Calpain and Other Cytosolic Proteases Can Contribute to the Degradation of Retro-translocated Prion Protein in the Cytosol*

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Xinhe Wang‡, Fei Wang‡, Man-Sun Sy§, and Jiyan Ma‡¶

From the ‡Department of Molecular and Cellular Biochemistry, The Ohio State University, Columbus, Ohio 43210 and the §Institute of Pathology and Department of Neurosciences, Case Western Reserve University School of Medicine, Cleveland, Ohio 44120

PrP, a cell surface-localized N-linked glycoprotein, is required for the pathogenesis of prion diseases. Recent studies have revealed that prion protein (PrP) becomes neurotoxic and prone to aggregation when it is in the cytosol, suggesting that cytosolic PrP may play a role in the pathogenesis of prion disease. Retro-translocation of PrP from the endoplasmic reticulum to the cytosol for proteasome degradation offers a natural route for PrP to enter the cytosol, but whether PrP is subject to retro-translocation is controversial. In this study, we investigated the metabolism of endogenous wild-type PrP in several cell lines and in primary mouse cortical neurons. Our results suggest that a portion of the endogenous wild-type PrP is retro-translocated to the cytosol and degraded by the proteasome. Moreover, we also found that calpain and other cytosolic proteases could degrade PrP in the cytosol when the proteasome activity is compromised. These results provide the foundation for the hypothesis that cytosolic PrP may be involved in the pathogenesis of prion disease.

Prion diseases are a unique group of neurodegenerative disorders that can be sporadic, dominantly inheritable, or infectious. The “protein-only” model postulates that PrPSc, the misfolded form of normal prion protein (PrP0), is the infectious agent that can convert PrP0 to PrPSc in a self-templated reaction (1). Indeed, PrPSc is the major component of the infectious agent and is found only in infected tissues (1, 2). The central role of PrP in the transmission of prion diseases is supported by a variety of experimental evidence (1, 3, 4).

Aside from the infectious property of PrP, extensive efforts have focused on elucidating the mechanism of neurodegeneration in prion disease (5, 6). Accumulated evidence revealed the contribution of normal host PrPc to the neurodegenerative process in prion disease. Using a neurografting technique, it has been shown that PrPSc itself is not toxic to neurons that do not express PrPc (7). This conclusion is further supported by a recent study using conditional PrP knockout mice. Despite continuous accumulation of PrPSc in these mouse brains, depletion of PrPc in neurons after prion infection not only stops the development of neurodegeneration but also reverses the neuropathology (8). Because PrPc is expressed abundantly in the brains of all mammals (9, 10), the neurotoxic form of PrP has to be an abnormal form of host PrPc. Several forms of PrP have been implicated as the neurotoxic species in prion diseases, including transmembrane PrP (11, 12), cross-linked PrP (13), and cytosolically localized PrP (14). Further investigation of these candidates is essential to identify which form of PrP is responsible for the neurodegeneration in prion disease or whether different neurotoxic PrP forms are responsible for different types of prion disease.

Expression of PrP in the cytosol, instead of its normal plasma membrane-associated form, has been shown to cause rapid and extensive degeneration of cerebellar granular neurons in transgenic mice (14). This neuropathological phenotype is similar to that of transgenic mice expressing a disease-associated PrP mutant (15). Moreover, in cell culture experiments, a significant portion of PrP in the reducing cytosolic environment gains a conformation similar to PrPSc, being highly aggregated with a signature proteinase K-resistant pattern (16, 17). This observation is also consistent with results from biochemical studies of PrP folding, in which reducing conditions can fold recombinant PrP into an aggregated, proteinase K-resistant, amyloidogenic conformation (18–20). Based on these findings, we have proposed that cytosolic PrP is the neurotoxic species in prion disease, and it may also play a role in the initial formation of the PrPSc conformation in sporadic and inherited prion diseases.

