Mutant prion protein acquires resistance to protease in mouse neuroblastoma cells

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Conversion of the cellular isoform of the prion protein (PrPc) into the pathogenic isoform (PrPSc) is thought to be the causative event in prion diseases. Biochemically, PrPSc differs from PrPc in its partial resistance to proteinase K (PK). The amino acid sequence AGAAAGA, comprising residues 112–119 of the murine PrPc, has been shown to be amyloidogenic and evolutionarily conserved. To assess the effect of mutations at and around this hydrophobic sequence on protease resistance, the sequence was replaced either by alanines or by glycines and, in a third mutant, a large part surrounding this region was removed. The PrP mutant carrying substitutions of glycines for alanines showed PK resistance and aberrant proteolytic processing. Tetracycline-induced expression of this mutant indicated that resistance to protease is acquired concurrent with the synthesis of the protein. These findings indicate that mutations in the central hydrophobic region lead to immediate alterations in PrP structure and processing.

Introduction

Prion diseases (transmissible spongiform encephalopathies) are fatal neurodegenerative diseases that include Creutzfeldt–Jakob disease, kuru, Gerstmann–Sträussler–Scheinker syndrome and fatal familial insomnia in humans, scrapie in sheep and bovine spongiform encephalopathy in cattle (Prusiner et al., 1996). Prion diseases can be acquired or inherited or are of idiopathic origin. Inherited forms show an autosomal dominant trait and are linked to point or insertion mutations in the Prnp gene (Prnp in humans, Prnp in mice) encoding the prion protein (PrP) (Prusiner & DeArmond, 1994). Prion diseases are characterized by the accumulation of a proteinase K (PK)-resistant isoform (PrPSc) of the cellular protease-sensitive prion protein (PrPc).

PrPc, a neuronal glycoprotein, is anchored to the plasma membrane by a glycosylphosphatidylinositol (GPI) moiety. The function of PrPc, which is a copper-binding protein (Brown et al., 1997a), is still not clearly understood. Recent data point to a function in synaptic interaction (Herms et al., 1999), resistance of neurons to oxidative stress (Brown et al., 1997b) or signal transduction (Mouillet-Richard et al., 2000).

Studies investigating PrPc metabolism have demonstrated that PrPc turns over with a half-life of 3–6 h in mouse neuroblastoma cells (Borchelt et al., 1990; Caughey et al., 1989). Chicken PrPc has been shown to recycle between the plasma membrane and an endosomal compartment (Shyng et al., 1993). During its normal processing, PrPc is cleaved at the central hydrophobic region, yielding a C-terminal, GPI-anchored fragment that can be identified in brain tissue and in cultured cells (Chen et al., 1995; Harris et al., 1993; Jiménez-Huete et al., 1998; Pan et al., 1992).

PrPSc is derived from PrPc by a post-translational process that seems to involve species-specific molecular interactions between the two isoforms. PrPSc is necessary for infectivity and pathology, as demonstrated by experiments showing complete resistance of Prnp-knockout mice to infection with prions (Brandner et al., 1996; Brown et al., 1996; Büeler et al., 1993). PrPSc is rich in α-helices, while PrPSc exhibits a greater content of β-sheets (Caughey et al., 1991; Pan et al., 1993; Sfar et al., 1993). The nuclear magnetic resonance structure of full-length recombinant PrPc revealed that the region comprising amino acids 121–231 contains a high level of secondary structure, including three α-helices and a two-stranded antiparallel β-sheet, whereas the N terminus is highly flexible (Hornemann et
al., 1997; Riek et al., 1996, 1997). Copper binding seems to confer a distinct structure on the N terminus (Viles et al., 1999). Synthesized peptides that are homologous to residues 109–122 and 106–126 form β-sheets spontaneously (Forloni et al., 1993; Gasset et al., 1992), with the internal sequence AGAAAAA (residues 112–119) displaying the highest tendency to form amyloid (Gasset et al., 1992). Peptides containing the AGAAAAA motif are toxic to neurons in culture, whereby the sequence AGAAAAA was found to be necessary but not sufficient for the neurotoxic effect (Brown, 2000a; Forloni et al., 1993). A recent study using in vitro conversion assays of PrP C to PK-resistant PrPSc indicates that synthetic peptides derived from the central part of PrP C (amino acids 106–141) have an inhibitory effect on this conversion (Chabry et al., 1998). The presence of residues 119 and 120 (the two last residues within the motif AGAAAAA) seems to be crucial for this inhibitory effect, arguing for their involvement in the intermolecular interaction that leads to PK-resistant PrP.

