Cell Surface Accumulation of a Truncated Transmembrane Prion Protein in Gerstmann-Straussler-Scheinker Disease P102L*

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A familial prion disorder with a proline to leucine substitution at residue 102 of the prion protein (PrP102L) is typically associated with protease-resistant PrP fragments (PrPSc) in the brain parenchyma that are infectious to recipient animals. When modeled in transgenic mice, a fatal neurodegenerative disease develops, but, unlike the human counterpart, PrPSc is lacking and mice, a fatal neurodegenerative disease develops, but, transmissible to recipient animals. When modeled in transgenic species. To understand these discrepant results, we studied the biogenesis of human PrP102L in a cell model. Here, we report that cells expressing PrP102L show decreased expression of the normal 18-kDa fragment on the plasma membrane. Instead, a 20-kDa fragment, probably derived from transmembrane PrP (CtmPrP), accumulates on the cell surface. Because the 20-kDa fragment includes an amyloidogenic region of PrP that is disrupted in the 18-kDa form, increased surface expression of 20-kDa fragment may enhance the susceptibility of these cells to PrPSc infection by providing an optimal substrate, or by amplifying the neurotoxic signal of PrPSc. Thus, altered susceptibility of PrP101L mice to exogenous PrPSc may be mediated by the 20-kDa CtmPrP fragment, rather than PrP102L per se.

Familial prion disorders of humans segregate with mutations in the prion protein gene (PRNP). Based on the clinical-pathological presentation, these disorders are categorized as Gerstmann-Straussler-Scheinker disease (GSS),1 Creutzfeldt-Jakob disease, and fatal familial insomnia (reviewed in Refs. 1–4). A proline to leucine substitution at codon 102 of PRNP is one of the most frequent mutations associated with GSS, and also one of the earliest prion disorders to be ascribed a genetic etiology (5, 10). Most GSS cases present with cerebellar ataxia and dementia, and a prominent pathological feature is the presence of multicentric amyloid plaques composed of protease-resistant PrP fragments of 8, 15, and 21–30 kDa. Although the 21-kDa fragment has also been observed in Creutzfeldt-Jakob disease, the 8-kDa fragment appears specific to GSS (6–8). The 21–30-kDa fragments are more prominent in GSS102L with a spongiform change, and low molecular mass fragments of 8 or 7–15 kDa are predominant in cases with multicentric amyloid plaques (7–10). These fragments are generated in vivo and are believed to contribute to the neuropathology observed in these disorders.

Modeling of GSS102L in transgenic mice expressing the corresponding PrP mutation (MoPrP P101L) has produced unusual and provocative observations. Animals expressing high levels of the transgene showed spontaneous neurodegeneration at 85–300 days of age without the presence of detectable PK-resistant PrPSc (11). The observed neurodegeneration in these mice is believed to result from adverse effects of the mutant PrP rather than overexpression of the transgene (12). Intracerebral inoculation of tissue from diseased animals transmitted the disease to healthy P101L mice expressing low levels of the transgene, but not to mice expressing normal PrP (11, 12). Subsequently, mice expressing a single copy of the P101L allele were generated by homologous recombination to avoid artifacts because of transgene overexpression. These mice also lacked PrPSc deposits, but surprisingly, although none of the animals developed spontaneous disease, their susceptibility to exogenous PrPSc infection was altered dramatically (13, 14). Thus, it appears that PrP102L alters certain cellular characteristics or functions of neuronal cells that influence the replication or toxicity of exogenously introduced PrPSc. The reasons for these discrepant observations are presently unclear.

We investigated the biogenesis of PrP102L in transfected human neuroblastoma cells in an attempt to model the events that might occur in vivo, and uncover the biochemical pathways of neurotoxicity in GSS102L. In this report, we show that the metabolism of PrP102L is altered, resulting in accumulation of a 20-kDa fragment of PrP on the cell surface, with a concomitant decrease in the expression of 18-kDa fragment, a product of normal recycling of PrP from the plasma membrane (15). The 20-kDa fragment is likely derived from CtmPrP, a transmembrane form of PrP that has been implicated as a mediator of neurodegeneration in certain inherited and infectious prion disorders (21, 22). Our results suggest that the change in ratio of 18:20-kDa fragments on PrP102L cells may increase their vulnerability to PrPSc toxicity by unconventional pathways, thus accounting for the complex biological effects of PrP102L in vivo.

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The abbreviations used are: GSS, Gerstmann-Straussler-Scheinker disease; PRNP, mutant PrP; PrPSc, normal cell-associated PrP; PrPSc, conformationally transformed scrapie form of PrP; CtmPrP, transmembrane PrP with the C terminus in the endoplasmic reticulum lumen; NtermPrP, transmembrane PrP with the N terminus in the ER lumen; PrP102L, PrP with a Pro to Leu mutation at codon 102 of the prion protein; PK, protease K; PNGase-F, N-glycosidase F; GPI, glycosyl phosphatidylinositol; FI-FLC, phosphatidylinositol-specific phospholipase C; ER, endoplasmic reticulum; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; DMEM, Dulbecco’s modified Eagle’s medium; PMSF, phenylmethylsulfonyl fluoride; TRITC, tetramethyl rhodamine isothiocyanate.
Experimental Procedures

