The chaperone protein BiP binds to a mutant prion protein and mediates its degradation by the proteasome

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Running title: BiP mediated proteasomal degradation of mutant PrP.
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Summary

Familial prion diseases are thought to result from a change in structure of the mutant prion protein (PrP), which takes a pathogenic conformation. We have examined the role of molecular chaperones in the folding of normal and mutant PrP Q217R (PrP217) in transfected neuroblastoma cells. In a previous report we showed that although most of the PrP217 forms escape the endoplasmic reticulum (ER) quality control system and aggregate in post-Golgi compartments, a significant proportion of PrP217 retains the C-terminal glycosylphosphatidylinositol signal peptide (PrP32), and does not exit the ER (1). We have now studied the folding and turnover of PrP32 to understand the mechanism by which abnormal PrP forms cause cellular toxicity in our cell culture model, and in the human brain carrying the GSS Q217R mutation. In this report, we show that PrP32 remains associated with the chaperone BiP for an abnormally prolonged period of time, and is degraded by the proteasomal pathway. This study is the first demonstration that BiP is chaperoning the folding of PrP, and plays a role in maintaining the quality control in the PrP maturation pathway. Our data provides new insight into the diverse pathways of mutant PrP metabolism and neurotoxicity.
Introduction

Prion diseases or transmissible spongiform encephalopathies are neurodegenerative disorders that can be sporadic, inherited, or acquired by infection (2, 3). The underlying pathogenetic event in all prion diseases is a conformational modification of the normal or cellular prion protein (PrP<sup>C</sup>) from a soluble form with a predominant \( \alpha \)-helical conformation to the pathogenic form that is aggregated, rich in \( \beta \)-sheets, and partially resistant to proteinase-K (PK) digestion (PrP<sup>Sc</sup>) (2, 4). According to the ‘protein only’ hypothesis, PrP<sup>Sc</sup> interacts directly with the host-encoded PrP<sup>C</sup>, which is then converted to PrP<sup>Sc</sup>. PrP<sup>Sc</sup> accumulates in the nervous tissue, and when it reaches a critical threshold, causes disease (2).

Several lines of evidence indicate that the PrP<sup>C</sup> to PrP<sup>Sc</sup> conversion is mediated by chaperone(s) (5). Molecular chaperones Hsp104 and GroEL have been shown to promote the conversion reaction of mammalian PrP<sup>C</sup> in a cell-free system and the conversion of prion-like proteins in intact yeast cells (6-8). Several chemical chaperones have been shown to act as conversion inhibitors (6). Data from transgenic animal models argue for the requirement of an accessory protein for the refolding of PrP<sup>C</sup> to PrP<sup>Sc</sup>. This protein, temporarily named protein X, remains to be identified (2), and may very well be a cellular chaperone.

In the inherited forms of human prion diseases, which include Gerstmann-Sträussler-Scheinker (GSS) disease, Creutzfeldt-Jakob disease (CJD) and fatal familial insomnia, mutations in the prion protein gene (PRNP) are believed to destabilize the mutant PrP, which then easily converts to PrP<sup>Sc</sup> (2, 4). Our series of studies using human neuroblastoma cell models of inherited prion diseases are indeed consistent with this hypothesis (1, 9). Although these models do not lead to the spontaneous formation of PrP<sup>Sc</sup>, they show that PrP variants carrying a human pathogenic mutation either become insoluble and are trapped in intracellular compartments (1), or are...
rapidly degraded through the proteasomal system (9). If proteasomal function is impaired, the mutant PrP accumulates in an insoluble and weakly PK-resistant form in the endoplasmic reticulum (ER) and post-ER compartments (1, 9).

These findings argue that chaperones play a double role in inherited prion diseases. They are likely to be involved not only in the conversion of PrP to PrP<sub>Sc</sub> as in other forms of prion diseases (6), but may also play a role in the destabilizing effect that the PRNP mutations have on the mutant PrP. Interaction of mutant PrP with chaperones might promote its degradation and delay the conversion to PrP<sub>Sc</sub>, helping to explain the conundrum of why inherited prion diseases often manifest clinically only late in life although the mutation is present from conception.

