Journal of Neurochemistry, 2006, **98**, 677–689 doi:10.1111/j.1471-4159.2006.03906.x

Prion protein reduces both oxidative and non-oxidative copper toxicity

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Abstract

The prion protein is a membrane tethered glycoprotein that binds copper. Conversion to an abnormal isoform is associated with neurodegenerative diseases known as prion diseases. Expression of the prion protein has been suggested to prevent cell death caused by oxidative stress. Using cell based models we investigated the potential of the prion protein to protect against copper toxicity. Although prion protein expression effectively protected neurones from copper toxicity, this protection was not necessarily associated with reduction in oxidative damage. We also showed that glycine and the prion protein could both protect neuronal cells from oxidative stress. Only the prion protein could protect these cells from the toxicity of copper. In contrast glycine increased copper toxicity without any apparent oxidative stress or lipid peroxidation. Mutational analysis showed that protection by the prion protein was dependent upon the copper binding octameric repeat region. Our findings demonstrate that copper toxicity can be independent of measured oxidative stress and that prion protein expression primarily protects against copper toxicity independently of the mechanism of cell death.

Keywords: Copper, Glycine, Oxidative Stress, Prion, PrP.
J. Neurochem. (2006) **98**, 677–689.

Prion diseases include, among others, bovine spongiform encephalopathy, Creutzfeldt-Jakob's disease, scrapie, and a number of human inherited forms (Collinge 2001). They are all associated with the deposition of an abnormal isoform of the prion protein (PrP^{Sc}) in the brain. This abnormal protein is considered to be both the transmissible agent of the diseases and also the potential cause of the pathological changes chief of which is neurodegeneration (Prusiner 1982, 1998). The neurodegeneration has fatal consequences but the incubation period of prion diseases is often lengthy with only a short period of symptoms. Loss of neurones in the brain has been associated with the accumulation of PrP^{Sc}, activation of glia and oxidative damage. Although, PrP- knockout mice do not develop a prion like disease (Büeler *et al*. 1993) it has been suggested that the loss of function of the normal isoform of the protein could play a role in the disease pathogenesis (Brown 2001).

PrP^{Sc} is derived from the normal cellular isoform of the prion protein (PrP^{c}) by an unknown conversion mechanism (Cohen and Prusiner 1998). PrP^{c} is expressed in the nervous system and many cell types but is chiefly associated with neurones (Salès *et al*. 1998). There has been considerable debate about the normal function of this protein. If loss of function of PrP^{c} is in any way connected with prion diseases then it is essential to know what that function is. Some aspects of the normal activity of the protein are known; it is a glycoprotein of short half-life, which is located at the cell surface where it is tethered by a glycosylphospatidyl inositol (GPI) anchor. It is also a copper binding protein that can bind at least four atoms of copper (Brown *et al*. 1997a, Brown *et al*. 2000). Copper binding at the cell surface drives internalisation of the protein (Pauly and Harris 1998; Haigh *et al*. 2005).The suggested functions for the protein include signal transduction (Mouillet-Richard *et al*. 2000); cell adhesion (Schmitt-Ulms *et al*. 2001); copper uptake (Brown 1999) or sequestration; cell survival (Kuwahara *et al*. 1999) and as a copper dependent antioxidant.

Received December 10, 2005; revised manuscript received February 17, 2006; accepted February 23, 2006.

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Abbreviations used: BHT, butylated hydroxytoleune; DCF, dichlorofluorescein; DMEM, Dulbecco's Modified Eagles Media; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; GPI, glycosylphosphatidylinositol; HAE, 4-hydroxyalkenals; MDA, malondialdehyde; MTT, 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; PrP, the prion protein; PrP^{c}, cellular PrP; PrP^{Sc}, abnormal PrP; ROS, reactive oxygen species; s.e.m, standard error of the mean.

The ability to bind copper implies a potential to inhibit the oxidative properties of copper by preventing the metal ion from interaction with water and generating radicals such as the hydroxyl radical. Therefore, there is a natural link between the copper binding capacity of the protein and its suggested role in preventing oxidative damage. The evidence linking expression of PrP^c to increased resistance to oxidative stress comes from direct evidence of an antioxidant activity for the protein (Brown *et al*. 1999; 2002), and from indirect evidence, showing that cells are more susceptible to oxidative damage when PrP^c expression is ablated (Brown *et al*. 1997a; Walz *et al*. 1999; White *et al*. 1999; Brown *et al*. 2002; Zeng *et al*. 2003; Senator *et al*. 2004; Weise *et al*. 2004; Roucou and LeBlanc 2005; Watt *et al*. 2005), or that cellular levels of PrP^c change in response to oxidative stress (Frederikse *et al*. 2000; Voigtlander *et al*. 2001; Dupuis *et al*. 2002; McLennan *et al*. 2004; Williams *et al*. 2004). Additionally, there is evidence that oxidative damage occurs during the disease process (Guentchev *et al*. 2002, 2000; Milhavet *et al*. 2000; Wong *et al*. 2001; Petersen *et al*. 2005), implying that the neuronal loss in prion disease might be linked to oxidative damage. Whether this is due to either excess generation of radicals, loss of antioxidant protection or both is still to be determined. Although one study has concluded a lack of role for PrP^c in antioxidant protection (Hutter*et al*. 2003) the experiments were carried out under conditions where copper that would bind to PrP^c was rendered biologically unavailable.

In the current work we provide further evidence that expression of PrP^c prevents oxidative damage and inhibits cell death caused by copper and toxic radicals. We show that this is dependent upon copper binding to the protein. In addition we show that copper toxicity can be both a result of oxidative stress and another component that is independent of oxidation by the metal ion. The toxicity due to the nonoxidative component was also inhibited by expression of PrP^c. This finding is relevant not only to prion research but also to neuroscience as it indicates that neuronal protection from copper overload is not just dependent on oxidative defence.

Materials and methods

Creation of plasmid constructs

The open reading frame of mouse prion protein gene Prnp was cloned into pEGFP-C1 (Clontech, BD Biosciences, Oxford, UK) as previously described (Holme *et al*. 2003). Reagents were purchased from Sigma-Alderich (Poole, UK) unless otherwise stated. The GFP-GPI vector is the same construct but lacking all of the PrP sequence inserted after GFP (from codon 39) up to the GPI anchor signal sequence (Holme *et al*. 2003). Deletion mutations were introduced into GFP-PrP using site directed PCR mutagenesis (Figure 1). Several of the mutants used in this study were previously described in Haigh *et al*. (2005). In addition to these mutants, three further mutants were created by site directed PCR mutagenesis (as described in Haigh *et al*. 2005) using the following forward primers Δ112-136 5′-CCAAAAACCAACCTCAAGCATGTGATGATCC-ATTTTGGCAACGACTGGG-3′, Δ122-146 5′-GCAGCTGGGG-CAGTAGTGCGCTACTACCGTGAAAACATGTAC-3′, Δ135-150 5′-GGGAGCGCCGTGAGCGAAAACATGTACCGC-3′, and complementary reverse primers.

Cell culture

F14 cells (Holme *et al*. 2003) were cultured in Dulbecco's Modified Eagles Media (DMEM) supplemented with 10% foetal bovine serum (FBS), and final concentrations of 1 U/mL penicillin and 0.5 mg/mL streptomycin. Cells were maintained at 37°C and with an atmosphere containing 5% CO_2.