Cell Lines, Reagents, and Antibodies—The human neuroblastoma cell line M17 was obtained from Dr. J. Beidler (Memorial Sloan-Kettering Cancer Center, New York, NY). Opti-MEM, fetal calf serum, penicillin-streptomycin, methionine, and cysteine-free Dulbecco’s modified Eagle’s medium (DMEM), and LipofectAMINE were from Invitrogen; hygromycin B and G418 were from Calbiochem; Tran-[35S]S-label was from ICN (Costa Mesa, CA); protein A-agarose and N-ethyldimidazole (PnGase-F) were from Roche Molecular Biochemicals; phosphatidylinositol-specific phospholipase C (PI-PLC) was from GLYKO (Novato, CA). All other chemicals were purchased from Sigma. Transfected M17 cells expressing wild type (PrP*) or mutant (PrP102L) prion protein were generated as described in a previous report (17, 18). All cultures were maintained at 37 °C in Opti-MEM supplemented with 5% fetal calf serum, penicillin-streptomycin, methionine-cysteine-free Dulbecco’s modified Eagle’s medium (DMEM), and preincubated in the same medium containing 5% dialyzed serum. For immunoprecipitation as described above, the appropriate antibody to detect PrP

Enzymatic Deglycosylation—For deglycosylation, unlabelled, or radio-labeled immunoprecipitated proteins were deglycosylated with five volumes of cold methanol and resuspended in denaturing buffer (0.5% SDS, 1% β-mercaptoethanol). Samples were boiled for 10 min and deglycosylated with PNGase-F (1000 units in 1% Nonidet P-40, 25 μg sodium phosphate, pH 7.5) for 1–3 h at 37 °C. Proteins were reprecipitated with five volumes of cold methanol at −20 °C for 2 h, dissolved in sample buffer, and resolved by SDS-PAGE immunoblotting or fluorography.

Cell Homogenization and Treatment with Proteinase K—PrP*- and PrP102L-expressing cells were washed and homogenized on ice in a 10 mM EDTA/H9262 buffer containing 10 μg/ml leupeptin, 2.5 μM pepstatin, 10 μM 1,10-phenanthroline (PMSF), and 0.5 μg/ml diithiothreitol with 20 strokes of a Kontes all-glass Dounce homogenizer. The homogenate was checked microscopically for cell breakage and centrifuged to pellet nuclei. The resulting supernatant was centrifuged at 20,000 × g for 10 min at 4 °C and subjected to immunoprecipitation as above.

Membrane Preparation—Cells expressing PrP* or PrP102L were cultured on poly-d-lysine-coated glass coverslips overnight. One set of PrP*- and PrP102L-expressing cells were incubated with anti-PrP antibody 3F4 conjugated with the appropriate fluorochrome and two different sets of cultures were incubated with the same antibody at the same dilution for 10 or 60 min each at 37 °C in a humidified CO2 incubator. At the end of each incubation period, cells were washed three times with PBS and fixed with 4% paraformaldehyde for 30 min at room temperature. Free aldehyde groups were quenched with 50 μM NH4Cl (in PBS), and nonspecific sites were blocked with PBS containing 10% goat serum and 0.2% bovine serum albumin for 20 min at room temperature. Cells were then incubated with anti-mouse TRITC to detect the anti-PrP antibody bound to PrP molecules on the cell surface. Subsequently, the cells were incubated with 0.25 mg/ml unlabeled anti-mouse IgG for 40 min to block the remaining anti-PrP antibody sites on the cell surface, and permeabilized with 0.1% Triton X-100 for 4 min. The cells were rinsed in PBS, and the internalized anti-PrP antibody was detected with FITC-conjugated anti-mouse antibody to detect anti-PrP-PrP complexes that have been internalized from the cell surface. After a final wash in PBS, cells were mounted in gel mount (Biomedia Corp., Foster City, CA) and observed using a laser scanning confocal microscope (Bio-Rad).

Results

Surface Expression of 18- and 20-kDa C-terminal Fragments of PrP Is Altered in PrP102L Cells—The steady state expression of PrP* and PrP102L in transfected neuroblastoma cells was evaluated by immunoblotting cell-associated PrP with antibodies specific to PrP residues 109 and 112 (3F4), 145–180 (8H4), or 220–230 (2001). Immunoreaction with 3F4 showed, as expected, three glycoforms of PrP* comprising the unglycosylated 20-kDa fragment, but in contrast to PrP C, all the PrP 102L isoforms were immunoprecipitated with antibodies to the 20-kDa fragment, but in contrast to PrP C, all the PrP 102L isoforms were immunoprecipitated with antibodies to the 20-kDa fragment.
lysates could be the result of increased production resulting from abnormal metabolism of PrP<sup>102L</sup> or, conversely, decreased turnover of the 20-kDa fragment caused by misfolding or aggregation because it probably includes the PrP<sup>102L</sup> mutation. The decreased representation of 18-kDa fragment, on the other hand, could be caused by reduced expression of full-length PrP<sup>102L</sup> on the cell surface because of sequestration in an intracellular compartment or, conversely, normal surface expression but decreased endocytosis and/or recycling to the plasma membrane. These possibilities were investigated in the following experiments.

