Prion protein expression and superoxide dismutase activity

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The function of the prion protein (PrP^c) remains uncertain. It has been suggested that prion protein expression may aid cellular resistance to oxidative stress by influencing the activity of Cu/Zn superoxide dismutase (Cu,Zn SOD). The activity of Cu,Zn SOD was investigated in mice with different levels of PrP^c expression. Increasing levels of PrP^c expression were linked to increased levels of Cu,Zn SOD activity. Western-blot and Northern-blot analysis indicated that mice either lacking or overexpressing PrP^c had levels of Cu,Zn SOD mRNA equivalent to those expressed in wild-type mice. Mice overexpressing the prion protein had lower levels of resistance to oxidative stress but higher expression levels of glutathione peroxidase, probably due to increased levels of hydrogen peroxide produced by increased Cu,Zn SOD activity. When cells were metabolically labelled with radioactive copper, increased radioactivity was immunoprecipitated with Cu,Zn SOD from mice with higher levels of PrP^c. In addition, diethyl-dithiocarbamate, a copper chelator that inactivates Cu,Zn SOD by capturing copper from the molecule, is more able to inactivate Cu,Zn SOD expressed in animals with higher levels of PrP^c. As recent studies have suggested that PrP^c may regulate some aspect of copper metabolism, it is suggested that PrP^c expression may regulate Cu,Zn SOD activity by influencing copper incorporation into the molecule.

INTRODUCTION

The prion protein (PrP^c) is a cell surface glycoprotein expressed by neurones and glia [1,2]. An abnormal isoform of this protein (PrP^{Sc}) [3] is involved in prion disease. Prion diseases are characterized by neurodegeneration, gliosis and accumulation of extracellular deposits of PrP^{Sc}, sometimes in the form of plaques. Human prion diseases include Creutzfeldt–Jakob disease, Gerstmann–Sträussler–Scheinker syndrome and Fatal Familial Insomnia [4–6]. Diseases in animals include bovine spongiform encephalopathy [7] and scrapie [8].

A number of possible explanations exist for the neuro-degeneration seen in prion diseases. There is strong evidence that PrP^{Sc} is neurotoxic. Much work has focused on the mechanism of toxicity of a prion protein peptide (PrP106-126) [9–12]. It has also been suggested that cell death may follow as a result of loss of PrP^c function due to its conversion to PrP^{Sc}. The creation of PrP^c-knockout mice deficient in expression of PrP^c has counted against this hypothesis, as these mice do not show neuro-degeneration comparable with prion disease [13,14]. However, cell culture studies suggest that PrP106-126 has two effects on cells which lead to cell death [10,11]. Activation of microglia to produce toxic substances such as oxygen radicals is an indirect effect leading to cell death but in line with a toxicity model [10]. However, PrP106-126 also has a direct effect necessary for the induction of cell death [10]. PrP106-126 reduces cellular resistance to oxidative stress, possibly by inhibiting the activity of Cu/Zn superoxide dismutase (Cu,Zn SOD) [11]. In addition, PrP106-126 is not toxic to cells from PrP^c-deficient mice (Prnp$^{0/0}$ mice) and is unable to reduce resistance to oxidative stress in these cells [9–11]. Therefore, the indirect effect involving toxic bombardment of neurones with substances produced by microglia is not sufficient to induce cell death. A change in the metabolism of PrP^c-expressing neurones is required. Cells from Prnp$^{0/0}$ mice also show reduced resistance to oxidative stress, possibly due to decreased activity of Cu,Zn SOD [11]. Therefore, it is possible that a 'loss of function' hypothesis may be relevant to the mechanism of neurodegeneration in prion disease. Understanding PrP^c function may therefore be important in determining how PrP^{Sc} may cause neurodegeneration.

Previous investigations have determined three possible disturbances resulting from lack of PrP^c expression in Prnp$^{0/0}$ mice [13]. First, Prnp$^{0/0}$ mice exhibit altered synaptic behaviour manifesting as reduced long-term potentiation, reduced γ-aminobutyric acid-type inhibition [15] and increased sensitivity to exogenous copper [16]. Secondly, Prnp$^{0/0}$ mice have altered circadian rhythms [17]. Thirdly, in cultures, Prnp$^{0/0}$ cerebellar cells are more sensitive to oxidative stress and have reduced activity of the enzyme Cu,Zn SOD [10,11]. Disturbances such as changes in long-term potentiation or circadian rhythms are changes in complex systems and so cannot be used to explain the loss of function of a protein at the basic molecular level. However, altered resistance to oxidative stress represents a change at the level of single cells and is thus closer to a change dependent on molecular function. In addition, there is mounting evidence to suggest that PrP^c may bind copper [16,18–20]. PrP^c may regulate some aspect of copper metabolism.

Cu,Zn SOD is an enzyme whose activity is dependent on the availability of copper. We have shown previously that mice deficient in prion protein expression have reduced Cu,Zn SOD activity. We investigated this further to determine if this change was related to a decrease in incorporation of copper into Cu,Zn SOD. Our results suggest that PrP^c regulates incorporation of copper into Cu,Zn SOD. The ability of PrP^c to bind copper may modulate Cu,Zn SOD activity and consequently cellular resistance to oxidative stress.

EXPERIMENTAL PROCEDURES

Unless stated all reagents were from Sigma.

Abbreviations used: PrP^c, prion protein; PrP^{Sc}, an abnormal isoform of PrP^c; SOD, superoxide dismutase; Prnp$^{0/0}$ mice, PrP^c-deficient mice; PIPLC, phosphatidylinositol-specific phospholipase C; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; BCA, bicinchoninic acid; DDC, diethyl-dithiocarbamate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.
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Animals

The mice used for experiments were either Prnp[0/0] as described previously by Büeler et al. [13], wild-type mice that were descendants of an F1 generation produced by interbreeding C57Bl/6J and 129/sv(ev) mice, or Tg35 mice overexpressing PrP[c] via a cosmid construct as described by Fischer et al. [21].