As a glycosylphosphatidylinositol-anchored protein, PrP is co-translationally translocated into the lumen of endoplasmic reticulum (ER).1 Thereafter, it travels within the secretory pathway and is not exposed to the reducing environment in the cytosol (21). Retro-translocation of misfolded PrP from the ER to the cytosol for proteasomal degradation has been proposed as a natural route for PrP to enter the cytosol. But whether PrP is subject to retro-translocation and proteasome degradation has been controversial (22, 23). Accumulation of wild-type and mutant PrP during proteasome inhibition has been observed in various cell lines and in human primary neurons (24–28). Because many of these studies used transfected cells expressing PrP under the control of cytomegalovirus promoter, a recent study argued that the accumulation of PrP results from an artifact of increased expression of the cytomegalovirus promoter under proteasome inhibition (29).

Given the central role of PrP retro-translocation to the cytosolic PrP hypothesis, we performed a detailed analysis of endogenous wild-type PrP in several cell lines and in primary mouse neurons. We found that a portion of PrP is retro-translocated to the cytosol for degradation by the proteasome. Moreover, we also found that other cytosolic proteases can contribute to the degradation of retro-translocated PrP when

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1 The abbreviations used are: ER, endoplasmic reticulum; PNGase F, peptide-N-glycosidase F; Endo H, endoglycosidase H; HSF, heat shock transcription factor.

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‡ To whom correspondence should be addressed. Tel.: 614-688-0408; Fax: 614-292-4118; E-mail: ma.131@osu.edu.

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proteosome activity is compromised. These results support the model that cytosolic PrP may be involved in the pathogenesis of prion disease.

EXPERIMENTAL PROCEDURES

Cell Lines and Cell Culture—PC3M is a metastatic subline of PC3 human prostate cancer cell line (30). PC3M cells were cultured in RPMI 1640 with 10% fetal bovine serum, 10 mM HEPES, pH 7.2, 2 mM glutamine, and penicillin-streptomycin. DU145 cells were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, 10 mM HEPES, pH 7.2, 2 mM glutamine, and penicillin-streptomycin. NT2 cells were cultured in Opti-MEM with 5% fetal bovine serum, and penicillin-streptomycin. All of the cell culture media and reagents were purchased from Invitrogen.

Treatment of Cells and Immunoblot Analysis—All of the cells used for further analyses were cultured in 6-well tissue culture plates. The cells were cultured in a humidified incubator at 37 °C in 5% CO₂. After cells reached 90% confluence, the media were exchanged with labeling medium (Dulbecco’s modified Eagle’s medium without 10% fetal bovine serum, 10 mM HEPES, pH 7.2, 2 mM glutamine, and penicillin-streptomycin). The cells were incubated with labeling medium (Dulbecco’s modified Eagle’s medium without 10% fetal bovine serum, 10 mM HEPES, pH 7.2, 2 mM glutamine, and penicillin-streptomycin). NT2 cells were cultured in Opti-MEM with 5% fetal bovine serum, and penicillin-streptomycin. All of the cell culture media and reagents were purchased from Invitrogen.

When the cells reached 90% confluence, pulse-chase analysis was performed. The cells were incubated with labeling medium (Dulbecco’s modified Eagle’s medium without Met and Cys; Invitrogen) for 30 min at 37 °C in a CO₂ tissue culture incubator. Afterward, the media were replaced with labeling media containing Tran35S-Label (200 μCi/ml; MP Biomedicals Inc.) with or without 50 μM MG132. After chasing for indicated time, the cells were washed with phosphate-buffered saline and lysed in 1 ml of lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 2% SDS). To denature all proteins in the lysate, 1.5 ml of 8% guanidinium HCl (Sigma) was added to 1 ml of lysate, and all of the proteins were precipitated with 4 volumes of cold methanol (−20 °C). Precipitated proteins were sonicated into 1 ml of DLPC buffer (4.2 mg/ml l-α-phosphatidylcholine, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2% sarcosyl). PrP was immunoprecipitated with 3F4 antibody and protein A-conjugated Sepharose 4B beads (Sigma). The beads were washed twice with buffer A (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 0.5% deoxycholate, 0.5% sarcosyl), twice with buffer B (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.1% Triton X-100, 0.05% deoxycholate), and once with buffer C (50 mM Tris-HCl, pH 7.5, 0.5% Triton X-100, 0.05% deoxycholate). PrP was released from the beads by boiling in SDS sample buffer for 10 min and separated on a 12% SDS-PAGE. The signals were detected and quantified by a Storm phosphor imaging system. 

