Proteasomal Degradation and N-terminal Protease Resistance of the Codon 145 Mutant Prion Protein 

(Received for publication, October 14, 1998, and in revised form, April 8, 1999)

Gianluigi Zanusso, Robert B. Petersen, Taocong Jin, Yi Jing, Rima Kanoush, Sergio Ferrari, Pierluigi Gambetti, and Neena Singh

From the Division of Neuropathology, Institute of Pathology, Case Western Reserve University, 2085, Cleveland, Ohio 44106

An amber mutation at codon 145 (Y145stop) of the prion protein gene results in a variant of an inherited human prion disease named Gerstmann-Sträussler-Scheinker syndrome. The characteristic features of this disorder include amyloid deposits of prion protein in cerebral parenchyma and vessels. We have studied the biosynthesis and processing of the prion protein containing the Y145stop mutation (PrP^{145}) in transfected human neuroblastoma cells in an attempt to clarify the effect of the mutation on the metabolism of PrP^{145} and to gain insight into the underlying pathogenetic mechanism. Our results demonstrate that 1) a significant proportion of PrP^{145} is not processed post-translationally and retains the N-terminal signal peptide, 2) most PrP^{145} is degraded very rapidly by the proteasome-mediated pathway, 3) blockage of proteasomal degradation results in intracellular accumulation of PrP^{145}, 4) most of the accumulated PrP^{145} is detergent-insoluble, and both the detergent-soluble and -insoluble fractions are resistant to mild proteinase K (PK) treatment, suggesting that PK resistance is not simply because of aggregation. The present study demonstrates for the first time that a mutant prion protein is degraded through the proteasomal pathway and acquires PK-resistance if degradation is impaired.

The central pathogenetic event common to all three forms of prion diseases, sporadic, acquired by infection, and inherited, is thought to be a change in conformation involving the cellular prion protein (PrP^{c}). PrP^{c}, a 209-amino acid glycoprotein, linked to the plasma membrane by a C-terminal glycosylphosphatidyl inositol anchor, is converted into an isoform that is insoluble in nonionic detergents, resistant to protease degradation, and in some cases, can transmit the disease (PrP^{res}) (1–3). Recent NMR studies on recombinant PrP have shown that the N-terminal domain (23–120 in mouse recombinant PrP) is highly flexible and has a random coil structure, whereas the C-terminal region (129–219) contains two short b-sheet structures and three a-helical domains (4, 5). The major conformational change that causes PrP^{c} to become the pathogenic and infectious PrP^{res} isoform is thought to involve refolding of the region between residues 90 and 112, which would lead to conversion of the region containing the two short b-sheet structures and of the first a-helix into a large b-sheet formation (3, 6). However, the remaining C-terminal structures including the two other a-helices and the disulfide bond need to be preserved for PrP^{res} to be infectious (3, 6).

The events leading to the conversion of PrP^{c} to PrP^{res} are, at present, not fully understood. In the inherited prion diseases, which have only been associated with mutations in the PrP gene (PRNP), the mutation is believed to destabilize the mutant PrP (PrP^{M}), which then undergoes a spontaneous conformational change into the protease-resistant and pathogenic form (7–9). Twenty-three pathogenic mutations in PRNP have been reported to date, which are associated with three phenotypes: Creutzfeldt-Jakob disease, fatal familial insomnia, and Gerstmann-Sträussler-Scheinker disease (GSS) (8, 9). Despite their congenital presence, all mutations cause diseases that become symptomatic in the adult or advanced age. Of the seven PRNP mutations associated with GSS, a chronic cerebellar ataxia and dementia characterized by the presence of prominent amloid plaques containing internal PrP^{M} fragments, all are missense mutations except for the mutation at codon 145 that replaces tyrosine (TAT) with a stop (TAG) codon (Y145stop) (10–12).

The 145 mutation is especially challenging because it results in the premature termination of protein synthesis and yields a truncated PrP (PrP^{145}) that lacks the C-terminal 146–231 amino acids including the glycosylphosphatidyl inositol anchor, which links PrP to the cell surface, and the two sites for N-glycosylation, which are known to stabilize the PrP molecule (13, 14). Thus, although PrP^{145} includes the 90–112 segment where the major conformational changes take place and almost all the 80–147 internal fragment that is found in PrP amyloid, it lacks the C-terminal region containing the two a-helices and the disulfide bond, which are required for the PrP^{c}–PrP^{res} conversion (15, 16). Moreover, most of the PrP^{145} is likely to include the 23–90-residue N-terminal region of PrP, which has been shown to be superfluous for conversion to PrP^{res} (15–17). Yet, the Y145stop mutation is associated with a phenotype not basically different from that of the GSS subtypes associated with other mutations, which do not result in the truncation of PrP^{M} (10). The only significant difference is the presence of numerous PrP-amloid deposits in the cerebral vessels rather
Degradation of Mutant Prion Protein by Proteasomes

than in the brain parenchyma as in the other GSS subtypes (10–12).