Cell culture

Preparation of cerebellar cells from 6 day old mice and culture in serum containing media was as described previously [10]. Cells used for experiments with radioactive copper were maintained in a serum-free media composed of Dulbecco's modified Eagle's medium (Biochrom) supplemented with 1 % antibiotics (penicillin, streptomycin; Biochrom) and TCM supplement (ICN). The survival assay and xanthine oxidase treatment were as described previously [10]. For some experiments, cerebellar cells were treated with phosphatidylinositol-specific phospholipase C (PIPLC) for 3 h at 0.2 Units/ml in serum-free media.

Northern blotting

Total RNA from mouse brain (10 day or 5 month old) was isolated using an RNA isolation kit (Qiagen) and quantified using an Ultrospec 3000 spectrophotometer (Pharmacia). RNA (10–15 μg) was electrophoresed on a 1 % agarose gel containing 6.6 % formaldehyde. The RNA was blotted to Hybond N membrane (Amersham) using a passive transfer method. Hybridization was using standard techniques. Detection was via chemiluminescence using CDP-Star (Tropix Inc.) and exposure to X-Omat film (Kodak) for 16–18 h. Individual Northern blots were hybridized three times using different probes. The blots were stripped between hybridizations. Three different probes were used. The cDNA for Cu,Zn SOD was a gift from the laboratory of Dr. G. Bewley (Department of Genetics, North Carolina State University, NC, U.S.A.) and was as described previously [22]. The DNA probe for Mn SOD was generated by reverse-transcriptase PCR from mRNA extracted from mouse liver and subsequent subcloning of the PCR product into the Bluescript vector (Stratagene). The primers used for the PCR reaction were 5′-TGAATTCATGTTTCGGGCGGCGTG-3′ and 3′-TAGGATCCCTTCTTGCAAGTGTAT-5′ based on the mouse sequence of Mn SOD as published previously [23]. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was a gift from Dr. B. Hogan (Department of Cell Biology, Vanderbilt University Medical School, Nashville, TN, U.S.A.) and was as described previously [24]. Quantification of Northern blots was carried out following scanning the X-ray films with a Hewlett Packard ScanJet Plus with Picture Publisher Version 3 (Micrografx). The intensities of the bands were determined densitometrically using Pcbas Version 2.09g (Raytest). The intensities of the bands for different extracts on filters hybridized with the PrP or Cu,Zn SOD probes were related by relating the intensity of the bands to that produced by hybridization with the GAPDH probe.

Western blotting

Protein was extracted from 3 month old mouse brains by homogenization in an extraction buffer (PBS, 1 % Nonidet P40; Sigma). After a 20 min incubation, the homogenate was centrifuged at 12 500 g for 10 min. The supernatant was taken as the extract. A protein determination was carried out using a BCA (bicinchoninic acid) protein-assay kit (Sigma) to ensure identical amounts of protein were loaded on to gels. Extracts were boiled and then electrophoresed on a 12.5 % acrylamide gel in a Bio-Rad Mini-Protean 2 system. After electrophoresis, the protein was transferred to a membrane (Immobilon, Millipore) with a semi-dry blotter (Bio-Rad). Cu,Zn SOD was detected using a polyclonal antibody (Europa Research Products) followed by an alkaline phosphatase-conjugated goat anti-rabbit IgG secondary antibody. Visualization was carried out by incubation of the blot with Nitro Blue Tetrazolium (Boehringer) and 5-bromo-4-chloro-3-iodolyl phosphate (Boehringer). Densitometric quantification of the visualized bands was carried out as described for Northern blots.

Enzymic assays

Adult mice were 3–6 months of age. Extracts were prepared by homogenizing mouse brain in a buffer of 50 mM sodium phosphate/100 mM NaCl/1 % Nonidet P40. The protein concentration was determined by a standard BCA assay.

Two different assays were used to assay SOD activity. A gel assay was used to visualize Cu,Zn SOD activity. Extracts at 50 μg of protein per lane were electrophoresed on 10 % acrylamide non-denaturing gels and assayed for SOD activity as described previously [25]. Secondly, a spectrophotometric assay was used to quantificate both total (Cu,Zn and Mn) SOD activity and Mn SOD activity alone. This assay was also described previously [26]. Each protein extract (10 μg) was assayed and compared with 1 Unit of bovine Cu,Zn SOD (Sigma) activity. The assay was carried out for 2 min after addition of the radical producer, xanthine oxidase (Boehringer). The SOD activity was expressed as percentage inhibition of the formazan produced in control reaction without SOD or protein extracts. 100 % formazan product formation is the amount of Nitro Blue Tetrazolium reduced by radicals formed by xanthine oxidase in 2 min. For Mn SOD activity, extracts were incubated at room temperature in 4 mM KCN for 20 min before assaying. Cu,Zn SOD activity was calculated by the subtraction of Mn SOD activity from total SOD activity. Assays were performed in triplicate or duplicate.

The assay for glutathione peroxidase was carried out according to the method of Flohé and Günzler [27]. Extracts were prepared as for SOD assays and balanced for protein content using the BCA assay.

Immunoprecipitation

Cerebellar cells were incubated with Hank's balanced salt solution supplemented with 400 μM L-histidine (Sigma) and 0.2 μM Cu[67] (Paul Scherrer Institute, Zürich, Switzerland) with a specific activity of approximately 37 Mbq/μg at the end of production. The cells were incubated in this solution for 3 h at 37 °C. Some cells were also incubated with 0.2 Units PIPLC for this 3 h. Protein was extracted from cerebellar cells by homogenization in PBS and 1 % Nonidet P40. Extracts were incubated with either Cu,Zn SOD antibody (Biotrends) (1:100 dilution) or synaptophysin (1:100 dilution) (Quartet) for 60 min on ice. The resulting mixture was then incubated for 60 min on ice with 10 mg of Protein A–Sepharose (Pharmacia) prepared in PBS. The Sepharose A was pelleted and washed with PBS three times. The pellet was then taken for gamma counting. The counts/min were related to the protein concentration in the starting material.

