Proteasomes and ubiquitin are involved in the turnover of the wild-type prion protein

Yifat Yedidia, Lior Horonchik, Salit Tzaban, Anat Yanai and Albert Taraboulos¹

Department of Molecular Biology, The Hebrew University-Hadassah Medical School, PO Box 12272, Jerusalem 91120, Israel

¹Corresponding author
e-mail: taraboul@cc.huji.ac.il

Prion diseases propagate by converting a normal glycoprotein of the host, PrP^C, into a pathogenic 'prion' conformation. Several misfolding mutants of PrP^C are degraded through the ER-associated degradation (ERAD)–proteasome pathway. In their infectious form, prion diseases such as bovine spongiform encephalopathy involve PrP^C of wild-type sequence. In contrast to mutant PrP, wild-type PrP^C was hitherto thought to be stable in the ER and thus immune to ERAD. Using proteasome inhibitors, we now show that ~10% of nascent PrP^C molecules are diverted into the ERAD pathway. Cells incubated with *N*-acetyl-leucinal-leucinal-norleucinal (ALLN), lactacystin or MG132 accumulated both detergent-soluble and insoluble PrP species. The insoluble fraction included an unglycosylated 26 kDa PrP species with a protease-resistant core, and a *M_r* 'ladder' that contained ubiquitylated PrP. Our results show for the first time that wild-type PrP^C molecules are subjected to ERAD, in the course of which they are dislocated into the cytosol and ubiquitylated. The presence of wild-type PrP molecules in the cytosol may have potential pathogenic implications.

Keywords: aggregation/ERAD/folding/quality control/spongiform encephalopathies

Introduction

The cellular prion protein PrP^C (Bolton *et al.*, 1982; Oesch *et al.*, 1985; Basler *et al.*, 1986) is a glycoposphatidyl inositol (GPI)-anchored glycoprotein that plays a key role in the transmissible spongiform encephalopathies (TSE), or prion diseases (Prusiner, 1982, reviewed in Prusiner, 1998). The function of PrP^C has not been completely established but it may function in copper transport (reviewed by Kretzschmar *et al.*, 2000; Brown, 2001). During prion diseases, PrP^C is converted into a pathological conformer, PrP^{Sc}, the scrapie isoform of the prion protein, which in turn is the only known component of infectious prions. TSEs can be either infectious, familial or sporadic. The infectious mode usually involves the transformation of wild-type (wt) PrP^C into PrP^{Sc}. Familial prion diseases are linked to pathogenic mutations in PrP.

The cell biology of PrP has been outlined. In cells not infected with prions, PrP^C is synthesized and processed in

the secretory pathway and is found at steady state primarily on the cell surface, where it is attached to cholesterol-rich 'rafts' (Taraboulos *et al.*, 1995; Naslavsky *et al.*, 1997). PrP^C molecules recycle to the interior of the cells, at least in part through clathrin-coated pits (Shyng *et al.*, 1994). PrP^C is degraded in a two-step process with a half-life of ~6 h (Caughey *et al.*, 1989; Taraboulos *et al.*, 1992). It is first N-terminally trimmed by an unidentified neutral protease, yielding a series of GPI-anchored intermediates with molecular weights of ~17–18 kDa (excluding the carbohydrates). These degradation intermediates are then completely degraded in an acidic environment, presumably in endosomes (Taraboulos *et al.*, 1992). While the metabolism of PrP^C in post-Golgi compartments has been delineated, much less is known about the processing of this protein in the earlier compartments of the secretory pathway. One question is whether ER-associated degradation (ERAD) plays a role in the quality control of wt PrP molecules.

The ERAD–proteasome pathway is involved in the degradation of incorrectly folded proteins, which are forbidden to exit the ER through vesicular traffic. Details of this pathway have been worked out for several luminal and transmembrane proteins (reviewed in Kopito, 1997; Bonifacino and Weissman, 1998; Brodsky and McCracken, 1999; Plemper and Wolf, 1999). The following model has emerged: polypeptides that fail to pass the ER quality control are translocated through the ER membrane, possibly through a modified sec61p translocon (Pilon *et al.*, 1997). The translocated molecule is then deglycosylated by a cytosolic *N*-glycanase, usually ubiquitylated, and presented to the 26S proteasome for degradation. Ubiquitin is a highly conserved, 8.6 kDa protein. Covalent attachment of ubiquitin or polyubiquitin chains to lysine residues of proteins often results in the formation of a series of species that are separated by *M_r* increments of 8.6 kDa, thus forming a *M_r* ladder.

Several recent studies have shown that the ERAD pathway is involved in the degradation of pathogenic PrP mutants that are linked with familial prion diseases. The stop codon mutant Y145stop and the mutant Q217R are both associated with Gerstmann–Sträussler–Scheinker syndrome (GSS), a familial TSE. Y145stop is rapidly and completely degraded through ERAD and its degradation is slowed by the proteasomal inhibitor *N*-acetyl-leucinal-leucinal-norleucinal (ALLN) (Zanusso *et al.*, 1999). In contrast to the complete failure of Y145stop to exit the ER by vesicular traffic, most Q217R molecules are successfully exported down the secretory pathway. A minority of Q217R molecules, however, is retained in the ER, where it interacts with BiP for an unusually long time, and is then degraded by the proteasome pathway (Jin *et al.*, 2000).

