PrP^{Sc}-like prion protein peptide inhibits the function of cellular prion protein

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Mice lacking expression of the prion protein are protected against infection with prion disease. Neurodegeneration in prion disease requires the formation of the abnormal isoform of the prion protein (PrP^{Sc}) from host prion protein. Therefore expression of normal host prion protein is necessary for prion disease. In the present investigation, it was demonstrated that PrP^{Sc} and a peptide resembling PrP^{Sc}, PrP106–126, both bind to cellular prion protein at amino acid residues 112–119. Interaction between PrP106–126 and the prion protein strips the prion

protein from cells. Direct interaction of PrP106–126 with the prion protein was found to make cells more susceptible to copper toxicity, inhibited copper uptake into cells and inhibited the superoxide dismutase-like activity of the prion protein. Direct inhibition of prion protein function by PrP^{Sc} may be necessary for neurodegeneration in prion disease.

Key words: copper, oxidative stress, re-folding, superoxide dismutase.

INTRODUCTION

Prion diseases are fatal neurodegenerative conditions, such as Creutzfeldt–Jakob disease of humans or bovine spongiform encephalopathy and scrapie of animals [1–4]. These diseases are characterized by accumulation of an abnormal isoform of the prion protein (PrP^{Sc}) in amyloid deposits [4,5]. PrP^{Sc} differs from the normal cellular isoform (PrP^C) on the basis of conformation, the ability to form fibrils *in vitro*, and possibly in that PrP^{Sc} can be infectious and transmit prion disease [4]. Mice lacking expression of PrP^C cannot be infected with prion disease [6,7] and neurons lacking PrP^C expression cannot be killed by the toxicity of PrP^{Sc} [8,9]. These findings indicate that PrP^C expression is an essential requirement for the disease. Understanding PrP^C function and activity is therefore an essential requirement for understanding the aetiology of the prion diseases.

Expression of the prion protein enhances the survival of neurons, at least in culture [10,11]. Although some strains of mice lacking PrP^C expression remain healthy [6,12], others do not [13,14]. Recent evidence indicates that the PrP^C protects cells against both oxidative stress [10,15] and the toxicity of copper [16]. There is now a large body of evidence suggesting the PrP^C binds copper [17–21], and that the presence of copper both enhances the rate of turnover of PrP^C [22] and endows the protein with a superoxide-dismutase-like activity [21,23], which might explain its apparent antioxidant function [10]. Additionally, expression of PrP^C enhances copper uptake at nanomolar concentrations [24]. The assays used to study the antioxidant activity of PrP^C and the ability of PrP^C to enhance copper uptake provide a way to determine by assay whether interaction between PrP^C and PrP^{Sc} or a peptide derivative leads to a loss of PrP^C function.

Investigation of the neurotoxicity of PrP^{Sc} and a synthetic peptide based on its sequence (PrP106–126) indicates that the toxic mechanism of PrP^{Sc} in neurons may involve a change or loss of function of PrP^C. Evidence from cell-culture studies suggests that loss of cellular resistance to oxidative stress is an essential component of the toxicity of either PrP^{Sc} or its peptide-mimic, PrP106–126 [9,10,25]. Cells from mice lacking PrP^C expression (Prnp^{0/0}) are more sensitive to oxidative stress [10,26]

and the toxicity of copper [16], whereas some cells expressing increased levels of PrP^C show an increased resistance to oxidative stress and copper toxicity [15]. The similarities between changes in cells treated with PrP106–126 and the novel characteristics of cells lacking prion protein expression provide indirect evidence implying that PrP106–126, and perhaps PrP^{Sc}, induce a loss of function.

The aim of the present investigation was to address the question as to whether PrP^{Sc} and PrP106–126 can bind directly to PrP^C, and whether this binding inhibits functions of PrP^C. The results indicate that PrP^C does bind to PrP^{Sc} and PrP106–126 at the same region of the PrP^C molecule. Interaction with this region (amino acid residues 112–119) inhibits the uptake of copper into cells, and prevents the superoxide-dismutase-like activity of the recombinant protein. These results provide evidence that PrP^{Sc} and PrP106–126 directly inhibit the function of PrP^C.