To begin to unravel the role of chaperones in prion diseases, PRNP-transfected neuroblastoma cells were used to examine the role of ER-localized molecular chaperones in the processing of normal and mutant PrP. The mutation at PRNP codon 217 resulting in the substitution of glutamine to arginine (Q217R) in the mutant PrP (PrP<sup>217</sup>) is associated with a GSS disease phenotype that presents in the seventh decade and is characterized by a slowly progressive dementia associated with cerebellar and extrapyramidal signs (10). The histopathological hallmark of the Q217R GSS variant is the presence of PrP<sup>217</sup> containing amyloid deposits and neurofibrillary degeneration in the brain tissue (10). In a previous study carried out on a transfected neuroblastoma cell model of GSS Q217R, we showed that a significant proportion of PrP<sup>217</sup>, which we identified as PrP32, retains the anchor C-terminal signal peptide, fails to acquire the glycosyl phosphatidylinositol (GPI) anchor, and is retained in the ER by the cellular quality control system. In this study, we provide the first evidence that the ER chaperone BiP binds to PrP. It binds transiently to PrP<sub>C</sub> and to some forms of PrP<sup>217</sup>, but it remains associated for an extended period of time to PrP32, which is subsequently degraded by the proteasomal
pathway. At variance with the anchored forms of PrP\textsuperscript{217} that are aggregated, insoluble in non-ionic detergents, and partially resistant to proteinase-K (PK) digestion (1), a relatively large portion of PrP32 remains soluble and sensitive to PK digestion. We postulate that the association of PrP32 with BiP sequesters PrP32 and prevents the formation of homoaggregates, maintaining PrP32 relatively soluble and PK-sensitive.
Experimental procedures

Materials, cell culture conditions, and production of transfected cell lines: The human neuroblastoma cell line M17 was obtained from Dr. J Beidler (Memorial Sloan-Kettering Cancer Center, New York). Opti-MEM, fetal bovine serum, penicillin/streptomycin, methionine and cysteine-free Dulbecco’s modified Eagle’s medium (DMEM), and Lipofectamine were from Life Technologies Inc.; hygromycin B and lactacystin were from Calbiochem; Trans\textsuperscript{35}S-label was from ICN (Costa Mesa, CA); protein A-agarose, N-glycosidase-F and endoglycosidase-H were from Boehringer Mannheim (Indianapolis, IN). All other chemicals were purchased from Sigma. Transfected M17 cells expressing wild type or mutant prion protein (Q217R) were generated as described (1). The endogenous PrP in M17 cells is expressed at very low levels, and is barely detectable by Western blotting and immunoprecipitation, whereas the transfected wild type and mutant PrP expression is at least ten-fold higher. All cultures were maintained at 37\textdegree C in OptiMEM supplemented with 5% fetal calf serum and penicillin-streptomycin, in a humidified atmosphere containing 5% CO\textsubscript{2}. Cultures of transfected cells were supplemented with 500\mu g/ml of hygromycin. Experiments were performed on bulk-selected cells at different times post-transfection. For all experiments, cells were re-plated overnight and used at 90-95% confluency. Plasmids containing normal and mutant (T34G) hamster BiP cDNA were a kind gift of Linda Hendershot (St. Jude’s Children’s Research Hospital, Memphis) and James Gaut (University of Michigan). The following antibodies were used in this study: anti-KDEL (a monoclonal antibody to the peptide KDEL that recognizes Grp94, BiP, and two other proteins of 64 and 47kDa), anti-hamster BiP, anti-calreticulin, and anti-Grp94 from StressGen, anti-PrP (3F4), a monoclonal antibody to PrP residues 109-112 from R. Kascasak (New York State Institute for Basic Research in Developmental Disabilities), anti-calnexin rabbit immune serum from A. Helenius (Swiss
Federal Institute of Technology, Zurich), and anti-GPI-SP mouse serum raised in our facility against a synthetic peptide representing the GPI-SP of PrP.

