Expression of Prion Protein Increases Cellular Copper Binding and Antioxidant Enzyme Activities but Not Copper Delivery*

Walid Rachidi‡§, Didier Vilette‡, Pascale Guiraud‡, Marie Arlotto†, Jacqueline Riondel‡, Hubert Laude©, Sylvain Lehmann©, and Alain Favier**

From the ¶Laboratoire de Biologie du Stress Oxydant (LBSo), Faculté de Pharmacie, Domaine de La Merici, 38706 La Tronche-Grenoble cedex 9, France, the §Unité de Virologie Immunologie Moléculaires, Institut National de la Recherche Agronomique (INRA), 78350 Jouy-en-Josas, France, the ¶Laboratoire de Génétique Humaine, CNRS U.P.R. 1142, 141, rue de la Cardonille, 34396 Montpellier Cedex 5, France, and the **Laboratoire des Lésions des Acides Nucleiques, UMR CNRS/CEA/UJF, 5046, Avenue des Martyrs, 38000 Grenoble, France

The N-terminal region of the prion protein PrPc contains a series of octapeptide repeats. This region has been implicated in the binding of divalent metal ions, particularly copper. PrPc has been suggested to be involved in copper transport and metabolism and in cell defense mechanisms against oxidative insult, possibly through the regulation of the intracellular CuZn superoxide dismutase activity (CuZn-SOD) or a SOD-like activity of PrPc itself. However, up to now the link between PrPc expression and copper metabolism or SOD activity has still to be formally established; particularly because conflicting results have been obtained in vivo.

In this study, we report a link between PrPc, copper binding, and resistance to oxidative stress. Radioactive copper (64Cu) was used at a physiological concentration to demonstrate that binding of copper to the outer plasma cell membrane is related to the level of PrPc expression in a cell line expressing a doxycycline-inducible murine PrPc gene. Cellular PIPLC pretreatment indicated that PrPc was not involved in copper delivery at physiological concentrations. We also demonstrated that murine PrPc expression increases several antioxidant enzyme activities and glutathione levels. Prion protein may be a stress sensor sensitive to copper and able to initiate, following copper binding, a signal transduction process acting on the antioxidant systems to improve cell defenses.

* This work was supported by the European Community QRT-2000-02353. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Supported by le centre evian pour l’Eau. To whom correspondence should be addressed: Laboratoire de Biologie du Stress Oxydant (LBSo), Façulté de Pharmacie, Domaine de La Merici, 38706 La Tronche, France. Tel.: 33-4-76-63-74-56; Fax: 33-4-76-63-74-55; E-mail: walid.rachidi@ujf-grenoble.fr.

§ The abbreviations used are: PrPc, cellular isoform of prion protein; SOD, superoxide dismutase; dox, doxycycline; PIPLC, phosphoinositol phospholipase C; ROS, reactive oxygen species; GPX, glutathione peroxidase; GR, glutathione reductase; MDA, malondialdehyde; SIN-1, 3-morpholinosydnonimine; PrPSc, scrapie isoform of prion protein; MTT, 3-(4,5-di-methyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; TBARS, thiobarbituric acid reactants.

Received for publication, November 20, 2002
Published, JBC Papers in Press, December 23, 2002, DOI 10.1074/jbc.M211830200

Prion diseases form a group of fatal neurodegenerative disorders including Creutzfeldt-Jakob diseases, Gerstmann-Sträussler Syndrome, Kuru and Fatal Familial Insomnia in humans, and scrapie and bovine spongiform encephalopathy in animals (1). All these disorders are characterized by the occurrence of an abnormally folded isoform of the cellular prion protein PrPc, denoted PrPSc, which represents the major component of infectious prion diseases (2). The formation of PrPSc from PrPc is accompanied by profound changes in structure and biochemical properties. PrPc rich in α-helical regions is converted into a molecule with highly β-sheeted structures and partial resistance to proteolytic digestion (2, 3). The conversion of PrPc into PrPSc remains enigmatic. Biosynthesis of PrPc is necessary for PrPSc formation, as mice lacking PrPc are resistant to scrapie infection (4).