Mutant PrP molecules carrying deletions of amino acids 108–121 or 114–121 are not convertible to PrPSc when overexpressed in scrapie-infected neuroblastoma cells (Holscher et al., 1998; Muramoto et al., 1996). The central hydrophobic region, spanning all or most of the sequence AGAAAAA, therefore plays an important role in the conversion process.

For further insight into the function of this region, we decided to establish a cell culture model. To develop such a system, we expressed PrP molecules carrying mutations at and around this hydrophobic sequence in murine neuroblastoma cells and analysed the mutant proteins for proteolytic processing and resistance to protease. We report here that one of these mutant PrP molecules acquired protease resistance in comparison with wild-type PrP.

Methods

Reagents and antibodies. DNA-modifying enzymes were purchased from Promega and dideoxy-sequencing reagents from Amersham. Cell-culture reagents were obtained from Biochrom KG. N-Glycosidase F (PNGase F) was purchased from New England Biolabs. All other reagents were from Sigma. The following PrP-specific antibodies were used: mouse mAb 3B5, directed against amino acids 68–84 of human PrP (Krasemann et al., 1996), mAb 6H4, directed against amino acids 155–163 of bovine PrP (Korth et al., 1997), mAb 13A5, directed against an epitope of Syrian hamster PrP (Rogers et al., 1991), and polyclonal antibody Kan72, directed against amino acids 89–103 of murine PrP (mPrP) (Holscher et al., 1998). Alkaline phosphatase (AP)-conjugated goat anti-mouse antibody was from Dianova. TRITC-coupled goat anti-mouse antibody was purchased by Dianova.

Brain tissues and N2a cells. Tissues were obtained from brain hemispheres of 8–12 mice (Scott et al., 1989), hamster and hamster infected with the scrapie strain 263K. The murine neuroblastoma cell line Neuro2a (Klebe & Ruddle, 1969) was obtained from the ATCC. N2a cells were grown in DMEM containing 10% foetal calf serum and penicillin/streptomycin in an atmosphere of 10% CO2.

Mutant Prnp genes. The generation of the plasmid pCI-Prnp has been described previously (Windl et al., 1999). The hamster-specific 13A5 epitope was inserted into the wild-type Prnp allele via overlap-extension (OE)-PCR (Higuchi et al., 1988) by using the outer primers T3 (5′-AATTAAACCTGCTAAAGGC-3′) and T7neo (5′-TAATACGACCT- ACTATAGG-3′) and the overlapping primers 13ASup (5′-CCAATAAGGCATCATGCGGCC-3′) and 13ASdo (5′-CCCATGATCGATTGGCGCA-3′). After cleavage with XbaI and Xhol, the PCR product was cloned into the corresponding restriction sites of pCI-neo (Promega) to give pCI-Prnp13AS. Based on pCI-Prnp13AS, plasmid pCI-PrnpASm1, containing a Prnp gene without its original Smal site, was established. The following primers were used in an OE-PCR: ΔSmalup (5′-CCCTGGCCGAGGATACCCGCG-3′) and ΔSmaldo (5′-CGGTATCTCGGCGAGGAAGG-3′).

Plasmid pCI-PrnpASm1 was the starting point for pCI-PrnpΔ105–125, pCI-PrnpΔ112–119 and pCI-PrnpG112–119. Plasmid pCI-PrnpΔ105–125 was generated via OE-PCR by using the following overlapping primers: Δ105–125up (5′-CCAGCAGTGACCGGCGGGGG-3′) and Δ105–125do (5′-CAGCAACCCCCGGGATCCATGCGGGAGCGG-3′). In addition to the deletion (codons 105–125), this construct carried a new Smal restriction site by changing codons 104 and 126 silently from CCA to CCC and from GCC to GGG. Constructs pCI-PrnpΔ112–119 and pCI-PrnpG112–119 were generated based on pCI-PrnpΔ105–125. The primers 112Δ119up (5′-ACCAAGGCAGCCCGCCCATGCGGGAGG-3′) or G112–119do (5′-ACCAAGGGGCGCGGCGGGAGG-3′) were obtained after 4 weeks. The level of PrP overexpression in the transfected with the different pCI-Prnp alleles was assayed by Western blot analysis (see below). Construction of recombinant cell lines. N2a cells were stably transfected with each of the pCI-Prnp constructs using Effectene or Superfect (both from Qiagen) as transfection reagents according to the manufacturer’s instructions. After transfection, antibiotic-resistant cells were selected in 400 μg/ml gentamicin (G418) and polyclonal cell lines were obtained after 4 weeks. The level of PrP overexpression in the different cell lines was assayed by Western blot analysis (see below).