**Metabolic labeling and immunoprecipitation:** In a typical experiment, $9 \times 10^6$ cells were used for each cell line. Immunoprecipitation was performed essentially as described (1, 9), with the following modifications. For co-immunoprecipitation experiments for the detection of PrP-chaperone complexes, cells were radiolabeled with 50$\mu$Ci/ml of Trans$^{35}$S-label for two hours in methionine-cysteine-free DMEM with 5% dialyzed serum, washed with PBS, and lysed in a buffer containing 2% Triton X-100 in Tris-buffered saline (TBS; Tris 20mM, NaCl 150mM, pH 7.4), containing a cocktail of protease inhibitors. For co-immunoprecipitation with anti-BiP, lysates were depleted of ATP by incubation with 50units/ml of Apyrase (Sigma) for 10 minutes prior to immunoprecipitation. Cell debris was cleared by centrifugation at 800xg, and the clarified cell lysates 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 bound to protein-A-agarose were washed four times with 0.5 ml of wash buffer (150 mM NaCl, 10 mM Tris-HCl pH 7.8, 0.1% N-lauryl sarcosine and 0.1 mM PMSF), and bound protein was eluted by boiling in sample buffer (Tris-HCl pH6.8, 3% SDS, 10% glycerol, 5% β-mercaptoethanol). For re-immunoprecipitation with the second antibody, eluted protein was diluted 10-fold in lysis buffer and subjected to immunoprecipitation as above. Both the first and second immunoprecipitates were analyzed by SDS-PAGE-fluorography. For pulse-chase experiments, cells were pre-incubated in the presence or absence of the indicated inhibitors (lactacystin 80$\mu$M, ALLN 80$\mu$M) for one hour before labeling with 0.166 $\mu$Ci/ml of Trans$^{35}$S-label in methionine-cysteine-free DMEM containing 5% dialyzed serum. Where indicated, appropriate inhibitors were included in the labeling and chase medium. At the indicated times,
cells were washed with PBS, lysed, and subjected to immunoprecipitation as above.

**SDS-PAGE and Western Blotting:** For detecting co-immunoprecipitated BiP by Western blotting, radiolabeled cell lysates of PrPC and PrP217 cells were subjected to immunoprecipitation with the anti-GPI-SP antibody. Proteins were eluted from protein-A-agarose beads by sample buffer, and fractionated by SDS-PAGE in duplicate. One half of the gel was processed for fluorography, whereas the other half was electrophoretically transferred to Immobilon-P (Millipore) for 2.5 hours at 70 volts at 4°C. Membranes containing transferred proteins were blocked in TBS containing 10% non-fat dry milk and 0.1% Tween-20 for 1 hour at 37°C, and probed with the anti-KDEL antibody (diluted 1:1000) in dilution buffer (TBS, 1% normal goat serum and 0.05% BSA). Immunoreactive bands were detected with anti-mouse antibody conjugated to HRP (diluted 1:4000), and visualized on an autoradiographic film by ECL (Amersham).

**Cell homogenization and Enzymatic deglycosylation:** PrP217 cells were radiolabeled in the absence or presence of ALLN for 2 hours, and the membrane and soluble fractions were separated by standard procedures. Briefly, cells were washed and homogenized on ice in the same buffer containing 10mM HEPES (pH7.9), 1.5mM MgCl2, 10mM KCl, and 0.5mM DTT by 10 strokes of a Kontes all glass Dounce homogenizer. The homogenate was checked microscopically for cell lysis and centrifuged to pellet nuclei. The resulting supernatant was centrifuged at 100,000g to separate the membrane and soluble fractions, which were supplemented with 1% Triton X-100, 1% BSA, 0.1% N-laurylsarcosine, and subjected to immunoprecipitation with anti-KDEL or 3F4 antibodies.

For deglycosylation, radiolabeled, immunoprecipitated proteins were re-precipitated with 5 volumes of cold methanol and re-suspended in denaturing buffer (0.5% SDS, 1% β-
mercaptoethanol). Samples were boiled for 10 minutes and deglycosylated with PNGase-F (1000 units in 1% NP-40, 25mM sodium phosphate, pH 7.5), or endoglycosidase-H (1000 units in 50mM sodium citrate, pH 5.5) for 1-3 hours at 37°C. Proteins were re-precipitated with 5 volumes of cold methanol at -20°C for 2 hours, dissolved in sample buffer, and resolved by SDS-PAGE-fluorography.

**Cell permeabilization with SLO and proteinase-K treatment:** Permeabilization of cells with streptolysin-O (SLO) was performed as described (11). In short, 4 units/ml of SLO were activated in a buffer containing 115mM KOAc, 25mM HEPES pH 7.4, 2.5mM MgCl₂, and 10mM DTT at 37°C for 30 minutes. Cells were then washed with transport buffer (115mM KOAc, 25mM HEPES pH 7.4, 1mM CaCl₂, 0.5mM MgCl₂), and incubated with 3ml of activated SLO for 30 minutes on ice. The cells were washed three times with cold transport buffer, and incubated at 37°C for 15 minutes in the same buffer for pore formation. Optimum permeabilization was assessed by determining lactate dehydrogenase (LDH) activity released into the transport buffer (70% release was considered optimal).