Human PrPc has 253 amino acids and is mainly expressed on neurons (5, 6). In its N-terminal region, a repeated sequence of five octapeptides can be found, which was shown to bind copper and zinc (7–9). The protein may have some superoxide dismutase-like activity and therefore a possible protective function against oxidative stress (10). Wild-type mouse brains have a significantly higher level of membrane-associated copper than PrPc-deficient mice. Treatment with phosphatidyl-inositol phospholipase C (PIPLC) specifically reduced the copper content from wild type mice but had no effect on the copper content of PrPc knockout mice (8). However, these results have not been confirmed (11). Incorporation of radioactive-labeled copper into CuZn-SOD was found to be proportional to the level of PrP expression (12). Pattison and Jebbett (13) noticed more than 30 years ago the similarity between prion histopathology to the histopathology induced by a copper chelator, cuprizone. The incidence of chronic Wasting disease (CWD), a sporadic prion disease of deer and elk, was observed to be higher in regions where the soil had a low copper content (14). Therefore, prion diseases may be related to an alteration of copper transport and a loss of copper-enzyme activities.

In a previous work, we demonstrated that neuronal cells infected with prion strains resulted in an alteration of the molecular mechanism promoting cellular resistance to ROS (15). The same alteration of antioxidant enzymes was shown in infected animals (16, 17). In the present study, we used a transfected transgenic cell line with a doxycycline-inducible murine PrPc gene to investigate the involvement of PrPc in copper metabolism and in the resistance mechanism to toxic stress.

EXPERIMENTAL PROCEDURES

Cell Culture and Construction—Murine PrPc was cloned in the pPRE plasmid (Clontech), and the resulting plasmid was transfected by the LipofectAMINE method (Invitrogen) into rabbit kidney epithelial cells (RK13) (18, 19). Stable transfectants were selected in the presence of puromycin (1 μg/ml), and one (A74) was amplified for further study. RK13 and A74 cells were grown at 37 °C in a 5% CO2-enriched atm...
cellular PrPC expression using ECL visualization. For the Western blot analysis, confluent cells were washed twice with cold phosphate-buffered saline, calcium- and magnesium-free, and lysed for 30 min at 4°C in Trition-deoxycholate lysis buffer (1× buffer is 150 mM NaCl, 0.5% Triton X-100, 0.5% sodium deoxycholate, and 50 mM Tris-HCl, pH 7.5) plus protease inhibitors. After 1 min of centrifugation at 2000×g supernatant was collected, and its protein concentration was measured by the BCA assay (Pierce). The equivalent of 20 μg of total protein in SDS loading buffer was subjected to 12% SDS-PAGE electrophoresis followed by electroblotting on polyvinylidene difluoride in Tris-glycine buffer containing 20% methanol. The membrane was blocked with 5% nonfat dry milk in TBST (0.1% Tween 20, 100 mM NaCl, 10 mM Tris-HCl, pH 7.8) for 1 h at room temperature, and MoPrP was detected by immunoblotting with P45-66 antibody previously described (20). After adding the second antibody (horseradish peroxidase-conjugated rabbit IgG), immunoreactive proteins were detected with ECL Western blot system. Quantification was achieved by densitometric scanning.