Currently, there are no animal or cellular models of the Y145stop mutation. Expression of the truncated PrP Pan was not detected in transgenic animals and transfected neuroblastoma cells following deletion of the 144–231 region, which results in a PrP isoform identical in primary structure to that generated by the Y145stop mutation (17, 18). To examine the effects of this mutation on the metabolism of PrP Pan, hence to gain a better understanding of the mechanisms involved in the pathogenesis of this GSS variant, we have transfected human neuroblastoma cells with PRNP constructs carrying the Y145stop mutation or wild type PRNP. We report, for the first time, that mutant PrP Pan is degraded through the proteosomal pathway. Inhibition of proteosomal degradation results in the accumulation of PrP Pan in intracellular compartments, including the endoplasmic reticulum (ER), the cis-medial-Golgi compartment, and the nucleus. Most of the accumulated PrP Pan is aggregated and partially resistant to mild proteinase K treatment. Protease-resistant PrP Pan is also present in the detergent-soluble fraction, suggesting that the PrP Pan protease resistance is not simply because of aggregation.

EXPERIMENTAL PROCEDURES

Materials, Cell Culture Conditions, and Production of Transfected Cell Lines—Opti-MEM, fetal bovine serum, penicillin/streptomycin, methionine, and cysteine-free Dulbecco’s modified Eagle’s medium, and Lipofectin were from Life Technologies Inc.; hybridrogycin B and lactacystin were from Calbiochem; Tran35S-label was from ICN; proteinase inhibitors. The lysate was subjected to immunoprecipitation with 5 volumes of cold methanol at –20 °C, fractionated by SDS-PAGE, and electrophoretically transferred to Immobilon-P (Millipore) for 2.5 h at 70 volts at 4 °C. Membranes containing transferred proteins were blocked in Tris-buffered saline containing 10% nonfat 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 diluted 1:3000) dissolved in antibody dilution buffer (Tris-buffered saline, 1% normal goat serum, and 0.05% bovine serum albumin). Immunoreactive bands were detected with the appropriate secondary antibody conjugated to horseradish peroxidase (anti-rabbit diluted 3:100, anti-mouse diluted 1:3000) and fractionated by SDS-PAGE to check co-immunoprecipitation with 3F4. The immunoprecipitated proteins were fractionated by SDS-PAGE and electrophoretically transferred to Immobilon-P, and the biotinylated PrP was detected by horseradish peroxidase-conjugated streptavidin and ECL.

Detection of Associated Chaperone Proteins with PrP Pan—Cells expressing PrP Pan or PrP145 were radiolabeled for 2 h in the presence or absence of 80 µM ALLN and lysed with a nondenaturating buffer containing 1% CHAPS or 2% Triton X-100 in the presence of a mixture of protease inhibitors. The lysate was subjected to immunoprecipitation with anti-KDEL (Stressgen), anti-calnexin, or anti-Gp 94 antibodies (Stressgen) and fractionated by SDS-PAGE to check co-immunoprecipitation of any of the PrP Pan or PrP145 forms with the above ER chaperones. In a parallel experiment, the PrP Pan was immunoprecipitated from the presence of any associated chaperones was evaluated by immunoblotting the electrophoretically transferred proteins by specific antibodies.

Detection of Ubiquitinated PrP—For detecting ubiquitinated PrP Pan, untreated and ALLN-treated PrP Pan, and PrP145, expressing cell lysates were immunoprecipitated with 3F4 as above. After fractionating on SDS-PAGE, the immunoprecipitated proteins were transblotted and probed with the anti-PrP antibody (3F4, diluted 1:50,000, or anti-C diluted 1:3000) dissolved in antibody dilution buffer (Tris-buffered saline, 1% normal goat serum, and 0.05% bovine serum albumin). Immunoreactive bands were detected with the appropriate secondary antibody conjugated to horseradish peroxidase (anti-rabbit diluted 1:3000, anti-mouse diluted 1:3000) and fractionated by SDS-PAGE to check co-immunoprecipitation with any of the PrP Pan or PrP145 forms with the above ER chaperones.

In Vitro Transcription and Translation—The cDNA 145 mutant was originally isolated from the cDNA library from the plasmid pVS1, which contains both T7 and SP6 bacteriophage RNA polymerase promoters, using oligonucleotide-directed mutagenesis. Using a BamHI-cleaved template, run-on capped RNA was produced using the Cap-scribe system (Roche Molecular Biochemicals) as recommended by the manufacturer. Transcripts were analyzed using ethidium bromide-stained gels to assess their purity. The in vitro transcription products were translated into protein using the Promega message-dependent rabbit reticulocyte system with or without added canine pancreatic microsomes (Promega) to cleave the signal peptide. To control for microsome activity, a transcript derived from β-lactamase was used. The conditions used in the translation reaction were essentially as described by the manufacturer.