**Confocal immunofluorescence microscopy:** For immunofluorescent staining, PrP⁰ or PrP²¹⁷ cells were grown on poly-D-lysine coated glass coverslips, and either cultured in complete medium (control), or treated with ALLN in the same medium for two hours. One sample of each was permeabilized with SLO as described above. Untreated, and ALLN treated SLO-permeabilized cells were fixed in 4% paraformaldehyde for 30 minutes at room temperature, and processed for immunostaining. Another set of ALLN treated samples was fixed as above, and permeabilized with 0.1% Triton X-100 for 4 minutes. Both SLO- and Triton-permeabilized cells were processed for immunostaining with anti-PrP antibody 3F4 (1:20 dilution) followed by FITC-conjugated anti-mouse secondary antibody (1:20 dilution) (green), and anti-calnexin C-
terminus immune serum (1:40 dilution) followed by RITC-conjugated anti-rabbit antibody (1:25 dilution) (red). Samples were then observed using a Laser Scanning Confocal Microscope (BioRad).
**Results**

*Highly abnormal forms of PrP*$_{217}$* are retained in the endoplasmic reticulum and are associated with the chaperone BiP*

As we have reported in a previous study, mature PrP$^C$ migrates as three distinct bands, which correspond to the PrP$^C$ with two, one, and no glycans respectively. In contrast, PrP*$_{217}$* shows an additional band of 32kDa (PrP32), which migrates at ~30kDa (rather than at 27kDa as PrP$^C$) following deglycosylation (Figure 1, lane 3 vs 6). We have previously shown that PrP32 lacks the GPI anchor and retains the 22 amino acid GPI C-terminal signal peptide (GPI-SP) (1). Furthermore, PrP32, as well as two other forms of PrP*$_{217}$* migrating at 29-30kDa, remain Endo-H sensitive, indicating that they are not transported out of the ER (Figure 1, lane 5).

To determine if the PrP32 that is retained in the ER is bound to a molecular chaperone, Trans$^{35}$S-methionine labeled PrP$^C$ and PrP*$_{217}$* cells were lysed under non-denaturing conditions and immunoprecipitated with anti-KDEL (Figure 2A, lanes 1 and 3), or anti-PrP antibody 3F4 (Figure 2A, lanes 2 and 4) prior to analysis by SDS-PAGE. Anti-KDEL is a monoclonal antibody raised to the carboxy-terminal ER-retention signal KDEL of proteins, and reacts with BiP (Grp78), Grp94, and Hsp47. After immunoprecipitation with anti-KDEL, four major bands are detected in both PrP$^C$ and PrP*$_{217}$* lysates, which include a band of 94kDa corresponding to Grp94 (not shown), a 78kDa band (BiP), and two additional bands of 64kDa and 47kDa (Figure 2A, lane 1). However, in the PrP*$_{217}$* lysates, an additional band that migrates at 32kDa is detected (Figure 2A, lane 3). Immunoprecipitation of lysates with the anti-PrP antibody 3F4 shows that only the 32kDa PrP isoform of PrP*$_{217}$* lysates co-migrates with a similar form detected in anti-KDEL immunoprecipitates (Figure 2A, lanes 4 and 3 respectively). To characterize this 32kDa band immunoprecipitated by the anti-KDEL antibody, anti-KDEL immunoprecipitates from both
PrP^C and PrP^{217} lysates were re-immunoprecipitated with two anti-PrP antibodies: one directed to the conserved sequence of PrP, the other to the C-terminal GPI-SP (anti-GPI-SP), which we have previously shown to be uncleaved in PrP32 (1). With both antibodies, only the 32kDa band is recovered from PrP^{217} immunoprecipitates, while no bands are recovered from the PrP^C preparation (Figures 2A and B, lanes 5 and 6, and 2, respectively). Furthermore, when PrP^C and PrP^{217} cell lysates are immunoprecipitated with an anti-PrP antibody after prolonged radiolabeling, BiP is recovered, along with the various PrP forms, but only from the PrP^{217} preparations (Figure 2A, lanes 7 vs. 8). Combined, these experiments clearly show that the 32kDa band recovered following immunoprecipitation with antibodies to KDEL contains PrP32, and that PrP32 is associated with BiP in the Q217R transfected neuroblastoma cells. Finally, immunoblotting of proteins eluted from the GPI-SP immunoprecipitates with the anti-KDEL antibody identified only the 78kDa BiP band (Figure 2B, lane 3). Immunoprecipitation under similar conditions with anti-calnexin, anti-Grp94 or anti-calreticulin antibodies did not co-immunoprecipitate PrP32 or any other isoform of PrP^C or PrP^{217} (data not shown). Therefore, PrP32 is associated with BiP, but not with any of the other ER chaperones.