**Over-representation of 20-kDa Fragment in PrP<sup>102L</sup> Cells Is Not the Result of Increased Production**—To evaluate whether accumulation of the 20-kDa fragment is the result of increased production as a result of aberrant metabolism of PrP<sup>102L</sup>, PrP<sup>C</sup>, and PrP<sup>102L</sup>-expressing cells were labeled with Tran<sup>35S</sup>-label for 2 h and subjected to immunoprecipitation with 3F4 or 8H4 antibodies. The three glycoforms of PrP and the 20-kDa fragment were detected with 3F4 in both PrP<sup>C</sup> and PrP<sup>102L</sup> lysates, and as observed in Fig. 1, PrP<sup>102L</sup> glycoforms and the 20-kDa fragment migrated ~1 kDa faster than PrP<sup>C</sup> forms (Fig. 2A, lanes 1 and 2). The 20-kDa fragment was prominent in both samples, but in contrast to the results obtained in Fig. 1 at steady state, the 20-kDa fragment was not over-represented in PrP<sup>102L</sup> lysates (Fig. 2A, lanes 1 and 2). Similar results were obtained with 8H4 antibody (Fig. 2A, lanes 3 and 4). A small amount of 18-kDa fragment could be detected in 8H4 immunoprecipitates, but there was no significant difference in the amount detected in PrP<sup>C</sup> and PrP<sup>102L</sup> lysates (Fig. 2A, lanes 3 and 4). Because the difference in the amount of 18- and 20-kDa fragments in PrP<sup>C</sup> and PrP<sup>102L</sup> lysates was detected at steady state but not after a 2-h pulse, it probably arose as a result of a cumulative process that becomes apparent over time, and not because of an acute abnormality in the metabolism of PrP<sup>102L</sup>.

To confirm the above results, the synthesis and turnover of PrP<sup>C</sup> and PrP<sup>102L</sup> were compared in a pulse-chase paradigm. Cells were radiolabeled with Tran<sup>35S</sup>-label for 30 min and chased for 0, 2, and 4 h. At the end of each chase period, radiolabeled GPI-linked surface proteins were cleaved with PI-PLC at 37 °C for 1 h, and the medium was collected. Subsequently, cell lysates and the PI-PLC-cleaved proteins were subjected to immunoprecipitation with 3F4 and fractionated by SDS-PAGE. Lysates treated with PI-PLC for 1 h soon after the pulse show the three glycoforms of PrP<sup>C</sup> and the 20-kDa fragment (Fig. 2B, lane 1). With increasing chase time, the amount of full-length PrP<sup>C</sup> forms and the 20-kDa fragment decreased in the lysate samples, and a corresponding increase was observed in the PI-PLC-cleaved samples (Fig. 2B, lanes 1–3 and 7–9). PrP<sup>102L</sup> samples showed similar kinetics of synthesis and transport of full-length and 20-kDa forms to the plasma membrane (Fig. 2B, lanes 4–6 and 10–12), except for a significant decrease in full-length PrP<sup>102L</sup> forms after 4 h of chase both in the lysate and PI-PLC-cleaved samples (Fig. 2B, lanes 6 and 12). In addition, a 14-kDa form was detected in the PrP<sup>102L</sup> lysates soon after the pulse, which decreased gradually with chase (Fig. 2B, lanes 4–6). Although the 14-kDa form of PrP<sup>102L</sup> was not detected in the PI-PLC-cleaved samples, subsequent experiments with long term labeling showed that it was indeed secreted into the culture medium (see Fig. 4B).

The above results highlight important characteristics of the 20-kDa fragment: 1) it is generated soon after pulse and does not increase with chase, indicating that it is not a metabolic product of full-length PrP, 2) it is transported to the cell surface and is cleavable by PI-PLC, and 3) it does not accumulate intracellularly or on the cell surface after 4 h of chase.

Together, these results show that the 20-kDa fragment
If the 20-kDa fragment arises from C\textsubscript{tm}PrP, it must be GPI-linked (20). To check this assumption, PrP\textsuperscript{C} and PrP\textsuperscript{102L} cells labeled overnight with the anchor component \([\textsuperscript{3}H]\)ethanolamine were lysed, immunoprecipitated with 3F4, and treated with PNGase-F to remove all glycans. The samples were fractionated on a long SDS-PAGE gel to accentuate the difference in migration of the 20-kDa fragment from PrP\textsuperscript{C} and PrP\textsuperscript{102L} lysates. As expected, the 27-kDa full-length form and the 20-kDa fragment of PrP\textsuperscript{102L} migrated ~1 kDa faster on SDS-PAGE (Fig. 2C, lane 1 versus lane 2). More importantly, the 20-kDa fragment from both PrP\textsuperscript{C} and PrP\textsuperscript{102L} lysates was labeled with \([\textsuperscript{3}H]\)ethanolamine, confirming that it is linked with the GPI anchor (Fig. 2C, lanes 1 and 2). As observed in Fig. 1 above, the 20-kDa was 4-fold more in PrP\textsuperscript{102L} lysates as compared with PrP\textsuperscript{C}.

The 20-kDa Fragment Is Generated from Transmembrane PrP in the Endoplasmic Reticulum—To evaluate whether the 20-kDa fragment arises from C\textsubscript{tm}PrP, PrP\textsuperscript{C} and PrP\textsuperscript{102L} cells were treated with the proteasomal inhibitor MG132 for 2 h, and subjected to immunoblotting with 3F4. There was a marked increase in the 20-kDa fragment after proteasomal inhibition, whereas the full-length PrP glycoforms were virtually unchanged (Fig. 3A, lane 1 versus lane 2 and lane 3 versus lane 4). Because C\textsubscript{tm}PrP is GPI-linked and degraded by the proteasomal pathway (20), an increase in a GPI-linked 20-kDa fragment (Fig. 2C) after proteasomal inhibition indicates its origin from C\textsubscript{tm}PrP. A significant increase in a 26-kDa band was also noted after proteasomal inhibition, especially in PrP\textsuperscript{C} lysates. The identity of this PrP form is currently under investigation.