Transfections

Stable cells lines were created by transfection using Fugene 6 reagent (Roche, East Sussex, UK) as descibed in the manufacturers guidelines. Cells were selected using 0.1 mg/mL G418 (Gibco, Paisley UK) and maintained in 0.05 mg/mL during culture.

Live cell imaging experiments

Cells were cultured in chambered coverslips (Nunclon – Fisher, Loughborough UK), at aproximately 50% confluency. Images were captured using a Zeiss LSM 510 confocal microscope (Zeiss, Zürich, Switerland). A minimum of three cells were studied per experiment, and experiments were repeated a minimum of four times.

Survival assay

Cells were plated at approximately 40% confluency for metal assays and 80% confluency for oxidative stress assays, and incubated overnight before addition of the test reagent. The cells were then returned to the incubator until the time of the assay (4 days for metal assays, 24 h for oxidative stress assays). At the time of the assay the media was removed from the cells and replaced by 200 μL of 200 μg/mL MTT [3, (4,5 dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide] reagent in Hanks. Plates were incubated for 30 min before the reagent was removed and 750 μL of dimethyl sulfoxide (DMSO) was added to dissolve the precipitate. Samples were read at 570 nm in a Cary 50 UV-visual spectrophotometer (Varian, Palo Alto, USA).

Direct measurement of free radicals – microplate assay

Direct measurement of reactive oxygen species (ROS) within cells was made using a microplate assay utilising CM-H_2DCFDA, which is a chemically reduced, acetmethoxy ester of 2′,7′-dichlorofluorescein (DCF). This compound is colourless until the acetate groups are removed by intracellular esterases and oxidation occurs within the cell. CM-H_2DCFDA is cell permeable until it is oxidised to its fluorescent product inside the cell. Oxidation may be induced by hydrogen peroxide (with the assistance of endogenous metal ions), organic hydroperoxides, nitric oxide and peroxynitrite.

F14 and F21 cells were plated in 96-well plates at 90–95% confluency and returned to the incubator overnight. Media was removed from test wells, and replaced by 50 μL of 5 μM probe in PBS, and incubated in the dark at 37°C for 20 min. Probe was removed from the cells and replaced by 100 μL of pre-warmed

OptiMEM. Test reagent was added to four wells per experiment, and fluorescence intensity was measured using a microplate reader with excitation and emission wavelengths of 488 and 534 nm, respectively, at time 0, 30 min, and 2 h.

Direct measurement of free radicals – RedoxSensor^{TM} Red

RedoxSensor^{TM} Red (Invitrogen, Paisley, UK) is a probe that passively enters live cells. Once inside the cell the non-fluorescent probe is oxidised to a red fluorescent product, which then accumulates in the mitochondria. Alternatively the non-fluorescent probe may be transported to lysozymes where it is oxidised to the fluorescent product. The intensity of the fluorescent signal is determined by the redox potential of the cytosol, with increased free radicals in the cytosol increasing the degree of oxidation of the probe.

Cells transfected with the GFP-PrP constructs were plated in chambered coverslips and allowed to adhere. Prior to the start of the assay, cells were incubated with 5 μM probe in normal media for 10 min, this was removed and cells washed once in Hanks, before addition of OptiMEM. Cells were observed by confocal microscopy at the start of the assay and 5, 10 and 15 min after the addition of test reagent.

Lipid peroxidation assays

Lipid peroxidation is a mechanism of cellular injury caused by, and therefore a useful indicator of, oxidative stress. Lipid peroxides break down to form aldehydes, which may themselves be reactive and cause further oxidative stress insults. Break down products include malondialdehyde (MDA) and 4-hydroxyalkenals (HAE). The measurement of these is used to demonstrate oxidative stress injury of cells (Esterbauer *et al*. 1991; Oxis product guide). Reaction of MDA or HAE with N-methyl-2-phenylindole results in a coloured product with an absorption maxima of 585 nm. The intensity of the colour is directly related to the extent of the lipid peroxidation.

A homogenate of 5×10^7 cells was required per assay condition. This was achieved by using 4–6 T75 cell culture flasks of cells per condition. Cells were grown to be confluent at the time of extraction. Test reagent was added 2 days prior to this time. Cells were collected by centrifugation at 1200 r.p.m. for 10 mins, washed in Hanks, then centrifuged again as before. The pellet was resuspended in 1.5 mL of 20 mM phosphate buffer (pH 7.4) and this was sonicated for 15 s to produce the homogenate. Immediately after sonication, 15 μL of 0.5 M butylated hydroxytoluene (BHT) in acetonitile, to a final concentration of 0.5 mM, was added to the homogenate to prevent further lipid peroxidation occurring. Samples were then stored at −80°C until use.

The lipid peroxidation assay was carried out using the Bioxytech® LPO-586$^{TM.}$ kit, as described in the product handbook. Following this a bicinchoninic acid assay was carried out (as described in the Sigma product handbook) on each sample to account for differences in protein concentration.

Intensity measurements and statistical analysis

Intensity measurements of live cell experiments were made using the Zeiss LSM software (Zeiss). Statistical analyses were performed using Minitab 12 statistical software. Direct linear ANOVA was used for comparing trends and one-way ANOVA was used to compare point differences of multivariate normally distributed data. Tukey's secondary test identified significant results.

Results

Prion protein expression and oxidative stress

The cell lines F14 and F21 differ in their level of expression of PrP^c. These cell lines have previously been fully characterised by Holme *et al*. (2003). F14 cells are a knockout cell line with no expression of PrP^c while F21 cells have a high level of PrP^c expression. In order to assess the level of radicals within the two different cell lines the radical detecting dye, CM-H_2DCFDA was loaded into both cell lines and the change in the fluorescent emission at 534 nm determined at 30 min and 2 h (Fig. 2). The intensity of the emitted fluorescence is directly proportional to the level of radicals generated in the cells and therefore the level of oxidative stress in the cells. As can be seen F14 cells maintained a higher level of oxidative stress than F21 cells ($F = 6.17$, $p = 0.006$). Copper is able to generate oxidative stress by Fenton chemistry. In order to assess the cellular oxidative stress levels in F14 and F21 cells when exposed to copper, 100 μM $CuSO_4$ was applied to the cells either on its own or in combination with 400 μM glycine (Cu-Gly). The glycine chelated copper was tested because under physiological conditions copper is unlikely to exist as a free ion. Glycine on its own had no effect on the oxidative stress levels in cells ($F = 0.02$, $p = 0.884$). Free copper increased levels of oxidative stress far more in F14 cells than in F21 cells ($F = 4.31$, 0.026), although the levels of oxidative stress in both cells were significantly increased ($F = 26.62$, $p < 0.001$). Interestingly, there was no difference in the increase in oxidative stress in F21 cells caused by copper in the presence of glycine ($F = 0.13$, $p = 0.72$). However, glycine chelation significantly protected F14 cells from increased oxidative stress caused by copper ($F = 7.1$, $p = 0.003$).