These data were reproduced using PrP^C and PrP^{217} cells that had been re-transfected with a construct expressing hamster BiP. Following immunoprecipitation of radiolabeled lysates from these cells with the anti-hamster BiP antibody, a band co-migrating with PrP32 is recovered from the PrP^{217} lysates (Figure 2C, lanes 3 and 4), while no bands co-migrating with any of the PrP^C forms are detected from the PrP^C preparations (Figure 2C, lanes 1 and 2).

**PrP32 is degraded by the proteasomal pathway**

Pulse-chase analysis of PrP^{217} following immunoprecipitation with an anti-PrP antibody shows that with no treatment, about 40% of the PrP32 present at zero time following a ten-minute pulse
remains after one hour of chase (Figures 3A, lanes 1-3, and 3B). In contrast, in the presence of ALLN, an inhibitor of proteasomal degradation, all of the PrP32 and three other bands of ~27-30kDa are recovered after the same chase time (Figures 3A, lanes 4-6, and 3B). Highly glycosylated PrP forms of 32-42kDa are also present, that probably represent glycosylated 27-30kDa forms rescued from degradation by ALLN (lanes 5 and 6). Similar results are obtained when immunoprecipitation is carried out under the same pulse-chase conditions with the antibody to KDEL, rather than to PrP (Figures 3A, lanes 7-12, and 3B). In the absence of ALLN, only 23% of PrP32 is recovered after one hour of chase (Figure 3A, lanes 7-9). In the presence of ALLN PrP32 is not degraded and the 27-30kDa bands are also detected (Figures 3A, lanes 10-12, and 3B). In experiments with a longer chase in the presence of ALLN, a significant amount of PrP32 could be co-immunoprecipitated with the anti-KDEL antibody even after 6 hours (data not shown). Thus, PrP32 remains associated with BiP for an abnormally prolonged period of time, and is degraded by the proteasomal pathway (Figure 3B).

Separation of the membrane bound and soluble PrP217 fractions shows that while all the PrP217 glycoforms and 76% of PrP32 are detected in the membrane-rich pellet fraction, a small amount of PrP32 is recovered in the membrane free supernatant (Figure 3C, lanes 1 and 2). Following ALLN treatment, the amount of PrP32 in the pellet increases by only 2.4%, whereas the fraction detected in the supernatant shows a significant increase of 30% compared to untreated samples (Figure 3C, lanes 1 vs 3 and 2 vs 4). Moreover, an additional form of ~28kDa is detected in the soluble fraction in the presence of ALLN (Figure 3C, lane 4) which may represent an intermediate product of PrP32 generated during proteasomal degradation. This form is unglycosylated, since it is insensitive to PNGase treatment (Figure 3, lanes 5 and 6). Thus, soluble, membrane-free forms of PrP32 increase after proteasomal inhibition, indicating that
PrP32 is retrotranslocated out of the ER. We did not detect ubiquitination of PrP32, or of the other PrP32-related forms (Data not shown).

We used immunofluorescence to determine whether PrP32 or its degradation product(s) accumulate in the cytosol and form an aggresome-like structure following proteasomal inhibition (Figure 4). Control and ALLN treated PrPC and PrP217 cells were treated with streptolysin-O (SLO) that selectively permeabilizes the plasma membrane (11), leaving all intracellular membrane organelles intact. Thus, the externally added antibodies have access only to cytosolic proteins and to epitopes of proteins exposed on the cytosolic side of the membranes. Following SLO treatment, PrPC and PrP217 are detected only at the surface of control and ALLN-treated cells (green), whereas immunoreactivity to the C-terminus of calnexin which is located at the cytosolic side of the ER membrane, is readily detected (red) (Figure 4, panels 1-4). In contrast, following treatment with detergent that permeabilizes all cellular membranes, a small amount of PrPC is observed in the Golgi region, while PrP217 is present in much larger quantity, and for the most part co-distributes with the ER (panels 5 and 6). Therefore, following inhibition of proteasomal degradation, PrP32 does not form an aggresome-like structure in the cytosol, as has been reported for other secretory proteins degraded by the proteasome (12, 13).
Discussion