RESULTS

We have used Tg35 mice overexpressing the prion protein to further examine its role in resistance to oxidative stress. Tg35 mice express approximately 10 times the wild-type level of mouse
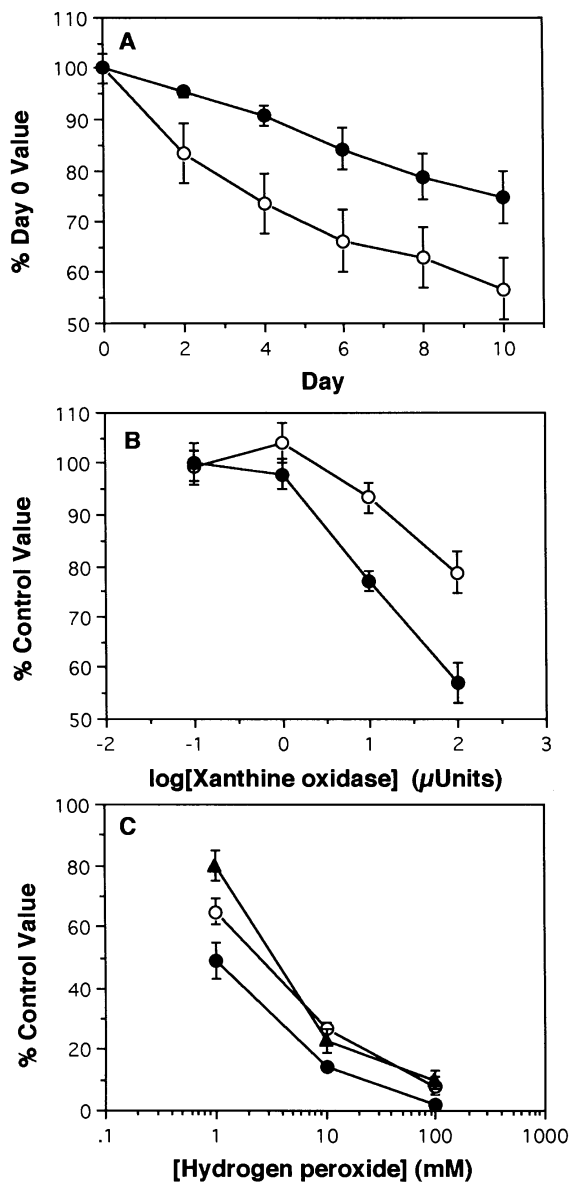
Figure 1   Cerebellar cell cultures from 6 day old Tg35 and wild-type mice were compared

(**A**) Cerebellar cells were cultured under identical conditions for 10 days and relative survival was determined using an MTT assay at 2 day intervals. Assay values were compared as a percentage of the values determined on day 0, 2 h after plating. All Tg35 values from 2 days onwards were significantly greater than wild-type values (Student's t test, $P < 0.05$). (**B**) Cerebellar cell cultures were treated with varying concentrations of xanthine oxidase and 5 μM xanthine as substrate. Survival was determined after 24 h with an MTT assay. Values were related as a percentage of untreated culture value (100%). Xanthine oxidase at 0.1–1.0 mUnits/ml was significantly ($P < 0.05$) more toxic to Tg35 cells than to wild-type cells. (**C**) Cerebellar cell cultures were treated with varying concentrations of hydrogen peroxide. After one day of treatment survival was determined with an MTT assay. Hydrogen peroxide was significantly ($P < 0.05$) more toxic to Tg35 cerebellar cells than wild-type cells at the concentrations tested. In all cases: ●, Tg35 cerebellar cells; ○, wild-type cells; ▲, Prnp$^{0/0}$ cells; the means $\pm$ S.E.M. for a minimum of 4 experiments with 3 determinations each are shown.

PrP^c [21]. Previously we showed that cerebellar cells from Prnp$^{0/0}$ mice survive less well in culture when compared with wild-type mice [11]. Cerebellar cell cultures from 6 day old wild-type and Tg35 mice were prepared under identical conditions and their
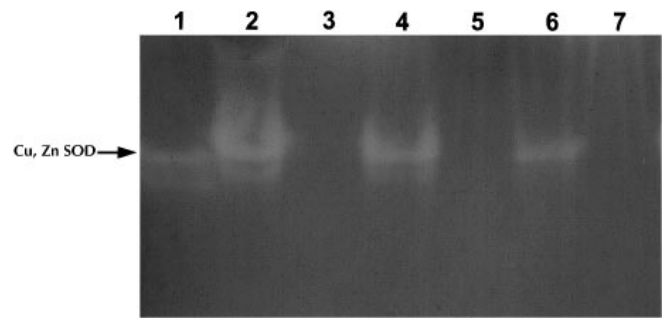

Figure 2   The activity of SOD in adult mice varies with PrP^c expression

Extracts of Tg35 (lanes 2 and 3), wild-type (lanes 4 and 5) and Prnp$^{0/0}$ (lanes 6 and 7) mouse brains were electrophoresed. SOD was detected in the gel as described [25]. Bovine SOD (lane 1) was used as a positive control. KCN pre-treatment (lanes 3, 5 and 7) of the extracts abolished the major band visible in lanes 2, 4 and 6, suggesting that this band represents Cu,Zn SOD. The SOD activity is highest in Tg35 mice and lowest in Prnp$^{0/0}$ mice, suggesting that the level of Cu,Zn SOD activity is related to PrP^c expression. Five individual experiments were performed.

relative survival traced over 10 days using an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. Wild-type cells die more rapidly in culture than Tg35 cells (Figure 1A). This suggests that cerebellar cells that overexpress PrP^c survive normal cell-culture conditions better than wild-type cells.