The toxicity of copper and Cu-Gly were also tested. F14 and cells were treated with a range of concentrations of both free copper and Cu-Gly. As can be seen in Fig. 3a free copper and Cu-Gly was similarly to F21 cells. However, both forms of copper were more toxic to F14 cells than F21 cells and Cu-Gly was more toxic to F14 cells than free Cu. Glycine showed no toxicity (data not shown). In order to test this further, F14 cells were transfected with a plasmid expression vector construct to expressing mouse PrP^c tagged with the green fluorescence protein (GFP-PrP). In addition F14 cells were transfected to express GFP with the N-terminus of PrP (amino acid residues 1–38) and the GPI signal sequence from PrP (Fig. 1). This latter construct termed GFP-GPI was previously shown to localise to the plasma membrane with a similar topology to GFP tagged PrP (Holme *et al*. 2003). The two cell lines thus differ only in

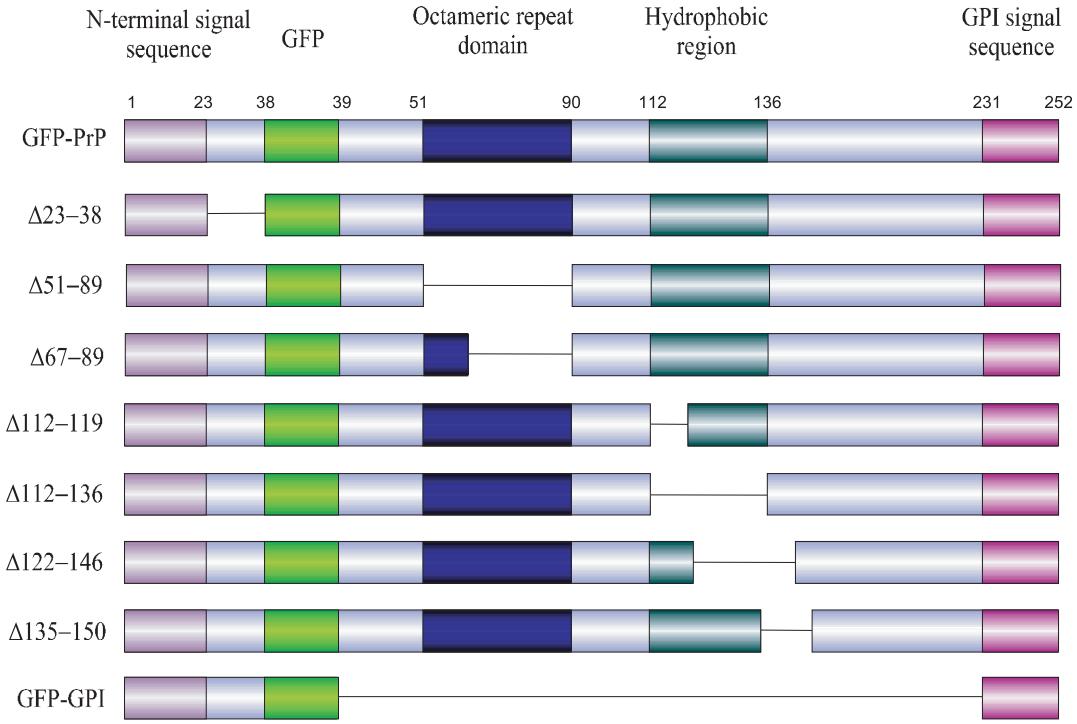

Fig. 1 PrP expression constructs. Schematic representations of mutant prion proteins expressed in cells lines from a plasmid containing the open reading frame of PrP^c. Numbers indicate amino acid residues in the mouse sequence. The missing domain, deleted by mutation is indicated by a line. GPI = glycosylphosphatidyl inositol anchor, GFP = green fluorescent protein.

that GFP-PrP expresses amino acid residues 38–231 of mouse PrP^c and GFP-GPI does not. These two cell lines were treated with free copper and Cu-Gly (Fig. 3b). After four days the survival was assessed with an MTT assay. Copper in any form was more toxic to cells expressing GFP-GPI than those expressing GFP-PrP. While there was no significant difference in the toxicity of free copper and Cu-Gly to GFP-PrP expressing cells ($F = 1.32$, $p = 0.256$), Cu-Gly was more toxic to GFP-GPI expressing cells ($F = 13.61$, $p = 0.001$).

The GFP-PrP and GFP-GPI expressing cell lines were also tested for their relative resistance to the toxicity of other metals and also other substances that cause oxidative stress. In particular, DMSO and xanthine oxidase were tested. GFP-PrP expressing cells were more resistant to the toxicity of both DMSO and xanthine oxidase than GFP-GPI expressing cells ($F = 4.8$, $p < 0.001$ and $F = 4.32$, $p = 0.001$, respectively; Fig. 3c and d). The toxicity of manganese was tested with and without the addition of a four molar excess of glycine. Manganese at concentrations of 50 μM and above was less toxic to the cell line expressing PrP (GFP-PrP) than that expressing the null GFP-GPI construct ($F = 14.72$, $p < 0.001$), but glycine did not alter the toxicity to either cell line (Fig. 3e). The toxicity of zinc, iron and nickel was also tested but there was no difference in the toxicity to the two cell lines ($F = 0.3$, $p = 0.587$, $F = 0.58$, $p = 0.451$, and $F = 1.85$, $p = 0.112$, respectively; Fig. 3f and g). These results suggest that the expression of PrP^c protects against the toxicity of copper, manganese and substances that cause oxidative stress. However, the protection of PrP^c against copper toxcity could be partially independent of the ability of copper to generate free radicals.

PrP domains necessary for protection against toxicity

Specific coding domains of PrP^c were deleted from the full length GFP-PrP construct (Fig. 1). These domains were chosen for their potential effect on the function of PrP^c. The domains included the whole octameric repeat region (Δ51–89), most of the octameric repeat region but leaving a single octameric repeat intact (Δ67–89), the N-terminus before GFP (Δ23–38), the palendromic region (Δ112–119) and the hydrophobic domain (Δ112–136). In addition mutants not directly linked to particular domains were also prepared (Δ122–146 and Δ135–150). All of these mutants were transfected into the null F14 cells and stable cell lines created. Most of these constructs have been characterised before (Haigh *et al*. 2005). The levels of expression of the constructs in these cell lines were shown to be equivalent (Haigh *et al*. 2005). However, in this study we also characterised the localisation of the new GFP-tagged PrP mutants located in different cellular compartments. Although most of the constructs were correctly processed by the cell