BiP has been shown to promote proper folding of newly synthesized polypeptides by keeping the precursors in a soluble state (12, 14-17). It interacts transiently with proteins, which presumably are properly folded. In contrast, it binds misfolded or unassembled proteins in relatively stable complexes and may mediate their retrograde translocation for proteasomal degradation (18-26). Our data provide the first evidence that BiP plays these roles in the processing of PrP. BiP briefly interacts in the ER with PrP\textsuperscript{C} (Figure 3A; unpublished observations). This brief interaction is consistent with BiP involvement in the folding of nascent PrP\textsuperscript{C}, which, presumably, promptly assumes the correct folding state. BiP interaction with the PrP carrying the Q217R mutation is more complex. In our neuroblastoma cell model, this mutant PrP is expressed in two major variants. The first variant, here referred to as PrP\textsuperscript{217}, includes three glycoforms and carries a GPI anchor as PrP\textsuperscript{C}. The second variant, PrP\textsuperscript{32}, which accounts for approximately 11% of the total Q217R mutant PrP, is glycosylated but lacks the GPI anchor and carries the uncleaved GPI anchor signal peptide (1). Unlike PrP\textsuperscript{C}, PrP\textsuperscript{217} is unstable and approximately 50% of it becomes insoluble and aggregates in a compartment distal to the \textit{cis}-Golgi. Furthermore, the instability leading to aggregation is very likely due to misfolding since it is largely corrected by exposure of the nascent PrP\textsuperscript{217} to low temperatures known to favor correct folding (1). However, despite the likely misfolding, PrP\textsuperscript{217} interacts with BiP only briefly in a way that is indistinguishable from that of PrP\textsuperscript{C}, and, like PrP\textsuperscript{C}, exits the ER (1). In contrast, BiP interaction with PrP\textsuperscript{32} is sustained and results in the retention of this form in the ER, but eventually PrP\textsuperscript{32} is translocated into the cytosol and degraded in the proteasomal system. Only BiP, among a variety of protein folding chaperones present in the ER, shows this association with PrP\textsuperscript{32}. The mechanism leading to the different BiP interaction and cellular routing between PrP\textsuperscript{217} and PrP\textsuperscript{32} is unclear. It might be
due to the lack of the GPI anchor and/or the presence of the GPI signal peptide that makes it impossible for PrP32 to enter the secretory pathway (27-32). Other mechanisms are also possible. For example, the different BiP binding might be related to intrinsic conformational differences between the two Q217R PrP variants. The conformation of some of the mutant PrP might be altered to such an extent that the anchor signal peptide cannot be cleaved, blocking the anchor addition and resulting in the formation of the PrP32 variant. Alternatively, the sustained association of PrP32 with BiP by itself might interfere with the anchor addition process. Regardless of the mechanism, it is remarkable that two major types of Q217R mutant PrP, PrP217 and PrP32, interact differently with BiP, and following this interaction are processed through different routes. PrP217 aggregates in a post-Golgi compartment, most likely the endosomal-lysosomal system, and eventually is degraded since it does not accumulate. In contrast, PrP32 remains largely soluble, and is degraded by the proteasome. However, inhibition of proteasomal function, which has no effect on PrP217, results in increased amounts of PrP32 in the ER and the cytosol. Presumably, the impairment of the endosomal-lysosomal degradation leads to the accumulation of PrP217. Thus, each of the two forms of Q217R mutant PrP has the potential to perturb the cellular metabolism, but by distinct mechanisms.

We have previously shown that another mutant PrP caused by the presence of a stop codon at PRNP position 145 (PrP145) is also degraded by the proteasomal pathway (9). PrP145 is C-terminus truncated, lacks the anchor and is associated with a GSS phenotype. Therefore, proteasomal degradation is shared by mutant PrP variants lacking the GPI anchor. However, unlike PrP32, PrP145 is not bound to BiP, perhaps because part or the whole C-terminal region is needed for this binding.
In addition to PrP, several other secretory and membrane proteins implicated in causing disease are degraded by the proteasomal pathway when they are mutated, and aggregate in intracellular compartments following the inhibition of proteasomal function (12, 25, 26, 33-37). The proteins implicated in neurodegeneration include huntingtin that forms intranuclear aggregates in Huntington’s disease, ataxins 1 and 3 associated with forms of spinocerebellar ataxia (38-41), and α-synuclein, the major component of the cytoplasmic Lewy bodies in Parkinson’s disease (42). The central theme in these disorders, as in prion diseases, is the conversion of a normally soluble and functional protein into a β-sheet rich structure that is insoluble, aggregates, and forms intracellular deposits. Contrary to previous assumptions, it appears now that intranuclear aggregates of huntingtin may have a protective role rather than causing neuronal damage (40, 41). Mutant huntingtin has been shown to be toxic in the absence of aggregates (41) and it is argued that by capturing the toxic protein the aggregate may protect the cell from this toxicity. Thus, the neurotoxic cascade might start before the formation of aggregates, possibly by soluble intermediates that escape degradation (43). Our results on PrP32 are consistent with this scenario. Although BiP prevents the aggregation and facilitates degradation of PrP32, it may in fact render PrP32 more pathogenic by maintaining it in a partially unfolded state in the ER, especially if BiP promotes the re-folding of PrP32 into an alternative, more pathogenic form, instead of its aggregation when proteasomal function is compromised.