Primary Mouse Cortical Neuronal Culture—Frozen mouse cortical neurons were purchased from QBM Cell Science. These neurons were cultured exactly according to the protocol supplied by the manufacturer. 

Subcellular Fractionation Using Iodixanol Gradient—PC3M cells were cultured in 10-cm plates and treatments were performed when cells were 90% confluent. The culture media were replaced with fresh complete medium, 10% FBS, 10% HEPES, and 10% penicillin-streptomycin supplements were purchased from Invitrogen as suggested by the manufacturer. These neurons were brought up and cultured for 7 days before the experiments were performed.

RESULTS

Accumulation of Unglycosylated and Aggregated PrP upon Proteasome Inhibition—To determine whether PrP is subject to retro-translocation to the cytosol for degradation by the proteasome, we analyzed endogenous wild-type PrP in a human prostate cancer cell line, PC3M. These cells express high levels of PrP that is fully glycosylated, soluble in mild detergents, and localized primarily to the plasma membrane (Ref. 31 and data not shown). When the proteasome inhibitor MG132 was added to these cells, PrP started to accumulate. Notably, the majority of accumulated PrP is unglycosylated and insoluble with mild detergent extraction (Fig. 1A). After 8 h of proteasome inhibition, the level of soluble, fully glycosylated PrP remained about the same level as that of control cells (Fig. 1B). In contrast, the level of unglycosylated PrP increased to about 4-fold that of control cells (Fig. 1B). After 24 h of proteasome inhibition, the level of soluble and fully glycosylated PrP was significantly decreased, presumably because of the effect of long term proteasome inhibition. How-
ever, the accumulation of unglycosylated PrP was dramatically increased (Fig. 1B), with the total unglycosylated PrP about 6-fold of that in control cells, reflecting continuous accumulation of PrP destined for proteasome degradation. Because many N-terminally truncated PrP fragments are present in normal cells (32), it is possible that the accumulated PrP could be a PrP fragment instead of full-length PrP. To differentiate between these two possibilities, we performed immunoblot analysis with 8B4 monoclonal antibody (Fig. 1C). This antibody recognizes an epitope at amino acid 37–44 of PrP, which is removed in almost all N-terminally truncated PrP fragments (32, 33). In the supernatant fraction, the signal detected by 8B4 antibody was much weaker comparing to the signal detected by 3F4 antibody (comparing the supernatant fractions between panels A and C of Fig. 1), indicating that most of the soluble and fully glycosylated PrP in PC3M cells was N-terminally truncated PrP fragments. However, the PrP in the pellet fraction was clearly detected by the 8B4 antibody (Fig. 1C, pellet). The presence of 8B4 epitope indicated that the PrP accumulated upon proteasome inhibition contains full-length PrP.

**Moderate Increase of Endogenous PrP mRNA Levels upon Proteasome Inhibition**—To ensure that the accumulation of unglycosylated PrP is not due to an increase in PrP expression, we performed an RNase protection assay to measure the mRNA levels of PrP in PC3M cells treated with proteasome inhibitor. The probe against cyclophilin was included in the assay as an internal control for total RNA levels. Upon proteasome inhibition, we detected a small but consistent increase of PrP mRNA level. After 8 h of MG132 treatment, PrP mRNA
Standard deviations are reflected by error bars. Level of endogenous PrP mRNA increases upon proteasome inhibition. A, RNase protection analysis of PrP mRNA levels in PC3M cells treated with 50 μM MG132 for indicated times. Endogenous cyclophillin mRNA levels were used as an internal control. B, quantification of PrP mRNA levels treated with 50 μM MG132. The y axis represents the fold of changes after MG132 treatment with control samples set as 1-fold. Standard deviations are reflected by error bars.

