Inherited prion diseases are linked to mutations in the prion protein (PrP) gene, which favors conversion of PrP into a conformationally altered, pathogenic isoform. The cellular mechanism by which this process causes neurological dysfunction is unknown. It has been proposed that neuronal death can be triggered by accumulation of PrP in the cytosol because of impairment of proteasomal degradation of misfolded PrP molecules retrotranslocated from the endoplasmic reticulum (Ma, J., Wollmann, R., and Lindquist, S. (2002) Science 298, 1781–1785). To test whether this neurotoxic mechanism is operative in inherited prion diseases, we evaluated the effect of proteasome inhibitors on the viability of transfected N2a cells and primary neurons expressing mouse PrP homologues of the D178N and nine octapeptide mutations. We found that the inhibitors caused accumulation of an unglycosylated, aggregated form of PrP exclusively in transfected N2a expressing PrP from the cytomegalovirus promoter. This form contained an uncleaved signal peptide, indicating that it represented polypeptide chains that had failed to translocate into the ER lumen during synthesis, rather than retrogradely translocated PrP. Quantification of N2a viability in the presence of proteasome inhibitors demonstrated that accumulation of this form was not toxic. No evidence of cytosolic PrP was found in cerebellar granule neurons from transgenic mice expressing wild-type PrP, resulting in the absence of PrP in the cytosol. In contrast, no accumulation of PrP in the cytosol was observed that mislocation of PrP in the cytosol is neurotoxic, and argues against the hypothesis that perturbation of PrP metabolism through the proteasomal pathway plays a pathogenic role in prion diseases.
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In this study we have systematically assessed the effect of proteasome inhibitors on the viability of N2a cells that express endogenous PrP or overexpress wild-type or mutant PrP molecules carrying the D177N substitution or a nine-octapeptide insertion (PG14) under the control of the CMV promoter. Additionally, we have analyzed the cellular localization of wild-type, D177N, and PG14 PrP in primary cerebellar granule neurons from Tg mice expressing the protein from its natural promoter, and compared their viability in the presence of proteasome inhibitors to that of neurons from PrP knock-out mice. Our analysis fails to confirm observations made by other laboratories, and argues against the hypothesis that cytosolic PrP plays a primary pathogenic role in inherited prion diseases.

EXPERIMENTAL PROCEDURES

Mice—Production of transgenic mice expressing wild-type and PG14 mouse PrPs tagged with an epitope for the monoclonal antibody 3F4 has been reported previously (19). In this study, we used transgenic mice of the TgWT-ES line (20) that express 4% of the endogenous PrP level, referred throughout the text as Tg(WT), as well as Tg(PG14-A3-V128) mice expressing PG14 PrP at the endogenous level. Tg(D177N) mice expressing 3F4-tagged mouse PrP carrying the D177N/V128 mutation under the control of the mouse PrP promoter were generated as described previously (19). (Detailed characterization of these mice will be presented elsewhere.) For this study, we used Tg(D177N/M128/W129) and Tg(D177N/V128/W129) mouse lines that express transgenic PrP at the endogenous level. All these transgenic lines were originally generated on a C57BL/6J X CBA/J hybrid and were subsequently bred with the Zurich I line of Prnp(+/−) mice (C57BL/6J X 129 background) (20), resulting in animals that express Tg PrP but not endogenous mouse PrP. The Prnp(+/−) mice used in this study were neurologically littermates of Tg(D177N) and Tg(PG14-A3-V128).

Cell Culture—Cerebellar granule neurons were prepared according to the procedure of Miller and Johnson (21), with the exception that cerebella were from mice at postnatal day 6. Briefly, cerebella were dissected, sliced into ~1-mm pieces and incubated in HBSS (Invitrogen, Life Technologies, Inc.) containing 0.3 mg/ml trypsin (Sigma) at 37 °C for 15 min. Trypsin inhibitor (Sigma) was added to a final concentration of 1 mg/ml, and the tissue was mechanically dissociated by passing through a flame-polished Pasteur pipette. Cells were plated at 350–400,000 cells/cm² on poly-L-lysine (0.1 mg/ml)-coated plates. Cells were maintained in Basal Medium Eagle (BME, Invitrogen, Life Technologies, Inc.) supplemented with 10% dialyzed fetal bovine serum (FBS, Sigma), penicillin/streptomycin, and KCl 25 mM (R25 + S), at 37 °C in an atmosphere of 5% CO₂, 95% air. To reduce the number of non-neuronal cells, cladiroblastin (3.3 μg/ml, Sigma) was added to the medium 36 h after plating. Non-neuronal contamination of the cultures was assessed as described, and found to be less than 3% (21).