In contrast to these mutant PrP molecules, wt PrP^C appears to be stable when retained in the ER. This was shown by two previous kinetic studies performed on widely different time scales. To study the metabolism of PrP in the ER, we previously performed pulse–chase metabolic labeling experiments, using brefeldin A (BFA) to prevent vesicular export from the ER (Taraboulos *et al.*, 1991). With a pulse period of 2 h, we detected no decrease in the PrP signal over a chase of several hours. We confirmed these results in BFA-treated cells using a 15 min pulse period (our unpublished data). In a recent paper, a short term pulse–chase experiment in cells that were maintained at 15°C (a temperature where vesicular export to the Golgi is stalled) failed to detect PrP degradation over a period of 4 h (Zanusso *et al.*, 1999).

In preliminary experiments we observed a dramatic accumulation of PrP in cells treated with inhibitors of proteasomes (Y.Yedidia, unpublished data). A similar observation was recently reported by Stewart *et al.* (2001). Because this accumulation occurred even in the presence of BFA, we surmised that some PrP^C may indeed be subjected to ERAD, but that this process may not have been detectable in the experimental conditions used in the previous studies cited above (Taraboulos *et al.*, 1991; Zanusso *et al.*, 1999). Here we studied the involvement of proteasomes in the metabolism of wt PrP^C in mouse neuroblastoma N2a and Chinese hamster ovary (CHO) cells. We show that: (i) in the presence of proteasome inhibitors (with or without BFA), as many as 10% of PrP^C molecules formed large detergent insoluble aggregates that resisted stringent proteolysis with proteinase K; (ii) detergent-insoluble PrP molecules were unglycosylated and included ubiquitylated conjugates. These results show that some PrP molecules fail to pass the ER quality control and are subjected to degradation in the ERAD pathway. This is the first demonstration of PrP molecules modified by ubiquitylation.

Results

Increased amounts of PrP^C in cells treated with proteasome inhibitors

To see whether proteasomes are involved in the processing of PrP, we used three different proteasome inhibitors. Chinese hamster ovary cells that stably express the MHM2–PrP gene (CHO-MHM2 cells) were treated for 12 h with lactacystin (10 µM), or with the peptide aldehyde ALLN (150 µM) or MG132 (10 µM), and their PrP content was analyzed in western immunoblots developed with the mAb 3F4. All three inhibitors induced a large increase in the total amount of PrP^C (Figure 1A). In N2a-C10 cells, the PrP accumulation was already prominent after 6 h treatment with ALLN (Figure 1B). Particularly pronounced was the increase in a band at 26 kDa (Figure 1B). Since this polypeptide resisted digestion with endo H (Figure 1C) and comigrated with PrP^C in cells treated with tunicamycin (Figure 4B), we identified it as the unglycosylated isoform of PrP. Larger PrP species (denoted 33–35 kDa in Figure 1) were also increased in cells treated with proteasome inhibitors. The 33–35 kDa species resisted endoH digestion (Figure 1C) and were thus probably complex glycoforms of PrP^C.

Fig. 1. Increased PrP in cells treated with proteasome inhibitors. (A) CHO-MHM2 cells were either untreated (lane 1), or treated for 12 h with 10 µM lactacystin (lane 2), 150 µM ALLN (lane 3) or 10 µM MG132 (lane 4), before lysis and analysis on western blots developed with the PrP mAb 3F4. All three inhibitors caused a large increase in the total amount of PrP^C. (B) N2a-C10 cells were incubated with 150 µM ALLN for 0–12 h before western blotting as in (A). An increase in PrP immunoreactivity was noticeable after 6 h. Two species of PrP were increased: a sharp band at 26 kDa (representing unglycosylated PrP), and a broad ‘33–35 kDa’ band, which includes complex PrP glycoforms. (C) N2a-C10 cells were either untreated (lanes 1 and 2) or incubated for 12 h with 150 µM ALLN (lanes 3 and 4), and then analyzed by western blotting as in (A). In lanes 2 and 4, the lysates were enzymically deglycosylated with endoH before the western blot analysis. Most PrP species that accumulated with ALLN resisted this digestion.

Proteasome inhibition caused an intensive accumulation of PrP throughout the cytoplasm and a milder increase in cell surface PrP

To further characterize the PrP species that were increased by the proteasomal inhibitors, we used immunofluorescent microscopy. CHO-MHM2 cells were treated with ALLN for 12 h, and stained with monoclonal antibody (mAb) 3F4 to detect surface PrP. There was a mild increase in cell surface PrP after this ALLN treatment (Figure 2A and B). This was also confirmed in cell surface biotinylation experiments (not shown). Interestingly, the patching pattern of cell surface PrP was not modified by ALLN (Figure 2B), indicating that no gross changes occurred in the association of cell surface PrP with membranal ‘rafts’.