The use of the tetracycline-inducible expression system required the use of the tetracycline-inducible expression system required the use of the tetracycline-inducible expression system. The use of the tetracycline-inducible expression system required the use of the tetracycline-inducible expression system. The use of the tetracycline-inducible expression system required the use of the tetracycline-inducible expression system.
previously (Windl et al., 1999). One of the resulting N2a–rtTA clones (clone 1) was subjected to a second round of transfection, a co-transfection with either pCMV+PnpG112–119 or pCMV+PnpG112–119 combined with pHAS5 (Pnp plasmid and hygromycin-resistance plasmid in a 19:1 mixture) using Effectene as the transfection reagent. Cells were selected in the presence of hygromycin (200 µg/ml) and genitcin (200 µg/ml).

**Immunocytochemistry.** Cells were grown in 24-well plates and were fixed with 10% paraformaldehyde in PBS for 10 min or with methanol for 30 min at -4 °C. After rinsing with PBS, the cells were incubated with mouse mAb 3B5 at a dilution of 1:5 for 1 h at room temperature. Cells were rinsed and then incubated with TRITC-conjugated secondary antibody at a concentration of 1:200 for 30 min at room temperature. After rinsing, the cells were embedded in PBS and examined with a fluorescence microscope (Olympus).

**Western blot analysis.** Cells grown in 75 cm² tissue culture flasks were lysed in 500 µl ice-cold lysis buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM sodium chloride, 0.5% NP-40 and 0.5% sodium deoxycholate supplemented with 1 mM PMSF. Brain tissue was homogenized in 9 vols ice-cold lysis buffer. Where indicated, lysates or brain homogenates were treated with PNGase F according to the manufacturer’s instructions. Proteins were separated by SDS–PAGE (12 or 15% acrylamide) transferred to a PVDF membrane and processed further as described elsewhere (LeGendre, 1990). For detection of PrP and its fragments, the membrane was incubated with one of various primary antibodies at a dilution of either 1:2000 (13A5, 6H4 and Kan72) or 1:50 (3B5). This was followed by incubation with AP-conjugated goat anti-mouse or goat anti-rabbit antibody (diluted 1:1000). Immunopositive signals were detected by using the chemiluminescence substrate CDP-Star (Tropix) according to the manufacturer’s instructions. The light signals were monitored, documented and quantified with a CCD camera system (Raytest) and the accompanying software (Diana, TINA, Raytest).

**Assay of protease resistance.** Cell lysates were treated with PK at different concentrations (3–3, 10, 20 or 50 µg/ml) for 10 min at 37 °C or they were digested with 3–3 µg/ml PK at 37 °C for 0, 10, 20, 40 or 60 min, as described previously (Lehmann & Harris, 1999). Proteolytic digestions were terminated by adding 2 mM PMSF and analysed directly by Western blot. The protease resistance of PrP in brain tissue from scrapie-infected hamster was analysed by digesting the homogenized brain with PK at a concentration of 20 µg/ml for 1 h at 37 °C. Digestion was terminated by the addition of 2 mM PMSF. Proteins in the brain homogenate were methanol-precipitated, denatured in 7 M guanidine hydrochloride and methanol-precipitated again. The resulting pellet was resuspended in lysis buffer.

**Results**

**Overexpression of wild-type and mutant moPrPs in N2a cells**

The wild-type moPrP contains the highly amyloidogenic sequence AGAAAAGA (positions 112–119) in the central region comprising residues 105–125 (Fig. 1). We generated several mutations in this sequence motif of PrP to study the fate of these mutant prion proteins at the cellular level. Two mutant moPrPs carried exclusively either glycine or alanine residues instead of the AGAAAAGA sequence (designated G112–119 or A112–119). A third mutant form of moPrP (designated Δ105–125) had a deletion in the central region including AGAAAAGA. Wild-type and mutant moPrPs were epitopically tagged by substituting a methionine residue for an isoleucine at position 138 (Fig. 1). The introduction of this epitope into moPrP allowed the detection of the protein by the mAb 13A5 (Rogers et al., 1991). The wild-type and mutant moPrP genes were positioned under the control of the strong hCMV promoter and stably transfected into N2a cells.