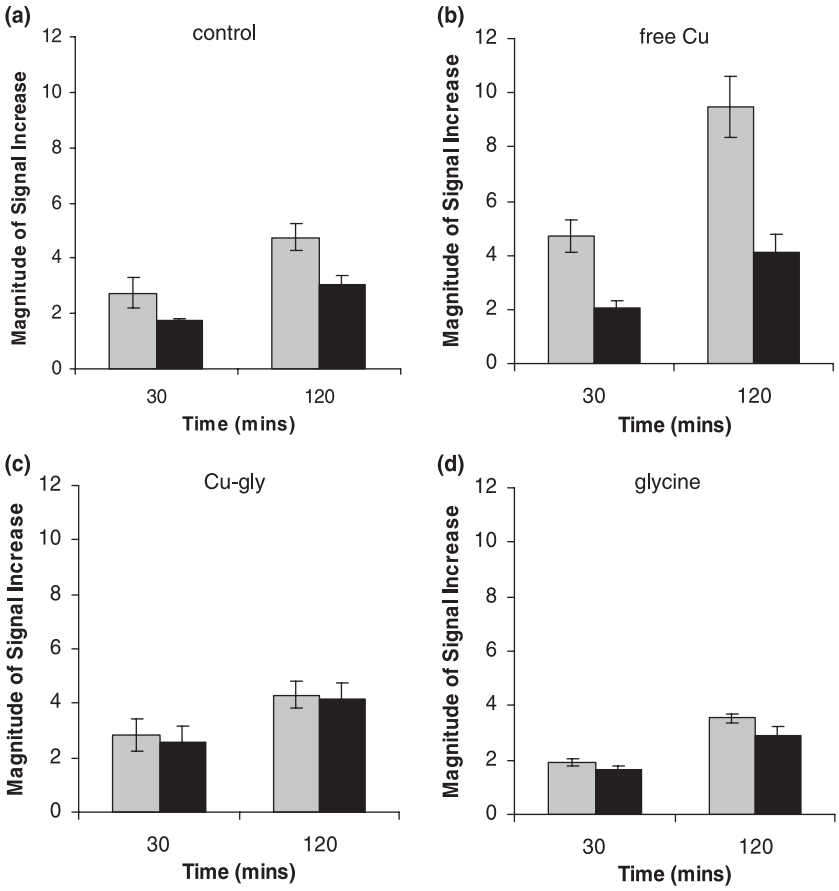

Fig. 2 Oxidative stress in cell lines. F14 (grey bars) and F21 (black bars) cell oxidative stress measured by DCFDA assay when cells are (a) untreated, or treated from time 0 with (b) 100 μM CuSO4, (c) 100 μM Cu-4xGly, or (d) 400 μM glycine alone. The increase plotted has been calculated as a ratio of time point value:time 0-value, to show the magnitude of the increase comparable to the time 0 reading. Shown are the mean and standard error of the mean (s.e.m.). $n = 5$.

and reached the cell surface Δ112–136, Δ122–146 and Δ135–150 were not. Δ112–136 and Δ135–150 remained trapped in the endoplasmic reticulum and Golgi compartments and Δ122–146 also localised to the nucleus (Fig. 4).

The cells expressing these mutants were treated with DMSO, xanthine oxidase or Cu-Gly and the survival compared to that of wild-type GFP-PrP. The cells were treated for 24 hours with DMSO or xanthine oxidase, or four days with Cu-Gly and survival was assessed with an MTT assay (Fig. 5). The mutants were assessed in terms of the ability of the expressed protein to protect against the toxic agent as compared to wild-type PrP. Those mutants that were significantly less protective than wild-type PrP at most concentrations tested were grouped together (Fig. 5a, c, e) while those that showed no significant difference to wild-type PrP were placed in a separate group (Fig. 5b, d, f). For cells treated with Cu-Gly only two mutants differed significantly to wild-type (Δ122–146 and Δ51–89; $F = 7.85$, $p < 0.001$). Some of the mutants tested (Δ51–89 and Δ112–136) showed reduced protection against the toxicity of both DMSO ($F = 4.92$, $p < 0.001$) and xanthine oxidase ($F = 5.14$, $p < 0.001$). Expression of Δ23–38 did not protect against the toxicity of DMSO but offered similar protection to wild-type against xanthine oxidase toxicity. Protection by Δ112–119 against both DMSO and xanthine oxidase toxicity was equivalent to that of wild-type GFP-PrP. Expression of Δ135–150 did not protect against the toxicity of xanthine oxidase but largely showed protection against DMSO similar to wild-type PrP. Other mutants showed little difference to wild-type PrP although some showed a significant difference to wild-type PrP at 2% DMSO in terms of protection against toxicity. However, as there was no difference at other concentrations it was deemed that the mutations did not produce a significant change in the protectiveness of GFP-PrP against oxidative stress.

Mechanism of PrP Protection Against Copper

GFP-PrP and the mutants studied in the previous section were also studied for their ability to protect against levels of oxidative stress in cells. F14 cells transfected to express GFP-PrP, mutants of GFP-GPI and GFP-GPI were loaded with the redox sensor probe RedoxSensor Red (Fig. 6). The RedoxSensor Red probe is sensitive to less radical species than the previously described CM-H_2DCFDA probe, but was used in preference for these assays because the latter is measured at the same wavelengths as the GFP tag. In other words a red fluorescent probe was necessary to contrast to the green fluorescence of GFP. Cells were then treated with