Metabolic Labeling, Immunoprecipitation, and Western Blots—In a typical experiment, 9 × 10⁶ cells were used for each condition. Equal amounts of total protein was used from cells expressing either normal or mutant PrP. Immunoprecipitation and Western blots were performed essentially as described (19), with the following modifications. For pulse-chase experiments, cells were preincubated in the presence or absence of the indicated inhibitors (lactacycin 80 µM, N-acetyl-leucyl-leucyl-norleucinal (ALLN) 80 µM, brefeldin A 5 µg/ml) for 1 h before labeling with 0.1 µCi/ml of [35S]methionine. Cultures of transfected cells were washed in 20 mM Tris, 10 mM NaCl, pH 7.4 containing a mixture of protease inhibitors. Cell debris was cleared by centrifugation at 290 × g, and the clarified cell lysate and medium samples were subjected to immunoprecipitation with the appropriate antibodies in the presence of 1% bovine serum albumin and 0.1% N-lauryl sarcosine. Protein-antibody complexes were bound to protein-A-Sepharose (Amersham Pharmacia Biotech) washed five times with 1 ml of wash buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.8, 0.1% N-lauryl sarcosine, and 0.1 mM phenylmethylsulfonyl fluoride), and bound protein was eluted by boiling in sample buffer (Tris-HCL, pH 6.8, 3% SDS, 10% glycerol, 5% β-mercaptoethanol) and analyzed by SDS-PAGE fluorography. PrP bands were quantitated by PhosphorImager analysis (Molecular Dynamics) and analyzed by quantitating the appropriate bands, exposing autoradiographic film by ECL (Amersham Pharmacia Biotech). To quantitate the relative density of immunoreactive bands, exposed autoradiographic film was scanned at 42-nm resolution with a GE10 densitometer and quantitatively analyzed using Quantity One software (PDIG20, QS30).

For biotinylation of surface proteins, untreated, or cells treated with ALLN for 2 h were biotinylated with 0.2 mg/ml of sulfo-N-hydroxysuccinimide-biotin in PBS for 15 min on ice. Excess biotin was quenched with 50 mM glycine (in PBS), and after three more washes with PBS, the cells were lysed as above and subjected to immunoprecipitation with 3F4. The immunoprecipitated proteins were fractionated by SDS-PAGE and electrophoretically transferred to Immobilon-P, and the biotinylated PrP was detected by horseradish peroxidase-conjugated streptavidin and ECL.

Detection of Associated Chaperone Proteins with PrP Pan—Cells expressing PrP Pan or PrP145 were radiolabeled for 2 h in the presence or absence of 80 µM ALLN and lysed with a nondenaturating buffer containing 1% CHAPS or 2% Triton X-100 in the presence of a mixture of protease inhibitors. The lysate was subjected to immunoprecipitation with anti-KDEL (Stressgen), anti-calnexin, or anti-Gp 94 antibodies (Stressgen) and fractionated by SDS-PAGE to check co-immunoprecipitation of any of the PrP Pan or PrP145 forms with the above ER chaperones. In a parallel experiment, the PrP Pan was immunoprecipitated from the presence of any associated chaperones was evaluated by immunoblotting the electrophoretically transferred proteins by specific antibodies.

Detection of Ubiquitinated PrP—For detecting ubiquitinated PrP Pan, untreated and ALLN-treated PrP Pan, and PrP145, expressing cell lysates were immunoprecipitated with 3F4 as above. After fractionating on SDS-PAGE, the immunoprecipitated proteins were transblotted and probed with the anti-PrP antibody (3F4, diluted 1:50,000, or anti-C diluted 1:3000) dissolved in antibody dilution buffer (Tris-buffered saline, 1% normal goat serum, and 0.05% bovine serum albumin). Immunoreactive bands were detected with the appropriate secondary antibody conjugated to horseradish peroxidase-conjugated streptavidin and ECL.
Degradation of Mutant Prion Protein by Proteasomes