In order to determine whether the mutant PrP molecules were expressed and delivered to the cell surface, we performed immunofluorescence microscopy with stably transfected polyclonal lines of N2a cells. Only overexpressed exogenous PrP (Fig. 2a–d), and not endogenous PrP of untransfected N2a cells (Fig. 2e), was detectable. Overexpressed wild-type, A105–125, G112–119 and A112–119 moPrPs were all detected on the surface of intact cells. The staining pattern around the cell periphery was similar before and after permeabilization (data not shown).

The size and glycosylation pattern of the mutant proteins were examined by Western blot (Fig. 2f). All four recombinant PrP molecules migrated as a series of three bands, which represent the un-, mono- and diglycosylated forms of the proteins. Wild-type, G112–119 and A112–119 moPrPs (Fig. 2f, lanes 1–3) were the same size as PrP C from other sources, whereas the Δ105–125 protein bands were shifted to a lower molecular mass (Fig. 2f, lane 4; also see Fig. 3). The levels of expression of A112–119, G112–119, Δ105–125 and wild-type moPrPs in stably transfected N2a cell lines were comparable, but were much higher than the expression of endogenous moPrP in normal N2a cells (see Fig. 3b).
Overexpression of wild-type, Δ105–125, G112–119 and A112–119 moPrPs. (a)–(e) N2a cell lines overexpressing wild-type (WT) (a), A112–119 (b), G112–119 (c) and Δ105–125 moPrP (d) as well as normal N2a cells (e) were labelled with mouse mAb 3B5 and stained with a fluorescein-coupled secondary antibody. Cells were observed by immunofluorescence microscopy. The exposure time was chosen to detect only overexpressed exogenous PrP. (f) Lysates of stably transfected N2a cell lines overexpressing wild-type (lane 1) and mutant (lanes 2–4) moPrPs were analysed by immunoblotting with mouse mAb 3B5. Equal amounts (100 µg) of protein were loaded in all lanes of the polyacrylamide gel. Positions of marker proteins are indicated.

Examination of PrP-specific fragments following deglycosylation

The sugar moieties of wild-type PrP<sub>C</sub> are N-linked and can be cleaved from their respective asparagines by PNGase F, giving rise to a protein of 27 kDa (Haraguchi et al., 1989). In order to study the PrP-specific fragments of the mutant moPrPs following deglycosylation, we performed Western blot analysis with different antibodies. Mouse mAb 13A5 immunoreacted with the 13A5-tagged wild-type, Δ105–125, G112–119 and A112–119 moPrPs (Fig. 3a, lanes 3–6) as well as with hamster PrP<sub>Sc</sub> in normal hamster or tg81 mice (Fig. 3a, lanes 1 and 2), but could not detect untagged endogenous PrP<sub>Sc</sub> of normal N2a cells (Fig. 3a, lane 7). mAb 6H4, which reacted with normal moPrP, also failed to detect endogenous PrP<sub>Sc</sub> (Fig. 3b, lane 5) due to the low level of expression.

Treatment of cell lysates with PNGase F reduced the heterogeneity of the PrP-specific bands in 13A5-tagged wild-type, G112–119 and A112–119 moPrPs to a fragment of 27 kDa [indicated by PrP<sub>Sc</sub> in Fig. 3a (lanes 3–5) and b (lanes 1–3)], which is consistent with the molecular mass of full-length deglycosylated PrP containing the GPI anchor. Δ105–125 moPrP showed the expected difference in molecular mass of about 2–3 kDa in comparison with wild-type moPrP (Fig. 3a, lane 6; b, lane 4). A prominent band of 18 kDa (designated C1) was recognized by mAbs 13A5 and 6H4 in blots of wild-type and A112–119 moPrPs [Fig. 3a (13A5), lanes 3 and 4; b (6H4), lanes 1 and 2]. It corresponded in size with the major C-terminal fragment of PrP<sub>Sc</sub> in hamster and tg81 mice (Fig. 3a, lanes 1 and 2). The PrP-specific fragment of 18 kDa was not detectable or was only weakly detectable with both antibodies in blots of Δ105–125 and G112–119 moPrPs [Fig. 3a (lanes 5 and 6), b (lanes 3 and 4) and c]. This finding was highly significant and reproducible. These experiments showed that, while all mutants carried N-linked glycosylation, the amount of the potentially proteolytic fragment C1 was dependent on the mutation introduced.