To study the intracellular sites of PrP accumulation in ALLN-treated cells, we used immunofluorescence microscopy in permeabilized cells (Figure 2C and D). CHO-MHM2 cells were treated for 12 h with 150 µM ALLN, fixed with formaldehyde and permeabilized with Triton X-100 before the immunostaining. In untreated cells (Figure 2C), intracellular PrP assumed its usual ER–Golgi pattern presumably representing molecules in transit to the cell surface (Taraboulos *et al.*, 1990). In ALLN-treated cells, the PrP pattern was strikingly different (Figure 2D). First, the intracellular signal was much stronger. Secondly, there was a prominent shift in the intracellular locations of PrP immunostaining, which was

Fig. 2. PrP accumulates in the cytoplasm and on the surface of ALLN-treated cells. Stably transfected CHO-MHM2 cells were processed for PrP immunofluorescence detection with mAb 3F4. (A and B) Staining performed on live cells to detect cell surface PrP. Cells were either untreated (A) or treated with 150 μ M ALLN for 12 h (B) before staining. The PrP surface signal was increased after treatment with ALLN. (C and D) Intracellular PrP in permeabilized cells. In untreated cells (C), PrP displayed the usual ER–Golgi pattern. When cells were treated with 150 μ M ALLN for 12 h (D) before staining, a strong PrP immunoreactivity was observed throughout the cytosol, and especially in perinuclear structures.

observed throughout the cytoplasm. The PrP signal was especially pronounced around the nucleus. This signal partially overlapped with both ER (calnexin, BiP and concanavalin A) and Golgi (wheat germ agglutinin) markers (not shown). Of note, there was no ‘aggre-some’-like (Johnston *et al.*, 1998; Wigley *et al.*, 1999) PrP accumulation in the centrosomal region.

Insoluble PrP in ALLN-treated cells

That proteasome inhibitors increased the amount of PrP^C suggested that proteasomes may be involved in the metabolism of this protein, perhaps through the ERAD pathway. For ERAD, ER luminal or membrane proteins are back-translocated to the cytosol where they are deglycosylated, ubiquitinated and then degraded (reviewed by Kopito, 1997; Bonifacino and Weissman, 1998; Brodsky and McCracken, 1999; Plemper and Wolf, 1999). In some cases where proteasomal proteolysis is prevented by inhibitors, the molecules poised for degradation accumulate within the cytosol and form large aggregates that are insoluble in detergents and often resist proteolysis. We performed a series of experiments to check if these ERAD characteristics apply to PrP. To determine the fraction of the PrP increase that occurs in the ER, we used BFA. While BFA completely stops the vesicular export of secretory proteins from the ER (Misumi *et al.*, 1986; Oda *et al.*, 1987), it does not prevent the degradation of proteins through the ERAD pathway (Klausner and Sitia, 1990; Zanusso *et al.*, 1999). N2a-C10 cells were incubated for 12 h with or without BFA and/or ALLN (Figure 3A) or lactacystin (not shown). Post-nuclear supernatants (PNSs) were then made 1% with Sarkosyl and separated into high speed supernatants and pellets, which were further analyzed for PrP in western

Fig. 3. Rapid accumulation of aggregated 26 kDa PrP species in cells treated with ALLN and BFA. (A) N2a-C10 cells were incubated for 12 h either with 150 μ M ALLN (lanes 3 and 4), with 10 μ g/ml BFA (lanes 5 and 6) or with both inhibitors (lanes 7 and 8). Untreated cells served as a control (lanes 1 and 2). The cells were lysed, and PNSs were made 1% with Sarkosyl, incubated for 30 min on ice and then spun for 1 h at $g_{av} = 109\,000$ at 4°C. Supernatants (odd numbered lanes) and pellets (even lanes) were analyzed by western blotting developed with the mAb 3F4. In ALLN-treated cells, the 26 kDa band appeared in the pellet, even when BFA was added. In addition, a PrP-reactive M_r ladder was also observed in these cells (arrowheads in lanes 4 and 8). (B) CHO-MHM2 cells were incubated for 0, 1, 2 or 4 h with 10 μ g/ml BFA with or without 150 μ M ALLN, as indicated, and the PNS fractions were then separated into high speed supernatants and pellets as in (A). A strong accumulation of aggregated 26 kDa species was already noticeable after 1 h (lane 6). (C) Densitometric analysis of the Sarkosyl-insoluble 26 kDa species in cells treated with ALLN. The intensities of the 26 kDa band in the untreated samples (lanes 1–4) were averaged. This background was subtracted from the intensities of the 26 kDa band in the ALLN-treated samples, yielding P_{ALLN} . The values of P_{ALLN} are depicted in arbitrary units. The 26 kDa band accumulates linearly and without lag time.

immunoblots developed with the mAb 3F4. With no ALLN, PrP was mostly soluble (Figure 3A, lanes 1, 2, 5 and 6). In cells treated with ALLN, we observed two types of effect. First, with ALLN alone, the amount of the soluble PrP in the supernatant increased (Figure 3A, compare lanes 3 and 1). These soluble PrP molecules comprised both the 26 kDa band and the larger (33–35 kDa) PrP. When the cells were treated with BFA alone, PrP^C was essentially soluble, and the size of the heavier glycoforms (33–35 kDa) was reduced, as expected (since BFA prevents the production of complex N-linked carbohydrates). When ALLN was added to the cells together with BFA, there was no increase in the soluble PrP (lanes 5 and 7). Thus, the increase in soluble PrP observed in ALLN-treated cells (Figure 3A, lane 3) is probably due to a post-Golgi event. Possible mechanisms leading to the accumulation of the heavier PrP (33–35 kDa) are proposed in the Discussion.