Prnp$^{0/0}$ cerebellar cells are more sensitive than wild-type cells to oxidative stress generated by xanthine oxidase [11]. Tg35 and wild-type cells were exposed to increasing concentrations of xanthine oxidase, an oxygen-radical producer. Relative survival was determined after 24 h by MTT assay. Xanthine oxidase is more toxic to Tg35 cerebellar cells than wild-type cells (Figure 1B). The toxicity of hydrogen peroxide (Aldrich) was also tested. Hydrogen peroxide was also more toxic to cerebellar cells from Tg35 mice than to wild-type cerebellar cells (Figure 1C). There was no difference in the toxicity of hydrogen peroxide to Prnp$^{0/0}$ as compared with wild-type cerebellar cells (Figure 1C). As xanthine oxidase activity can also lead to the generation of hydrogen peroxide, it is possible that our observations suggest that Tg35 cerebellar cells are more sensitive to the toxicity of hydrogen peroxide. To test this, 50 mUnits of murine catalase were incubated with cerebellar cells treated with 100 μUnits of xanthine oxidase/50 μM xanthine for 24 h. The survival of xanthine oxidase-treated wild-type cells was 80 ± 4% of untreated control values without catalase and 82 ± 4% of untreated controls with catalase ($n = 4$). Tg35 cultures treated with xanthine oxidase alone survived to 53 ± 4% of control values but with additional catalase treatment the Tg35 cerebellar cells survived to 67 ± 3% of control values ($n = 4$). This suggests that only catalase can significantly ($P < 0.05$) reduce the toxicity of xanthine oxidase to Tg35 cerebellar cells, suggesting the increased toxicity of xanthine oxidase to these cells is due to increased sensitivity to hydrogen peroxide.

As we have shown previously that both adult brains and cerebellar cells from Prnp$^{0/0}$ mice have reduced Cu,Zn SOD activity as compared with wild-type mice [11], we investigated whether PrP^c-overexpressing mice also have altered Cu,Zn SOD activity. Homogenates of Tg35, wild-type and Prnp$^{0/0}$ mouse brains were prepared. The relative activity of Cu,Zn SOD in these extracts was visualized after electrophoresis on a non-denaturing polyacrylamide gel (Figure 2) by the ability of Cu,Zn SOD to prevent a blue staining reaction, i.e. to prevent free-radical formation and thus reduction of Nitro Blue Tetrazolium.
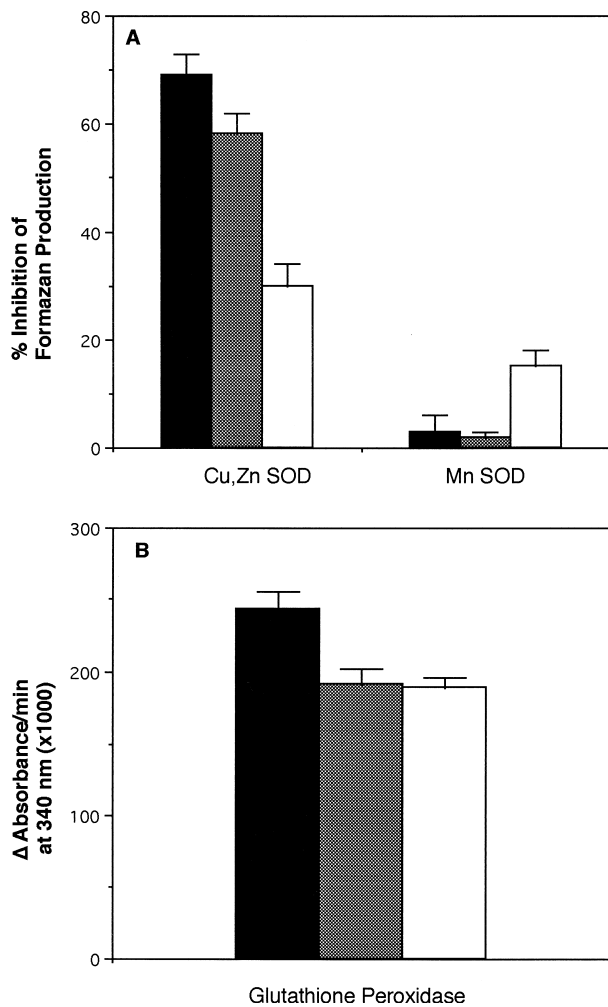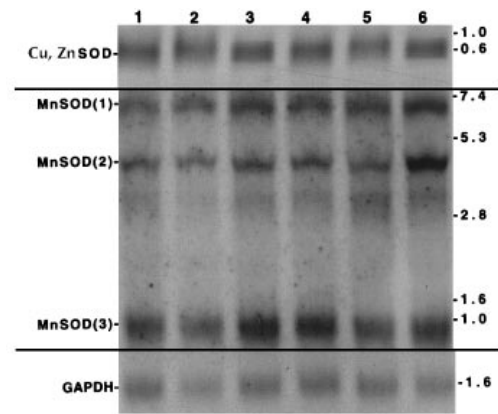
Figure 4   Northern blotting was used to detect expression of Cu,Zn SOD (upper panel), Mn SOD (middle panel) and GAPDH (lower panel) in Tg35 (lanes 1, 4), wild-type (lanes 2, 5) and $Prnp^{0/0}$ (lanes 3, 6) mouse brain

Total RNA was extracted from either 10 day old (lanes 1–3) or 5 month old (lanes 4–6) mouse brain and electrophoresed on a denaturing agarose gel before being transferred to a filter. Individual filters were hybridized with DNA probes for all three RNA species. Between hybridizations the filters were stripped before the next round of hybridization. Signals were detected by chemiluminescence. All samples expressed an RNA species of 1.6 kb similar to that of GAPDH and an RNA species of approximately 0.7 kb similar to that of Cu,Zn SOD. All samples expressed three RNA species of approximately 7.4, 4.8 and 1.0 kb similar to the Mn SOD probe.

Figure 3   Anti-oxidant enzyme activity

(**A**) The level of Cu,Zn SOD activity and KCN-insensitive Mn SOD activity was quantified for the extracts of Tg35 (black bars), wild-type (grey) and $Prnp^{0/0}$ (white) mouse brain. Extracts were pre-incubated with KCN to block Cu,Zn SOD activity, allowing Mn SOD activity to be quantified. Mn SOD activity was elevated significantly ($P < 0.05$) in $Prnp^{0/0}$ mice. Extracts were also assayed in the absence of KCN. This assay gave total SOD activity, from these values Mn SOD activity was subtracted to give the relative Cu,Zn SOD activity for the different extracts. Cu,Zn SOD activity of Tg35 extracts was significantly greater ($P < 0.05$) than the activity in wild-type mice. However, the Cu,Zn SOD activity was much lower in $Prnp^{0/0}$ mice. (**B**) The activity of glutathione peroxidase was determined for extracts from adult mouse brain. There was no observed difference between wild type and $Prnp^{0/0}$ in the activity of glutathione peroxidase. However, Tg35 mice had significantly higher ($P < 0.05$) levels of glutathione peroxidase than the other mouse strains. The means $\pm$ S.E.M. of assays of 4 mice of each strain are shown.