G112–119 moPrP is resistant to protease

In order to test the mutant moPrPs for protease resistance, the lysates were digested for 10 min with 3·3 µg/ml PK and treated with PNGase F to simplify the evaluation. Wild-type, A112–119 and endogenous moPrPs, as well as PrP of tg81 mice, were completely degraded under these conditions (Fig. 4a, lanes 2, 8, 10 and 12), but the mutant G112–119 moPrP gave a clear protease-resistant signal that migrated in the 22 kDa range (Fig. 4a, lane 6).

To assess the extent of protease-resistance of G112–119, we subjected lysates of G112–119 and wild-type moPrPs to digestion with 3·3 µg/ml PK for 10, 20, 40 and 60 min at 37 °C. In contrast to the complete degradation of wild-type moPrP (Fig. 4b, lanes 2–5), significant amounts of immunoreactive G112–119 moPrP remained after 10 and 20 min (Fig. 4b, lanes 7 and 8). Comparison with the protease-resistant core of hamster PrP<sup>Sc</sup> (Fig. 4b, lane 12) showed that the fragments of both hamster PrP<sup>Sc</sup> and G112–119 moPrP remaining after proteinase digestion migrated at a similar size range and faster than respective untreated samples (Fig. 4b; compare lanes 6 and 7 as well as lanes 11 and 12).

Doxycycline-induced expression of wild-type and G112–119 moPrPs

The protease resistance of G112–119 moPrP was examined further with the reverse tetracycline-inducible expression
system. The two stably transfected cell lines rtTA–wild-type and rtTA–G112–119 displayed inducibility (Fig. 5a; compare lane 2 with lane 3 and lane 4 with lane 5) when tested for background expression before induction and overexpression after induction with doxycycline. The level of expression increased about 2- to 3-fold after induction with 2 µg/ml doxycycline in the medium for 20 h. Comparison of the amount of wild-type or G112–119 moPrP in the uninduced state with the level of endogenous PrP in untransfected N2a cells shows a background of wild-type and G112–119 moPrPs (Fig. 5a, lanes 2 and 4).

In order to examine whether G112–119 became protease-resistant in the tetracycline-inducible system, we performed a digestion with 3-3 µg/ml PK for 10 min with lysates of cells before and after induction for 20 h with 2 µg/ml doxycycline. In contrast with the complete degradation of wild-type (Fig. 5b, lanes 8 and 10) and endogenous (Fig. 5b, lane 6) moPrP, we found protease-resistant G112–119 moPrP in the induced as well as in the uninduced state (Fig. 5b, lanes 2 and 4). The amount of protease-resistant G112–119 moPrP correlated with the amount of total G112–119 moPrP before induction (Fig. 5b, lanes 1 and 2) as well as after induction (Fig. 5b, lanes 3 and 4) and was about 50% of the total G112–119 moPrP in both expression states. The clear increase in the amount of protease-resistant PrP was therefore acquired within 20 h after induction. In the induced state, G112–119 moPrP showed a clear shift of the diglycosylated, monoglycosylated and unglycosylated bands after digestion with PK (Fig. 5b, lane 4).
amounts (100 µg) of lysate from normal N2a cells was added as a control (lane 1). Equal and 5) moPrPs was analysed by Western blot with the mAb 3B5. A cell 20 h. Expression of wild-type (lanes 2 and 3) and G112–119 (lanes 4 and 5) moPrPs was analysed by Western blot with the mAb 3B5. A cell lysate from normal N2a cells was added as a control (lane 1). 1–5 moPrPs was analysed by Western blot with the mAb 3B5. A cell lysate from normal N2a cells was added as a control (lane 1). (a) Stably double-transfected N2a cell lines were tested before (-) and after (+) induction with doxycycline (2 µg/ml medium) for 20 h. Expression of wild-type (lanes 2 and 3) and G112–119 (lanes 4 and 5) moPrPs was analysed by Western blot with the mAb 3B5. A cell lysate from normal N2a cells was added as a control (lane 1). Equal amounts (100 µg) of protein were loaded in all lanes. (b) Protein lysates of stably double-transfected N2a cell lines expressing wild-type (lanes 7–10) or G112–119 (lanes 1–4) and protein lysates of normal N2a cells (endogenous; lanes 5 and 6) were treated with PK (3±3 µg/ml) at 37 °C for 10 min or left untreated as indicated before (lanes 1, 2, 7 and 8) or after (lanes 3, 4, 9 and 10) induction with doxycycline (Dox; 2 µg/ml medium) for 20 h. Equal amounts (100 µg) of protein were then subjected to immunoblotting using Kan72 antiserum. (c) Proteins in lysates of cells expressing wild-type (lanes 6–8) or G112–119 (lanes 1–5) moPrPs after induction with doxycycline (2 µg/ml medium) were treated with PK at different concentrations (3±3, 10, 20, 50 µg/ml) at 37 °C for 10 min (lanes 2–5, 7 and 8) or left undigested (lanes 1 and 6). Equal amounts (100 µg) of protein were analysed by Western blot and the rabbit polyclonal antiserum Kan72. Positions of marker proteins are indicated.