EXPERIMENTAL

Protein purification and mutagenesis

Recombinant mouse prion protein was expressed in bacteria and purified using a histidine-tagging method, as described previously [23]. Refolding of the protein using urea and the binding of copper to the protein were also performed as described previously [23]. A PCR-based method of mutagenesis was used to delete amino acid residues 112–119 of the mouse prion protein sequence, as described in [23]. The splint oligonucleotides used to make the deletion were CCTCAAGCATGTGGTAGTGGGGGGCC and GGCCCCCACTACCACATGCTTGAGG. Additionally, a substitution mutant was also prepared using the oligonucleotides AAGCATGTGGGAGGCGGTGGGGGTGGAGGCGGGAAGCATGTG and CCCCCTACCCCGCCTCCACCC-CCACCGCCTCCCACATGCTT to substitute octaglycine for amino acid residues 112–119 (PrP-R112–119). The deletion mutant PrPΔ51–89 was as previously described [23]. N-terminal sequencing and MS analysis were performed in-house according to standard procedures by the PNAC facility.

Abbreviations used: PrP^{Sc}, the abnormal isoform of the prion protein; PrP^C, the normal cellular isoform of prion protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; TXRF, total X-ray reflection fluorescence; PI-PLC, phosphatidylinositol-dependent phospholipase C; BCS, bathocuproine sulphonate.

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Figure 1 PrP106–126 binds to PrP^{Sc}

An ELISA-based assay was used to assess the binding of recombinant PrP^{Sc} to immobilized PrP106–126 (A) or PrP^{Sc} (B). ○, Wild-type PrP^{Sc}; ●, PrPΔ112–119; ▲, PrP-R112–119; (C) A similar ELISA-based assay was used to assess the binding of biotinylated PrP106–126 to immobilized recombinant PrP^{Sc}. △, Wild-type PrP^{Sc}; ▲, PrPΔ112–119 PrP^{Sc}; ○, PrP-R112–119; ●, PrPΔ51–89. Detection in (A) was with DR1, in (B) with DR3, and in (C) with streptavidin–horseradish peroxidase. Shown are the means ± S.E.M. for four experiments.

ELISA

PrP or PrP106–126 was bound to 96-well ELISA plates in water at 100 ng/well overnight at 4 °C. After extensive washing with PBS, the wells were reacted with serial dilutions of wild-type recombinant PrP^{Sc}, mutant PrP^{Sc} or biotin-labelled PrP106–126. After incubation for 1 h at room temperature, the non-bound material was washed off with PBS. Antibodies raised against PrP were then applied to the plates, and their detection was ac-

Figure 2 PrP106–126 harvests PrP^{Sc} from cells

(A) Western blot of recombinant prion protein. Either wild-type PrP^{Sc} or PrPΔ112–119 PrP^{Sc} was reacted with PrP106–126 and electrophoresed on a polyacrylamide gel. PrP was detected with the antibody DR1. (B and C) Peptides applied to cells were collected from the supernatant by centrifugation. The pellets were dissolved in urea and electrophoresed on a polyacrylamide gel. The resulting gel was either stained with Coomassie Blue or blotted, and PrP was detected with DR1 antibody, which does not recognize PrP106–126. Lanes 1, no peptide; lanes 2, 100 µM scrambled peptide; lanes 3, 20 µM PrP106–126; lanes 4, 100 µM PrP106–126; lanes 5, 100 µM βA25–35.

complished using either horseradish-peroxidase-conjugated anti-rabbit antibody or streptavidin–horseradish peroxidase (Amersham International, Little Chalfont, Bucks., U.K.). After the colorimetric reaction had reached completion, the level of horseradish peroxidase activity in each well was quantified using a plate reader (Pharmacia, Milton Keynes, U.K.) with a 492 nm filter. Peptides used in the present study were as described previously [27,28], or PrP^{Sc} was prepared from mice infected with the ME7 strain of scrapie using the method described in [29]. The antibodies used for detection were purified from rabbit polyclonal sera produced against either a peptide based on amino acid residues 89–103 of the mouse prion protein sequence (also referred to as DR1) or amino acid residues 37–53 (DR3).

Cell culture

Cerebellar cell cultures were prepared as described previously [25]. Survival of cerebellar cells was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT)-based assay, as previously described [25]. The preparation of astrocytes and co-culture of astrocytes with cerebellar cell cultures was performed as described in [30].

Copper uptake

Radioactive copper (⁶⁷Cu) was purchased from Brookhaven Laboratories, New York, NY, U.S.A. The determination of specific radioactivity and the method of assaying uptake by cerebellar cells were performed as described previously [24].

Total X-ray reflection fluorescence (TXRF) spectroscopy

Analysis of copper content using TXRF spectroscopy was as described previously [17,23].