Fig. 4. Ubiquitylated PrP^C accumulates after treatment with ALLN. (A) CHO cells (lane 1), and CHO cells transiently expressing MHM2-PrP (lane 2) were treated with ALLN (150 μ M, 12 h), and high speed Sarkosyl pellets were then analyzed in a western blot developed with 3F4. Both the 26 kDa band and a M_r ladder separated by $\sim$ 8 kDa appeared only in the transfected cells. (B) CHO-MHM2 cells were treated with tunicamycin (5 μ g/ml) with or without 150 μ M ALLN, and high speed pellets were analyzed as in (A). (C) CHO (lanes 1, 2, 5 and 6) and stably expressing CHO-MHM2 (lanes 3, 4, 7 and 8) cells were treated with or without ALLN (150 μ M, 12 h) as indicated. High speed pellets were then denatured (0.3% SDS, 65°C, 10 min), and PrP was immunoprecipitated using PrP mAb 3F4 covalently bound to protein A-Sepharose beads. The immunoprecipitates were then analyzed in western blots developed first with a ubiquitin mAb (lanes 5–8), and then stripped and reacted with the PrP mAb 3F4 (lanes 1–4). Several bands in the M_r ladder, but not the 26 kDa species, were recognized by the ubiquitin Ab (compare lanes 4 and 8).

The high speed pellets were also strongly affected by ALLN. In both untreated and BFA-treated cells, addition of ALLN yielded strong PrP bands in the pellets (Figure 3A, lanes 4 and 8). This insoluble material consisted primarily of the unglycosylated 26 kDa band (in contrast, the higher M_r bands remained essentially soluble after ALLN treatment). In addition, pellets of ALLN-treated cells also contained a M_r ladder (denoted by arrowheads in lanes 4 and 8). The molecular identity of this ladder will be discussed below.

These results indicate that the accumulation of 26 kDa aggregates occurs at least in part in a pre-Golgi compartment, and suggest that classical ERAD is involved in the clearance of the 26 kDa PrP species when proteasomes are not inhibited. Whether the 26 kDa polypeptide is formed due to a failure in N-glycosylation or by the deglycosylation of PrP^C following dislocation into the cytosol remains to be determined.

Insoluble PrP after short treatments with ALLN

The above results were all obtained after long ($\geq$ 6 h) incubations with proteasome inhibitors. We next studied

shorter treatments with proteasome inhibitors. In order to improve our ability to discern changes that occur during a short ALLN treatment, we turned to the insoluble PrP fraction (Figure 3B). N2a-C10 cells were treated with BFA (10 μ g/ml) with (lanes 5–8) or without (lanes 1–4) the addition of ALLN (150 μ M) for 0, 1, 2 or 4 h (as indicated), and then solubilized and separated into high-speed pellets and supernatants as described for Figure 3A. In cells treated with BFA alone, the amount of insoluble 26 kDa PrP was small and remained almost constant (Figure 3B, lanes 1–4). In contrast, in cells that were treated with both BFA and ALLN, there was a time-dependent accumulation of the 26 kDa PrP species (lanes 5–8).

To quantify this accumulation, we turned to densitometry. We first calculated the average intensity of the bands in lanes 1–4 (Figure 3B). This average, P_{bg} , represents the ‘background’ material that sediments in the absence of ALLN. P_{bg} was then subtracted from the densitometric values of the bands in lanes 5–8. The subtracted values, called P_{ALLN} , are depicted in Figure 3C as a function of time. The graph shows that the insoluble 26 kDa species (which represents PrP molecules whose ERAD has been stalled by ALLN) increased linearly with time, with no detectable lag period. This militates in favor of a direct effect of ALLN on the accumulation of PrP.

We used the data depicted in Figure 3C to provide a crude evaluation of the fraction of nascent PrP molecules that are diverted towards ERAD. To this end, we compared the hourly increase of P_{ALLN} with the total level of PrP present in these cells (P_{total}). The latter data (not shown) were obtained from additional lanes run in the same western blot. We found that the ratio P_{ALLN}/P_{total} increased at a rate of 1.5% per hour. Thus, the PrP molecules diverted towards the ERAD pathway during 1 h represent $\sim$ 1.5% of all the PrP molecules found in the cells. However, because the $t_{1/2}$ of PrP is $\sim$ 6 h (Taraboulos *et al.*, 1992), the total amount of PrP molecules synthesized each hour is about one-sixth of the total amount of PrP found in the cell. We thus conclude that $\sim$ 1.5% $\times$ 6 = 9% of all nascent PrP molecules at any time are diverted towards the ERAD pathway.