N2a cells (ATCC CCL-131) were grown in Dulbecco’s modified Eagle’s medium and minimal essential medium α:1 supplemented with 10% FBS, non-essential amino acids and penicillin/streptomycin, and maintained in an atmosphere of 5% CO₂, 95% air. cDNAs encoding wild-type, PG14, D177N, and PG14 derivatives derived from the Prn-pa allele and containing the 3F4 epitope tag (22) were cloned into the pCDNA3 expression plasmid (Invitrogen). N2a cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Two days after transfection, cells were split in complete medium supplemented with the antibiotic G418 (750 μg/ml, Clontech). Resistant colonies were isolated after 2 weeks of exposure to G418, expanded, and tested for moPrP expression by immunoblotting using the 3F4 monoclonal antibody.

Treatment of the Cultures and Quantification of Cell Viability—Cerebellar granule neurons were exposed to proteasome inhibitors 6 days after plating. N2a cells were plated at 20,000 cells/cm² and treated with the inhibitors for the indicated times. Cell viability was assessed by measuring the level of cellular reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to formazan (23). Cells were incubated for 3 h at 37 °C with 0.4 mg/ml MTT, dissolved in 0.04 N HCl in 2-propyl alcohol, and analyzed spectrophotometrically at 540 nm with an automatic microplate reader (Labsystems Multiskan MS).

Antibodies—Monoclonal antibody 3F4 (24) was used at dilutions 1:5,000 for Western blotting and 1:500 for immunofluorescence staining. Polyclonal antibody P45–66 (25), raised against a synthetic peptide encompassing residues 45–66 of mouse PrP (25), was used at 1:2,500 for Western blot. An antibody (anti-SP) that selectively recognizes forms of murine PrP containing an uncleaved signal peptide was used at 1:500 for Western blot (26). Anti-giantin (Covance) and anti-trap (Upstate Biotechnologies) antibodies were used, respectively, at 1:1,000 and 1:1,000 for immunofluorescence staining.

Biochemical Analysis—To assay detergent insolubility, cells were lysed in 10 mM Tris pH 7.5, 100 mM NaCl, 0.5% sodium deoxycholate, and 0.5% Nonidet P-40 containing protease inhibitors (pepsatin and leupeptin, 1 μg/ml; phenylmethylsulfonlfyl fluoride, 0.5 mM; and EDTA, 2 mM). After a brief centrifugation to remove debris, lysates corresponding to 300 μg of protein were centrifuged at 186,000 × g for 40 min in a Beckman Optima Max-E ultracentrifuge. Proteins in the...
pellet and supernant were separated by SDS-PAGE and electrotransferred onto polyvinylidene fluoride membranes (Immobilon P, Millipore). Membranes were incubated with 5% nonfat dry milk in 100 mM Tris pH 7.5, 150 mM NaCl and 0.1% Tween 20 (TTBS). Membranes were then incubated with anti-PrP antibody overnight at 4 °C or 1 h at room temperature, rinsed three times with TTBS and incubated 1 h at room temperature with horseradish peroxidase-conjugated secondary antibody (diluted 1:5000; Santa Cruz Biotechnology). Immunoreactivity was visualized by enhanced chemiluminescence (ECL, Amersham Biosciences).

RESULTS

Transfected N2a Synthesize a Signal Peptide-bearing Form of Cytoplasmic PrP That Is Not Toxic—To investigate the potential neurotoxicity of cytoplasmic accumulation of mutant PrP, we generated stably transfected clones of N2a cells overexpressing D177N or PG14 PrP, and compared their viability in the presence of proteasome inhibitors to that of untransfected or transfected N2a expressing wild-type PrP. In preliminary experiments we investigated the effect of proteasome inhibitors on the metabolism and biochemical properties of PrP. Western blot analysis of N2a cells treated with MG132 for 24 h showed that the inhibitor caused a dramatic change in the distribution of PrP glycoforms, with a striking increase of a low molecular mass band resembling unglycosylated PrP, which at the highest concentration of inhibitor was the only species detected (Fig. 1A, top panel, lanes 2 and 3). This molecular species was induced also by treatment with epoxomicin and ALLN (Fig. 3C and data not shown); however, it was observed only in transfected cells. In untransfected N2a expressing low levels of endogenous PrP this band was not induced, and the inhibitors caused a striking reduction of all PrP glycoforms (Fig. 1A, top panel, lanes 4–6). In transfected cells the effect of proteasome inhibitors on induction of the low molecular mass species was highly variable. In some experiments, the low molecular mass band was the primary PrP species detected (Fig. 1A, top panel, lane 9), while in other experiments there was accumulation of an additional PrP band of higher molecular mass (Figs. 1B and 3, A and C, see description below). The lower molecular species that accumulated upon proteasome inhibition had an apparent mass of ~27 kDa, and was ~2 kDa larger than the mature, unglycosylated form (Fig. 1A, top panel, compare lanes 2 and 3 with lane 1). Its electrophoretic mobility did not change after digestion with either endo H or PNGase F (not shown), indicating that it did not represent an altered glycoform of PrP.

