates as well as by the expression of aberrant PrP\textsuperscript{M} forms. The aberrant truncated forms of PrP\textsuperscript{M} are indicative of an abnormal cleavage that normally occurs 14 amino acids downstream, at residue 231. It is unlikely that this is simply due to misfolding of the nascent PrPM to make it unfit to receive the anchor since the mutation at residue 217 interferes with the anchor linkage and the Quality Control System of the Secretory Pathway—properly folded or oligomerized glycoproteins are prevented from exiting the ER. This process is accomplished by a chaperone-mediated interplay between folding and glycosylation processes, and the misfolded proteins are eventually degraded (38–42). Some mutant glycoproteins, however, are stopped in post-ER compartments or are allowed to reach their destination at the plasma membrane (43).

The aberrant PrP\textsuperscript{M} forms caused by the Q217R mutation appear to be variously affected by the quality control system. Thus, while the anchorless 32-kDa form is retained in the ER-cis Golgi compartment, anchored forms that are either unglycosylated or carry one oligosaccharide chain appear to escape the ER quality control system and aggregate in post-ER compartments. In contrast, most of the fully glycosylated H form is transported to the plasma membrane. However, even this form may have an abnormal conformation since it is likely to generate the abnormal 17- and 14-kDa peptides by an aberrant proteolytic cleavage (44–46). Transport of an abnormal glycosylated PrP\textsuperscript{M} conformer to the cell surface is consistent with the notion that N-glycosylation does not ensure proper folding but rather stabilizes conformation and prevents aggregation (42).

The Q217R neuroblastoma cells thus provide a striking model of the complex changes that a PRNP point mutation can introduce into the processing of PrP\textsuperscript{M}, presumably engendering various degrees of protein misfolding. In the inherited prion disease, long incubation, aging, and cell diversity of the human brain are likely to add to this complexity.

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