Ubiquitylated PrP in ALLN-treated cells

In addition to the 26 kDa band, the pellets of the ALLN treated cells also contained a discrete M_r ladder of PrP immunoreactive species (Figure 3A, lanes 4 and 8, arrowheads). That this ladder consisted of PrP species was confirmed by its presence in cells transiently expressing MHM2-PrP but not in mock-transfected cells (Figure 4A). That the M_r s of the polypeptides in this ladder were separated by $\sim$ 8 kDa, suggested that some of them might represent ubiquitin conjugates of the 26 kDa PrP species. To see if these bands consist of PrP glycoforms, we used tunicamycin (Figure 4B). CHO-MHM2 cells were treated with tunicamycin (5 μ g/ml) for 12 h, with or without the addition of ALLN (150 μ M), and high speed Sarkosyl pellets were then analyzed in western blots developed with 3F4. Tunicamycin did not prevent the formation of the PrP M_r ladder in ALLN-treated cells (Figure 4B). This shows that the higher M_r species did not consist of N-linked glycoforms of PrP.

To study the possibility that the PrP M_r ladder contains ubiquitylated species, we performed serial immunoprecipitation–western blotting experiments on the pellet fraction (Figure 4C). Following a 12 h incubation with ALLN, CHO and CHO-MHM2 cells were lysed, and Sarkosyl-insoluble pellets were prepared. Because aggregated PrP is poorly immunoreactive (Serban *et al.*, 1990; Zanusso *et al.*, 1999), we denatured the pellet fraction before PrP immunoprecipitation. The pellets were denatured by incubation in 0.3% SDS at 65°C for 10 min, and PrP species were immunoprecipitated with 3F4 as described in Materials and methods. Immunoprecipitates were then analyzed by western blotting. The blot was first developed with a ubiquitin mAb (lanes 5–8). It was then stripped and reprobbed with 3F4 (Figure 4C, lanes 1–4). When the films were aligned and compared, we found that several bands in the M_r ladder reacted with both antibodies (labeled with arrowheads in the figure). No ubiquitin signal was detected in either non-transfected CHO cells (lanes 5 and 6), or in cells not treated with ALLN (lanes 5 and 7). Identical results were obtained in similar experiments where separate western blots were run for 3F4 and the ubiquitin Ab (not shown). We conclude that those bands in the M_r ladder that react with the ubiquitin antibody consist of PrP–ubiquitin conjugates.

Insoluble PrP in ALLN-treated cells is partially protease resistant

Protein aggregation is often accompanied by increased resistance to proteolysis, and several proteins have been shown to become partially protease resistant when proteasomes were inhibited. To see whether this is also the case for PrP, we incubated N2a-C10 cells with ALLN for 12 h. Lysates were subjected to proteolysis (10 µg/ml proteinase K, 30 min, 37°C) before western immunoblotting with 3F4 (Figure 5A). While PrP in untreated cells was completely digested (lane 2), protease-resistant PrP species appeared in ALLN-treated cells. Especially prominent was a band of apparent size ~19 kDa (lane 4). This band comigrated with the 19 kDa protease-resistant core of the prion isoform PrP^{Sc}, which was obtained from prion-infected ScN2a-C10 cells and was included in the same gel as a M_r reference (lane 5). Interestingly, a 19 kDa band was present in the cell lysate even before proteolysis (lane 3). To check whether the protease-resistant PrP species are aggregated, we treated N2a-C10 cells with ALLN for 12 h and separated their lysates into high speed pellets and supernatants before proteolysis and western analysis (Figure 5B). The soluble PrP species were sensitive to proteolysis (lanes 5 and 7). In the pellets of ALLN-treated cells, the 26 kDa band that was predominant before proteolysis (lane 4) was largely, but not entirely, replaced by a 19 kDa species (lane 8).

Discussion

Proteasome inhibitors caused both a large increase in total PrP and the appearance of detergent-insoluble, protease-resistant and partially ubiquitylated PrP. These results indicate that ERAD is involved in the routine quality control of PrP. This is the first report of ERAD participating in the metabolism of wt PrP^C.

Fig. 5. Insoluble PrP in ALLN-treated cells is partially protease resistant. N2a-C10 cells were treated for 12 h with 150 µM ALLN. In (A), cell lysates were incubated with proteinase K (10 µg/ml, 37°C, 30 min, lanes 2 and 4) before western analysis with 3F4. A protease-resistant PrP species with a molecular weight of 19 kDa appeared in ALLN-treated cells (lane 4). In lane 5, the protease-treated lysate of prion-infected ScN2a-C10 cells was added for size comparison. The protease-resistant band in lane 4 co-migrated with the lower (unglycosylated) glycoform of PrP27–30 (lane 5). (B) The lysates of N2a-C10 cells were separated into high speed supernatants and pellets before proteolysis. The protease-resistant species were insoluble in Sarkosyl (lane 8). Proteolysis appeared to partially convert the 26 kDa aggregate into the 19 kDa band.