To test whether the 27-kDa band corresponded to the untranslocated form of PrP induced by the proteasome inhibitors in transfected CHO and PC12 cells (16), Western blots of N2a lysates were reacted with an antibody (anti-SP), which selectively recognizes the PrP signal peptide (26). We found that the 27-kDa band was labeled by this antibody (Fig. 1A, lower panel, lanes 2 and 3), indicating that the main form of PrP that accumulated in N2a cells upon treatment with MG132 corresponded to untranslocated polypeptide chains. We noticed that sometimes the anti-SP antibody recognized a doublet of bands. However, this was not a consistent finding even within the same experiment, and was probably caused by post-lysosomal degradation of signal peptide-bearing PrP.

It has been shown that cytoplasmic PrP that accumulates in the presence of proteasome inhibitors is detergent-insoluble and partially protease-resistant (13, 14). Based on this and other pieces of evidence, it was claimed that retrotranslocated PrP is converted to a PrPSc-like isoform in the cytosol through a self-sustained process (27, 28). To test whether this putative scrapie-like isoform represented untranslocated PrP, we subjected the cell lysates to ultracentrifugation at 186,000 × g, and analyzed the proteins in the supernatants and pellets by Western blot with antibody P45-66 (top panel) or anti-signal peptide (α-SP1 antibody (bottom panel)). B, stably transfected N2a cells expressing 3F4-tagged wild-type (lanes 1–6), or D177N/M128 (lanes 7–12) PrP were treated with MG132 at the indicated concentrations for 24 h. After treatment, cells were lysed in a buffer containing non-denaturing detergent, and the cleared lysates were centrifuged at 186,000 × g for 60 min. PrP in the supernatant (S) and pellet (P) fractions was visualized by immunoblotting with antibody 3F4 (top panel) or anti-signal peptide antibody (lower panel). The arrow and asterisk indicate bands corresponding to signal peptide-bearing PrP and endo H-sensitive PrP, respectively. Molecular mass markers are in kilodaltons.
digestion (not shown). Analysis of detergent insolubility revealed that this immature PrP glycoform was also partially aggregated (Fig. 1B, lanes 3–6).

As previously found for D177N PrP expressed in CHO cells (22), this mutant protein was partially insoluble when expressed in N2a cells (Fig. 1B, top panel, lanes 7 and 8). MG132 induced strong accumulation of insoluble PrP species identical to those observed in N2a cells expressing wild-type PrP (lanes 9–12). In some cases, high molecular mass bands were also visible in the pellet fraction (Fig. 1B, top panel, lane 10), probably corresponding to SDS-resistant PrP aggregates, or untranslocated PrP that has been ubiquitinated prior to proteasomal degradation. Analogous results were observed when we analyzed the effect of proteasome inhibitors on N2a cells expressing PG14 PrP, with the difference that because of the nine-octapeptide insertion, the insoluble, signal-peptide bearing form of PrP that accumulated after treatment with the inhibitors had an apparent molecular mass of ~33 kDa (not shown) (16).

To test whether accumulation of untranslocated PrP was cytotoxic, we compared the viability in the presence of proteasome inhibitors of untransfected and transfected N2a expressing either wild-type or mutant PrPs. For these experiments, we used stably transfected cells overexpressing untagged (one clone) or 3F4-tagged (two independent clones) wild-type PrP, as well as two independent clones of each mutant, selected for having similar expression levels. Consistent with our previous findings (29, 30), expression of D177N and PG14 PrPs had no detectable effect on the morphology or viability of N2a cells, indicating that expression of mutant PrP per se was not cytotoxic. Cells were exposed to either 10 or 50 μM MG132, and their viability was assessed after 2–24 h by MTT assay. MG132 induced cell death by apoptosis, as demonstrated by the presence of pyknotic and fragmented nuclei by bisbenzimide staining, and positive TUNEL (TdT-mediated dUTP-X nick-end labeling) staining (not shown). 10 μM MG132 induced time-dependent reduction in cell survival that was similar for all lines, with no statistically significant differences between untransfected and transfected N2a (Fig. 2A). At 50 μM MG132, transfected N2a overexpressing PG14 PrP, and to a lesser extent those transfected with wild-type and D177N PrP, were significantly more resistant to the toxicity of the inhibitor than untransfected cells (Fig. 2B). This protective effect of PrP overexpression was also observed when cells were exposed to ALLN or epoxomicin (Fig. 2C).