This activity was completely blocked by KCN at 4 mM, indicating that the bands present were due to Cu,Zn SOD and not to mitochondrial Mn SOD. The relative intensity of the visualized bands varied thus: Tg35 > wild type > $Prnp^{0/0}$, suggesting that the activity of Cu,Zn SOD varies with the level of PrP^c expression. This was quantified further by a spectrophotometric assay based on oxygen radical formation by xanthine oxidase. The measured activity of Cu,Zn SOD was significantly higher (Student's t test, $P < 0.05$) in Tg35 extracts and significantly lower in $Prnp^{0/0}$ mouse brain extracts than for wild-type extracts (Figure 3A). Tg35 mice have approximately twice the activity of Cu,Zn SOD as $Prnp^{0/0}$ mice. In extracts from wild-type and Tg35

mice the level of Mn SOD was very low. In comparison, $Prnp^{0/0}$ mice have significantly ($P < 0.05$) elevated Mn SOD levels as reported previously [11]. In addition, we assayed the activity of glutathione peroxidase in similar extracts. Glutathione peroxidase activity was equivalent in wild-type and $Prnp^{0/0}$ mice but elevated in Tg35 mice (Figure 3B).

The Northern-blot technique was used to quantificate the mRNA for Cu,Zn SOD and Mn SOD from the brains of 10 day old and 5 month old mice. Specific DNA probes were generated for Cu,Zn SOD, Mn SOD and GAPDH. Total RNA was extracted from mouse brains and equivalent amounts electrophoresed and transferred to filters. Individual filters were hybridized with all three probes. An example is shown in Figure 4. The filters were densitometrically quantified and the intensity of bands, generated by hybridization with Cu,Zn SOD and Mn SOD probes, were normalized by comparison of the levels of bands detected by the GAPDH probe. Hybridization with an Cu,Zn SOD produces a single band of 0.7 kb. For 10 day old mouse brain, the bands for Tg35 mice were $81 \pm 30\%$ of the wild-type level and the $Prnp^{0/0}$ signal was $74 \pm 36\%$ of wild-type ($n = 5$). For 5 month old mice the signal for Tg35 mouse brain was $129 \pm 24\%$ and for $Prnp^{0/0}$ mouse brain was $130 \pm 31\%$ of the wild-type level ($n = 4$). These difference were not significant (Student's t test, $P < 0.05$). This suggests that at the level of transcription there is no difference in the production of Cu,Zn SOD in the different mouse strains. Similar Western-blot analysis of protein expression in mice with different levels of PrP^c expression showed no difference in the level of Cu,Zn SOD expression ($n = 4$ each, Figure 5). The quantification following densitometric scanning showed that the level of $Prnp^{0/0}$ mouse brain Cu,Zn SOD protein as indicated by Western blot was $98.2 \pm 2\%$ that of wild-type mouse brain, and Tg35 Cu,Zn SOD protein was $102 \pm 5\%$ of wild-type Cu,Zn SOD protein, indicating no significant differences in the amount of protein detected.
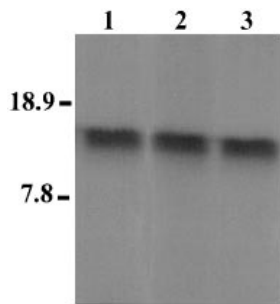
Figure 5   Western-blot analysis of Cu,Zn SOD protein expression in Tg35 (lane 1) wild-type (lane 2) and Prnp$^{0/0}$ (lane 3) adult mouse brain protein extracts

Equal amounts of protein were loaded. Intensity of bands for several Western blots were quantificated. There was no difference in the level of expression of Cu,Zn SOD protein in Tg35, wild-type and Prnp$^{0/0}$ mouse brain extracts.

Total RNA extracted from mouse brain hybridized with an Mn SOD probe showed three homologous bands at 7.4, 4.8 and 1.0 kb. In terms of Mn SOD activity seen for mouse brain extracts, the only significant difference was seen for the comparison of Prnp$^{0/0}$ mice and wild-type mice (Figure 4). For the Northern-blot analysis the bands for 5 month old Prnp$^{0/0}$ mice were 145 ± 31, 144 ± 24 and 141 ± 29 % of the equivalent bands for wild-type mice. The intensity of the 4.8 kb Prnp$^{0/0}$ band was significantly higher than that for wild-type mice ($n = 4$, $P < 0.05$). This is in keeping with the results for activity and suggests
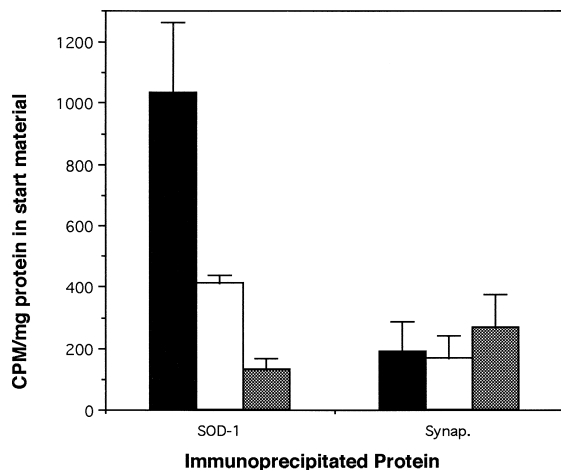

Figure 6   Cu^{67} incorporation into SOD of cerebellar cells with different PrP^c levels