Accumulation of Unglycosylated PrP Results from Inhibition of Its Degradation—An alternative explanation of unglycosylated and aggregated PrP accumulation would be that these PrP molecules were destined to the proteasome for degradation. To test this possibility, we performed pulse-chase analysis in PC3M cells with 3P4 anti-PrP antibody. Two pitfalls could have potentially interfered with pulse-chase analysis of retrotranslocated PrP. First, the pulse labeling generally is performed in a short time period, such as 30 min. Even with the PC3M cells that express a very high amount of endogenous wild-type PrP, only about 5% of total PrP accumulated after 4 h of continuous proteasome inhibition (Fig. 1), and only about 15% of total PrP accumulated after 8 h of proteasome inhibition. (Quantification was performed using a Storm phosphorus imaging system.) Therefore, short pulse labeling will not accumulate detectable amount of PrP molecules destined to proteasome degradation. Second, the majority of the accumulated PrP is insoluble in mild detergents (Fig. 1). Immunoprecipitation of aggregated proteins is generally inefficient, and these proteins tend to be removed during the preclar centrifugation step of immunoprecipitation. To overcome these two potential pitfalls, we pulse-labeled cell in the presence or absence of the proteasome inhibitor MG132 for 6 h and chased under the same condition for 12 or 18 h. After cell lysis, all of the proteins were denatured with 4.8 M guanidinium HCl and precipitated with methanol. A buffer used previously to solubilize aggregated PrP (34) was used to solubilize methanol-precipitated proteins. In cells treated with MG132, a band migrated at the position of unglycosylated PrP was clearly detectable after pulse labeling (Fig. 3A, indicated by an arrow). This band did not appear in the control sample that was treated with MG132, pulse-labeled, and immunoprecipitated under the same procedure except without incubation with anti-PrP antibody (data not shown), suggesting that it represents the accumulated unglycosylated PrP after proteasome inhibition. After 12 h of chase, majority of the fully glycosylated PrP were degraded in control cells (Fig. 3, A and B), consistent with previous reports that PrP is a short-lived protein (34, 35). In MG132-treated cells, the majority of the fully glycosylated PrP was also turned over after 12 h of chase (Fig. 3A, indicated by a bracket). In contrast, the portion of accumulated unglycosylated PrP in the same MG132-treated samples remained even after 18 h chase (Fig. 3A, bands indicated by a arrow). Taken together, these results suggest that the accumulation of unglycosylated PrP results from the inhibition of proteasome degradation rather than increased expression. Moreover, the normal turnover rate of fully glycosylated PrP in MG132-treated cells indicated that proteasome inhibition did not change the metabolism of PrP molecules going through the secretory pathway as previously reported (29).

Cytosolic Localization of Accumulated PrP upon Proteasome Inhibition—In mammalian cells, signal peptides are generally very efficient in directing the co-translational translocation of protein into the ER lumen. If the increase of PrP levels upon
proteasome inhibition results from increasing PrP expression, accumulated PrP, even if aggregated, should be within the secretory pathway and associated with membrane. To determine the subcellular localization of accumulated PrP, we used a discontinuous iodixanol gradient to separate membrane fractions from cytosolic proteins. In this gradient, membrane fractions will flow to the top of the gradient, whereas cytosolic protein will remain in the sample-loading region. The successful separation of membrane and cytosolic fractions was confirmed by the enrichment of endogenous PrP and a membrane-bound chaperone, calnexin, in the top three fractions of the gradient (Fig 4A, Control). The cytosolic chaperone protein Hsp70 was used to identify fractions containing cytosolic proteins (the bottom five fractions). A very small amount of Hsp70 co-migrated with the membrane fraction (Fig. 4A, fraction 2), reflecting its association with membrane-bound co-chaperones (as reviewed in Ref. 36). When the homogenate from cells treated with MG132 was applied to the same gradient, the fully glycosylated and membrane-bound PrP still migrated to the top three fractions as well as calnexin (Fig. 4B). However, accumulated unglycosylated PrP migrated in the same fractions as cytosolic chaperone Hsp70, demonstrating that these PrP molecules are localized in the cytosol (Fig. 4iB). Cytosolic accumulation of unglycosylated PrP further supports that these PrP molecules are destined to the proteasome for degradation.