PrP<sup>145</sup> Is Expressed as Two Isoforms at Steady State—On blots immunostained with the anti-PrP antibody 3F4 (to residues 109–112), the normal or cellular prion protein (PrPC) migrates as three bands corresponding to the unglycosylated (U), intermediate (I), and highly glycosylated (H) forms of 27, 33–42 kDa, respectively (Fig. 1A), except that PrP<sub>15.5</sub> accounts for only 55% of the total PrP<sub>145</sub> expressed in the steady state. In contrast, the cell surface PrP<sub>145</sub>, which is approximately 9 times smaller than that of PrPC, is mostly distributed at the cell surface and in the Golgi region (Fig. 1E, panel 1) (19), a very small amount of PrP<sub>145</sub> is detected in the intracellular compartment, and it co-stains with the Golgi marker α-mannosidase II (Fig. 1E, panel 3). No ER localization is observed with the ER marker calnexin (Fig. 1E, panel 2), probably because the small quantity of PrP<sub>145</sub> that escapes degradation transits through the ER very rapidly. No PrP<sub>145</sub> is detected on the cell surface. The very low expression of PrP<sub>145</sub> compared with PrPC suggests that PrP<sub>145</sub> turns over very rapidly. The under-representation of PrP<sub>15.5</sub> after radiolabeling and immunoprecipitation (25%) when compared with the steady state level by Western blot analysis (66%) (Figs. 1A versus B) raises the possibility that this form is unable to undergo proteasomal degradation because of a change in its conformation, as previously observed with the PrP<sub>145</sub> Q217R (19) (see below).

PrP<sub>145</sub> Is Rapidly Degraded in a Pre-Golgi Compartment by the Proteasomal Pathway—To determine whether PrP<sub>145</sub> is degraded by the lysosomes or in a pre-Golgi compartment, we carried out pulse-chase analysis either in the presence of various lysosomal inhibitors (leupeptin, ammonium chloride, or chloroquine) or by blocking transport beyond the ER-cis-Golgi compartment by incubating cells at 15 °C or treating them with brefeldin A (20). Neither the inhibition of lysosomal activity (data not shown) nor the block of vesicular transport at low temperature (Fig. 2A) or brefeldin A (Fig. 2B) blocked the degradation of PrP<sub>145</sub> although, as expected, the rate of degradation was slower at 15 °C as compared with 37 °C (see Fig. 1D). A similar analysis at 37 °C shows that although PrP<sub>C</sub> matures into various glycoforms and is stable, 70% of the total being present after 2 h of chase, most of the PrP<sub>C</sub> disappears rapidly, and only 12% remains after 2 h (data not shown). Taken together, these results demonstrate that PrP<sub>145</sub> turns

RESULTS

PrP<sub>145</sub> Is Expressed as Two Isoforms at Steady State—On blots immunostained with the anti-PrP antibody 3F4 (to residues 109–112), the normal or cellular prion protein (PrPC) migrates as three bands corresponding to the unglycosylated (U), intermediate (I), and highly glycosylated (H) forms of 27, 33–42 kDa, respectively (Fig. 1A). In contrast, PrP<sub>145</sub> migrates as two bands, a lower band of 14 kDa (PrP<sub>14</sub>) and a higher band of 15.5 kDa (PrP<sub>15.5</sub>) that accounts for 66% of the total PrP<sub>145</sub> (Fig. 1A). As expected, both bands are readily detected with the 3F4 and anti-N antibodies but not with anti-C-terminal antibody (data not shown). Immunoprecipitation of PrP<sub>145</sub> (which includes both PrP<sub>14</sub> and PrP<sub>15.5</sub>) from cells radiolabeled with [35S]methionine and cysteine for 2 h with the 3F4 antibody shows a similar pattern (Fig. 1B), except that PrP<sub>15.5</sub> accounts for only 25% of the total PrP<sub>145</sub>. The cell-associated pool of PrP<sub>145</sub> at steady state is approximately 9 times smaller than that of PrP<sub>C</sub> (Fig. 1A). Moreover, <1% of the PrP<sub>145</sub> is recovered from the medium, suggesting that the low expression of PrP<sub>145</sub> is not because of secretion.