In conclusion, the present finding that the two mutant PrP variants generated by the Q217R PRNP mutation are processed through entirely different metabolic pathways underscores the complexity and diversity of the pathogenic perturbation that may be caused by a simple point mutation. Only a detailed study of the metabolism of the mutant protein can bring to light this
complexity, which must be understood before the optimal therapeutic intervention can be established.

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References


Figure legends

Figure 1. Highly abnormal forms of PrP\textsuperscript{217} are retained in the endoplasmic reticulum: PrP\textsuperscript{C} or PrP\textsuperscript{217} cells radiolabeled for 2 hours were immunoprecipitated with the anti-PrP antibody 3F4, and analyzed by fluorography. PrP\textsuperscript{C} lysates show three distinct bands corresponding to the three glycoforms of PrP: a highly glycosylated diffuse band of 33-42kDa, intermediate bands of 29 and 30kDa with one or two glycans, and a discrete band of 27kDa representing the unglycosylated form of PrP (lane 1). PrP\textsuperscript{217} shows a band of 32kDa (PrP32) in addition to the three glycoforms observed in PrP\textsuperscript{C} (lane 4, marked\textsuperscript{*}). As expected, all PrP\textsuperscript{C} and PrP\textsuperscript{217} forms co-migrate with the 27kDa form upon deglycosylation with N-glycosidase-F except PrP32, which migrates at ~30kDa (lanes 3 vs 6). Treatment of lysates with Endoglycosidase-H shows that all the PrP\textsuperscript{C} forms are resistant to cleavage (lane 2). In contrast, PrP32 and the PrP\textsuperscript{217} bands migrating at ~29-30kDa remain Endo-H sensitive, suggesting that they are not transported from the ER (lane 5).

Figure 2. PrP32 is associated with the chaperone BiP in the endoplasmic reticulum: (A) Cells expressing PrP\textsuperscript{C} or PrP\textsuperscript{217} were radiolabeled for two hours, and immunoprecipitated with either anti-KDEL (lanes 1 and 3), or anti-PrP (lanes 2 and 4) antibodies. Fluorography shows that only PrP32 co-immunoprecipitates with anti-KDEL, and co-migrates with a similar form detected in anti-PrP immunoprecipitates (lanes 3 and 4). No PrP\textsuperscript{C} forms co-immunoprecipitate with anti-KDEL (lanes 1 and 2). Proteins immunoprecipitated from PrP\textsuperscript{C} and PrP\textsuperscript{217} lysates with anti-KDEL were eluted from agarose beads, and re-immunoprecipitated with anti-PrP. No protein band is detected in the PrP\textsuperscript{C} sample (lane 5), whereas from PrP\textsuperscript{217}, only PrP32 is re-immunoprecipitated (lane 6). Over-exposed anti-PrP immunoprecipitates of PrP\textsuperscript{C} and PrP\textsuperscript{217} lysates show that only a 78kDa band corresponding to BiP co-immunoprecipitates with PrP\textsuperscript{217}.
(lanes 7 and 8). (B) Radiolabeled PrP<sup>C</sup> and PrP<sup>217</sup> cells were immunoprecipitated with anti-GPI-SP antibody and fractionated by SDS-PAGE in duplicate. One set of samples was analyzed by fluorography, whereas the other set was transblotted and probed with anti-KDEL antibody. No PrP form is detected in PrP<sup>C</sup> immunoprecipitates, and only PrP32 is detected in the PrP<sup>217</sup> sample (lanes 1 and 2). Immunoblotting with anti-KDEL shows only the 78kDa BiP band in the PrP<sup>217</sup> immunoprecipitates (lane 3). (C) PrP<sup>C</sup> and PrP<sup>217</sup> cells were transfected with a plasmid encoding hamster BiP, and transient transfectants were radiolabeled for 2 hours and immunoprecipitated with anti-hamster-BiP antibody. PrP32 is detected in the PrP<sup>217</sup> immunoprecipitates (lanes 3 and 4), but not in PrP<sup>C</sup> samples (lanes 1 and 2).