It was shown that transient proteasome inhibition with the reversible inhibitor MG132 is sufficient to induce accumulation of insoluble, unglycosylated PrP in the cytosol, which continues for a number of hours after the inhibitor is removed (28). Based on this evidence it was argued that cytosolic PrP once formed is able to nucleate aggregation of additional PrP molecules through a self-sustained process. To test whether formation of untranslocated, cytosolic PrP in the absence of proteasome inhibition was sufficient to trigger cell death, N2a cells overexpressing wild-type PrP were treated with MG132. After 4 h of treatment the inhibitor was removed by replacing the culture medium (wash-out), and cell viability was evaluated after 24 h and compared with that of cells cultured in the continuous presence of MG132. Western blot analysis of cells treated with MG132 for 4 or 24 h showed that the inhibitor induced accumulation of a 27-kDa species corresponding to untranslocated PrP, based on its reactivity to the anti-signal peptide antibody (data not shown), as well as low amount of the higher molecular mass species corresponding to the endo H-sensitive, immature glycoform described above (Fig. 3A, lanes 2 and 3). In contrast to that observed previously (28), we found that accumulation of the unglycosylated 27 kDa species was reversed by removal of the inhibitor (Fig. 3A, lane 4), indicating that its formation required continuous proteasome inhibition. To confirm that accumulation of the abnormal PrP species was dependent on permanent proteasome impairment, we performed the same experiment using the irreversible inhibitor epoxomicin. We found that epoxomicin caused accumulation of the untranslocated and the endo H-sensitive forms of PrP, which continued even after the inhibitor was removed (Fig. 3C, lanes 3 and 4). Evaluation of cell viability demonstrated that removal of the inhibitors significantly rescued N2a cells from death, independently of whether they continued accumulating untranslocated PrP (Fig. 3, B and D), indicating dissociation between accumulation of this cytosolic species and cell death.

PG14 and D177N PrPs Display Altered Intracellular Distri-

tions in Cerebellar Granule Neurons—We have previously reported that PG14 and D177N PrPs expressed in transfected cells are delayed in their export from the ER, and that at the steady state they are present on the cell surface at reduced levels compared with wild-type PrP, and accumulate in intra-

**Fig. 2. Overexpression of wild-type or mutant PrPs protects N2a cells from proteasome inhibitor toxicity.** Stably transfected N2a cells expressing wild-type, D177N, or PG14 PrPs, as well as untransfected cells, were exposed to 10 μM (A) or 50 μM (B) MG132, and cell viability was evaluated after 2–24 h by MTT assay. Data are the mean ± S.E. of 20–42 determinations from 3–6 independent experiments in A; *F w = n.s. (analysis of variance, ANOVA 2 × 6 test); or 12–18 determinations from 3–4 independent experiments in B. *p < 0.05; **p < 0.01 versus untransfected (Tukey-Kramer test). F w = 3.512 p < 0.01 (analysis of variance, ANOVA 2 × 8 test); C: cell viability of N2a cells was evaluated after 24 h of exposure to 100 μM ALLN or 5 μM epoxomicin. Each bar represents the mean ± S.E. of 15–18 determinations from three independent experiments. *, p < 0.05; **, p < 0.01 versus control group (Tukey-Kramer test). Data represent pooled values from experiments performed with three independent transfected N2a lines expressing wild-type PrP, and two lines of each mutant.
cellular compartments, including the ER and Golgi (9, 16). To investigate the distribution of these mutants in primary neurons, we performed immunofluorescence confocal analysis of PrP in cerebellar granule neurons (CGN) from Tg mice. CGN were prepared from the cerebella of 6-day-old mice and maintained in culture for 5–8 days before immunofluorescence staining. To localize PrP, cells were fixed, permeabilized, and stained with antibody 3F4. PrP staining was followed by staining with trap and giantin (Figs. 4 and 5, panels A, B, D, and E). No colocalization was observed between wild-type PrP and the ER marker trap (Fig. 4, panels A, C, E, and I). In some cells a perinuclear distribution of PrP was also observed, which colocalized with the marker giantin, as expected for PrP molecules in transit in the Golgi compartment (Fig. 5, panel I, yellow color). In CGN from Tg(PG14) mice, PrP was barely detectable on the cell membrane and along neurites, and was mainly found in intense perinuclear patches, colocalizing with trap and giantin (Figs. 4 and 5, panels B, F, and L). Low expression on the cell surface and intense intracellular immunofluorescence colocalizing in part with giantin and trap, were observed also in CGN expressing the D177N/V128 mutant (Figs. 4 and 5, panels C, G, and M), although the number of cells displaying this altered intracellular PrP distribution was reduced compared with PG14 neurons. The polymorphic variant D177N/M128 showed more intense surface fluorescence compared with PG14 and D177N/V128 PrPs, and the number of neurons in which D177N/M128 PrP displayed an altered intracellular distribution was lower compared with the other mutants (Figs. 4 and 5, panels D, H, and N). These data indicate that, similar to that observed in transfected cells (7–12, 16, 32), also in primary neurons, the pathogenic mutations alter the trafficking of PrP molecules and cause a portion of them to reside abnormally in intracellular compartments.