ERAD is involved in the metabolism of wt PrP^C

We present here several lines of evidence that show that ERAD is involved in the normal metabolism of PrP^C. First, inhibiting proteasomes caused the appearance of a 26 kDa, unglycosylated, Sarkosyl-insoluble PrP species (Figures 1 and 3). This 26 kDa species started to accumulate after treatments as short as 1 h (Figure 3B), suggesting a specific effect of the proteasome inhibitors. Identical effects were observed with three different inhibitors, including the highly specific lactacystin (Figure 1A). Secondly, several bands in the Sarkosyl-insoluble M_r PrP ladder contained ubiquitylated PrP, since they reacted with a ubiquitin antibody and displayed size increments of ~8 kDa (Figure 4). As expected, the 26 kDa PrP was not ubiquitylated. Of note, PrP denaturation in hot SDS was essential for detecting the ubiquitylated bands (our unpublished observations). The existence of ubiquitin conjugates indicates that some PrP molecules have been retro-translocated into the cytosol, where ubiquitylation occurs. Thirdly, both the 26 kDa band and the M_r ladder appeared in ALLN-treated cells even in the presence of BFA (Figure 3), confirming that they result from an ER-specific mechanism. Taken together, these results strongly suggest that the ALLN-induced 26 kDa band represents PrP molecules in the process of classical ERAD. Presumably, after failing to pass the ER quality control, these molecules are back-translocated to the cytosol where they are deglycosylated by a cytoplasmic *N*-glycanase

(Suzuki *et al.*, 1994) and, in part, ubiquitinated. Many details of this degradation remain to be established.

Whether the 26 kDa band originates exclusively from deglycosylation of PrP molecules, or also contains molecules that have never been glycosylated, is unknown. For some proteins, the N-linked glycans have been shown to play a role in engaging the retro-translocation mechanism (Ellgaard *et al.*, 1999). This is probably not the case for PrP, because we obtained similar accumulation of detergent-insoluble PrP in cells treated with tunicamycin in addition to ALLN (Figure 4B). Possible interactions of PrP^C with chaperones and folding enzymes also remain to be characterized. In the case of the unstable PrP mutant Q217R, interaction with BiP has been demonstrated (Jin *et al.*, 2000).

It is important to mention that the stably transfected cells utilized in this work were only moderate expressors of MHM2-PrP. Whether some of the PrP ERAD characteristics depend on the level of PrP expression remains to be seen.

Why was PrP ERAD not observed before?

We evaluate that ~10% of all nascent PrP molecules at any time are diverted towards the ERAD pathway (see Figure 3C and accompanying text). That such a small fraction of PrP is subjected to ERAD may explain why previous kinetic experiments, including our own, have failed to detect an ER-associated PrP degradation (see Introduction) (Taraboulos *et al.*, 1991; Zanusso *et al.*, 1999).

Our results do not provide information as to the kinetics of this ERAD of nascent PrP^C molecules. If this degradation were rapid, occurring mainly during the pulse period, then no further change would take place during subsequent chase periods where the stability of PrP is assayed. It is interesting to note that the half-life for ERAD varies enormously between proteins, with examples as short as 10 min and as long as 8 h (Klausner and Sitia, 1990).

Possible implications of cytosolic PrP

That PrP is translocated to the cytosol during routine quality control may have important implications. PrP contains a NH₂-proximal nuclear localization signal (NLS) that could be brought to bear in the cytosol (Basler *et al.*, 1986). PrP has been reported in the nucleoli of prion-infected cells (Pfeifer *et al.*, 1993). The pathogenic mutant Y145stop has also been detected in the nucleus following proteasome inhibition (Zanusso *et al.*, 1999). Possible consequences of PrP in the nucleus remain to be determined. PrP could also exert deleterious effects in the cytosol. For instance, the affinity of PrP for cytoskeletal elements has been documented. In particular, PrP interacts *in vitro* with glial fibrillary acidic protein (GFAP) (Oesch *et al.*, 1990). While the 'classical' secretory topology of PrP precludes such interactions *in vivo*, a cytosolically located PrP could bind to GFAP and initiate important interactions. It is pertinent to note that one of the hallmarks of prion diseases is the activation of glia and the increased synthesis of GFAP (Jendroska *et al.*, 1991) (see Kretzschmar, 1993; DeArmond and Prusiner, 1995 for reviews).

An interesting question is whether cytosolic ubiquitin conjugates of PrP could eventually access the

endomembrane system, for instance through autophagy. In this context, it is pertinent to recall that both PrP^{Sc} and ubiquitin conjugates have been observed in multivesicular endosomes in the brain of mice and hamsters with experimental scrapie (Arnold *et al.*, 1995).

'Prion-like' PrP

The 26 kDa PrP band induced by ALLN strongly resembles bona fide PrP^{Sc} in its biochemical properties. In particular, proteolysis replaced this band with a 19 kDa, protease-resistant core. The question of how well such 'prion-like' (Lehmann and Harris, 1995) properties correlate with prion infectivity is still deliberated in the literature. Indeed, studies have repeatedly shown that the C-terminal region of wt PrP^C is more resistant to proteolysis than the rest of the polypeptide (Capellari *et al.*, 2000), so that a 19 kDa PrP species that resists mild proteolysis does not necessarily indicate PrP^{Sc}. In addition, many authors have reported that prion-like PrP can be induced by metabolic disturbances. For instance, tunicamycin (Lehmann and Harris, 1997), dithiothreitol (DTT) and a combination of both inhibitors (Ma and Lindquist, 1999) cause the accumulation of insoluble, protease-resistant PrP in treated cells. In view of this confusion, we do not suggest here that malfunctioning or overloaded proteasomes can produce infectious prions. In this respect, it is interesting to report here that the immunoreactivity of PrP towards 3F4 in ALLN-treated cells was not enhanced by denaturation with guanidine thiocyanate (our unpublished results). Since guanidine-enhanced PrP immunoreactivity is a hallmark of bona fide PrP^{Sc} (Serban *et al.*, 1990; Taraboulos *et al.*, 1990; Safar *et al.*, 1998), this finding argues against a prion identity of the ALLN-induced PrP.