To further confirm the origin of 20-kDa fragment from C\textsubscript{tm}PrP, microsomes prepared from PrP\textsuperscript{C} and PrP\textsuperscript{102L} cells were treated with 20 \(\mu\)g/ml PK on ice for 30 min and subjected to immunoblotting with 3F4 or anti-calnexin antibodies. Because the N terminus of C\textsubscript{tm}PrP faces the cytosol, it would be cleaved by PK treatment, releasing a C-terminal fragment of ~20 kDa in the ER, whereas the fully translocated PrP in the ER lumen would be protected from protease digestion and therefore show no change in migration or intensity (21, 22). As shown in Fig. 3B, there was a small but significant increase in the 20-kDa fragment after protease digestion of PrP\textsuperscript{C} and PrP\textsuperscript{102L} microsomes, whereas the full-length PrP glycoforms were virtually unchanged (Fig. 3B, lane 1 versus lane 2 and lane 3 versus lane 4). Similar analysis of cell homogenates prepared from cells cultured with the proteasomal inhibitor MG132 for 2 h showed increased expression of the 26- and 20-kDa fragments in both PrP\textsuperscript{C} and PrP\textsuperscript{102L} preparations (Fig. 3B, lanes 5–8). PrP\textsuperscript{102L} samples, in addition, showed a doublet in the region of 14 kDa when proteasomal function was inhibited (Fig. 3B, lane 7). PK treatment of microsomes prepared from these cells showed an increase in the 20-kDa fragment in both PrP\textsuperscript{C} and PrP\textsuperscript{102L} samples, whereas the 26-kDa fragment and full-length PrP forms were unaffected (Fig. 3B, lane 5 versus lane 6 and lane 7 versus lane 8). Simultaneous cleavage of calnexin by PK to produce a faster migrating species lacking the cytosolic C-terminal domain confirms the efficacy of PK treatment and intactness of the microsomes in this experimental system (Fig. 3B, lanes 2, 4, 6, and 8). The above results strongly suggest that the 20-kDa fragment in our cell model is a product of C\textsubscript{tm}PrP. The small increase in the amount of 20-kDa fragment after PK treatment is consistent with previous reports indicating that only ~2% of PrP is in the transmembrane orientation in cultured cells (23).

To evaluate whether up-regulation of C\textsubscript{tm}PrP synthesis in PrP\textsuperscript{102L} cells accounts for the over-representation of 20-kDa fragment, PrP\textsuperscript{C} and PrP\textsuperscript{102L} cells were radiolabeled in the
The 20-kDa fragment is generated from transmembrane PrP in the endoplasmic reticulum. A, PrPC and PrP102L cells were cultured in the presence of the proteasomal inhibitor MG132 for 2 h and subjected to immunoprecipitation with 3F4. There is a significant increase in the 20-kDa fragment after proteasomal inhibition, whereas the full-length PrP glycoforms are virtually unchanged (lane 1 versus lane 2 and lane 3 versus lane 4). B, microsomes prepared from PrPC and PrP102L cells were treated with 20 μg/ml PK on ice for 30 min and subjected to immunoblotting with 3F4 or anti-calnexin antibody. A significant increase in the 20-kDa fragment is observed after proteasome digestion of PrPC and PrP102L microsomes, whereas the full-length PrP glycoforms are virtually unchanged (lane 1 versus lane 2 and lane 3 versus lane 4). Simultaneous cleavage of calnexin by PK confirms the efficacy of PK treatment and intactness of the microsomes in this experimental system (lanes 2 and 4). PrPC and PrP102L cells treated with MG132 for 2 h show accumulation of 26-kDa (arrow) and 20-kDa forms (lanes 5–8), and a 14-kDa species in PrP102L lysates (lane 7). Only the 20-kDa shows an increase after PK treatment, indicating its origin from a transmembrane form of PrP (lane 5 versus lane 6, and lane 7 versus lane 8). C, PrPC or PrP102L cells were radiolabeled in the absence or presence of the proteasomal inhibitor MG132 for 2 h at 37 °C, or at 15 °C to block transport of proteins from the ER. Cell lysates were subjected to immunoprecipitation with 3F4 and analyzed by SDS-PAGE fluorography. There is a small increase in all full-length PrP forms in the presence of MG132 both at 37 °C and at 15 °C (lanes 1–4 versus lanes 5–8). Cells labeled at 15 °C show glycoforms with only high mannose core glycans. The highly glycosylated forms that are acquired in post-ER compartments are absent (Fig. 3C, lanes 2, 4, 6, and 8). The 20-kDa fragment was detected even at 15 °C in both PrPC and PrP102L lysates, confirming that it is generated in the ER (Fig. 3C, lanes 2 and 4). Surprisingly, an additional fragment of 14 kDa was detected at 15 °C in PrP102L lysates (Fig. 3C, lane 4). Both the 14- and 20-kDa fragments increased in amount in the presence of the proteasomal inhibitor MG132, indicating either increased production caused by a sparring of their precursor molecule or decreased turnover of these fragments by the proteasomes.