Cerebellar cells from 6 day old mice were loaded with Cu^{67} for 3 h by incubation in Hank's balanced salt solution containing 400 μM histidine and 0.2 μM Cu^{67}. A sheep-derived antibody was used to immunoprecipitate Cu,Zn SOD from cerebellar cells. As a control, immuno-precipitations were also carried out using a mouse anti-synaptophysin (Synap.) antibody. The bound material was then reacted with Protein A–Sepharose and pelleted. The pelleted material was gamma counted and counts/min per mg of protein in the starting material determined. Immunoprecipitation from Tg35 cells (black bars) with the Cu,Zn SOD antibody also led to the immunoprecipitation of significantly more ($P < 0.05$) Cu^{67} than for wild-type cells (white bars) and the immunoprecipitation from wild-type cells contained more Cu^{67} than for Prnp$^{0/0}$ cells (grey bars). There was no significant difference for the Cu^{67} contents of immuno-precipitations with synaptophysin. The means $\pm$ S.E.M. for a minimum of 5 experiments are shown.

that Mn SOD activity in Prnp$^{0/0}$ mice may be upregulated at the level of transcription.

Cu,Zn SOD activity can also be regulated post-translationally by the incorporation of copper [28,29]. Copper-starved animals have reduced activity of Cu,Zn SOD. Experiments were carried out with radioactive copper (Cu^{67}) to determine if copper is incorporated into Cu,Zn SOD to a different degree. Cultured cerebellar cells from 6 day old animals of different strains were exposed to 0.2 μM Cu^{67} in Hank's balanced salt solution solution containing 400 μM histidine. After 3 h exposure, an extract was made in PBS containing 0.1 % Nonidet 40 and Cu,Zn SOD immunoprecipitated using a specific antibody. The amount of Cu^{67} present in the immunoprecipitated material was quantificated by gamma counting. Significantly more (Student's t test, $P < 0.05$) Cu^{67} was present in material precipitated from Tg35 cultures than wild-type cultures and significantly more Cu^{67} was present in material immunoprecipitated from wild-type cultures than Prnp$^{0/0}$ cultures (Figure 6). Immunoprecipitation with a control antibody (synaptophysin) did not show the same tendency. This suggests that incorporation of Cu^{67} into Cu,Zn SOD depends on the level of PrP^c expression.

PIPLC cleaves PrP^c from the surface of cells [30]. Cerebellar cells from wild-type and Prnp$^{0/0}$ mice were incubated with Cu^{67} buffer for 3 h concurrent with a 3 h pre-treatment with 0.2 Units/ml of PIPLC. Following this, Cu,Zn SOD was immuno-precipitated and the Cu^{67} content determined by gamma counting. After PIPLC treatment of wild-type cells, 246 ± 6 counts/min per mg of starting-material protein was present in Cu,Zn SOD antibody-immunoprecipitated material as compared with 410 ± 27 counts/min per mg of starting-material protein without PIPLC treatment. This suggests that PIPLC treatment significantly reduces ($n = 4$, $P < 0.05$) the amount of Cu^{67} incorporated into Cu,Zn SOD. In comparison, when similar experiments were carried out with Prnp$^{0/0}$ cerebellar cells, 139 ± 43 counts/min per mg of starting-material protein was in-corporated in anti-Cu,Zn SOD-immunoprecipitated material after PIPLC treatment and 131 ± 34 counts/min per mg of starting-material protein without PIPLC treatment. This suggests that PIPLC has no effect on the incorporation of Cu^{67} into Cu,Zn SOD from Prnp$^{0/0}$ cerebellar cells. These results suggest that Cu^{67} incorporated into Cu,Zn SOD was taken up after binding to PrP^c.

Further experiments were carried out to determine if the different activity of Cu,Zn SOD in extracts of brains from different mouse strains is a result of differences in the copper content of Cu,Zn SOD. Diethyldithiocarbamate (DDC) is known to inactivate Cu,Zn SOD by capturing copper from the molecule [31,32]. Extracts from Tg35, wild-type and Prnp$^{0/0}$ mouse brain were incubated with increasing amounts of DDC. The results were expressed as percentage inhibition of Cu,Zn SOD activity. Low concentrations of DDC inhibited Cu,Zn SOD activity most in Tg35 extracts and least in Prnp$^{0/0}$ extracts (Figure 7), suggesting that these concentrations of DDC are more able to capture Cu from Tg35 or wild-type extracts than Prnp$^{0/0}$ extracts. As higher concentrations also inhibited Cu,Zn SOD in Prnp$^{0/0}$ extracts, then copper was incorporated into Cu,Zn SOD independently of PrP^c expression.

DISCUSSION

The results presented here represent a continuation of three aspects of our research. These are the relations between; (i) PrP^c expression and cellular resistance to oxidative stress, (ii) PrP^c expression and Cu,Zn SOD activity, and (iii) PrP^c expression and copper metabolism. All three aspects are also linked in that
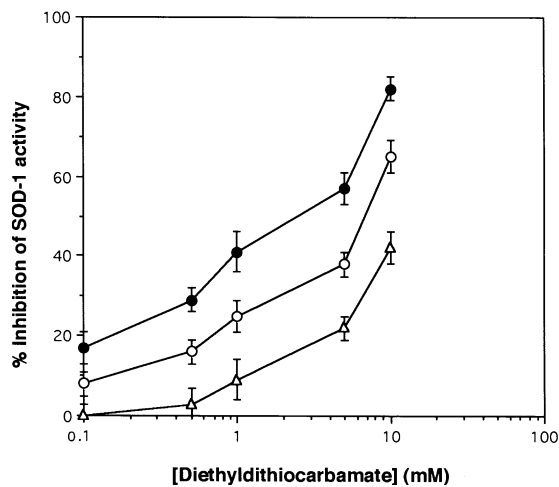
Figure 7 Cu,Zn SOD activity was determined for extracts of wild-type, Tg35 and Prnp$^{0/0}$ adult mouse brains

Details are given in Figure 3A. Varying concentrations of DDC were added to the assay mixtures and incubated for 20 min before addition of xanthine oxidase to commence the assay. Inhibition of formazan formation was measured for extracts with and without DDC treatment. KCN-sensitive activity of Cu,Zn SOD was then calculated and the ability of DDC to block this inhibition of formazan production expressed as a percentage of the total inhibition for the different extract types, i.e. Tg35 (●), wild-type (○) and Prnp$^{0/0}$ (△). DDC inhibited Cu,Zn SOD activity most effectively for Tg35 and least effectively for Prnp$^{0/0}$ extracts. Means ± S.E.M. for 4 individual experiments are shown.

copper is necessary for the activity of Cu,Zn SOD, which in turn is a major anti-oxidant enzyme. Our results confirm our previous findings that altering levels of PrP^c expression alters Cu,Zn SOD activity. However, here we demonstrate that this alteration is a result of changes in the level of incorporation of copper into the enzyme.