Accumulation of 30–35 kDa PrP following long ALLN treatment

One interesting finding in our experiments is the vast increase in heterogeneous M_r PrP species, denoted '33–35 kDa' in Figures 1 and 2, which accumulated only after prolonged (>6 h) treatment with ALLN. These 33–35 kDa species were soluble in Sarkosyl (Figure 3) and sensitive to proteolysis (Figure 5). They were found primarily in post-Golgi locations, since (i) they resisted enzymic deglycosylation with endoglycosidase H (endoH) (Figure 1C), and (ii) cell surface PrP was amplified by prolonged ALLN treatment (Figure 2), a finding that was confirmed by cell surface biotinylation (our unpublished data). That the soluble species are indeed post-Golgi is in line with the fact that they were not amplified when ALLN-treated cells were also treated with BFA (Figure 3A), since this inhibitor prevents protein export to post-Golgi compartments.

That ALLN, MG132 and lactacystin amplified high M_r PrP species found in post-ER compartments is a surprising result that is not easily reconciled with current models of ERAD. Post-Golgi species are not natural candidates for proteasomal degradation, which is thought to occur exclusively in the ER. We envisage two mechanisms through which these species could be amplified by ALLN or lactacystin.

One possible explanation is that these inhibitors directly or indirectly slow the post-Golgi turnover of PrP. In this

respect, it is provocative to note the similarities between the overall $t_{1/2}$ of PrP and the accumulation time of the 33–35 kDa species in ALLN-treated cells (both ~6 h). Previous work has shown that mature PrP is degraded in two steps (Taraboulos *et al.*, 1992). The first step is the N-terminal removal of about one-third of the polypeptide to form a series of GPI-anchored intermediates of M_r ~17 kDa (unglycosylated M_r). This trimming occurs outside the ER, since it is inhibited by BFA (Taraboulos *et al.*, 1992). The trimming protease has not yet been identified.

Because their M_r corresponds to that of full-length PrP, the amplified 33–35 kDa PrP species in ALLN-treated cells do not appear to have undergone this trimming. Whether the trimming step could be directly inhibited by ALLN or lactacystin remains to be seen. It should be noted that in addition to their usual proteasomal inhibition, both ALLN and MG132 also inhibit non-proteasomal proteases such as cathepsins and calpains (Lee and Goldberg, 1998). Even the more specific inhibitor lactacystin interferes with non-proteasomal cathepsin A (Ostrowska *et al.*, 1997, 2000). Kinetic experiments will be needed to determine whether proteasome inhibitors slow the N-terminal trimming of PrP^C.

A more remote explanation for the accumulation of the 33–35 kDa PrP species in cells treated with ALLN or lactacystin is that proteasomes are indeed directly involved in the degradation of these post-Golgi species. This would require that the 33–35 kDa PrP species be dislocated into the cytosol, a process that is believed to occur only in the ER. Presumably, then, the mature PrP species should be retro-transported into the ER before accessing the proteasomes. Although such a process appears highly hypothetical, it is important to note that other proteins recycle into the ER from locations as remote as the trans-Golgi stack (Nehls *et al.*, 2000).

In summary, two pathways of PrP degradation were inhibited by proteasome inhibitors. (i) The ERAD pathway, which involves ~10% of nascent PrP molecules, and (ii) the post-Golgi turnover of PrP^C. Whether the involvement of proteasomes in the quality control of PrP molecules plays a role in prion diseases remains to be determined.

Materials and methods

Materials

Cell culture reagents were purchased from Biological Industries (Bet Haemek, Israel). Tissue culture plates were from Miniplast (Ein Shemer, Israel) or Nunc (Denmark). Lactacystin (#426100), MG132 (#474790) and G418 were from Calbiochem (San Diego, CA). ALLN was either from Calbiochem (#208719) or from Sigma (#A-6185). Tunicamycin (T-7765) and all other chemicals were from Sigma (St Louis, MO). Stock solutions of inhibitors were prepared in dimethylsulfoxide (DMSO) (ALLN, lactacystin, MG132 and tunicamycin) or ethanol (BFA) at 1000× working concentration. Stocks were kept at –20°C.

Cells and transfections

Cells were grown at 37°C in DMEM16 (low glucose) supplemented with 10% fetal calf serum. N2a-C10, ScN2a-C10 (Naslavsky *et al.*, 1997) and CHO-MHM2 cells stably express moderate levels of the MHM2–PrP chimera (Scott *et al.*, 1992). For some experiments, cells were transiently transfected with PrP constructs. Transfection was performed with the non-liposomal reagent FuGene 6 (Roche Molecular Biochemicals, Germany) and cells were harvested 72 h after the transfection.