Quantitative estimation of the above results in terms of percentage increase of 20 kDa in comparison to full-length PrP forms shows increased accumulation of the 20-kDa fragment at 15 °C, and in the presence of MG132 in both PrPC and PrP102L lysates. The percentage of 20 kDa in PrP102L lysates was less than PrPC under all four conditions examined, i.e. at 37 °C or 15 °C, and in the absence or presence of MG132. The 14-kDa fragment appears to be highly unstable, because it was detected only at 15 °C or in the presence of MG132 and was significantly increased in PrP102L cells. This fragment did not increase in amount after PK treatment of microsomes, arguing against its origin from the N-transmembrane form of PrP (N terminus PrP) (21, 22). The origin of this fragment is presently under investigation.

Together, the above results show that: 1) the 20-kDa fragment is generated in the ER, most likely from proteolytic cleavage of C-terminus PrP at the ER membrane, 2) inhibition of C-terminus PrP degradation by proteasomal inhibition results in an increase in the generation of 20-kDa fragment, 3) an additional N-terminal fragment of 14 kDa is detected in PrP102L cells, the identity of which is presently unclear, and 4) increased representation of 20-kDa fragments in PrP102L is not because of increased synthesis of C-terminus PrP, but rather because of decreased turnover of this fragment, probably at the cell surface. In the following experiments, cell surface expression and turnover of the 20-kDa fragment were investigated to understand the cellular processes that lead to its accumulation in PrP102L cells.

The 20-kDa Fragment Accumulates on the Surface of PrP102L Cells—As noted in Fig. 1, not only is the 20-kDa over-represented in PrP102L at steady state, but the 18-kDa fragment of PrP is significantly decreased. To study the metabolism of 18- and 20-kDa fragments under conditions resembling steady state, PrPC, and PrP102L-expressing cells were labeled with Tran35S-label overnight in a 3:1 ratio of methionine-cysteine-free and normal DMEM containing 5% dialyzed serum. After 18 h of radiolabeling, the labeling medium was collected to check for any secreted proteins, and cells were washed with PBS and incubated with PI-PLC for 1 h to cleave cell surface GPI-linked proteins. The cells were subsequently lysed, and the lysates, PI-PLC-cleaved proteins, and medium samples were subjected to immunoprecipitation with 3F4 or 8H4 and analyzed by SDS-PAGE-fluorography.

As expected, in the lysate and PI-PLC-cleaved PrPC samples, the three glycoforms representing the highly glycosylated, intermediate, and unglycosylated forms were detected. The 20-kDa fragment was mostly recovered in the PI-PLC-cleaved material rather than the lysate (Fig. 4A, lanes 1 and 3). PrP102L lysates showed under-representation of the unglycosylated form as compared with PrPC, and all the PrP102L forms migrated ~1 kDa faster than PrPC (Fig. 4A, lane 2). As observed in the PrPC samples, the 20-kDa fragment was mostly detected in the PI-PLC-cleaved material, and was over-represented in PrP102L as compared with PrPC (Fig. 4A, lanes 3 and 4). The difference in the amount of 20-kDa fragment between PrPC and
Deglycosylation of PI-PLC cleaved samples showed 5-fold less difference in the amount of 20-kDa between PrPC and PrP102L samples (lanes 7 and 8). An additional 18-kDa fragment is detected in PrP C lysates (lane 7), majority of which is in the PI-PLC-cleaved sample (lane 9). The 18-kDa fragment is under-represented, and the 20-kDa fragment is over-represented, in PrP102L lysates (lanes 8 and 10). Deglycosylation of PI-PLC cleaved samples accentuates the difference in 18- and 20-kDa fragments in PrP C and PrP102L (lanes 11 and 12). B, medium collected from overnight labeling of PrP C and PrP102L cells was immunoprecipitated with 3F4 and analyzed by SDS-PAGE fluorography. PrP102L lysates (lanes 1 and 2) and PrP102L samples (lanes 5 and 6). An additional band of 14 kDa is detected in the PI-PLC-cleaved sample of PrP102L (lane 4). Immunoprecipitation with 8H4 shows similar PrP C and PrP102L glycoforms as observed with 3F4 (lanes 7 and 8). An additional 18-kDa fragment is detected in PrP C lysates (lane 7), majority of which is in the PI-PLC-cleaved sample (lane 9). The 18-kDa fragment is under-represented, and the 20-kDa fragment is over-represented, in PrP102L lysates (lanes 8 and 10). Deglycosylation of PI-PLC cleaved samples accentuates the difference in 18- and 20-kDa fragments in PrP C and PrP102L (lanes 11 and 12). B, medium collected from overnight labeling of PrP C and PrP102L cells was immunoprecipitated with 3F4 and analyzed. A small amount of the full-length PrP C and PrP102L forms (lanes 1 and 2) and a 14-kDa fragment are detected in PrP102L samples (lane 2).

From the pulse-chase experiments, it is clear that PrP102L was transported to the plasma membrane normally. The marked decrease in 18-kDa may therefore arise because of inefficient endocytosis or recycling of PrP102L at the plasma membrane. In addition, it is clear from the above data that the over-representation of 20 kDa in PrP102L is not the result of up-regulation of C-PrP, or of an increase in the proteolytic processing of C-PrP. Instead, the 20-kDa may accumulate at the plasma membrane because of decreased turnover, probably as a result of inefficient internalization and lysosomal degradation. These possibilities were investigated below.