Cellular 64Cu Binding—Cells (RK13 and A74) were cultured in 35-mm Petri dishes. Culture medium was replaced by 2 ml of fresh complete medium containing different concentrations of dox (0–500 ng/ml) to stimulate murine PrP expression in A74 cells, and 1.6 μM 64Cu (CIS biointernational, Gif-sur-Yvette, France; specific activity 20 mCi/mg) to evaluate copper binding to cells as a function of the level of murine PrPC expression. Non-transfected RK13 cells were used as control and treated under the same conditions. Cells were incubated at 37°C under 5% CO2. The radioactive medium was removed after 30–40 min, 2, 4, 8, 10, 24, and 26 h. Cells were rinsed twice with 2 ml of diluted Puck’s saline A solution (Innovenetech), and harvested after addition of 1 ml of 0.25% trypsin. After harvesting, each dish was rinsed with 1 ml of Puck’s saline A solution. The final 2 ml obtained for each dish were counted for 2 min using a Packard Cobra III, mono well gamma counter (Packard Instrument Company, Meriden, CT). Protein content was assayed with the BCA protein assay reagent kit. Data were analyzed using a “self made” computer half-life calculation program, to obtain results as μCi of 64Cu incorporated or retained per mg of protein (μCi/mg protein). Each sample was assayed twice, and results were expressed as SOD units and normalized to the cell protein content.

RESULTS

Prion Expression Increases PrP64Cu Copper Binding

Characterization of Glutathione-dependent Antioxidant System—For the determination of total glutathione levels, confluent cells in 25-cm2 flasks were washed three times in phosphate-buffered saline and collected in 10 ml of isotonic, trace-element-free Tris-HCl buffer (400 mM, pH 7.3), and lysed in hypotonic Tris-HCl buffer (20 mM) by five freeze-defrost cycles. After 10 min of centrifugation at 4000 rpm, 4°C, the lysate was assayed for metalloenzyme activities and soluble protein content. Total SOD, Mn-SOD, and CuZn-SOD were determined using the pyrogallol assay following the procedure described by Marklund and Marklund (23), based on the competition between pyrogallol oxidation by superoxide radicals and superoxide dismutation by SOD, and spectrophotometrically read at 420 nm. Briefly, 50 μl of the sample were added to 1870 μl of Tris (50 mM)-DTPA (1 mM)-cadocyclic acid buffer, pH 8.3, and to 80 μl of pyrogallop (10 mM) in order to induce an absorbance change of 0.02 in the absence of SOD. The amount of SOD inhibiting the reaction rate by 50% in the given assay conditions was defined as one SOD unit. The specific CuZn-SOD inhibition by KCN (60 μl of KCN, 54 mM) added to 300 μl of lysate allowed the Mn-SOD determination under the same conditions. Each sample was assayed twice, and results were expressed as SOD units and normalized to the cell protein content.

Analysis of Glutathione-dependent Antioxidant System—For the determination of total glutathione levels, confluent cells in 25-cm2 flasks were washed three times in phosphate-buffered saline and collected in 10 ml of isotonic, trace-element-free Tris-HCl buffer (400 mM, pH 7.3), and lysed in hypotonic Tris-HCl buffer (20 mM) by five freeze-defrost cycles. Samples of whole lysate were deproteinized by adding metaphosphoric acid (5°C, 10 ml of 40% metaphosphoric acid, pH 3.5), and the supernatants were assayed for total glutathione content according to the Akerboom and Sies method (24).

The glutathione peroxidase (GPX) activity was assayed by the method of Gunzler et al. (25). GPX was measured in a coupled reaction with glutathione reductase (GR), using tert-butylhydroperoxide as substrate. Briefly, 25 μl of the sample were added to 900 μl of Tris (50 mM), EDTA, sodium azide buffer, pH 7.6 (azide was included in the assay mixture to inhibit interference of catalase) and 20 μl of glutathione (0.15 M), 20 μl of glutathione reductase (200 units/m), 20 μl of NADPH (0.4 mM) in order and incubated for 1 min for mixture equilibration. Then 20 μl of tert-butylhydroperoxide (500 μM) were added, and the decrease of the absorbance was monitored at 340 nm for 200 s. The difference in absorbance per minute was used to calculate the enzyme activity, and results were expressed as GPX units/mg of protein.

 guest based on fluorescence of thiobarbituric acid reactants measured with a stock solution of 1,1,3,3-tetraethoxypropane prepared in alcohol. The results were expressed as TBARS, μmol/g of protein.