Cerebellar Granule Neurons Expressing Either Wild-type or Mutant PrPs Do Not Accumulate Detectable Levels of Cytosolic PrP, nor Are They More Vulnerable to the Toxicity of Proteasome Inhibitors Than PrP Knock-out Neurons—To investigate whether the proteasome inhibitors induced accumulation of insoluble, cytosolic PrP in primary neurons, CGN from Tg mice expressing wild-type, PG14 or D177N PrP, were exposed to lactacystin β-lactone (5 and 10 μM), MG132 (5, 10 and 50 μM), ALLN (150 μM) or epoxomicyn (5 μM) for 24 h. After incubation, detergent extracts of the cells were subjected to ultracentrifugation at 186,000 g for 40 min, and PrP in the soluble and insoluble fractions was visualized by Western blot using the antibody 3F4. As shown in Fig. 6, treatment with the inhibitors did not induce accumulation of the insoluble, unglycosylated form indicative of cytosolic PrP in CGN from Tg(WT) mice (top panel). Mutant PrP molecules expressed in granule neurons from Tg(PG14) and Tg(D177N) mice were partially insoluble, similar to what has been observed when these proteins were expressed in transfected cells or in the cerebral and peripheral tissues of the mice (19, 33, 34). Treatment with the inhibitors caused an increase in the amount of PrP that partitioned in the insoluble fraction (Fig. 6B). However, this involved all PrP glycoforms and was not restricted to the unglycosylated form, as it would be expected if a significant number of mutant PrP molecules accumulated in the cytosol because of abortive translocation or retrograde transport from the ER. Incubation of the blots with the antibody against the PrP signal peptide did not reveal any reactive band, indicating that in primary neurons PrP was efficiently cotranslationally translocated into the ER lumen (not shown). Immunofluorescence confocal analysis with antibody 3F4 demonstrated that treatment with the inhibitors (0.5–25 μM MG132 and lactacystin β-lactone, and 50–250 μM ALLN, for 16 and 24 h) did not induce accumulation of detectable levels of PrP in the cytoplasm of neurons (not shown).

Experiments in transgenic mice support the contention that even very low levels of cytosolic PrP may be sufficient to kill neurons (4). To explore the possibility that undetectable amount of cytosolic PrP was generated in primary neurons, which might be neurotoxic if not efficiently degraded by the proteasome, we analyzed the viability of CGN after proteasome impairment. CGN from Tg(WT), Tg(PG14), Tg(D177N) and PrP knock-out mice were exposed to MG132 or lactacystin β-lactone (0–10 μM), and their viability was evaluated after 24 h of treatment. We found that the susceptibility of neurons expressing either wild-type or mutant PrPs was not significantly different from that of PrP knock-out neurons (Fig. 7, A and B). Although there were not statistically significant differences in the dose-response curves of the different lines, the Tukey-Kramer test revealed statistically significant differences at some of the doses used. To better explore whether this result reflected a biological difference between neurons expressing different types and levels of PrP, we performed additional experiments by exposing CGN to several different doses of the inhibitors (0.1 and 5 μM MG132 and 0.5 and 5 μM lactacystin β-lactone) for different times (8, 16, 24, and 48 h). These analyses failed to highlight consistent differences between CGN that expressed or did not express PrP (data not shown). Moreover, no increased susceptibility of transgenic neurons compared with PrP knock-out cells was observed when we analyzed the effect of two additional proteasome inhibitors (ALLN and epoxomicyn); in fact, consistent with that observed in N2a cells, neurons expressing PrP were significantly more resistant to the toxic effect of these inhibitors than PrP knock-out cells (Fig. 7C).

DISCUSSION

In the present study we have tested the hypothesis that impairment of proteasome function and consequent accumulation of retrotranslocated PrP in the cytosol of neurons may be a primary neurotoxic culprit in prion diseases. The data presented here, in conjunction with our previous observations,
indicate that PrP accumulates in the cytosol exclusively in transfected cells under conditions of elevated PrP expression, mainly because of abortive polypeptide chain translocation during biosynthesis, rather than retrograde transport of abnormally folded molecules from the ER. Our observations also indicate that accumulation of untranslocated PrP in the cytosol is not toxic, and that the effect of proteasome impairment on neuronal viability is independent of PrP expression. These results argue that accumulation of abnormal PrP species in the cytosol is unlikely to play a primary pathogenic role in prion diseases.