Endocytosed PrP102L Is Targeted to Lysosomes Instead of Recycling Back to the Plasma Membrane—The kinetics of PrP C and PrP102L endocytosis and recycling were examined by immunofluorescence analysis. PrP C and PrP102L cells were incubated with anti-PrP antibody 3F4 in complete culture medium for 30 min on ice, or for 10 and 60 min each at 37 °C in a CO2 incubator. At the end of each time point, cells were washed with PBS, fixed, and immunostained with anti-mouse RITC to visualize PrP-antibody complexes on the plasma membrane. Excess antibody on the cell surface was quenched with 0.25 mg/ml unlabeled anti-mouse antibody, and the cells were washed and permeabilized with 0.1% Triton X-100. Subsequent immunostaining with FITC-conjugated anti-mouse antibody revealed intracellular PrP-antibody complexes. Thus, with this procedure, PrP molecules on the cell surface stained red, whereas the PrP-antibody complexes internalized during the incubation period stained green.

Following 30 min of incubation with 3F4 on ice, almost all of the PrPC and PrP102L were detected on the cell surface (Fig. 5A, red, panels 1 and 2). On the other hand, if the cells were incubated at 37 °C for 10 min, most of the PrPC was detected in intracellular vesicles, probably recycling endosomes (Fig. 5A, panel 2). In contrast, the intracellular vesicles in PrP102L were concentrated in a perinuclear location rather than close to the plasma membrane (Fig. 5A, panel 4), a difference that became more apparent after 60 min of incubation with 3F4 (Fig. 5A, panel 5 versus panel 6). Fig. 5B shows confocal images of the same experiment at a higher magnification to emphasize the difference between intracellular localization of endocytic vesicles loaded with PrPC (Fig. 5B, panels 1, 3, and 5) or PrP102L (Fig. 5B, panels 2, 4, and 6). In addition, there are significant differences in the fluorescence intensity of PrPC and PrP102L at the cell surface. After 10 min of incubation at 37 °C, the surface expression of PrPC and PrP102L is similar (Fig. 5, A and B, panels 3 and 4). However, after 60 min, the surface expression of PrPC and PrP102L was significantly different. Immunoreaction on the surface of PrPC cells showed red and green co-staining, indicating that some of the internalized PrP-antibody complexes recycled back to the plasma membrane. In PrP102L cells, only the red stain was prominent, suggesting that a fair amount of PrP102L did not get internalized even after 1 h of incubation at 37 °C, and that internalized PrP102L-antibody complexes that should have stained green did not resurface back to the plasma membrane (Fig. 5, A and B, panels 5 and 6).

To check whether the reduced surface expression of PrP102L is caused by targeting of internalized molecules to the lysosomes, PrPC and PrP102L cells incubated with 3F4 for 60 min as above were processed for co-immunostaining with cathepsin-D, a marker for late endosomes and lysosomes, or LysoTracker, a marker for lysosomes. As opposed to PrPC, a significant amount of co-staining was observed in PrP102L samples (data not shown).

Together, the above data indicate that, as opposed to PrPC, PrP102L is probably degraded by the lysosomes following endocytosis and not recycled back to the plasma membrane.
Kidney to emphasize the difference in intracellular localization of the recycling back to the plasma membrane. PrPC and PrP102L cells at 37 °C for more than PrP102L (mouse antibody reveals intracellular PrP-antibody complexes. Triton X-100. Subsequent immunostaining with FITC-conjugated anti-antibody, and the cells were washed and permeabilized with 0.1% PrP-antibody complexes on the plasma membrane. Excess antibody on conditions. At the end of each time point, the cells were washed with 

FIG. 5. Endocytosed PrP102L is targeted to lysosomes instead of recycling back to the plasma membrane. PrP102L and PrP102L cells were incubated with anti-PrP antibody 3F4 in complete culture medium for 30 min on ice or for 10 and 60 min at 37 °C under normal culture conditions. At the end of each time point, the cells were washed with PBS, fixed, and immunostained with anti-mouse RTIC to visualize PrP-antibody complexes on the plasma membrane. Excess antibody on the cell surface were quenched with 0.25 mg/ml unlabeled anti-mouse antibody, and the cells were washed and permeabilized with 0.1% Triton X-100. Subsequent immunostaining with FITC-conjugated anti-mouse antibody revealed intracellular PrP-antibody complexes. A, following 30 min of incubation with 3F4 on ice, almost all of the PrP102L and PrP102L are detected on the cell surface (panels 1 and 2). Incubation at 37 °C for 10 min shows most of the PrP- in intracellular vesicles close to the plasma membrane (panel 3). In contrast, intracellular vesicles in PrP102L are concentrated in a perinuclear location (panel 4). This difference is more apparent after the 60-min time point (panel 5 versus panel 6). B, confocal images of the above experiment at a higher magnification to emphasize the difference in intracellular localization of PrP102L (panels 1, 3, and 5) and PrP102L (panels 2, 4, and 6). After 60 min of incubation with 3F4 the surface expression of PrP102L is significantly more than PrP102L (panels 5 and 6).