Cell Viability Assay—Cell viability was determined by a modified MTT assay with glutathione reductase (GR), using tert-butylhydroperoxide as substrate. Briefly, 25 μl of the sample were added to 900 μl of Tris (50 mM), EDTA, sodium azide buffer, pH 7.6 (azide was included in the assay mixture to inhibit interference of catalase) and 20 μl of glutathione (0.15 M), 20 μl of glutathione reductase (200 units/m), 20 μl of NADPH (0.4 mM) in order and incubated for 1 min for mixture equilibration. Then 20 μl of tert-butylhydroperoxide (500 μM) were added, and the decrease of the absorbance was monitored at 340 nm for 200 s. The difference in absorbance per minute was used to calculate the enzyme activity, and results were expressed as GPX units/mg of protein.

RESULTS

Doxycycline-inducible Expression of Murine PrP in A74 Cells—We used the tetracycline-inducible (tet-on) system (18, 27) to achieve regulated high-level expression of the murine PrP64Cu. After transfection of several cell lines, a strong inducible expression of murine PrP64Cu was obtained in most of the clones derived from a rabbit kidney epithelial cell line (RK13). Data obtained with a representative clone (A74) are presented in this article. Expression of murine PrP64Cu was related to dox concentration in the culture medium, detectable at 10 ng/ml dox and reaching a maximum at 500 ng/ml of dox (Fig. 1). No PrP64Cu was detected in either unstimulated A74 cells (Fig. 1) or non-transfected RK13 (data not shown), confirming that ex-
expression of endogenous, rabbit PrP was undetectable in these cells (18). We also studied the induction kinetics of PrP<sup>C</sup> expression in A74 cells stimulated with 500 ng/ml dox. Expression of PrP<sup>C</sup> can be detected 8 h after induction, and a plateau was obtained at 24 h (Fig. 2). Not all, although up to 32% of cells, produced PrP<sup>C</sup> at a high level and expressed it on the outer face of the plasma membrane (Fig. 3).

**PrPC induction is doxycycline-dependent.** Dox was added at different concentrations (0, 10, 25, 50, 100, 500, and 1000 ng/ml) to medium for 24 h, and PrP<sup>C</sup> expression was determined in A74 cells by Western blot. The equivalent of 20 μg of protein (as determined with the BCA protein assay kit) were loaded to a 12% polyacrylamide gel, transferred onto polyvinylidene difluoride membrane, and PrP<sup>C</sup> was detected with antibody P45–66 raised against the N terminus of the protein. PrP<sup>C</sup> expression reaches a maximum at 500 ng/ml of dox; after this concentration we have a plateau. Specific murine PrP<sup>C</sup> bands were quantified by densitometry and plotted as a percentage of maximum signal of PrP<sup>C</sup> expression in A74 cells. Molecular mass markers are indicated on the left in kDa.

To study the influence of PrP<sup>C</sup> cleavage on copper binding, the cells were treated for 2 h with 0.2 units/ml PIPLC at 37 °C before measuring copper binding. PIPLC pretreatment dramatically decreases <sup>64</sup>Cu binding in stimulated A74 cells (500 ng/ml dox) but had no effect on unstimulated cells (Fig. 4C). However, there was a small difference in the kinetics of copper binding between stimulated cells treated with PIPLC and unstimulated cells presumably because PIPLC might not have cleaved all PrP<sup>C</sup> from membranes. PIPLC pretreatment had no effect on stimulated or unstimulated non-transfected RK13 cell controls (Fig. 4D).