Three experiments were performed to determine whether PrP<sub>15.5</sub> represents a form of PrP<sub>145</sub> with an uncleaved N-terminal signal peptide of 22 amino acids: 1) metabolic labeling with [35S]cysteine, a residue that is present only in the signal peptide; 2) cell-free translation with radiolabeled methionine and cysteine or only cysteine in the absence or presence of microsomes to obtain a translation product with or without the signal peptide, respectively; 3) a short pulse of 30 s with [35S]labeled methionine and cysteine followed by a chase to investigate whether PrP<sub>15.5</sub> converts to the PrP<sub>14</sub> form. Metabolic labeling of cells with cysteine for 2 h shows only PrP<sub>15.5</sub>, whereas both forms are detected when cells are radiolabeled with methionine and cysteine (data not shown). After cell-free translation with radiolabeled methionine and cysteine in the absence of microsomes, only PrP<sub>15.5</sub> is retrieved, most of which is converted to the PrP<sub>14</sub> form when the microsomes are added co-translationally (Fig. 1C). Translation in the presence of radiolabeled cysteine yields only PrP<sub>15.5</sub>, which disappears when microsomes are added, confirming that PrP<sub>15.5</sub> includes the N-terminal signal peptide that is lost on addition of microsomes (Fig. 1C). The bands obtained in vitro co-migrate with PrP<sub>15.5</sub> and PrP<sub>14</sub> from radiolabeled cells (Fig. 1C). The short pulse-chase experiment shows that at the end of a 30-s pulse, PrP<sub>15.5</sub> is predominant and accounts for 55% of the total PrP<sub>145</sub> (Fig. 1D). The ratio between the two forms is reversed at the end of 2.5 min (Fig. 1D), whereas the total PrP<sub>145</sub> remains unchanged during this time period. This finding suggests that PrP<sub>15.5</sub> is converted into the PrP<sub>14</sub> form. Both forms then decrease rapidly during the chase, so that only 16% of total PrP<sub>145</sub> remains after 30 min, most of which consists of PrP<sub>14</sub> (Fig. 1D).

Immunofluorescence analysis by double immunostaining with anti-PrP and an antibody to calnexin, an endoplasmic reticulum (ER)-specific protein, shows that although PrP<sub>C</sub> is mostly distributed at the cell surface and in the Golgi region (Fig. 1E, panel 1) (19), a very small amount of PrP<sub>145</sub> is detected in an intracellular compartment, and it co-stains with the Golgi marker α-mannosidase II (Fig. 1E, panel 3). No ER localization is observed with the ER marker calnexin (Fig. 1E, panel 2), probably because the small quantity of PrP<sub>145</sub> that escapes degradation transits through the ER very rapidly. No PrP<sub>145</sub> is detected on the plasma membrane either by immunostaining (Fig. 1E, panels 2 and 3) or cell surface biotinylation (data not shown), excluding the possibility that PrP<sub>15.5</sub> is inserted into the cell membrane through the signal peptide.

Together, these results show that 1) PrP<sub>145</sub> is unstable and is not detected in the culture medium in significant amounts, 2) PrP<sub>15.5</sub> represents a PrP<sub>145</sub> form with an uncleaved N-terminal signal peptide even though it is apparently translocated efficiently into the ER (see below), 3) no PrP<sub>145</sub> is detected at the cell surface. The very low expression of PrP<sub>145</sub> compared with PrPC suggests that PrP<sub>145</sub> turns over very rapidly. The under-representation of PrP<sub>15.5</sub> after radiolabeling and immunoprecipitation (25%) when compared with the steady state level by Western blot analysis (66%) (Figs. 1A versus B) raises the possibility that this form is not immunoprecipitated efficiently because of a change in its conformation, as previously observed with the PrP<sub>C</sub> Q217R (19) (see below).
over in a pre-Golgi compartment and is not degraded through
the lysosomal pathway.

Because both membrane and secretory proteins can be
degraded through the proteasomal pathway (21–23), we evalu-
ated this possibility by treating cells expressing PrP145 with
the proteasomal inhibitor lactacystin or ALLN during pulse-
chase experiments. In cells treated with lactacystin or ALLN,
48 and 36%, respectively, of PrP145 remain after a 2-h chase, as
compared with 12% in untreated cells (Fig. 2C; *p < 3 \times 10^{-4}
at 1 h, and \( p < 6 \times 10^{-5} \) at 2 h; \( n = 3 \), PrP14 accounts for most of the protected PrP145 (Fig. 2C). There is no change in
the kinetics of turnover of PrP145 in the presence of ALLN or laca-
cystin under the same experimental conditions (data not shown). To check if the PrP145 that accumulates intracellularly following proteasomal inhibition is ubiquitinated, ALLN-
treated cells were immunoprecipitated with 3F4, and the im-
munoprecipitates were immunoblotted with a panel of anti-
ubiquitin antibodies to detect any ubiquitinated PrP forms.
Alternately, PrP145 cells were transfected with normal or a
dominant negative mutant of ubiquitin followed by immuno-
precipitation with 3F4. No ubiquitinated PrP145 was detected
in either case, and the co-expression of mutant ubiquitin did
not stabilize PrP145 (data not shown).