DISCUSSION

The perplexing lack of correlation among neurodegeneration, transmissibility, and PrPSc accumulation in GSS102L has been difficult to reconcile with the commonly held belief that PrPSc is the principal pathogenic agent in all prion disorders. In this report, we provide a detailed analysis of the metabolic consequences of PrP102L, mutation in transfected neuroblastoma cells. We show that the processing and turnover of PrP102L is altered, resulting in decreased expression of the normal 18-kDa fragment, and increased accumulation of the 20-kDa CtmPrP fragment on the surface of these cells. This change in phenotype may render PrP102L cells more susceptible to exogenous PrPSc infection and toxicity by two inter-related mechanisms. First, the 20-kDa fragment may be more conducive to a change in transformation to PrPSc because it includes an amyloidogenic region of PrP that is disrupted in the 18-kDa fragment. Second, the 20-kDa may amplify the neurotoxic signal of exogenous PrPSc as observed in the case of CtmPrP (22), without serving as a substrate for PrPSc. A similar change in the cellular phenotype of PrP101L transgenic mice may partially explain the confounding biological effects of this mutation in vivo.

In our cell model of PrP102L, the surface representation of 20-kDa CtmPrP fragment is 4-fold more than the 18-kDa 5-fold less, as compared with PrP102L cells. We believe that this difference arises from aberrant recycling of PrP102L from the cell surface. Normally, cell surface PrP102L undergoes constitutive recycling between the plasma membrane and endocytic compartments. During this process, PrP102L is cleaved at residues 111/112, and a C-terminal fragment of 18 kDa is transported back to the cell surface (15). Thus, under steady state conditions, 40–50% of PrP102L on the plasma membrane comprises the 18-kDa fragment rather than full-length PrP102L. This observation has important clinical implications, because the 18-kDa is disrupted at residues 111/112 and therefore lacks critical residues, especially 90–112, involved in the refolding of PrP102L to PrP102L (27–30). The significance of these residues is highlighted by the fact that, in PrP-null mice that are resistant to scrapie infection, transgenes encoding PrP with deletions up to residue 93, but not 106, restore susceptibility of the animals to PrPSc infection (25). Because the 20-kDa fragment, but not the 18-kDa fragment, includes most of this region, a change in the ratio of 20- to 18-kDa fragment on the surface of PrP102L-expressing cells could have profound implications for PrPSc replication and neurotoxicity in vivo.

In our cell model, PrP102L is neither aggregated nor PK-resistant. Although this observation is consistent with reports on PrP101L-overexpressing transgenic mice that develop neurologic disease in the absence of detectable PrPSc (11, 13, 14), the mechanism by which mutant PrP mediates neurodegeneration is unclear. Adding to this apparent paradox are studies on transgenic mice expressing only a single allele of PrP101L, and the PG14 mice with an expanded octapeptide repeat that fail to develop spontaneous neurodegenerative disease, but exhibit increased vulnerability to exogenous PrPSc infection (13, 14, 26). When considered in context with our data, it is reasonable to assume that enhanced susceptibility of PrP101L mice to PrPSc infection may be the result of increased expression of the potentially amyloidogenic 20-kDa fragment on the cell surface, providing an optimal substrate for the incoming PrPSc (26). Once a nidus of PrPSc is initiated on the cell surface, albeit comprising only the 20-kDa fragment, subsequent conversion of full-length PrP and additional 20-kDa fragments would occur exponentially, as reported in our earlier study (16). Such a scenario would account for the increased susceptibility of PrP101L transgenic mice to exogenous PrPSc infection, as reported by Barron et al. (14). Alternately, the 20-kDa fragment may am-
simplify the neurotoxic signal initiated by exogenous PrPSc without itself undergoing a transformational change, causing neurotoxicity by a CtmPrP-mediated phenomenon. The latter scenario is supported by studies where an inverse correlation between PrPSc accumulation and CtmPrP has been described in mice challenged with PrPSc, implicating CtmPrP as the principal modulator of neurotoxicity (22).

Based on the results reported here and in a previous study, the 20-kDa fragment is generated in the ER probably from CtmPrP (16), whereas the 18-kDa fragment is produced during recycling from the cell surface (15). Although PK treatment of PrPC remains in peripheral endosomes close to the plasma membrane, PrP102L mutation is probably a result of the amino acid change to leucine, allowing better binding to SDS than proline, which is a helix breaker. It is possible that the 20-kDa fragment with the PrP102L mutation is endocytosed less efficiently because of the similar change in secondary structure may account for the mechanism leading to over-representation of 20-kDa fragment because 18-kDa fragment must recycle back to the plasma membrane. Such an alteration in the targeting and turnover of full-length PrP102L and its 20-kDa fragment would reverse the ratio of 20- to 18-kDa fragment on the surface of these cells, thus altering their phenotype in a significant way. However, both full-length PrP102L and its 20-kDa fragment can be released from the cell surface by PI-PLC, excluding the possibility that these are grossly misfolded or aggregated on the cell surface.

In conclusion, this report highlights important differences in the metabolism of PrP102L as compared with PrPSc, implicating the 20-kDa metabolic product of CtmPrP in the pathogenesis of GSS102L. Although the underlying mechanism of the pathogenic process is presently unclear, this report provides the basis for future investigations to clarify the complex biological manifestations of this mutation in vivo.