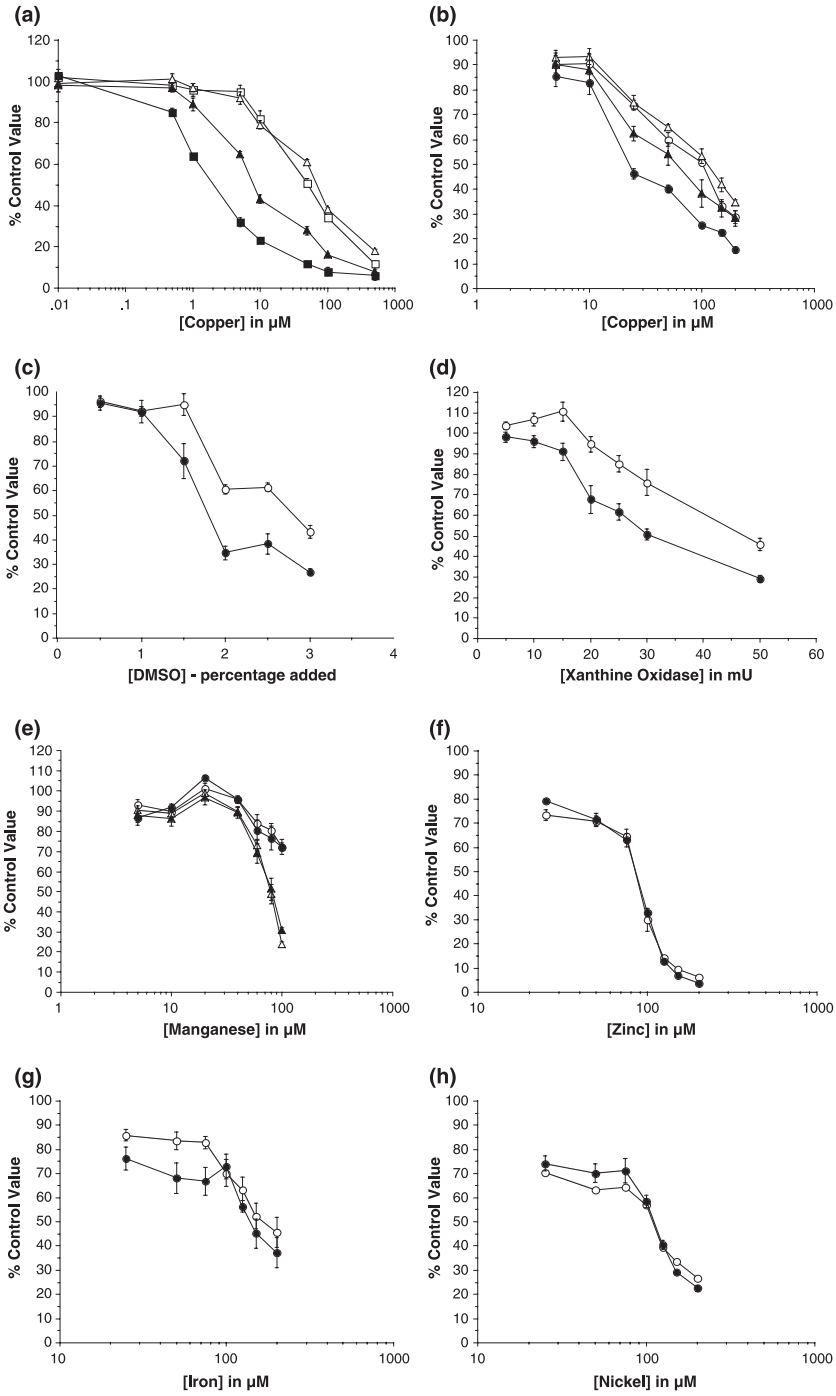

Fig. 3 PrP^c Expression protects against toxicity. Cell lines were tested for survival when treated with a variety of toxic agents. Cells were treated for four days with metals or 24 h with oxidants and the survival determined using an MTT assay. Values were then compared to those of the untreated control as a percentage. (a) The cell lines F14 (black symbols) and F21 (open symbols) were either treated with $CuSO_4$ (triangles) without chelator or Cu-Gly (squares). (b) Cells expressing GFP-GPI (black symbols) and GFP-PrP (open symbols) were either treated with $CuSO_4$ (triangles) without chelator or Cu-Gly (squares). (c) Treatment with DMSO (GFP-PrP = open circles, GFP-GPI = black circles). (d) Treatment with xanthine oxidase (GFP-PrP = open circles, GFP-GPI = black circles). (e) Cells expressing GFP-GPI (triangles) and GFP-PrP (circles) were either treated with $MnSO_4$ (open symbols) without chelator or Mn-gly (black symbols). The same cell lines (GFP-PrP = open circles, GFP-GPI = black circles) were also treated with increasing concentrations of (f) $ZnSO_4$, (g) $FeSO_4$, and (h) $NiSO_4$.Shown are the mean and s.e.m. for at least four experiments with three determinations each.

100 μM $CuSO_4$, Cu-Gly or 400 μM glycine on its own. The level of red fluorescence signal was then determined at 0, 5, 10 and 15 min after application. Without treatment of the cells there was no change in the basal level of the fluorescent probe during this time period for any of the proteins expressed. Similarly, glycine had no effect on basal levels of fluorescence (data not shown) for any of the proteins expressed. Exposure of-non-transfected cells to free copper resulted in an increase in fluorescence in the cells indicating that free copper caused an increase in detectable oxygen radicals in the cells ($F = 5.04$, $p = 0.007$; Fig. 7). Similarly, cells expressing GFP-GPI showed a similar increase in red fluorescence when exposed to free copper indicating that this construct did not protect the cells against oxidative stress ($F = 4.23$, $p = 0.043$). In contrast, cells expressing GFP-PrP showed no increase in red fluorescence levels over the time ($F = 0.85$, $p = 0.437$). This suggests the expression of GFP-PrP blocked the increased fluorescence in response to

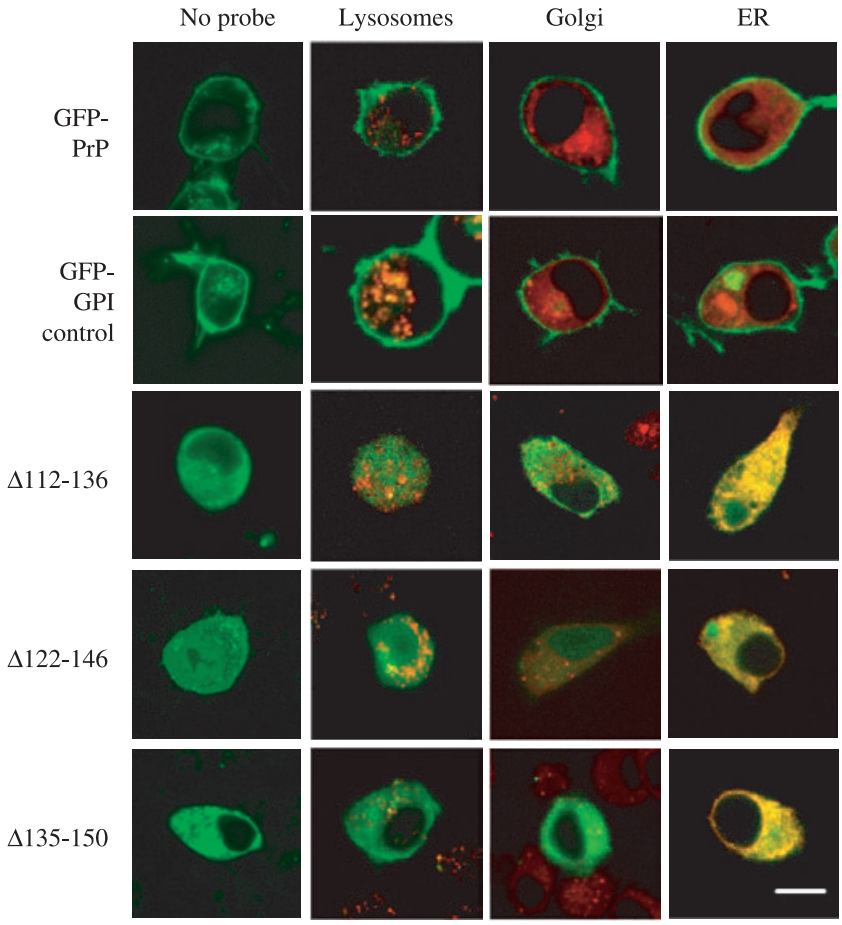

Fig. 4 Expression/Localisation of mutant PrP. Cells expressing GFP tagged wild-type and mutant PrP were visualised with a confocal microscope. GFP could be detected and corresponded to the localisation of PrP (green). The same cells were also labelled with vital dyes (red) specific for particular subcellular compartments (Golgi apparatus, endoplasmic reticulum and lysosomes). Co-localisation between mutant PrP and particular compartments could be assessed by the mixture of the two labels (yellow). Scale bar = 10 μm.

free copper. The only mutant that did not block the response to free copper was Δ51–89 ($F = 6.85$, $p = 0.008$; Fig. 6). The quantification of these results is shown in Fig. 7. When cells were treated with Cu-Gly there was no increase in the level of red fluorescence ($F = 1.41$, $p = 0.239$), suggesting that an oxidative stress response was not seen in the cells following treatment with Cu-Gly. As a result none of the proteins expressed by the cells showed any difference to the untransfected cells in terms of their response to Cu-Gly (Fig. 7).

It is possible that oxidative effects of Cu-Gly would only be evident at the cell surface. Therefore we used a second assay to assess effects of oxidation of membranes. Lipid peroxidation caused by copper in the chelated and-non-chelated form was assessed using a commercial kit that measures formation of malondialdehyde MDA and HAE. The cell lines expressing GFP-PrP and GFP-GPI were compared and showed no significant difference in the basal level of these lipid peroxidation products ($F = 0.23$, $p = 0.872$). However, exposure of the cells to 50 μM $CuSO_4$ resulted in a marked increase in lipid peroxidation products only for the cell line expressing GFP-GPI (MDA $F = 4.45$, $p = 0.001$; HAE $F = 3.9$, $p = 0.04$; Fig. 8a). This indicated that the expression of PrP protected the cells from lipid peroxidation caused by free copper. In contrast, glycine chelated copper at the same concentration as free copper showed a lesser effect on lipid peroxidation (Fig. 8a). This suggests that glycine protects against the lipid peroxidation effects of copper.