**Figure 3. PrP32 is degraded by the proteasomes:** (A) PrP<sup>217</sup> cells were either left untreated, or pre-incubated with ALLN for 1 hour, and radiolabeled for 10 minutes. Lysates were immunoprecipitated with either anti-PrP (lanes 1-6) or anti-KDEL (lanes 7-12) after a chase for the indicated times in the absence or presence of the same inhibitor. Following the pulse (T-0), PrP<sup>217</sup> glycoforms representing the unglycosylated (27kDa) and partially glycosylated (29 and 30kDa) forms, and PrP32 are seen in both ALLN untreated and treated samples (lanes 1 and 4). After a chase of 30 minutes (T-0.5), as expected, the highly glycosylated forms of PrP appear (lanes 2 and 5). While all the PrP<sup>217</sup> forms degrade slowly in untreated samples and only ~40% of PrP32 remains after 1 hour of chase (lanes 1-3), following treatment with ALLN, almost all of PrP32 remains. Furthermore, additional forms of 27-30kDa, and highly glycosylated forms of 32-42kDa are detected (lanes 4-6). Immunoprecipitation with the anti-KDEL antibody shows similar kinetics of degradation of PrP32 without and with ALLN treatment, and demonstrates an additional band of 30kDa (lanes 7-12). (B) The numerical data are expressed as percent of initial PrP32 synthesized at different chase times in the absence or presence of ALLN, and plotted as
the mean +/- S.D. (n=3). (Significance: 0.5hr, p<4^{-3}; 1h, p<5^{-3}). (C) From PrP^{217} cells radiolabeled for 2 hours in the absence or presence of ALLN, membrane and cytosolic fractions were isolated, immunoprecipitated with anti-PrP antibody, and analyzed by fluorography. While all the PrP^{217} glycoforms partition in the membrane fraction (P), a small amount of PrP32 is detected in the soluble fraction (S) (lanes 1 and 2). After treatment with ALLN, this fraction increases by 30% (lanes 2 vs 4), and an additional band of ~28kDa is detected (lane 4). The faster migrating 28kDa band is unglycosylated since it is insensitive to PNGase, as opposed to PrP32 which migrates at 30kDa after deglycosylation (lanes 5 and 6).

**Figure 4: PrP32 rescued from proteasomal degradation does not aggregate in the cytosol:**

PrP^{C} and PrP^{217} cells cultured in the absence or presence of ALLN for 2 hours were permeabilized with SLO and immunostained with anti-PrP (green) or anti-calnexin C-terminus antibody (red) in the absence or presence of detergent. In SLO-permeabilized PrP^{C} and PrP^{217} cells, only surface PrP staining is detected in both control and ALLN treated samples (panels 1-4). No intracellular PrP staining is seen. In the presence of detergent, PrP^{C} staining can be detected on the cell surface and in the Golgi area after ALLN treatment (panel 5). PrP^{217} cells show additional PrP immunoreactivity in the ER and other intracellular membrane organelles (panel 6). Immunoreactivity to the C-terminus of calnexin (red) is seen as a reticular ER pattern in all samples as expected, indicating that cytosolic proteins are accessible in both sets of samples.
Figure 1

A gel electrophoresis experiment showing the effects of Endo-H and PNGase on PrP and PrP217. Lane 1 and 2 show the untreated samples, while Lane 3 to 6 show the treated samples. The results indicate that Endo-H and PNGase have different effects on the two proteins, with Endo-H having a more pronounced effect on PrP217.
### Figure 2

#### A

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Figure 3
Figure 4

PrPC  PrP^{217}

1  2

3  4

5  6

SLO (+ALLN)
SLO (-ALLN)
SLO +Tx-100 (+ALLN)
The chaperone protein BiP binds to a mutant prion protein and mediates its degradation by the proteasome
Taocong Jin, Yaping Gu, Gianluigui Zanusso, ManSun Sy, Anil Kumar, Mark Cohen, Pierluigi Gambetti and Neena Singh

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