**Accumulated PrP Has Been Proteolytically Processed in the ER**—Cytosolic accumulated PrP could result from PrP molecules that has been in the lumen of ER, misfolded, and retrotranslocated. Alternatively, it may also come from PrP molecules that never enter the ER because of some aberrant effects of proteasome inhibition. To differentiate between these two possibilities, we took advantage of the fact that two proteolytic cleavages occur when PrP is in the lumen of ER, that is, cleavage of the N-terminal 22-amino acid signal peptide and cleavage of the C-terminal 22 amino acids for addition of the glycosylphosphatidylinositol anchor (21). Had accumulated PrP been in the lumen of ER, it should be through all these modifications and have the same molecular weight as that of deglycosylated wild-type PrP that has traveled through ER to the cell surface. To compare the size of accumulated PrP with deglycosylated PrP, we included control N2a cell line (moPrP cell line, N2a cells stably transfected with mouse PrP carrying a 3F4 epitope) that has been used in previous studies (14, 37). PrP from moPrP cells was sensitive to both Endo H and PNGase F digestions (Fig. 5A, compare lanes 1, 3, and 5), typical of N-linked oligosaccharides. PrP from PC3M cells was sensitive to PNGase F digestion but resistant to Endo H digestion (Fig. 5A, compare lanes 1, 3, and 5), characteristic of N-linked oligosaccharides being modified in the Golgi complex. Smaller molecular mass bands (Fig. 5A, lane 5) represented the N-terminal truncated PrP fragments as showed in
signal peptide antibody did not recognize accumulated PrP from PC3M cells even after prolonged exposure (Fig. 6, anti-signal peptide antibody). This result is in accordance with our previous finding that the accumulated PrP from PC3M cells has been proteolytically processed in the lumen of ER. In addition, the fact that the fast migrating species of accumulated PrP in PrP-3T3 cells did not contain the signal peptide (Fig. 6, solid arrow) suggests that this portion of PrP from this stably transfected cell line has also been processed in the lumen of ER. All together, these results suggest that the accumulated PrP has been translocated into the ER, processed, retro-translocated to the cytosol and accumulated in the cytosol when the proteasome is inhibited.

Accumulation of PrP in Different Cell Lines or Treated with Different Proteasome Inhibitors—To rule out the possibility that retro-translocation of PrP is a specific phenomenon of PC3M cell line, we tested two additional cell lines that express detectable levels of endogenous wild-type PrP. DU145 is a human prostate tumor cell line, and NT2 is a human teratocarcinoma cell line that has the characteristics of neuronal progenitor cells (38). The difference in the migration patterns of the soluble fully glycosylated PrP from these two cell lines represents the different modifications of N-linked oligosaccharides of PrP in these two cell lines (Fig. 7). Just as in PC3M cells, the accumulation of unglycosylated PrP in these two cell lines was detectable after 4 h of MG132 treatment and reached a higher level after 8 h of treatment (Fig. 7), and a significant portion of these accumulated PrP was aggregated in mild detergents.

To differentiate whether the accumulation of PrP is truly due to the effect of proteasome inhibition or to an aberrant effect of MG132, we tested several specific proteasome inhibitors. We treated PC3M cells with 10 μM epoxomicin, 10 μM lactacystin, or 50 μM MG132 for 8 h, and accumulation of unglycosylated PrP in the pellet fraction was clearly detected in cells treated with all proteasome inhibitors but not in untreated cells (Fig. 8). Accumulation of PrP in different cell lines and with treatment of different proteasome inhibitors suggests that retro-translocation of PrP to the cytosol for proteasome degradation is a general phenomenon and not an artifact of a particular cell line or a particular proteasome inhibitor.
Additional cytosolic proteases may contribute to cytosolic PrP degradation.