We find that treatment with several different proteasome
antibody 3F4. D177N/V128 is shown in the

experiments. Because the percentage of insoluble D177N/M128 and

determined form of PrP in transfected N2a cells. This form is ~2 kDa

larger than mature, unglycosylated PrP and is recognized by an antibody specific for the PrP signal peptide, demonstrating unequivocally that it corresponds to untranslocated PrP molecules that have never entered the ER, rather than retrogradely translocated PrP as previously argued. We find that signal peptide-bearing PrP forms insoluble aggregates, which are resistant to digestion with low concentrations of proteinase-K (PK: total proteins = 1,000–4,000; 30 min at 37 °C) (Fig. 1B and data not shown). This is likely to reflect nonspecific aggregation of unprocessed PrP molecules carrying the N-terminal signal peptide and the C-terminal hydrophobic glycosylphosphatidylinositol addition sequences, rather than re-folding of PrP into a scrapie-like isoform in the cytoplasm. In fact, we find that accumulation of untranslocated PrP requires continuous proteasome impairment, arguing against the possibility that this form of cytosolic PrP folds into a scrapie-like isoform able to nucleate its own aggregation.

We have found that untranslocated PrP accumulates in transfected CHO and PC12 cells exposed to proteasome inhibitors, and that this form resides on the cytoplasmic face of the ER (16). As previously found in CHO and PC12 cells, also in

mouse neuroblastoma N2a untranslocated PrP is induced exclusively when the protein is constitutively synthesized at high level from the heterologous CMV promoter. In untransfected cells and in primary neurons that express PrP from the endogenous promoter, the untranslocated form is not induced, and the amount of endogenous PrP actually decreases following treatment with the proteasome inhibitors, most likely because down-regulation of mRNA and protein synthesis associated with programmed cell death induced by proteasome impairment (21, 35). Thus, the data presented here in conjunction with our previous observations support the conclusion that cytosolic PrP accumulates mainly because of impaired degradation of abortively translocated, signal peptide-bearing molecules synthesized from the heterologous CMV promoter. Consistent with this conclusion, it has been recently reported that the signal sequence of PrP is intrinsically inefficient in initiating translocation into the ER lumen (36).

We also find that treatment of transfected N2a cells with proteasome inhibitors induces accumulation of an immature, endo H-sensitive PrP glycoform, in addition to untranslocated PrP. Accumulation of endo H-sensitive PrP was observed also

FIG. 6. Proteasome inhibitors do not induce unglycosylated, insoluble PrP in cerebellar neurons. A, cerebellar granule neurons derived from Tg(WT), Tg(PG14), and Tg(D177N) mice were treated for 24 h with the vehicle alone (lanes 1 and 2), or with different proteasome inhibitors: 5 μM lactacystin β-lactone (β-Lac, lanes 3 and 4), or MG132 (lanes 5 and 6), and 150 μM ALLN (lanes 7 and 8). After treatment, cells were lysed in a buffer containing non-denaturing detergents, and the cleared lysates were centrifuged at 186,000 g for 40 min. PrP in the supernatants (S) and pellets (P) was visualized by immunoblotting with antibody 3F4. D177N/V128 is shown in the lower panel; similar results were obtained from the analysis of D177N/M128 (not shown). Molecular mass markers are in kilodaltons. B, amount of PrP in the pellet fractions was quantified by densitometric analysis of Western blots similar to the ones shown in A. Data are the mean ± S.E. of 3–5 independent experiments. Because the percentage of insoluble D177N/M128 and D177N/V128 PrP was similar, data from these two lines were pooled.

FIG. 7. Quantitative evaluation of the toxic effect of different proteasome inhibitors on cerebellar granule neurons from transgenic and PrP knock-out mice. Cerebellar granule neurons from Prnp<sup>+</sup> (KO), Tg(WT), Tg(PG14), Tg(D177N/M128), and Tg(D177N/V128) mice were exposed to the indicated concentrations of MG132 (A), or lactacystin β-lactone (B), for 24 h. Cell survival was quantified by MTT assay and expressed as a percentage of values for cells treated with the vehicle. Data are the mean ± S.E. of 12–30 replicates from 3–6 independent experiments. **, p < 0.01 versus KO by Tukey-Kramer test. Because no significant differences were found between D177N/M128 and D177N/V128 neurons, data from these two lines were pooled.

A

PrP

MG132 (μM)

WT

PG14

D177N

0

0.1

0.5

1

2.5

10

% Survival

B

β-Lactone (μM)

WT

PG14

D177N

0

0.1

0.5

1

2.5

10

% Survival

C

ALLN

EPOXOMICIN

1:1,000–4,000; 30 min at 37 °C) (Fig. 1 A–C).