Superoxide and superoxide dismutase assays

The Nitro-Blue-Tetrazolium/xanthine-oxidase-based assay was used to assess superoxide dismutase-like activity. The use of this assay for assessing prion protein activity was as previously described [23]. To assess superoxide production by PrP106–126 and PrP^{Sc}, the same assay was used, except that xanthine oxidase and xanthine were not added to the assay mixture. The amount of formazan was determined after incubating 10 μ g of PrP106–126 or PrP^{Sc} in the assay mixture for 10 min.

RESULTS

In order to demonstrate more directly that PrP^{Sc} or PrP106–126 inhibits the function of PrP^C, we first had to demonstrate that both PrP^{Sc} and PrP106–126 bind to PrP^C specifically at the same site. We prepared recombinant mouse PrP^C using a histidine-tag purification system [23]. In addition, we also generated three mutants of PrP^C. The first (PrP Δ 112–119) lacked the amino acid residues 112–119, which form the palindromic region AGAAAGAA, a region of the protein that is universally conserved [31]. The second had a substitution of GGGGGGGG for this region (PrP-R112–119). The third had the octa-repeat region deleted (PrP Δ 51–89). Using an ELISA-based system, PrP^{Sc} or PrP106–126 was bound to a 96-well plate and the recombinant protein was incubated with the bound protein or peptide. After thorough washing, bound PrP^C was detected using a monoclonal antibody

that recognizes the N-terminal region of PrP^C not present in PrP106–126 or PrP^{Sc} (Figures 1A and 1B). Full-length PrP^C and PrP Δ 51–89 bound to both PrP106–126 and PrP^{Sc} in a dose-dependent manner. There was no binding to either PrP Δ 112–119 or PrP-R112–119. Additionally, PrP^C and the three mutant recombinant proteins were bound to the ELISA plate and reacted with PrP106–126 that had been biotinylated. The biotin-labelled peptide was detected with a streptavidin–horseradish peroxidase method (Figure 1C). PrP106–126 only bound to full-length PrP^C or PrP Δ 51–89. These results suggest that PrP^{Sc} and PrP106–126 can bind specifically to PrP^C via the AGAAAGAA region.

PrP106–126 was reacted with 10 μ g/ml PrP^C or PrP Δ 112–119 for 2 h at room temperature in PBS. The resulting protein/peptide solution was electrophoresed on an SDS-denaturing gel (Figure 2A). PrP was detected with an antibody that does not recognize PrP106–126. Reaction with PrP106–126 caused a proportion of PrP^C to be shifted to a higher molecular mass, and caused the formation of what appeared to be dimers of protein. However, the peptide did not have this effect on PrP Δ 112–119.

Cerebellar cells from wild-type mice that have been shown to express high levels of PrP^C [30] were exposed to 100 μ M PrP106–126 or β A25–35, another amyloid-forming peptide, for 24 h. This period of peptide treatment is insufficient to cause neuronal death. After this time, the medium from the cells was collected. The peptides applied in these solutions form large aggregates,

Figure 3 PrP106–126 enhances copper toxicity

(A) Cerebellar cells from either wild-type or Prnp^{0/0} mice were treated with 80 μ M PrP106–126 with or without prior treatment with PI-PLC. Survival was determined with an MTT assay after 5 days. The values were compared with that of untreated controls as a percentage. (B) Wild-type cerebellar cells were treated with PrP106–126 and increasing concentrations of one of six different antibodies. Survival of the cerebellar cells was determined after 5 days with an MTT assay. The antibodies used were DR1 ($\circ$), DR2 ($\square$), DR3 ($\bullet$), DM1 ($\blacksquare$), DM2 ($\triangle$) and DM3 ($\blacktriangle$). (C) Wild-type cerebellar cells were grown in either serum-supplemented ($\bullet$) or serum-free ($\circ$) conditions, and treated for 4 days with 40 μ M PrP106–126. Concurrently, increasing concentrations of CuSO₄ were applied to the cerebellar cells. Relative survival was determined in comparison with untreated controls using an MTT assay. (D) Cerebellar cells from wild-type ($\circ$) and Prnp^{0/0} mice ($\bullet$) were treated with 40 μ M CuSO₄ and increasing concentrations of PrP106–126. After 4 days, relative survival was determined with an MTT assay. Shown are the means $\pm$ S.E.M. for a minimum of four experiments (three determinations each).