Accumulation of PrP in Primary Mouse Cortical Neurons upon Proteasome Inhibition—Because the main pathological changes during prion disease occur in neurons, retro-translocation of PrP has to occur in neurons for cytosolic PrP to be a valid candidate for the neurotoxic form in prion disease. To test whether PrP in neurons is subject to retro-translocation, we treated mouse cortical neuron primary cultures with or without MG132 or with MG132 plus brefeldin A (an ER stress-inducing agent) (41). Upon proteasome inhibition, there was a clear increase of unglycosylated PrP appearing in the pellet fraction (Fig. 10). Higher amounts of PrP accumulation were detected in cells treated with MG132 plus brefeldin A (Fig. 10), suggesting that brefeldin A increases PrP retro-translocation from the ER. Accumulation of PrP after proteasome inhibition in primary neurons suggests that retro-translocation of PrP occurs in neurons.

**DISCUSSION**

Retro-translocation of PrP from the ER to the cytosol is a natural route for membrane-associated PrP to enter the cytosol. Our results suggest that a portion of endogenous wild-type PrP is retro-translocated to the cytosol for degradation by the proteasome. Moreover, retro-translocated PrP is also subject to the digestion by other cytosolic proteases when the proteasome is inhibited. If escaped proteolysis, accumulated PrP in the cytosol will aggregate, and aggregated PrP molecules are apparently resistant to further degradation.

Whether the cytosolic PrP accumulation after proteasome inhibition results from retro-translocation of PrP from the ER or from the portion of PrP that never enters the ER has been controversial (29). Our previous results suggested that the accumulated PrP in the cytosol is from the PrP from the ER (27). Here, we showed that the accumulated PrP is without the N-terminal signal peptide (Fig. 6). Because the cleavage of the signal peptide occurs in the ER lumen, these PrP molecules must have entered the ER and retro-translocated to the cytosol. In addition, the accumulated PrP has exactly the same size as that of deglycosylated PrP from different cell lines or from mouse brain (Fig. 5), suggesting these molecules have been through the same processing in the lumen of ER. Therefore, we conclude that the cytosolic PrP accumulation in these cells results from PrP molecules that have been in the lumen of ER.

An unexpected finding of this study is the increase of mRNA level of endogenous PrP upon proteasome inhibition. This small but very consistent increase of PrP mRNA levels is likely due to the fact that there are two heat shock transcription factor (HSF)-binding sites in the PrP promoter (42). Because PrP expression is up-regulated during heat shock treatment, these HSF binding sites are obviously functional inside cells (42, 43). Proteasome inhibition is known to increase the half-lives of various short-lived transcription factors, including HSF (44, 45). Increased HSF protein levels may cause the PrP mRNA level increase during proteasome inhibition. But the small increase of PrP mRNA level cannot explain the massive accumulation of unglycosylated and aggregated PrP in the cytosol upon proteasome inhibition. The accumulation of unglycosylated and aggregated PrP results from the accumulation of retro-translocated PrP in the cytosol.

Retro-translocation of protein from ER to the cytosol is a major cellular pathway to remove misfolded and misassembled proteins in the secretory pathway (46). Accumulated evidence suggests that, except for a few transmembrane proteins, most of these proteins are exported from ER through a protein channel (46–48). To fit through the pore of the channel, retro-
translocated proteins are believed to be at least partially unfolded (46, 49). ER resident chaperones have been proposed to participate in recognizing and delivering the substrate to the channel for retro-translocation (49). As a lumenal glycosylphosphatidylinositol-anchored protein, PrP is likely to be retro-translocated through the pore of the protein channel. To pass through this pore, the retro-translocated PrP has to remain unfolded. ER chaperones may play a role in maintaining the unfolded-status of PrP. Indeed, a disease-associated mutant PrP has been found to remain associated with ER chaperone BiP for a prolonged time and degraded by the proteasome (26).