Antibodies

MAB 3F4 (Kascsak *et al.*, 1987) binds to amino acid residues Met108 and Met111 (Rogers *et al.*, 1992) in chimeric MHM2–PrP but does not recognize the mouse PrP endogenous to N2a cells (Scott *et al.*, 1992). It was used for western blots at a dilution of 1:5000 of the ascitic fluid. The ubiquitin mAb (#MMS-258R) was from Babco (Richmond, CA) and was used at a 1:25 000 dilution. Secondary antibodies were from Jackson ImmunoResearch (West Grove, PA).

PrP isoforms and PrP analysis

The PrP isoforms were characterized and separated as described (Meyer *et al.*, 1986). Cells were lysed in ice-cold lysis buffer (0.5% Triton X-100, 0.23% Na-deoxycholate, 150 mM NaCl, 10 mM Tris–HCl pH 7.5, 10 mM EDTA) and the lysates were immediately centrifuged for 30 s at 14 000 r.p.m. in a microfuge. All biochemical analyses were performed on this PNS, also referred to as the ‘cell lysate’. Protease resistance of PrP^C fractions was assayed with proteinase K (Roche) (10 µg/ml, 37°C, 30 min). The same proteolytic conditions were used to prepare PrP27–30 from ScN2a-C10 cells in Figure 5. SDS–PAGE and western immunoblotting the PrP isoforms were carried out as described (Taraboulos *et al.*, 1995; Naslavsky *et al.*, 1997).

Aggregated PrP was separated from soluble fractions by high speed centrifugation. PNS was brought to 1% Sarkosyl, incubated on ice for at least 30 min and then spun for 1 h at 45 000 r.p.m. at 4°C in a TLA45 rotor (g_{av} = 109 000). The pellets were resuspended either in lysis buffer (for western blots) or in a SDS buffer (for immunoprecipitation, see below).

Western blots were developed using an ECL system. Band intensities were quantified by densitometry and analyzed using the Scion program (Scion corporation, Frederick, MD).

Polyvinylidene fluoride membranes were stripped by incubation in 100 mM glycine–HCl pH 2.7 [3 min, room temperature (RT)], followed by neutralization in 1 M Tris–HCl pH 8.

Immunoprecipitation

PrP was immunoprecipitated from insoluble pellets as follows. Pellets were kept frozen overnight at –20°C, thawed, resuspended in 200 µl SDS buffer (0.3% SDS, 10 mM Tris–HCl pH 7.5, 150 mM NaCl) and incubated at 65°C for 10 min to denature PrP. After addition of Triton X-100 to a final concentration of 2%, the tubes were further incubated at 65°C for 2 min and then placed on ice. After the addition of 0.5 ml TNS (1% Sarkosyl, 10 mM Tris–HCl pH 7.5, 150 mM NaCl), protein A beads with covalently bound 3F4 were added and incubated at 4°C for 18 h. The beads were rinsed six times in TNS, and PrP was recovered by boiling in SDS sample buffer (in non-reducing conditions) and then analyzed by SDS–PAGE. 3F4 was covalently attached to protein A beads (Pharmacia, Sweden) with dimethylpimelimidate (Sigma D-8388) as described previously (Harlow and Lane, 1988).

Immunofluorescence

To detect PrP on the surface of cells, we took advantage of the patching effect caused by the polyvalent secondary antibody (Rothberg *et al.*, 1990; Taraboulos *et al.*, 1990; Mayor *et al.*, 1994). Live cells were cooled to 4°C and then sequentially incubated with the mAb 3F4 followed by a secondary anti-mouse IgG coupled to FITC. The Abs were diluted at 1:2000 and 1:200, respectively, in HEPES buffer (20 mM HEPES pH 7.4, 150 mM NaCl, 1 mM MgCl₂, 0.1 mM CaCl₂) containing 1% bovine serum albumin (BSA). Following extensive washes, the cells were fixed with formaldehyde [8% formalin in phosphate-buffered saline (PBS), 4°C, 15 min] and then quenched by several rinses with 2% NH₄Cl in PBS before mounting. To detect total PrP, the cells were fixed (8% formalin in PBS, 4°C, 30 min), rinsed and quenched with 2% NH₄Cl in PBS. The cells were then permeabilized (0.1% Triton X-100 in PBS, 5 min, RT) and blocked with 50% low-fat milk in HEPES buffer before their incubation with 3F4 and the secondary antibody (diluted in blocking solution). Cells were mounted in an anti-fading preparation (0.5% *n*-propyl gallate, 100 mM Tris–HCl pH 9, 70% glycerol) (Giloh and Sedat, 1982) and viewed in a Zeiss Axioplan microscope equipped with epifluorescence.

Acknowledgements

We thank Dr A. Yaron and the members of our laboratory for numerous discussions and for critically reading the manuscript. This work was supported by a generous grant from the Israel Center for the Study of Emerging Diseases.

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Received June 13, 2001; revised and accepted August 9, 2001