Figure 4 Chelation of copper inhibits PrP106–126 toxicity

(A) BCS was applied to wild-type cerebellar cells at increasing concentrations with 100 μM PrP106–126. After 5 days of treatment, survival was determined with an MTT assay and the results were expressed as a reduction in toxicity for either serum-supplemented conditions (●) or serum-free conditions (○). (B) Chelators of other metals were also tested for their ability to inhibit the toxicity of 100 μM PrP106–126 to wild-type cerebellar cells grown in serum-supplemented conditions. After 5 days of treatment, survival was determined with an MTT assay, and the results were expressed as a reduction in toxicity. Shown are dose–response curves for dipicnic acid (●), bathophenanthroline disulphonic acid (○) and cyclohexanediaminetetraacetic acid (△). (C) Prnp^{0/0} cerebellar cells were grown alone (open bars), co-cultured with wild-type astrocytes (black bars) or co-cultured with Prnp^{0/0} astrocytes (grey bars). The cells were treated with 40 μM CuSO₄ alone or in combination with 40 μM PrP106–126 for 5 days. The relative survival of the cerebellar cells after removal of the astrocytes was determined using an MTT assay. PrP106–126 is not toxic to Prnp^{0/0} cerebellar cells on its own (see Figure 2A). Shown are the means $\pm$ S.E.M. for a minimum of four experiments (three determinations each).

which can be collected by high-speed centrifugation. Peptide collected this way was compared with pelleted material from control cultures. The collected peptide was denatured with 8 M urea, and analysed following SDS/PAGE. Two distinct bands

could be detected on a Coomassie-Blue-stained gel only in the pelleted material from PrP106–126-treated cells (Figure 2B). Western blotting and immunodetection with a specific antibody (DR1), which does not detect PrP106–126, identified these bands as being prion protein (Figure 2C). The bands present on the Coomassie-Blue-stained gel were analysed by both N-terminal sequencing and MS, and were identified as the prion protein truncated at amino acid residue 89 or 90. A small amount of prion protein was also present in the pellet from $\beta\text{A25–35}$ -treated cells. These results suggest that PrP106–126 binds to PrP^c, can cause it to aggregate and can remove it from the surface of cells such as cerebellar cells without causing cell death.

PrP106–126 and PrP^{Sc} are toxic to cell via the same mechanism [9,25]. Therefore PrP106–126 mimics PrP^{Sc} toxicity and provides a valid model for its action. The necessity for interaction of PrP106–126 with PrP^c at the cell surface to bring about PrP106–126 toxicity was tested. Phosphatidylinositol-specific phospholipase C (PI-PLC) is an enzyme that has been shown to cleave PrP^c from the surface of cells [32]. Cells were treated with 0.2 unit/ml PI-PLC once every 6 h for 5 days. This treatment stripped PrP^c from the cells, as indicated by Western blotting (results not shown). During this time, the cells were also treated with 80 μM PrP106–126. The cells used for these experiments were either wild-type cells expressing PrP^c or cells lacking PrP^c expression (Prnp^{0/0}). Neither PI-PLC or PrP106–126, nor a combination of both, had any effect on the survival of Prnp^{0/0} cerebellar cells (Figure 3A). PI-PLC reduced the survival of wild-type cerebellar cells slightly, but this treatment did not block the toxicity of PrP106–126.

Purified antibodies specific for PrP^c were tested to see if they could block the toxicity of PrP106–126. Six different antibodies were purified as an IgG fraction. The antibodies recognize the following amino acid residues of the mouse prion protein: 89–103 (DR1), 94–109 (DR2), 37–53 (DR3), 68–84 (DM1), 121–136 (DM2) and 142–160 (DM3). Of these, only two antibodies blocked toxicity (Figure 3B). The epitopes of these antibodies were within the amino acid residues 94–109 and 121–136. Analysis using ELISA indicated that these antibodies cannot detect the peptide PrP106–126 (results not shown). Because none of the antibodies used can interact with the peptide, then any effect of the antibodies must be due to interaction with prion protein at the cell surface. The implications of this are that the two antibodies prevented interaction of the peptide with the prion protein, and that this prevented PrP106–126 toxicity. This suggests that the peptide interacts within the region of amino acid residues 94–136 of the prion protein. These results suggest that, although the interaction of PrP106–126 and PrP^c is necessary for PrP106–126 toxicity, it is not necessary that PrP^c remains anchored to the cell surface.