The appearance of unfolded PrP molecules in the cytosol will lead to several consequences explaining our observations. First, it can be a substrate for proteasome degradation as other retro-translocated proteins. Second, when proteasome activity is compromised, unfolded PrP molecules will accumulate in the cytosol, an environment that is not natural for PrP. Therefore, these unfolded PrP molecules will be susceptible to digestion by various cytosolic proteases. Third, the conformational change of an unfolded PrP exposed to the reducing cytosolic environment is similar to the folding of a recombinant PrP under reducing conditions. It has been shown that recombinant PrP will fold into a β-sheet rich, proteinase K-resistant, aggregated conformation under the reducing conditions (18–20). Thus, the majority accumulated PrP becomes aggregated in the reducing cytosolic environment (more than two-thirds of accumulated PrP was aggregated). Fourth, detection of aggregated PrP in cells treated only with very specific proteasome inhibitors (such as epoxomicin) indicates that some PrP molecules can escape other cytosolic proteases digestion. This is likely due to the inaccessibility of cytosolic proteases to these PrP molecules once they are aggregated.

We found that calpain is one of the cytosolic proteases contributing to the degradation of retro-translocated PrP in the

PrP in the pellet or supernatant fractions were detected by immunoblot analysis with 3F4 anti-PrP antibody. B, PC3M cells were treated with different combinations of inhibitors as indicated. Analysis was performed exactly as in A. C, quantification of accumulated PrP in the pellet fractions of PC3M cell treated with different inhibitors as indicated. Standard deviations are reflected by error bars. The asterisk indicates that the difference between cells treated with epoxomicin alone or with epoxomicin plus MDL28170 is statistically significant (p = 0.0035). Concentrations of inhibitors used here: epoxomicin, 10 μM; lactacystin, 10 μM; MG132, 50 μM; NH4Cl, 1 mM; cloroquine, 200 μM; MDL28170, 100 μM.
cytosol when proteasome activity is compromised. The calpain proteases are a family of Ca\(^{2+}\)-activated cysteine proteases localized in the cytosol (50). Interestingly, inhibition of calpain-dependent endoproteolytic cleavage has been shown to inhibit the accumulation of PrP\(^{Sc}\) in scrape-infected cells and reduce infectivity associated with these cells (51). Whether cytosolic PrP plays any role in these calpain-mediated effects is unknown, but the contribution of calpain to the cytosolic PrP degradation could potentially explain these effects. Proteasome activity decreases during prion disease (52). Therefore, it is reasonable to postulate that scrape-infected cells may also have a decreased proteasome activity. Under this circumstance, cytosolic proteases, like calpain, may play a role in removing cytosolic PrP. Inhibition of calpain activity could result in the accumulation of toxic cytosolic PrP molecules. 

In vitro, scrape does not uniformly infect all cells; only some of the cells are infected. Upon calpain inhibition, infected cells would be gradually selected out because of the accumulation of the toxic cytosolic PrP. As a result, an increased number of uninfected cells will be populated in the culture, which will be reflected by reduced PrP\(^{Sc}\) accumulation and infectivity.

In the course of our experiments, we observed increasing cell death (detached cells floating in the media) in primary neurons and neuronal like NT2 cells treated with proteasome inhibitor. Cell death was more obvious when proteasome inhibition lasted for a longer period of time. In contrast, no obvious cell death was observed in PC3M or DU145 cells even after 24 h of proteasome inhibition. These observations seem to correlate neuronal cell death with cytosolic accumulation of PrP. However, proteasome inhibition affects many aspects of cell metabolism, and events other than cytosolic accumulation of PrP could very well be the reason for neuronal cell death. Therefore, further investigation is required to determine whether cultured neurons can recapitulate the neurotoxicity caused by cytosolic PrP in transgenic mice (14).

Retro-translocation of PrP to the cytosol has been observed in human primary neurons by subcellular fractionation (28). Here, we observed accumulation of PrP in primarily cultured mouse cortical neurons with proteasome inhibition. The characteristic features of in vitro cultured neurons may vary depending on what kind of neurons is selected, which procedure is used, and which culture condition is applied. However, observations of PrP retro-translocation by using two different assays in two different kinds of neurons is selected, which procedure is used, and characteristics of retro-translocation by using two different assays in two different kinds of neurons is selected, which procedure is used, and characteristics of retro-translocation by using two different assays in two different kinds of neurons is selected, which procedure is used, and characteristics of retro-translocation by using two different assays in two different kinds of neurons.
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