Transfected CHO and PC12 cells expressing PrP from the constitutive CMV promoter, the untranslocated form is induced exclusively when the protein is constitutively synthesized at high level from the heterologous CMV promoter. In untransfected cells and in primary neurons that express PrP from the endogenous promoter, the untranslocated form is not induced, and the amount of endogenous PrP actually decreases following treatment with the proteasome inhibitors, most likely because of down-regulation of mRNA and protein synthesis associated with programmed cell death induced by proteasome impairment (21, 35). Thus, the data presented here in conjunction with our previous observations support the conclusion that cytosolic PrP accumulates mainly because of impaired degradation of abortively translocated, signal peptide-bearing molecules synthesized from the heterologous CMV promoter. Consistent with this conclusion, it has been recently reported that the signal sequence of PrP is intrinsically inefficient in initiating translocation into the ER lumen (36).

We also find that treatment of transfected N2a cells with proteasome inhibitors induces accumulation of an immature, endo H-sensitive PrP glycoform, in addition to untranslocated PrP. Accumulation of endo H-sensitive PrP was observed also

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in COS cells transiently transfected with wild-type or D177N PrP, and was argued to be indicative of molecules physiologically trigated by the ER quality-control system (14). However, analysis of PrP biosynthesis by pulse-chase labeling experiments has shown that maturation of mutant PrP from an endo H-sensitive to an endo H-resistant form is not impaired, even though delayed compared with wild-type PrP (16). Thus, a reasonable explanation for this finding is that endo H-sensitive forms of PrP accumulate at detectable levels only when the biosynthetic and protein trafficking capacity of the cell is exceeded.

In cultured, non-neuronal cells mouse PrP molecules carrying the D177N and nine-apeptide mutations adopt an abnormal conformation soon after synthesis in the ER (6), and at the steady state they appear to accumulate in the ER and Golgi because their transit out of these organelles is delayed (9, 16). Here we provide evidence that the same holds true for mutant PrPs synthesized in primary neurons. We find that PG14, D177N/V128, and D177N/M128 PrPs expressed in cerebellar granule neurons of transgenic mice are distributed in a pattern that overlaps with markers of the ER and Golgi, although we cannot rule out the possibility that some of the mutant protein is present in other cellular locations as well. In contrast, wild-type PrP is mainly distributed on the surface of cell bodies and along the neurites, and only in a small number of neurons it appears concentrated in the Golgi apparatus, as expected for molecules in transit in this compartment (37). We also find that, compared with wild-type PrP, PG14, D177N/V128, and D177N/M128 PrPs are present at lower levels on the surface of cerebellar granule neurons. This altered cell surface distribution of PrP, which was confirmed by immunofluorescence staining of unpermeabilized neurons (data not shown), was especially noticeable for PG14 PrP, which displayed a weaker surface immunofluorescence than D177N. Since all the mutants are expressed at similar levels, as judged by Western blot, the differences documented by immunofluorescence analysis are likely to reflect differences in their cellular trafficking and/or metabolism. We also noticed that D177N/M128 is more expressed on the cell surface than D177N/V128, and that in general D177N PrP displays an altered intracellular localization in a lower number of neurons compared with PG14 PrP. Interestingly, we find that D177N/M128 expressed in the brains of Tg mice is less PK-resistant than D177N/V128, and that both polymorphic variants are less detergent insoluble than PG14 PrP when expressed in the brains of Tg mice and in cultured cerebellar neurons (Fig. 6B).2 Similar differences were observed for PG14 and D177N PrPs synthesized in transfected CHO and BHK cells (9). Because mutant PrP becomes detergent-insoluble and protease-resistant in different locations along the secretory/endocytic pathway (6), it is possible that the effects of a mutation on the trafficking and biochemical properties of PrP are correlated.

In contrast to that observed in N2a cells, in mouse cerebellar neurons the proteasome inhibitors do not cause accumulation of detectable amounts of untranslocated or retrotranslocated PrP. In fact, we fail to detect either mature or signal peptide-bearing, unglycosylated PrP in whole cell lysates of neurons treated with a wide range of reversible or irreversible proteasome inhibitors. Moreover, we could not find unglycosylated PrP in enriched cytosolic fractions of cerebellar neurons treated with the inhibitors alone or in combination with brefeldin A (not shown), a procedure which favors accumulation of proteins in the ER (38), and that allowed other investigators to detect retrotranslocated PrP in cortical mouse and human neurons (18, 39). These results indicate that neither wild-type, nor mutant PrPs are subjected to ERAD in cerebellar granule neurons, and suggest that distinct neuronal cell types may differ in their capacity of trafficking and/or metabolizing PrP. Thus, although mutant PrP molecules misfold early in the secretory pathway (6) and reside longer in the ER of cerebellar neurons (16), they eventually escape the ER quality control system of these cells.