We have shown above that Cu-Gly is less toxic to cells expressing elevated levels of PrP^c. In order to assess whether this is due to increased antioxidant protection, F14 and F21 cell lines were treated with Cu-Gly as before and survival assessed after four days. The cells were also treated with the antioxidants, Cu/Zn superoxide dismutase, catalase, N-acetyl cysteine or all three combined. There was no evidence that the antioxidants protected the cells from the toxicity of Cu-Gly ($F = 0.72$, $p = 0.619$; Fig. 8b).

Discussion

The findings presented here demonstrated that the prion protein expressed by cultured cells protects against the toxic effects of copper and substances that cause oxidative stress. More significantly they show that copper toxicity can be independent of oxidative stress and that prion protein can

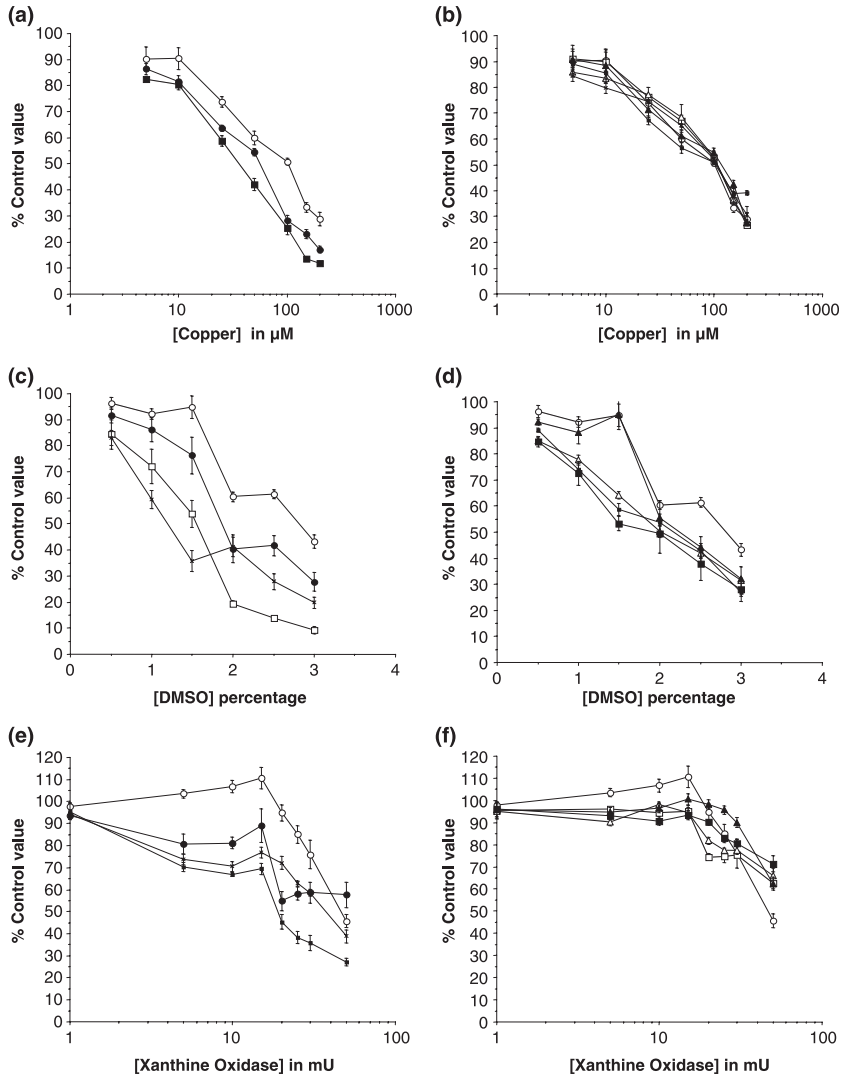

Fig. 5 Toxicity of copper and oxidants to GFP-PrP mutant expressing cells. Cells expressing tagged wild-type and mutant forms of PrP were treated either with Cu-Gly for four days (a, b), DMSO (c, d) or xanthine oxidase (e, f) for 24 h after which time the survival of the cells was determined with an MTT assay and compared to untreated control values as a percentage. For all parts of the figure the symbols correspond to the following proteins expressed by the cells. GFP-PrP = open circles, Δ23–38 = open squares, Δ51–89 = black circles, Δ67–89 = open triangles, Δ112–119 = black triangles, Δ112–136 = crosses, Δ122–146 = black sqaures and Δ135–150 = small squares. Shown are the mean and s.e.m. for at least four experiments with three determinations each.

protect against this form of copper toxicity as well. In terms of the toxicity of free copper applied to cells, the presence of an excess of the amino acid glycine is sufficient to block against the oxidative effects of copper but not against its toxicity. This suggests that the toxicity of copper is not necessarily related to its potential to generate free radicals by processes such as the Fenton reaction. The protective effect of glycine against the radical generating potential of copper is logical in that it would prevent copper interactions with water. There is evidence that there is little free copper in biological systems (Rae *et al*. 1999) and therefore free copper would only exist under abnormal circumstances. However, there has been little study of copperglycinate in terms of its biological role but one study suggested that it could act as a radical scavenger (Jagetia *et al*. 1993).

Using several techniques we have shown that expression of PrP^c protects against the toxicity of a variety of agents. This protection can come either from exogenous expression or through expression from a recombinant source. Most other studies have been carried out with primary neuronal cultures from wild-type mice and compared to the survival of similar neuronal cultures from PrP-knockout mice (Brown *et al*. 1997a; White *et al*. 1999; Kim *et al*. 2004). As most reports concerning the protection by PrP^c have focussed on the potential of the protein to protect against oxidative stress it has been largely assumed that the protection offered by PrP^c against copper toxicity is due to the ligation of the copper preventing oxidative damage caused by the generation of oxygen radicals via Fenton chemistry. Our new findings suggest that the toxicity caused by copper is not necessarily the result of oxidative stress but potentially through a non-oxidative mechanism. Simple chelation by glycine prevented the generation of oxygen radicals by copper but did not inhibit its toxicity. In contrast, PrP^c greatly reduced both the oxidative stress caused by free copper and also inhibited the toxicity caused by free copper and chelated copper. The implication is that PrP^c can inhibit the toxicity of copper by acting through two different mechanisms.