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To evaluate if PrP145 rescued from proteasomal degradatio

FIG. 1. PrP145 is synthesized as two forms, one of which retains the N-terminal signal peptide. A, immunoblotting with the monoclonal
antibody 3F4 to PrP reveals three major PrP145 forms of 33–42 kDa (H), 29–30 kDa (I), and 27 kDa (U). In contrast, PrP15.5 migrates as two bands,
PrP15.5 (15.5 kDa) and PrP14 (14 kDa), respectively, of which PrP15.5 is more prominent. B, radiolabeling of PrP145 and PrP14 for 2 h with
[^35]Smethionine and cysteine followed by immunoprecipitation with 3F4 reveals the same PrP145 and PrP14 pattern as in (A), but the ratio between
PrP14 and PrP15.5 is reversed. C, PrP145 synthesized with radiolabeled methionine and cysteine in a cell-free transcription-translation system in
the absence of microsomes (lane 2) co-migrates with PrP15.5 detected in intact cells (lane 1), confirming that this form includes the signal peptide.
Addition of microsomes results in the 14-kDa signal-cleaved product (PrP14) (lane 3). When translation is performed only in the presence of
radiolabeled cysteine, PrP15.5 is synthesized in the absence of microsomes, as expected (lane 4); on addition of microsomes, the PrP14 form is not
detected because the radiolabel-containing N terminus is lost subsequent to its translocation into the microsomes (lane 5). D, Following labeling
with[^35]Smethionine and cysteine for 0.5 min and immunoprecipitation, PrP15.5 accounts for 55% of the total PrP145. The ratio between the two
forms reverses after a 2.5-min chase, when PrP14 accounts for 60% of the total, consistent with the conversion of PrP15.5 to PrP14 (lane 1 versus
lane 2). After 30 min of chase, only 16% of the total PrP145 remains (lanes 1–6). E, immunofluorescent staining of PrP with 3F4 (green) and of
calnexin, an ER marker (red), shows that PrP145 is localized to the cell surface and the Golgi region (panel 1). Similar staining of PrP145-expressing
cells shows a small amount of PrP145 at steady state, mainly co-localizing with the Golgi marker a-mannosidase II (red; panel 3). No co-localization
of PrP145 is seen with the ER marker calnexin (red; panel 2).
is secreted, cells expressing PrP145 were radiolabeled for 2 h in the continuous presence of lactacystin or ALLN, and the cell lysate and culture medium were subjected to immunoprecipitation with 3F4. Only PrP15.5 was secreted, although the intracellular pool of PrP14 exceeded that of PrP15.5 (Fig. 2D). No PrP145 form was detected in the medium if cells were labeled in the presence of brefeldin A (data not shown).

Accumulated PrP145 Is Aggregated and Less Sensitive to Protease Digestion—To investigate whether the PrP145 that accumulates in the absence of proteasomal degradation is aggregated, untreated cells and cells treated with lactacystin for 2 h were lysed in a buffer containing the nonionic detergents Nonidet P-40 and sodium deoxycholate, conventionally used for detecting aggregated PrP. After pelleting the cell debris (P1) by a low speed centrifugation, the supernatant (S1) was centrifuged at 100,000 x g for 1 h to separate a soluble (S2) and an insoluble (P2) fraction. In untreated cells, almost all of the PrP145 is recovered in the high speed supernatant or soluble (S2) fraction, and a small amount of PrP15.5 is detected in the P2 fraction (Fig. 3A, upper panel). In lactacystin-treated cells, the amount of PrP145 is at least 4-fold higher, and a significant proportion of it is present in the pellet (P2) as an insoluble fraction. This fraction contains mostly PrP15.5 (Fig. 3A, lower panel) and increases in amount with extended chase time (data not shown). The insolubility of PrP15.5 and inefficient immunoprecipitation especially in the presence of proteasomal inhibitors was further confirmed when we subjected untreated and lactacystin-treated cell lysates to immunoprecipitation and immunoblotted the remaining supernatant with the same antibody. In untreated cells, virtually no PrP14 or 15.5 could be detected in the supernatant, whereas in lactacystin- and ALLN-treated cells, significant amounts of mostly PrP15.5 remained in the supernatant with nonimmunoprecipitated proteins. However, when the lysates were boiled in the presence of SDS before immunoprecipitation, no PrP15.5 remained in the supernatant in treated or untreated cells (data not shown). These findings may explain the preferential detection of PrP15.5 in immunoblots as compared with pulse-labeled immunoprecipitates (see Figs. 1, A and B) if the presence of the signal peptide induces a change in its conformation that leads to inefficient immunoprecipitation. None of the PrP145 forms (PrP14 or 15.5) were associated with any of the ER chaperones, Grp78, Grp94, or calnexin (data not shown).