Fig. 5. Doxycycline-induced overexpression of wild-type and G112–119 moPrPs. (a) Stably double-transfected N2a cell lines were tested before (-) and after (+) induction with doxycycline (2 µg/ml medium) for 20 h. Expression of wild-type (lanes 2 and 3) and G112–119 (lanes 4 and 5) moPrPs was analysed by Western blot with the mAb 3B5. A cell lysate from normal N2a cells was added as a control (lane 1). Equal amounts (100 µg) of protein were loaded in all lanes. (b) Protein lysates of stably double-transfected N2a cell lines expressing wild-type (lanes 7–10) or G112–119 (lanes 1–4) and protein lysates of normal N2a cells (endogenous; lanes 5 and 6) were treated with PK (3±3 µg/ml) at 37 °C for 10 min or left untreated as indicated before (lanes 1, 2, 7 and 8) or after (lanes 3, 4, 9 and 10) induction with doxycycline (Dox; 2 µg/ml medium) for 20 h. Equal amounts (100 µg) of protein were then subjected to immunoblotting using Kan72 antiserum. (c) Proteins in lysates of cells expressing wild-type (lanes 6–8) or G112–119 (lanes 1–5) moPrPs after induction with doxycycline (2 µg/ml medium) were treated with PK at different concentrations (3±3, 10, 20, 50 µg/ml) at 37 °C for 10 min (lanes 2–5, 7 and 8) or left undigested (lanes 1 and 6). Equal amounts (100 µg) of protein were analysed by Western blot and the rabbit polyclonal antiserum Kan72. Positions of marker proteins are indicated.

compared with the three full-length bands before digestion (Fig. 5b, lane 3). Treatment of G112–119 moPrP in the induced state with 3·3 µg/ml PK for different lengths of time yielded protease-resistant G112–119 moPrP after digestion for 10 and 20 min (data not shown), which corresponded to our results with the permanently expressed mutant moPrPs under the control of the hCMV promoter.

The high level of expression of wild-type and G112–119 moPrPs in the induced state gave us reason to test the extent of resistance to PK at higher concentrations. After induction for 20 h with 2 µg/ml doxycycline, we treated lysates of the N2a cell lines rtTA–G112–119 and rtTA–wild-type with PK at concentrations of 3·3, 10, 20 and 50 µg/ml for 10 min (Fig. 5c). Wild-type moPrP was degraded completely (Fig. 5c, lanes 7 and 8), whereas immunoreactive G112–119 moPrP still remained after digestion with 10 µg/ml PK (Fig. 5c, lane 3). The amount was still about 12% of full-length G112–119 moPrP (Fig. 5c, compare lane 3 with lane 1).

By employing both a permanent and an inducible expression system, we could show that the acquisition of protease resistance is an intrinsic property of G112–119 moPrP in N2a cells.

Discussion

Three different mutants of PrP were overexpressed in N2a cells and analysed alongside normal N2a cells and N2a cells overexpressing wild-type PrP. The three mutations were positioned in the central, evolutionarily highly conserved domain of the protein in order to reveal any consequences of changing or deleting the highly amyloidogenic ‘palindrome’ AGAAAGAGA (residues 112–119 in moPrP) (Gasset et al., 1992). By immunofluorescence analysis, we showed that all mutant prion proteins were transported to a large extent to the cell surface, as was the wild-type protein. Most mutant PrP that have so far been stably overexpressed in cells reach the cell surface, with the notable exception of proteins mutated at the first N-glycosylation site (Lehmann & Harris, 1997). In all mutant proteins presented here, both glycosylation sites were used. Therefore, we did not find any indication of a defect in the post-translational transport of the mutant PrPs to the cell surface, whereas a few pathogenic human mutations, such as P102L, D178N and F197, as well as an insertional mutation with nine additional octapeptides, were shown to have an effect on the delivery of the molecules to the cell surface (Ivanova et al., 2001).