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REFERENCES

1. Prusiner, S. B. (1996) Cold Spring Harbor Symp. Quant. Biol. LXXI, 473–493
2. Prusiner, S. B. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 13363–13383
3. Weissmann, C. (1999) J. Biol. Chem. 274, 3–6
4. Caille, J. (2001) Annu. Rev. Neurosci. 24, 519–550
5. Hsiao, K., Baker, H. F., Crow, T. J., Poulter, M., Owen, F., Terwilliger, J. D., Westaway D, Ott, J., and Prusiner, S. B. (1989) Nature 338, 342–345
6. Barbaretto, M., Fabreguet, G., Moreau, J., Fontanilles, P., Cardone, F., Maraz, B., Equestre, M., Machhi, G. L., and Pochiarri, M. (1996) Neurology 47, 743–741
7. Parchi, C., P. Chen, S. G., Brown, P., Zou, W., Capellari, S., Ugliengo, P., Hainfellner, J., Reyes, P. F., Golden, G. T., Hawe, J., J. Gudjusek, D. C., Bagnoli, P. C., and Gambetti, P. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 8222–8227
8. Yamada, M., Tomimori, H., Yokota, T., Tomi, H., Sunohara, N., Mukoyama, M., Itoh, Y., Suematsu, N., Otomo, E., Okada, R., Matsushita, M., and Mizunaga, H. (1999) Neurology 52, 260–265
9. Hsiao, K., P. Chen, S. G., Brown, P., Zou, W., Capellari, S., Ugliengo, P., Gambetti, L. (1995) J. Biol. Chem. 270, 19173–19180
10. Gu, Y., Fujioka, H., Mizusawa, R., Li, R., and Singh, N. (2000) J. Biol. Chem. 275, 2275–2286
11. Singh, N., Zanussi, G., Chen, S. G., Fujioka, H., Richardson, S., Gambetti, P., and Petersen, R. B. (1997) J. Biol. Chem. 272, 28461–28470
12. Telling, C. G., Haga, T., Torricelli, M., DeAmorin, S. J., and Prusiner, S. B. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 473–478
13. Mancini J. C., Dlouhy, S. R., Farlow, M. R., Cass, C., Da Costa, M., Conneally, P. M., Ghebrehiwet, B., and Harris, D. A. (2001) J. Biol. Chem. 276, 2220–2226
14. Jin, T., Gu, Y., Zanussi, G., Sy, M., Kumar, A., Cohen, M., Gambetti, P., and Singh, N. (1999) J. Biol. Chem. 274, 23386–23404
15. Hsiao, K., Groth, D., Scott, M., Yang, S. L., Serban, H., Rapp, D., Foster, D., Teller, J. K., Gambetti, P., and Autilio-Gambetti, P. (1999) J. Neuropathol. Exp. Neurol. 58, 979–988
16. Gu, Y., Fujioka, H., Mizusawa, R., Li, R., and Singh, N. (2000) J. Biol. Chem. 275, 2275–2286
17. Singh, N., Zanussi, G., Chen, S. G., Fujioka, H., Richardson, S., Gambetti, P., and Petersen, R. B. (1997) J. Biol. Chem. 272, 28461–28470
18. Zanussi, G., Petersen, R. B., Jin, T., Ting, Y., Kanashiro, R., Ferrari, S., Gambetti, P., and Singh, N. (1999) J. Biol. Chem. 274, 23386–23404
19. Jin, T., Gu, Y., Zanussi, G., Sy, M., Kumar, A., Cohen, M., Gambetti, P., and Singh, N. (2000) J. Biol. Chem. 275, 38699–38704
20. Stewart, R. S., Drisaldi, B., and Harris, D. A. (2001) Mol. Biol. Cell 12, 861–889
21. Hegde, R. S., Mistrianni, J. A., Scott, M. R., DeFea, K. A., Trembley, P., Torchia, M., DeAmorin, S. J., Prusiner, S. B., and Lingappa, V. R. (1998) Science 278, 827–834
22. Hegde, R. S., Trembley, P., Groth, D., DeAmorin, S. J., Prusiner, S. B., and Lingappa, V. R. (1999) Nature 402, 822–826
23. Stewart, R. S., and Harris, D. A. (2001) J. Biol. Chem. 276, 2212–2220
24. Yedidia, Y., Hongouhid, L., Tshuan, S., Yana, A., and Turahulous, A. (2001) EMBO J. 20, 5383–5391
25. Fischer, M., Rulicke, T., Eberle, A., Sailer, A., Mieser, M., Weichsel, F., S. Aguzzi, A., and Weissmann, C. (1999) EMBO J. 15, 1255–1264
26. Chiara, E., and Harris, D. A. (2001) Neurobiol. Dis. 8, 743–763
27. Kaneko, K., Perez, D., Pan, K.-M., Blochberger, T., Wille, H., Gabizon, R., Poronik, O. H., Cohen, P. E., Baldwin, M. A., and Prusiner, S. B. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 11160–11164
28. Chabry, J., Caughey, B., and Chesebro, B. (1998) J. Biol. Chem. 273, 12903–12923
29. Holscher, C., Delius, H., and Burke, A. (1998) J. Virol. 72, 1153–1159
30. Nguyen, J., Baldwin, M. A., Cohen, F. E., and Prusiner, S. B. (1995) Biochemistry 34, 4186–4192

2 R. S. Mishra and N. Singh, unpublished observations.
Cell Surface Accumulation of a Truncated Transmembrane Prion Protein in Gerstmann-Straussler-Scheinker Disease P102L
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