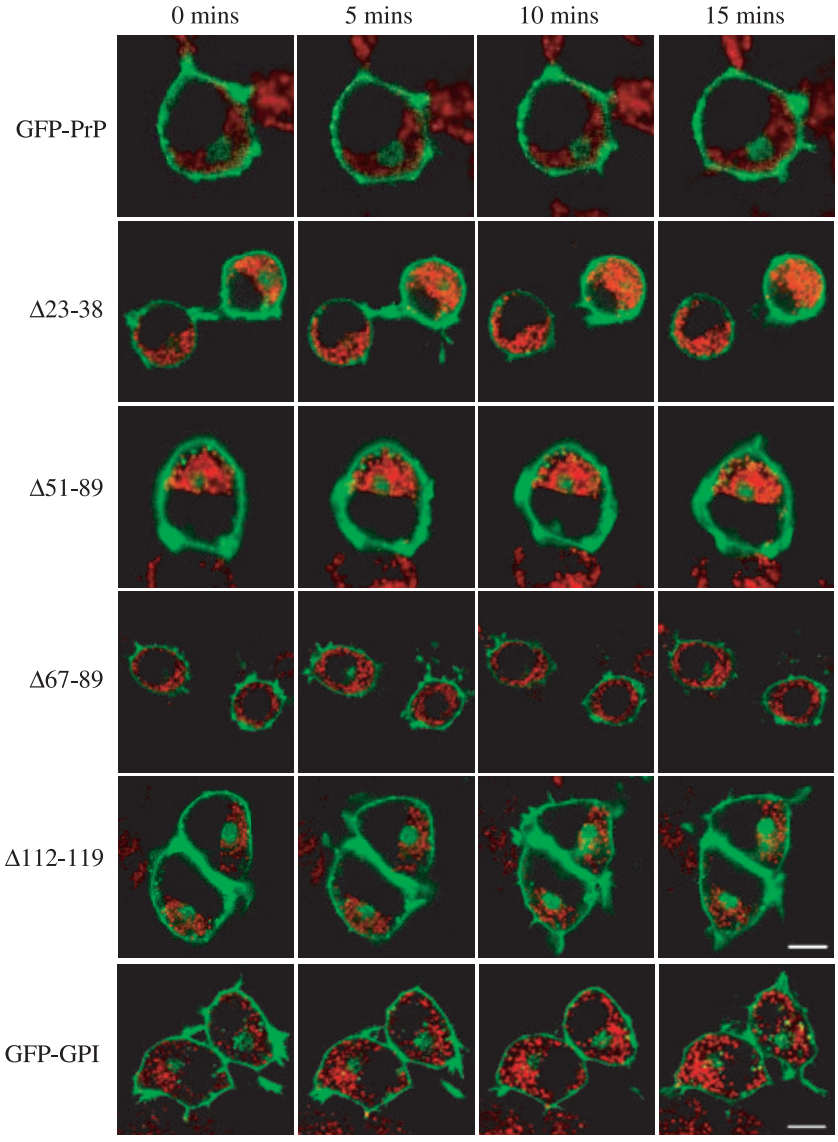

Fig. 6 Redox status of GFP-PrP expressing cells exposed to copper. Images of cells expressing GFP tagged wild-type and mutant forms of PrP. The Green label shows the localisation of the PrP, mostly at the cell surface. The cells were loaded with RedoxSensor Red and images capture at 0, 5, 10, and 15 min later. Scale bar = 10 μm.

The finding that PrP^c or fragments of PrP^c prevent the toxicity of copper is not new (Shaked *et al*. 1999). Several studies have now shown that the octarepeat region prevents copper toxicity when applied both *in vitro* (Brown *et al*. 1998; Nishimura *et al*. 2004) and *in vivo* (Chacon *et al*. 2003). As the protein binds copper through this domain (Brown *et al*. 1997a) then inhibition of toxicity is clearly associated with this capacity of the protein. Until now this has largely been assumed to relate to the accepted view that ligation of copper inhibits its oxidative potential. A view supported by the many findings suggesting that expression of PrP^c prevents oxidative damage (Brown *et al*. 1997b; White *et al*. 1999; Milhavet *et al*. 2000; Klamt *et al*. 2001; Brown *et al*. 2002; Dupuis *et al*. 2002; Zeng *et al*. 2003; McLennan *et al*. 2004; Weise *et al*. 2004; Roucou and LeBlanc 2005; Watt *et al*. 2005). Although the current findings also support the role of PrP^c in protection against oxidative stress, they also suggest that PrP^c protects against copper toxicity regardless of how this toxicity occurs.

Experiments on copper toxicity used a time period of four days. The half-life of PrP^c at the cell surface is in the order of an hour and exposure of copper alters the cell surface level of the protein by copper mediated internalisation (Haigh *et al*. 2005). We chose a four-day treatment time to rule out the possible interference of altered protein expression or copper trafficking that occur in these shorter time intervals. Although, it is possible that a shorter exposure to copper would have produced different results to those shown here, our own studies suggest that treatment for one day would produce a similar result to that for four.

In this study we used DNA constructs that generated GFP-PrP fusion proteins. Our previous studies with these constructs showed that GFP did not alter the normal

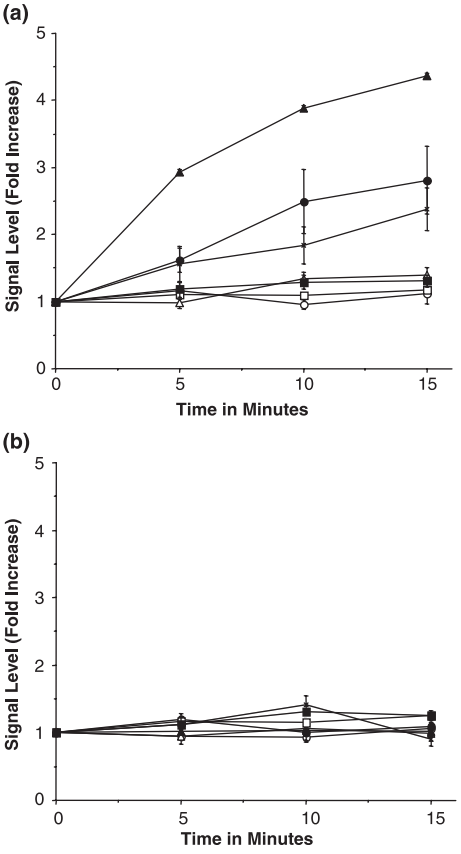

Fig. 7 Quantitation of redox status. Cell lines expressing GFP tagged wild-type and mutant PrP were labelled with RedoxSensor Red and the intensity of the signal measured over time using a confocal microscope after the application of either (a) free copper or (b) Cu-Gly. The relative change in intensity was compared to that of the 0 time point as a fold increase above the initial level (0 time point = 1). The proteins expressed were GFP-PrP (open circles), GFP-GPI (black circles), Δ23–38(open triangles), Δ51–89 (black triangles), Δ67–89 (open squares), Δ112–119 (black squares). Also shown are the results for the untransfected cell line (crosses). Shown are the mean and s.e.m. for a minimum of four experiments and a minimum of 12 cells.

trafficking of PrP^c (Holme *et al*. 2003; Haigh *et al*. 2005). Studies with bacterially expressed recombinant proteins also showed that the different mutations we introduced into the protein did not alter the secondary structure or copper binding potential of the protein (Cui *et al*. 2003). Therefore we are confident that our mutational analysis of the protection by PrP^c against oxidative stress and copper toxicity reflect the specific deletion and not any other change. Deletion of the copper binding octameric repeat regions caused a loss of protection in all cases indicating that copper binding to this domain is the likely functional link to the protective effect of PrP^c expression. No other deletion altered protection against increases in cellular oxidative stress. This protection effect could be due to simple chelation of copper rather than a true antioxidant activity of PrP^c. However, PrP^c has been suggested to have a superoxide dismutase activity (Brown *et al*. 1999). This activity does not explain the ability of PrP^c to protect against copper toxicity not related to oxidative damage.

It should be noted that the deletion of the octameric repeat region removes the main copper binding site from the protein. However, the so called fifth site (around the histidines at residues 95 and 110) remains intact. As the retention of this site did not protect cells from either copper toxicity or cell death from oxidative stress then this region plays no role in the protective activities of PrP^c.