Analysis of detergent insolubility of PrP in cerebellar granule neurons exposed to proteasome inhibitors reveals a significant increase in the amount of mutant PrP that partitions in the insoluble fraction, whereas the solubility of wild-type PrP is unaffected (Fig. 6B). This change in solubility involves all PrP glycoforms and is not restricted to the unglycosylated form, as it would be expected if a significant number of PrP molecules aggregated in the cytosol as a consequence of abortive translocation or retrograde transport from the ER. Thus, increased aggregation of mutant PrPs is likely to be an indirect consequence of proteasome inhibition on PrP metabolism; for instance, the inhibitors may alter the cellular level or localization of molecular chaperons and heat-shock proteins that might modulate aggregation of PrP (40–42).

In contrast to previous observations (4), several pieces of evidence provided here indicate that accumulation of PrP in the cytosol of neuronal cells is not toxic. First, we find that although the proteasome inhibitors cause accumulation of untranslocated PrP in the cytoplasm of transfected N2a cells, their rate of death is not increased compared with untransfected N2a, in which untranslocated PrP is not detected. This evidence contrasts with the report that a line of N2a cells overexpressing PrP died more rapidly than the untransfected parental line when exposed to MG132 (4). The reason for this difference is not clear. However, since we systematically analyzed the viability of a number of independently generated N2a lines, exposed to different doses of MG132, ALLN and epoxomicin, it is likely that if overexpression of PrP consistently enhanced the rate of cell death induced by the inhibitors we should have detect it. Secondly, we find that death of N2a cells induced by epoxomicin is reversed by removal of the inhibitors (Fig. 3D), despite the fact that cells treated with this inhibitor continue accumulating high levels of untranslocated PrP even after the inhibitor has been removed (Fig. 3C). Finally, we fail to detect significant differences in susceptibility to proteasome inhibitors’ toxicity between cerebellar granule neurons from transgenic mice expressing wild-type or mutant PrPs and PrP knock-out mice, arguing against the contention that neuronal death is induced by failure of the proteasome to degrade neurotoxic PrP species.

Our observations are consistent with those of Roucou et al. (18), who showed that expression of signal peptide-lacking cytosolic PrP is not toxic when expressed in a number of different neuronal cell lines, and that microinjection of cDNA constructs encoding wild-type or mutant PrP does not sensitize primary human neurons to the toxicity of epoxomicin. Despite its lack of toxicity in cultured neurons, artificial targeting of PrP to the cytosol by deletion of the N-terminal signal sequence causes selective loss of cerebellar granule neurons in Tg mice (4). Therefore, forced expression of PrP in the cytosol may indeed be toxic to certain neuronal population in vivo. In nontransgenic animals or in Tg mice expressing full-length PrP, however, cytosolic PrP has been observed only in subpopulations of neurons of the hippocampus, neocortex, and thalamus, and its presence was not associated with signs of neurodegeneration (43, 44). This observation, along with the evidence that expression of signal peptide-lacking PrP in cultured neurons protects against Bax-induced apoptosis (18), indicates that cytosolic PrP
is not toxic and may actually perform a normal physiological function in specific neuronal populations (45).

Unexpectedly, we found that expression of wild-type or mutant PrPs exerts a modest but significant neuroprotective effect toward proteasome inhibitor toxicity, which is most evident after long-term treatment with high doses of MG132, ALLN and epoxomicin (Figs. 2, B and C and 7C). Although the physiological function of PrP remains uncertain, several pieces of evidence indicate that PrP may play a role in protecting cells from hypoxic and oxidative injury by increasing antioxidant enzymes activities and glutathione level (46–50), and may protect from several apoptosis-inducing insults, possibly by inhibiting Bax-mediated pathways (18, 51–53). Moreover, several pieces of evidence suggest that PrP may function as a trophic receptor that leads to activation of a neuroprotective state (54–56). Interestingly, neuronal death induced by proteasome inhibitors is associated with increased generation of free radicals, and decreased glutathione levels, as well as with activation of Bax-mediated apoptosis (57, 58), suggesting that the protective effect of PrP against the toxicity of the inhibitors might be caused by the capacity of the protein to act on one or more of these pathways. If this were the case, then our observation that PG14 and D177N PrP exert a neuroprotective effect comparable to that of wild-type PrP (Figs. 2 and 7C) would support the contention that pathogenic mutations do not alter the physiological function of the protein (30, 59).

In conclusion, the data presented here argue that accumulation of PrP in the cytosol and/or perturbation of PrP metabolism through the proteasomal pathway are unlikely to be a general pathogenic mechanism of prion diseases. These findings have clinical implication. It was cautioned that the use of proteasome inhibitors in biomedical research and in clinical settings might increase the risk for development of prion disease (4, 60). The observation that proteasome inhibitors have no effect on the metabolism and biochemical properties of PrP expressed in cultured neurons, and that the neurotoxic effect of these compounds is independent of PrP expression requires a re-evaluation of this warning.

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Proteasome Inhibitors Toxicity and PrP
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