Cerebellar cells were grown either in serum-free conditions or serum-supplemented conditions. PrP106–126 at a concentration of 40 μM was applied to the cerebellar cells for 4 days, along with increasing concentrations of CuSO₄. PrP106–126 applied to cerebellar cells grown in serum-free conditions showed significantly less toxicity than when applied in serum-supplemented conditions (Student's *t* test, $P < 0.05$). However, under both conditions low concentrations of CuSO₄ enhanced peptide toxicity drastically (Figure 3C). Addition of CuSO₄ abolished the difference in toxicity seen with different media.

In another set of experiments, 40 μM CuSO₄ was applied to both wild-type and Prnp^{0/0} cerebellar cells (lacking PrP^c expression). PrP106–126 at low concentrations is not toxic. It has been shown previously that PrP106–126 is not toxic to Prnp^{0/0} cerebellar cells at any concentration [25]. The toxicity of 2 μM PrP106–126 to wild-type cerebellar cells after 2 days of treatment

Figure 5 PrP106–126 inhibits copper uptake

(A and B) Uptake of radioactive copper (^{67}Cu) by wild-type (○) and Prnp^{0/0} (●) cerebellar cells grown in serum-free conditions 2 days before experimentation. ^{67}Cu was applied as a histidine chelate for 2 h. PrP106–126 was applied at increasing concentrations before application of ^{67}Cu -histidine. The cells were extensively washed with Hanks solution, and separated into membrane-rich (A) and cytosolic (B) fractions. The relative level of ^{67}Cu uptake was determined by γ -radiation counting, and expressed as c.p.m./mg of protein of the original cells. (C–E) Copper content of wild-type and Prnp^{0/0} cerebellar cells grown in serum was determined either without treatment (open bars) or after treatment with 40 μM PrP106–126 (grey bars). TXRF analysis was used to determine the content of copper in the cells (C) and the medium (D), and the pelletable material was prepared from the medium of treated and untreated cells, and its copper content was also determined by TXRF analysis (E). Shown are the means $\pm$ S.E.M. for a minimum of three experiments.

was determined by MTT assay. The MTT assay value for this treatment was $101 \pm 1\%$ of the untreated control values ($n = 5$), indicating that this concentration is not toxic. The toxicity of CuSO_4 to cerebellar cells in the presence of PrP106–126 at low concentrations was tested. As shown previously [16], CuSO_4 is more toxic to cells lacking PrP^c expression (Figure 3D). Addition of increasing concentrations of PrP106–126 enhanced the toxicity of CuSO_4 to wild-type cells, but had no effect on the toxicity of CuSO_4 to Prnp^{0/0} cerebellar cells. These results suggest that cells that express PrP^c become more sensitive to copper toxicity when exposed to PrP106–126.

Bathocuproine sulphonate (BCS), a chelator specific for copper, was added to wild-type cerebellar cells at non-toxic concentrations directly before the addition of 100 μM PrP106–126. This amount of peptide caused a $> 50\%$ reduction in cell survival over 5 days. Cell survival was assayed after this time, and the relative inhibition of PrP106–126 toxicity was measured (Figure 4A). Increasing concentrations of BCS inhibited PrP106–126 toxicity. The presence or absence of serum had no significant effect ($P > 0.05$) on the protection provided by BCS.

Chelators of other metal ions were tested for their ability to inhibit the toxicity of PrP106–126. Chelators of manganese (cyclohexanediaminetetra-acetic acid), iron (bathophenanthro-

line disulphonic acid) and zinc (dipicnic acid) had no effect on the toxicity of PrP106–126 (Figure 4B). This suggests that the toxicity of PrP106–126 in this cell culture system involves only copper.

Astrocytes protect neurons from the toxicity of compounds such as glutamate. By virtue of the expression of metallothioneins and copper-storage proteins, astrocytes may also protect neurons from copper toxicity. In order to test this, Prnp^{0/0} cerebellar cells were co-cultured with wild-type or Prnp^{0/0} astrocytes. Wild-type astrocytes protected the cerebellar cells from the toxicity of 40 μM CuSO_4 , but Prnp^{0/0} astrocytes did not (Figure 4C). PrP106–126 was not toxic to Prnp^{0/0} cerebellar cells (40 μM ; $102 \pm 2\%$ of the untreated control value, $n = 4$). Application of 40 μM PrP106–126 inhibited the protective effect of wild-type astrocytes against 40 μM CuSO_4 . Since astrocytes also express PrP^c, it is possible that PrP106–126 inhibits the protective effects of astrocytes against copper toxicity by inhibiting the ability of PrP^c expressed by the cells to clear copper.