The sensitivity to PK of the PrP145 that accumulates follow-
The Y145stop mutation in the human prion protein gene, PRNP, is associated with a GSS variant of prion disease. Previous attempts to generate a model of this GSS variant in transgenic mice and transfected cells have failed because no expression of the mutant PrP145 could be detected in these models (18, 24). We now demonstrate that in a transfected cell model, PrP145 is expressed in two truncated forms, one of which conserves the signal peptide. Both forms are unstable and are rapidly degraded through the proteasomal pathway. However, both accumulate in significant quantities in intracellular compartments and become aggregated and weakly protease-resistant when proteasomal degradation is impaired. These findings
may resolve the dilemma posed by the previous models. They also widen the spectrum of pathogenetic mechanisms that may be involved in prion diseases and provide novel avenues of investigation toward the understanding of this puzzling GSS variant.

The Uncleaved Signal Peptide Predisposes PrP_{145} to Aggregation—Inefficient cleavage of the N-terminal signal peptide because of naturally occurring mutations within the signal has been shown to be pathogenic in various conditions, but it is unprecedented in prion diseases (25–28). This “proform” appears to accumulate intracellularly and tends to aggregate more readily than the signal-cleaved form. The inefficient immunoprecipitation of this form is probably because of a change in conformation of its “soluble” pool, in addition to the formation of detergent-insoluble aggregates (see below). Thus, under normal experimental conditions, the amount of the signal peptide containing PrP_{145} recovered in immunoblots is more than four times the amount recovered after immunoprecipitation. After lactacystin treatment, the signal peptide containing PrP_{145} accounts for almost all of the detergent-insoluble and weakly protease-resistant aggregates that accumulate intracellularly. With continuous lactacystin treatment, both the signal-uncleaved and -cleaved forms are secreted into the medium through a brefeldin A-sensitive pathway, although the signal-uncleaved form comprises the major secreted form. The preferential detection of the latter in the medium could be because of its greater stability. Thus, both forms translocate into the ER lumen, and the signal-uncleaved form is not inserted in the lipid bilayer through the signal peptide. This conclusion is consistent with the lack of detectable PrP_{145} on the cell surface by either immunofluorescence or biotinylation. None of the PrP_{145} forms were found to be bound to any of the major ER-specific chaperones, either in the presence or absence of lactacystin.

PrP_{145} Is Degraded by the Proteasome—The PrP_{145} has a half-life of ~10 min and at steady state is nine times less abundant than PrP_{C}. Therefore, it is by far the most unstable of all the forms of mutant PrP we have examined to date (13, 19). The lack of all major post-translational modifications and presence of the signal peptide, both of which target PrP_{145} for rapid degradation and aggregation, easily explain the marked instability of PrP_{145}.

The turnover of both PrP_{145} forms that persists at 15 °C and...
in the presence of brefeldin A point to a pre-Golgi site of degradation. Following inhibition of proteasomal degradation, PrP\textsuperscript{145} accumulates primarily in the ER, Golgi, and in the nucleus, but apparently not in the late endosomes or lysosomes. The precise site of PrP\textsuperscript{145} proteasomal degradation has not been established in this study. PrP\textsuperscript{145} might be degraded by proteasomes on the cytosolic face of the ER membrane, as has been reported for the T-cell receptor \(\alpha\)-chain and other ER luminal and secretory proteins (29–36). It would then accumulate upstream in the secretory pathway in the ER and Golgi and also diffuse to the nucleus from the cytosol when the degradation is blocked. Recently, cytosolic accumulation of two transmembrane proteins, presenilin-1 and cystic fibrosis transmembrane regulator, has been described upon inhibition of proteasomal function (37). We do not observe significant accumulation of PrP\textsuperscript{145} in the cytosol after proteasomal inhibition. Instead, PrP\textsuperscript{145} seems to be specifically targeted to the nucleus by a nuclear localization signal that becomes functional when the carboxyl end of the protein is truncated at residue 145. One type of nuclear localization sequence comprises one or more clusters of basic amino acid residues, which, however, lack tight consensus sequence (38). Interestingly, the N terminus of PrP has a cluster of amino acids (KKRPKP) similar to the SV-40 large T antigen nuclear localization signal (PKKKRKV). Studies are ongoing to establish if this sequence functions as a cryptic nuclear localization signal.

We did not detect ubiquitinated PrP\textsuperscript{145} even though our data prove conclusively that the proteasomal pathway degrades PrP\textsuperscript{145}. Whether PrP\textsuperscript{145} is degraded without ubiquitination as observed for other proteins (29) or is tagged by some other ubiquitin-like protein (39) remains to be determined.