There is increasing evidence that PrP^c expression regulates some aspect of copper metabolism. Recent biophysical studies have suggested that PrP^c can bind copper [16,18,19]. PrP^c contains an octameric repeat region that is rich in histidine and represents a putative copper-binding domain. Additionally, an affinity procedure in which copper is coupled to an immobilized metal-affinity-chromatography column can be used to selectively purify PrP^c [33]. Furthermore, when copper binds to a peptide representing the octameric repeat region of PrP^c, the binding induces a conformational change to the α-helix in the C-terminal region of the peptide [20]. Cell-biology studies also give evidence of a relation between copper metabolism and PrP^c expression. Exposure of PC12 cells to high concentrations of copper allows the production of a PC12 variant that has increased resistance to copper toxicity and increased levels of PrP^c expression [34]. This PC12 variant also has greater resistance to oxidative stress and higher levels of Cu,Zn SOD activity. The amplitudes of inhibitory currents in Purkinje cells can be reduced by exposing Prnp$^{0/0}$ cerebellar slices to 2 μM CuCl$_2$, but similar treatment has no effect on wild-type Purkinje cells [16]. Prnp$^{0/0}$ cerebellar cells are also more sensitive to the toxicity of copper-containing salts and this can be blocked by the presence of a peptide based on the sequence of the octameric repeat region of PrP^c [35]. The decreased activity of a copper-containing enzyme in Prnp$^{0/0}$ mouse brain is in itself suggestive of a link between copper metabolism and PrP^c expression [11].

Cleavage of PrP^c from the cell surface of cerebellar cells with PIPLC reduces their copper content [16]. PIPLC may also cleave

other glycosyl-phosphatidylinositol-anchored proteins that bind copper but, as this enzyme has little effect on the copper content of Prnp$^{0/0}$ cerebellar cells [16], it is more likely that this loss of copper is due to loss of PrP^c. Similarly, PIPLC treatment reduces the amount of copper incorporated into Cu,Zn SOD, as determined from immunoprecipation experiments on Cu^{67}-labelled wild-type cerebellar cells. The amount of Cu^{67} incorporated into Cu,Zn SOD is higher in cells expressing higher levels of Cu,Zn SOD. Although these results suggest that PrP^c expression regulates the incorporation of copper into Cu,Zn SOD, they do not prove that the copper so incorporated was taken up into the cell by binding to PrP^c. Although PrP^c may bind copper, it may act as a sensor that copper is present in the environment and signals passed into the cell may activate other uptake mechanisms. However, as PIPLC treatment selectively reduces cerebellar-cell copper content [16] then it is possible that a significant amount of copper enters the cell after binding to PrP^c and may then be passed to Cu,Zn SOD.

The changes in Cu,Zn SOD activity that we have detected can be related to the level of PrP^c expression, as overexpressing mouse brain extracts have the highest level whereas PrP^c-deficient mouse brain extracts have the lowest. Exhaustive analysis of the level of expression of the mRNA and protein for Cu,Zn SOD was carried out by us and we found no evidence that there is any change in the expression level of Cu,Zn SOD. This also confirms that the differences in activity measured by us are due to differences in the amount of copper incorporated, as availability of copper has been determined to be a major determinant of the activity of Cu,Zn SOD. This is supported by experiments with DDC, which captures copper from Cu,Zn SOD. DDC inhibits Cu,Zn SOD more in Tg35 mice and wild-type mice. As these extracts contain the same level of Cu,Zn SOD protein as Prnp$^{0/0}$ extracts, then the differences in the ability of DDC to inhibit Cu,Zn SOD are due to differences in the amount of copper captured from the enzyme. The lower ability of DDC to inhibit Cu,Zn SOD in Prnp$^{0/0}$ extracts suggests that the Cu,Zn SOD contained less copper that could be captured by DDC. Therefore we feel confident to suggest that the difference in Cu,Zn SOD activity in the different strains of mice we examined relates to differences in incorporation of copper into the enzyme. PrP^c may function to regulate incorporation of copper into Cu,Zn SOD.

The level of Cu,Zn SOD expression by cells is known to alter cell survival and resistance to oxidative stress [36,37]. Cells overexpressing Cu,Zn SOD show higher survival rates in culture when exposed to toxic substances [38,39]. Tg35 cerebellar cells have increased activity of Cu,Zn SOD and survive better in culture than wild-type cells. However, these cells also show an increased sensitivity to hydrogen peroxide. Although spontaneous dismutation of superoxide to hydrogen peroxide occurs in the absence of Cu,Zn SOD, the enzyme catalyses dismutation 10000 times faster than the spontaneous rate [40]. Tg35 mice show increased activity of glutathione peroxidase, the main enzyme involved in defence against hydrogen peroxide. The increased sensitivity of these cells to exogenous hydrogen peroxide may be because these cells must deal with metabolic consequences of altered oxygen radical metabolism from increased activity of Cu,Zn SOD. Such changes may include removal of superoxide from reactions that may react rapidly with and nullify the toxic effects of other molecules such as nitric oxide. Such results show that maintaining defence against oxidative molecules is complicated and levels of anti-oxidant enzymes must also be regulated to ensure effective clearance of the oxidative threat. Therefore, our results support the notion that the expression of PrP^c may aid cellular resistance to oxidative stress but also suggest that overexpression may have detrimental

or non-beneficial effects, such as increased susceptibility to hydrogen peroxide toxicity.