Assays were performed to test the effect of PrP106–126 on the copper content of cells. It has been shown previously that radioactive copper provided as a histidine chelate is taken up by cells expressing PrP^c at a rate higher than by those that do not. Cerebellar cells were exposed to 0.2 μM ^{67}Cu applied in a

Figure 6 PrP106–126 inhibits PrP^{Sc} antioxidant activity

(A) The Nitro Blue Tetrazolium/xanthine oxidase spectrophotometric assay used routinely to measure superoxide dismutase activity was used to assay the ability of PrP106–126 ($\circ$), PrP^{Sc} ($\bullet$) or the $\beta\text{A25-35}$ peptide ($\triangle$) to inhibit the superoxide dismutase-like activity of PrP^{Sc}. Increasing concentrations of each agent were added to a reaction tube containing 1 $\mu\text{g/ml}$ recombinant mouse wild-type PrP^{Sc}. The values obtained were expressed as % inhibition of the superoxide dismutase-like activity of PrP^{Sc}. (B) Copper content of recombinant wild-type PrP^{Sc} after incubation with PrP106–126, PrP^{Sc} or the $\beta\text{A25-35}$ peptide. The protein mixture was either dialysed (open bars) or digested with proteinase K, and dialysed to remove non-bound copper (black bars). Analysis of copper content was performed with TXRF spectroscopy. Values of copper content were on the basis of the original PrP^{Sc} content. (C) The ability of various agents to convert Nitro Blue Tetrazolium into formazan as determined by spectrophotometric assay. The absorbance at 560 nm was determined after exposure of the agents to the reaction mixture for 10 min. Shown are the means $\pm$ S.E.M. for a minimum of four experiments.

mixture with 40 μM histidine in the presence or absence of increasing concentrations of PrP106–126. Following a 2 h treatment, the cells were washed, and membrane and cytosolic fractions were prepared. As shown previously, ^{67}Cu retained by membrane fractions (Figure 5A) or taken up into a cytosolic fraction (Figure 5B) of wild-type cells was higher than that in the equivalent fraction from Prnp^{0/0} cells. Application of PrP106–126 did not alter the ^{67}Cu content of Prnp^{0/0} cerebellar cells, but decreased the ^{67}Cu content of fractions from wild-type cells. This suggests that PrP106–126 inhibits uptake into cells expressing PrP^{Sc}.

Cerebellar cells were grown for 2 days in the presence of 40 μM PrP106–126. The copper content of the cells and the medium was assayed by TXRF spectroscopy. In addition, the peptide from some samples was separated from the medium by centrifugation, and collected. The copper content of the pellet was also determined. Total cellular copper content of Prnp^{0/0} cerebellar cells was unaffected by PrP106–126 treatment (Figure 5C), as was the copper content of medium from these cells (Figure 5D). When the pelletable peptide from Prnp^{0/0} cerebellar cells exposed to PrP106–126 was assayed, there was little evidence of increased copper content as compared with the pellet from the medium of cells that was not exposed to the peptide (Figure 5E). In comparison, the pelletable peptide from wild-type cultures retained a very high copper content (Figure 5E). Peptide treatment of wild-type cerebellar cells decreased the amount of copper that could be detected in the cells (Figure 4C), but increased the amount of copper than could be detected in the medium (Figure 4D). These results suggest that PrP106–126 inhibits uptake of copper into the cells.

Once PrP^{Sc} binds at least two atoms of copper, the protein demonstrates an antioxidant activity similar to that of superoxide dismutase [21,23]. This activity is readily detectable by spectrophotometric assays. To produce PrP^{Sc} superoxide dismutase-like activity, the protein was refolded in the presence of copper, as described previously. Recombinant PrP^{Sc} was assayed for superoxide dismutase-like activity. Increasing the concentrations of either PrP106–126 or PrP^{Sc} inhibited the superoxide dismutase-like activity (Figure 6A). To determine whether this loss of activity was due to decreased copper content, PrP^{Sc} reacted with PrP106–126 or PrP^{Sc} was dialysed and assayed using TXRF spectroscopy. No difference in the amount bound was identified (Figure 6B). This implies that PrP106–126 or PrP^{Sc} does not induce loss of PrP^{Sc} activity by causing PrP^{Sc} to release copper. Samples of PrP^{Sc}-reacted PrP106–126 or PrP^{Sc} were also digested with proteinase K (10 units/ml), dialysed, and the copper content was assayed. This treatment removed virtually all of the bound copper, suggesting that the copper was not sequestered by the proteinase K-resistant protein (i.e. PrP106–126 or PrP^{Sc}). Finally, PrP106–126 and PrP^{Sc} were assayed for their ability to generate superoxide by applying them directly to the superoxide dismutase assay without including the oxygen radical generator. In comparison with 10 μM DMSO, neither PrP106–126 nor PrP^{Sc} generated significant levels of reactive oxygen species. Addition of 10 μM CuSO_4 with PrP106–126 likewise did not enhance the oxygen-radical formation by the peptide (Figure 6C).