DMSO and xanthine oxidase cause cell death through oxidative damage. It is therefore likely that protection against the toxicity of these agents is due to the antioxidant activity of PrP^c. The domains essential for this activity were not always the same for protection against DMSO or xanthine oxidase. The hydrophobic domain (112–136) was necessary for the protective effect but the smaller fragment of the same domain (112–119) was not. This is consistent with previous studies on the superoxide dismutase activity of PrP^c that showed that this domain was also essential for the activity (Cui *et al*. 2003). It is important to note that the hydrophobic domain and the octameric repeats are the two most conserved parts of the protein sequence (Wopfner *et al*. 1999) also in keeping with these domains being necessary for normal protein function. The deletion of 23–38 caused loss of protection against DMSO but not xanthine oxidase. This domain has been suggested to play a role in internalisation of PrP^c (Sunyach *et al*. 2003). A reduction in this capacity might keep a significant amount of PrP^c at the cell surface which might protect against the less cell permeable xanthine oxidase but not against the highly cell permeable DMSO. The opposite effect was seen with the deletion of 135–150. This mutant was not able to reach the cell surface and remained trapped within intracellular organelles. In this case the protein was not able to protect against xanthine oxidase which may have its effects due to radicals generated outside the cell while DMSO could potential generate radicals after entry into the cell. One mutant 122–146 did not protect against copper toxicity but did protect against oxidative stress. Possibly due to its cellular localisation it was likely to be unable to bind copper at the site where it would cause toxicity to the cell.

The mode of action of copper was verified by studies that showed that in a free form it caused oxidative damage such as lipid peroxidation and resulted in elevated radicals within the cell. As already mentioned chelation by glycine prevented both oxidative stress and lipid peroxidation. Furthermore, antioxidants applied to cells in the presence of glycine chelated copper did not inhibit its toxic effects further emphasising that this form of copper toxicity was independent of oxidative stress.

In this paper we tested the hypothesis that the cell damage caused by copper was independent of oxidative stress by the

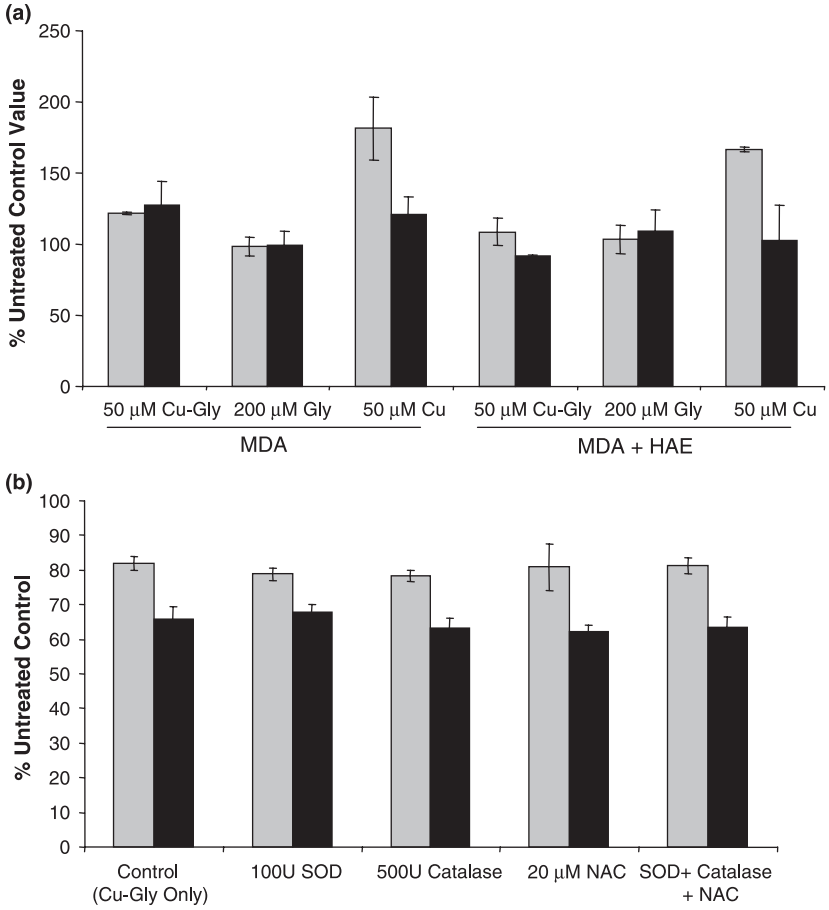

Fig. 8 Copper stimulated lipid peroxidation. (a) Lipid peroxidation was assessed for cell lines expressing either GFP-PrP (grey bars) or GFP-GPI (black bars). The cells were treated either with free copper, glycine or Cu-Gly for 48 h. Shown are the mean and s.e.m. for five experiments. (b) F21 (grey bars) and F14 (black bars) were treated with 50 μM Cu-Gly and cell survival assessed after four days. In parallel similarly treated cultures were also incubated with either 100 U of bovine Cu/Zn superoxide dismutase (SOD), 500 U catalase, 20 μM N-acetyl cysteine (NAC) or all three antioxidants at once. Shown are the mean and s.e.m. for 4 experiments.

use of assays that determine the level of oxidation products and the production of radicals in cells produced by exposure to copper. Our results clearly show that cell death can occur from exposure to copper in the absence of oxidative damage or generation of radicals. This supports our hypothesis. This could be further verified by measurements of the Cu-gly chelate in a stopped flow system that would directly assess the ability of Cu in this form to generate radicals. Therefore the use of Cu-Gly chelates provides a tool for determining the mechanism of toxicity of copper independently of oxidative damage. Additionally, the redox sensing probes we have used in this study could be used in other experimental models looking at the damaging effects of copper such as those that have been developed for Wilson's and Menke's disease.

Increasing copper load to cells does not necessarily increase the levels of oxidative stress (Armendariz *et al*. 2004). In Wilson's disease there is an increased accumulation of copper in neurones which leads to neurodegeneration (Cuthbert 1995). This is due to a failure of copper to be extruded from the cell (Voskoboinik *et al*. 2001). One possibility is that copper alters energy metabolism and particularly glycolysis (Lai and Blass 1984). In addition copper may saturate metal storage proteins such metallotheioneins, displacing other metals that may be more toxic for cells. Predki and Sarkar (1992) showed that copper can be substituted for zinc in zinc finger proteins and by doing so ablates their function. Such substitutions in essential metalloproteins would explain the non-oxidative toxicity of copper. In the presence of glycine, copper may be more toxic simply because of increased uptake as chelation of copper is known to increase uptake by neurones (Hartter and Barnea 1988). High levels of copper in cells could also lead to precipitation of cupric salt crystals (as occurs in Wilson's disease) which could damage cell organelles (Johnson and Campbell 1982).

In summary, we have confirmed that PrP^c protects against both copper toxicity and oxidative stress and we analysed the domains necessary for this effect as expressed by cultured cells. In addition, we have shown for the first time that PrP^c protects against non-oxidative toxicity of copper. This supports the previous hypothesis that PrP^c acts to protect cells from stress.

Acknowledgements

This work was supported by both a senior fellowship (DRB) and a studentship (CLH) from the Biotechnology and Biological Sciences Research Council of the UK.

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