In summary, we have provided evidence that PrP^c expression regulates copper incorporation into Cu,Zn SOD. This function has the consequence of altering cellular resistance to oxidative stress and may regulate other copper-dependent aspects of cell metabolism.

REFERENCES

1   Kretzschmar, H. A., Prusiner, S. B., Stowring, L. E. and DeArmond, S. J. (1986) Am. J. Pathol. **122**, 1–5

2   Moser, M., Colello, R. J., Pott, U. and Oesch, B. (1995) Neuron **14**, 509–517

3   Bolton, D. C., McKinley, M. P. and Prusiner, S. B. (1982) Science **218**, 1309–1311

4   Prusiner, S. B. (1991) Science **252**, 1515–1522

5   Hsiao, K. and Prusiner, S. B. (1990) Neurology **40**, 1820–1827

6   Kretzschmar, H. A. (1993) Arch. Virol. Suppl. **7**, 261–293

7   Hope, J., Multhaup, G., Reekie, L. J. D., Kimberlin, R. H. and Beyereuther, K. (1988) Eur. J. Biochem. **172**, 271–277

8   Prusiner, S. B. (1982) Science **216**, 136–144

9   Brown, D. R., Herms, J. and Kretzschmar, H. A. (1994) Neuroreport **5**, 2057–2060

10   Brown, D. R., Schmidt, B. and Kretzschmar, H. A. (1996) Nature (London) **380**, 345–347

11   Brown, D. R., Schulz-Schaeffer, W. J., Schmidt, B. and Kretzschmar, H. A. (1997) Exp. Neurol. **146**, 104–112

12   Brown, D. R., Herms, J. W., Schmidt, B. and Kretzschmar, H. A. (1997) Eur. J. Neurosci. **9**, 1162–1169

13   Büeler, H., Fischer, M., Lang, Y., Bluethmann, H., Lipp, H.-P., DeArmond, S. J., Prusiner, S. B., Aguet, M. and Weissmann, C. (1992) Nature (London) **356**, 577–582

14   Büeler, H., Aguzzi, A., Sailer, A., Greiner, R. A., Autenried, P., Auget, M. and Weissmann, C. (1993) Cell **73**, 1339–1347

15   Collinge, J., Whittington, M. A., Sidle, K. C. L., Smith, C. J., Palmer, M. S., Clarke, A. R. and Jefferys, J. G. R. (1994) Nature (London) **370**, 295–297

16   Brown, D. R., Qin, K., Herms, J. W., Madlung, Manson, J., Strome, R., Fraser, P. E., Kruck, T., A., von Bohlen, A., Schulz-Schaeffer, W., Giese, A., Westaway, D. and Kretzschmar, H. A. (1997) Nature (London) **390**, 684–687

17   Tobler, I., Gaus, S. E., Deboer, T., Achermann, P., Fischer, M., Rülicke, T., Moser, M., Oesch, B., McBride, P. A. and Manson, J. C. (1996) Nature (London) **380**, 639–642

18   Hornshaw, M. P., McDermott, J. R. and Candy, J. M. (1995) Biochem. Biophys. Res. Commun. **207**, 621–629

19   Hornshaw, M. P., McDermott, J. R., Candy, J. M. and Lakey, J. H. (1995) Biochem. Biophys. Res. Commun. **214**, 993–999

20   Miura, T., Hori-i, A. and Takeuchi, H. (1996) FEBS Lett. **396**, 248–252

21   Fischer, M., Rülicke, T., Raeber, A., Sailer, A., Moser, M., Oesch, B., Brandner, S., Aguzzi, A. and Weissmann, C. (1996) EMBO J. **15**, 1255–1264

22   Bewley, G. C. (1988) Nucleic Acids Res. **16**, 2728

23   DiSilvestre, D., Kleeberger, S. R., Johns, J. and Levitt, R. C. (1995) Mamm. Genome **6**, 281–284

24   Matsui, Y., Zsebo, K. M. and Hogan, B. L. M. (1990) Nature (London) **347**, 667–669

25   Beauchamp, C. and Fridovich, I. (1971) Anal. Biochem. **44**, 276–287

26   Oberley, L. W. and Spitz, D. R. (1984) Methods Enzymol. **105**, 457–464

27   Flohé, L. and Günzler, W. A. (1984) Methods Enzymol. **105**, 114–121

28   Pal, R., Nath, R. and Gill, K. D. (1993) Pharmacol. Toxicol. **73**, 209–214

29   Rossi, L., Ciriolo, M. R., Marchese, E., De Martino, A., Giorgio, M. and Rotilio, G. (1994) Biochem. Biophys. Res. Commun. **203**, 1028–1034

30   Stahl, N., Borchelt, D. R. and Prusiner, S. B. (1990) Biochemistry **29**, 5405–5412

31   Cocco, D., Calabrese, L., Rigo, A., Marmocchi, F. and Rotilio, G. (1981) Biochem. J. **199**, 675–680

32   Misra, H. P. (1979) J. Biol. Chem. **254**, 11623–11628

33   Pan, K.-M., Stahl, N. and Prusiner, S. B. (1992) Protein Sci. **1**, 1343–1352

34   Brown, D. R., Schmidt, B. and Kretzschmar, H. A. (1998) Int. J. Dev. Neurosci. **15**, 961–972

35   Brown, D. R., Schmidt, B. and Kretzschmar, H. A. (1998) J. Neurochem. **70**, 1686–1693

36   Rothstein, J. D., Bristol, L. A., Hosler, B. and Brown, R. H. (1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 4155–4159

37   Kinouchi, H., Epstein, C. J., Mizui, T., Carlson. E., Chen. S. F. and Chan, P. H. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 11158–11162

38   Elroy-Stein, O., Bernstein, Y. and Groner, Y. (1986) EMBO J. **5**, 615–622

39   Chan, P. H., Chu, L., Chen, S. F., Carlson, E. J. and Epstein, C. J. (1990) Stroke **21** (Suppl. III), III-80–III-82

40   Frinovich, I. (1975) Annu. Rev. Biochem. **44**, 147–159