DISCUSSION

Alteration of PrP^{Sc} function or activity is central to prion disease. Without PrP^{Sc}, mice cannot be infected with the disease [7], and therefore PrP^{Sc} expression is an essential requirement. Although mice lacking expression of PrP^{Sc} are healthy, they show subtle differences in behaviour [33] and, at the cellular level, there are indications of altered metabolism [10,11,16]. Some critics have

argued that because 'knock-out' of PrP^c does not lead to prion disease, loss of PrP^c function is not important to prion disease. However, the assumption that loss of PrP^c function would be all that is required to initiate disease is a false one, since prion disease is inseparable from the formation of PrP^{Sc} from PrP^c, and far more is biologically implicit in PrP^c expression than merely protein function alone (e.g. interaction of PrP^c or metabolic fragments with other proteins). Evidence that PrP^c function protects against prion disease came from work with transgenic mice expressing hamster PrP^c via a GFAP promoter to direct cellular expression to astrocytes only [34]. When the mice (wild-type) expressed both hamster and mouse PrP^c, infection with hamster prion proteins did not lead to prion disease. Hamster PrP^{Sc} cannot convert mouse PrP^c into PrP^{Sc}, and the mouse protein remains functional. However, when the PrP^c-deficient mice were modified to express hamster PrP^c via the GFAP promoter (i.e. lacking neuronal expression of PrP^c), it was possible to infect these mice with hamster prions, and the mice developed the pathology of prion disease [34]. One interpretation of these results is that mice lacking functional mouse PrP^c in neurons are susceptible to the toxicity of hamster PrP^{Sc}, whereas mice with functional mouse PrP^c in neurons were protected. Therefore loss of PrP^c function may be necessary for neurodegeneration in prion disease.

PrP106–126 has been used as a model for the neurotoxicity of PrP^{Sc} for a while now [10,25,27,28,35]. The basic mechanism of action of both PrP^{Sc} and PrP106–126 is the same *in vitro*. PrP^c expression is a requirement that is essential for toxicity of PrP106–126 and PrP^{Sc} *in vitro*, and for PrP^{Sc} *in vivo* [8,9,25]. Therefore, in assessing the ability of PrP106–126 to mimic PrP^{Sc}, it was essential to determine whether both proteins interact with PrP^c and, if so, to demonstrate that they do so at a similar region. It was found that both PrP^{Sc} and PrP106–126 interact with recombinant PrP^c at amino acid residues 112–119 of the mouse sequence. Additionally, both PrP^{Sc} and PrP106–126 directly inhibit the superoxide dismutase-like activity of recombinant PrP^c. This implies that, in assessing protein–protein interactions, PrP106–126 may serve as a good substitute for PrP^{Sc}. This fits in well with evidence that PrP^{Sc} and PrP106–126 have other similar characteristics, such as resistance to proteinase K, a similar content of β -sheet and the ability to form fibrils [35,36].

Antibodies that bound in the vicinity of the region of amino acid residues 112–119 inhibited the toxicity of PrP106–126 to cultured neurons. Antibodies that bound elsewhere did not. This is possibly because the antibodies prevented PrP106–126 from directly interacting with PrP^c. This is consistent with other studies on the interaction between hamster PrP and a peptide on the basis of the PrP^c sequence, which suggest that the well-known antibody 3F4, which recognizes amino acid residues surrounding residues 109–112, can block the interaction [37]. Evidence that PrP106–126 binds to PrP^c from cultured cells is drawn from experiments showing that aggregates of PrP106–126 harvest PrP^c from cultured cells (Figure 2C).