**Applicability of the Present Model to the 145 GSS Human Disease**—The PrP\textsuperscript{145} forms present several unusual characteristics when they are compared with the other mutant PrPs expressed in transfected cells (13, 14, 19). First, they are highly unstable and are for the most part rapidly degraded. Second, when degradation is impaired, they become partially resistant to protease digestion. Third, paradoxically, the PrP\textsuperscript{145} is more protease-resistant in the dispersed than in the aggregated form and, in this form, the protease-resistant fragments include the intact N terminus, whereas a 1.5–2-kDa sequence located at the C terminus remains protease-sensitive. This contrasts with the data from the other transfected cell models of inherited prion diseases in which the mutant PrP spontaneously becomes protease-resistant, the protease-resistant fraction is present only in the aggregated form, and the protease-resistant core includes residues \(\sim 90–231\) (14, 19, 41). The present findings argue that also the N-terminal region of PrP, including the signal peptide, may aggregate and become weakly resistant to proteases. Moreover, they indicate that because PrP\textsuperscript{145} is also resistant to protease treatment in the dispersed state, the protease resistance is not exclusively because of aggregation but to other mechanisms such as, for example, the presence of a protective ligand or the adoption of a \(\beta\)-sheet conformation in a monomeric or oligomeric state. A recent report supports this assumption (47). It has also been recently shown that the PrP 121–231 C terminus segment can adopt a \(\beta\)-sheet conformation at acidic pH, independently of other segments (40).

The salient histopathological features of the human Y145stop variant of GSS are the widespread PrP amyloid deposits in vessels and parenchyma of brain and the presence of intraneuronal fibrillary inclusions called neurofibrillary tangles, whereas spongiform degeneration is lacking (7, 11, 12). The amyloid deposits have been shown to immunostain with antibodies raised to the N-terminal 25 amino acids of PrP, indicating the presence of N-terminal fragment(s) of PrP\textsuperscript{145} (11). In addition, an N and C terminus-truncated \(\sim 7.5\)-kDa PrP fragment has been detected in monomeric and oligomeric...
forms, which by epitope mapping is believed to include amino acids 90–147 (12). A ~7.5-kDa PrP fragment has also been isolated from the amyloid deposits of other GSS variants associated with PRNP point mutations, and it has been found to be the only PK-resistant PrP form recovered from the brain when spongiform degeneration is absent (8, 10, 42). In the P102L GSS variant, the ~7.5-kDa fragment has been shown to span residues 78–82 to residues 147–150 by sequence and mass spectrophotometric analyses (42). Therefore, the 7.5-kDa fragment present in the amyloid deposits of Y145stop GSS variant is likely to include residues ~80 to ~145 and to be the only major PK-resistant PrP fragment present in the brain parenchyma of subjects affected by this disease.

It is not immediately evident how the findings of the human disease and the present findings can be reconciled. We did not find a 7.5-kDa PrP fragment or any fragment of smaller size. Data obtained from cell models of inherited prion diseases have been compared with those obtained from brains affected by the corresponding disease in a previous study (13). It was found that although the cell model does not form a PK-resistant PrP comparable with that of the disease, it reproduces the metabolic changes occurring in the mutant PrP in the brain (13). Therefore, it is possible to postulate that PrP14 and 15.5 forms are expressed in the brain of the subjects carrying the Y145stop PRNP mutation and are in large amount cleared through the proteasomal pathway. Effective proteasomal degradation of PrP145 along with the presence of the PrPβ encoded by the normal allele may prevent the expression of disease until adult age. However, a decrease in proteasomal function with advanced age or the low but continuous intracellular accumulation and secretion of the aggregated and weakly PK resistant PrP145 would result in the formation of the highly amyloidogenic ~7.5-kDa PrP fragment and formation of amyloid deposits. Future studies of Y145stop GSS variant-affected brains should search for the presence and distribution of the PrP14 and 15.5 forms. It would be important to determine whether PrP145 is present in aggregated and weakly PK-resistant form and whether some of it is located inside the nucleus. These findings would provide indirect evidence that proteasomal degradation is impaired in the human disease.

Other neurodegenerative diseases have also been shown to involve the proteasome. Recently, it has been shown that the proteasomal system participates in the metabolism of amyloid β peptide, the main component of the amyloid accumulating in Alzheimer’s disease (33). The presence of PrPβ in the nucleus also provides an interesting analogy with a group of inherited neurodegenerative diseases, which include Huntington’s chorea and forms of cerebellar ataxia. In each of these diseases, the presence of polyglutamine repeat expansions leads the mutated protein to adopt a β-sheet structure and to form insoluble, ubiquitinated aggregates in the nucleus (43–46), consistent with proteasomal involvement in these diseases as well. Studies aimed at evaluating changes in proteasomal function with advancing age will provide important information regarding the role of this organelle in the pathogenesis of these disorders and potential therapeutic approaches.

**Acknowledgments**—We thank Dr. A. Tartakoff (Case Western Reserve University) for helpful discussions and a critical evaluation of the manuscript. We also thank S. Bowen for secretarial help, Diane Kofsky and Anuradha Arora for technical help, and Dr. Kristin Defe and Dr. J. Anderson for use of the confocal microscope.