A certain amount of normal PrPC is cleaved physiologically, which is identifiable by the presence of a C-terminal fragment (Chen et al., 1995; Harris et al., 1993; Jiménez-Huete et al., 1998; Pan et al., 1992). It is the amount of this C-terminal fragment relative to the full-length PrP that is altered substantially in two of the mutant PrPs presented here. It is easily conceivable why this might be a consequence of the extensive deletion in mutant Δ105–125 moPrP, in which all residues N- and C-terminal to the putative cleavage site (residue 110 or 111) have been removed (Chen et al., 1995). The strongly inhibitory effect of the mutant G112–119 on the
efficacy of cellular cleavage is probably determined directly by a mutation-induced alteration in protein conformation, but there are several possible explanations for how proteolytic processing is influenced by the altered structure of G112–119 moPrP: (i) the mutated residues might themselves be a poor substrate for the putative cleaving enzyme; (ii) the structure of the whole protein might be changed and the cleavage site might be less accessible; or (iii) due to the mutation introduced, the protein might be distributed on the cell surface in a different manner and therefore be in infrequent contact with the processing enzyme.

Analysis of the protease resistance of the various PrP mutants showed that G112–119 moPrP displayed resistance against PK digestion at a concentration of 3-3 µg/ml for 10 min at 37 °C. These conditions are much milder than those used in the detection of PrPSc in brain homogenates of diseased animals and humans (e.g. 20 µg/ml for 50 min at 37 °C; Büeler et al., 1994), but were used by Lehmann & Harris (1997) in the analysis of PrP genes carrying human-pathogenic mutations. In the latter study, the authors found that, under such conditions, the introduction of the human-pathogenic mutations P102L, D178N, T183A and E200K and an insertion of six additional octapeptides in the ORF of Prnp at the respective locations (P101L, D177N, T183A and E199K) rendered the mutated proteins resistant to PK at the above concentration. The PK resistance was always accompanied by enhanced concentration of PK, the dependence of these results on the cell type used (Petersen et al., 1998; Singh et al., 1997). The general importance of PrPSc for the pathogenesis of prion diseases remains controversial (Stewart & Harris, 2001).

Structural data on full-length recombinant PrP define the N-terminal half of the protein (residues 23–120 in moPrP and residues 23–124 in hamster PrP) as flexibly disordered or random-coil (Donne et al., 1997; Riek et al., 1997). Copper binding seems to confer a distinctive structure on the N-terminal octapeptide repeats (Viles et al., 1999), but the motif AGAAAAAGA is positioned at the hydrophobic C terminus of this unstructured part of PrP, and a recent refinement of the structure by Liu et al. (1999) has confirmed that it does not adopt regular secondary structure. Yet, the latter authors found indications of multiple discrete conformations in this hydrophobic domain, which might imply that this region is metastable and could therefore play an essential role in the formation of PrPSc. It was suggested that PrPSc interacts with recombinant PrPSC at amino acid residues 112–119 of the mouse sequence (Brown, 2000b), which might indicate that the sequence encompassing residues 112–119 is of special importance to the conversion of PrPSC to PrPSc. Copper ions reversibly induce PrPSC to become protease-resistant (Quaglio et al., 2001), but it remains to be seen in what way the binding of copper to the N-terminal repeats and the concomitant adoption of structure influences the structural transitions that potentially take place in this more C-terminal hydrophobic domain of the N terminus. Our data demonstrate that a certain mutation in the hydrophobic region induces the ready formation of PK-resistant PrP. Therefore, it can be hypothesized that this mutation stabilizes a conformation that shares features with the infectious form of PrP. Whether prion infectivity was generated de novo in tissue culture will be tested in future by using infectivity assays and transgenic animals.
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References


Quaglio, E., Chiesa, R. & Harris, D. A. (2001). Copper converts the cellular prion protein into a protease-resistant species that is distinct from the scrapie isoform. Journal of Biological Chemistry 276, 11432–11438.


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