Stripping PrP^c from cells with PI-PLC does not protect them from the toxicity of PrP106–126, implying that the interaction with PrP^c does not directly cause toxicity to neurons via a signal-transduction mechanism leading directly from PrP^c as a result of PrP106–126 binding to it. Salmona et al. [38] have shown that PrP106–126 can interact with cells and alter properties of the membrane such as microviscosity. However, it has also been shown that PrP106–126 can interact with the membranes of neurons that lack expression of PrP^c [28]. Therefore it is possible that interactions between PrP106–126 and PrP^c are not necessary for the effects of PrP106–126 at the membrane. However, these

effects do not cause neuronal death, since PrP106–126 is not toxic to neurons lacking PrP^c expression [25]. Therefore membrane interactions, or even internalization of the peptide, are insufficient on their own to cause neurotoxicity. Preventing interaction of the peptide with PrP^c greatly reduced the toxicity of PrP106–126. This provides further support for the hypothesis that PrP106–126 is toxic to neurons by inhibiting PrP^c function.

PrP106–126 was less toxic to neurons in serum-free medium than in medium supplemented with serum. Although this would appear to be an unexpected result, there is a possible explanation for it: serum-free medium contains low levels of glutamate, copper and other potentially toxic substances. One of the advantages of serum replacements is that for some cell types, especially cerebellar cells, use of these supplements leads to cultures with higher viability. Therefore the explanation of this result is that the serum-free medium increases the viability of the cells. This, in turn, protects neurons from the toxicity of PrP106–126, which is known to be enhanced by toxic substances such as superoxide [25] and, as shown in this paper, copper.

PrP106–126 enhanced the toxicity of copper and inhibited copper uptake into neurons. Given that large amounts of PrP^c accumulated in aggregates of PrP106–126, then it is possible that PrP106–126 inhibited copper uptake by inhibiting PrP^c uptake into cells. PrP106–126 toxicity involves binding of the peptide to PrP^c on the cells' surface. Because aggregates of PrP106–126 accumulate large amounts of PrP^c from cells, it is possible that PrP106–126 exerts its effect on copper uptake by binding to PrP^c and tearing it from the cell surface to join the aggregate. This would result in inhibition of PrP^c uptake into the cells, and inhibition of the amount of copper taken up bound to PrP^c. A consequence of this further downstream in the signal-transduction pathway would be an increase in the copper content of the culture medium, which would subsequently enhance the level of copper toxicity.

The results of the experiments presented here suggest that PrP106–126 directly inhibits the superoxide activity of recombinant PrP^c by directly binding to it at amino acid residues 112–119. This protein still bound copper, implying that the loss of activity was not due to release of copper. This implies that this region is of importance to the superoxide dismutase-like activity of PrP^c, but that binding of the peptide to the protein does not inhibit its ability to bind copper. The peptide may tear PrP^c away from the cell surface, decreasing copper uptake. Copper taken up bound to PrP^c has previously been shown to be utilized by the cytosolic Cu/Zn superoxide dismutase [39]. Additionally, it has also been shown that PrP106–126 toxicity directly involves a decrease in neuronal resistance to oxidative stress. This might be as a result of loss of the antioxidant PrP^c itself. Copper bound to PrP^c stripped from the surface of cells by PrP106–126 might eventually be released by the action of proteases, and this may eventually lead to increased toxicity attributable to the copper itself. Copper can also generate reactive oxygen species. This implies that neurodegeneration caused by PrP106–126 is probably a result of direct inhibition of the functions of PrP^c in both copper homeostasis and resistance to oxidative stress.

The sequence encompassing residues 112–119 is of special importance to PrP^c and its role in prion disease. This region contains the palindrome AGAAAAGA. The region AGAA-AAGA has been implicated in the transmission of the disease. Cells transfected with a PrP transgene that express a mutant PrP with a deletion of this region of the protein cannot be infected with PrP^{Sc} [40]. This implies that this region is necessary for conversion of PrP^c into PrP^{Sc}. As the cells transfected with this construct also express wild-type PrP^c, then the transgene acted as a dominant-negative mutation, inhibiting conversion of the wild-

type protein. Furthermore, peptides generated that include the AGAAAAGA region inhibit the conversion *in vitro* of PrP^c into PrP^{Sc}, suggesting that these amino acid residues are essential for the interaction of the two molecules necessary for the conversion event [41]. This region of the protein, which has been highly conserved during evolution [31], may turn out to be the key to understanding the disease process.

In summary, PrP^{Sc} and PrP106–126 can bind to PrP^c at the same region. Binding of PrP106–126 to PrP^c inhibits copper-related functions of PrP^c. This inhibition could be part and parcel of the process of conversion of PrP^c into PrP^{Sc}. Further studies with animal models are necessary to verify that loss of PrP^c function by PrP^{Sc} is a part of the mechanism of neurodegeneration seen in prion diseases.

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