**PrPC binds copper at the outer side of the cell membrane at physiological concentrations.** To investigate the location of bound copper and check for a real entry, cells were rinsed twice with 2 ml of diluted Puck’s saline A solution (Invitrogen) 24 h after PrP<sup>C</sup> induction with 500 ng/ml of dox and incubated in a radioactive medium containing <sup>64</sup>Cu. Treatment with PIPLC decreased the amount of PrP<sup>C</sup> in stimulated cells (Fig. 5A) concomitantly with a high decrease in cell-associated radioactivity (Fig. 5C). Immunoblotting demonstrated that PrP<sup>C</sup> was found only in the medium when stimulated cells (500 ng/ml dox) were treated with PIPLC (Fig. 5B). The release of PrP<sup>C</sup> was correlated with a high increase of radioactivity in the medium (Fig. 5D). These findings indicate that PrP binds copper at the outer side of the cell membrane and that cleavage of PrP<sup>C</sup> liberates copper into the medium. The difference in copper binding between stimulated and unstimulated cells was abolished after treatment with PIPLC indicating that no copper had been taken up by the PrP<sup>C</sup>. Therefore, PrP<sup>C</sup> does not transport copper inside the cell at physiological concentrations. These results are confirmed by measuring cellular copper con-
tent by electrothermal atomic absorption spectrophotometry. Copper content was increased up to 2-fold in the total but not the soluble fraction of stimulated cells (see Table II). Thus, at physiological concentrations PrPC did not transport copper from the extracellular medium to cytoplasm since there was no difference in $^{64}$Cu content between the cytosolic fractions of stimulated and unstimulated cells.

**Effect of PrPC Expression on Transition Metal Toxicity**—As induction of PrPC expression did not increase the incorporation of physiological levels of copper, we decided to investigate whether PrPC-dependent bound copper could play a role in the protection of cells toward copper toxicity. Since it was suggested that manganese can compete with copper for the binding sites (28), the resistance to this metal was also tested. The data presented in Fig. 6, A and B clearly show that cells overexpressing PrPC (1000 ng/ml dox) withstood higher copper (but not manganese doses, Fig. 6B) than unstimulated (0 dox) cells or stimulated RK13 cells. This resistance to copper was more related to stimulated cells, with an LC50 of 540 and 341 μM $\text{CuSO}_4$ when they were compared with unstimulated cells. Therefore PrP increases cellular resistance to copper but not to manganese toxicity.

**PrPC Overexpression Increases Resistance to Oxidative Stress and Antioxidant Enzyme Activities**—Because it has been suggested that one of the physiological functions of PrPC could be in the protection of cells toward an oxidative stress (10), we investigated both the resistance to an oxidative stress and the activities or levels of the main antioxidant systems in cells overexpressing PrPC. To study the relationship between prion protein expression and resistance to oxidative stress, MTT assays were performed following 3-morpholinosydnonimine (SIN-1) treatments, which generates different free radicals: $\text{O}_2^\cdot$, $\text{NO}^\cdot$, and/or $\text{NO}_x$, and other potent oxidants such as ONOO$^-$. Stimulated cells (500 ng/ml dox) presented higher resistance (cell viability, 95%) to this oxidative stress when compared with unstimulated cells (0 dox) (cell viability, 59%) or control cells (RK13 0 or 500 dox) (Fig. 7A). In contrast, cells overexpressing PrPC were surprisingly more susceptible to hydrogen peroxide than unstimulated cells. Treatment for 3 h with different concentrations of $\text{H}_2\text{O}_2$ induced a more severe decrease in viability for doses exceeding 200 μM (Fig. 7B) in stimulated cells as compared with unstimulated cells. At 500 μM $\text{H}_2\text{O}_2$-stimulated cells revealed 50% lower viability than unstimulated cells. These data demonstrate that PrPC expression decreases resistance to peroxide toxicity.

We also evaluated the involvement of PrPC expression in the cellular defense against oxidative stress by measuring different antioxidant activities such as SOD, GPX, GR, and glutathione levels. Induction of PrP increases total SOD (−21%), CuZnSOD (−27%), GR (−64%) activities, and GSH levels (−78%), while the activities of GPX and mitochondrial Mn-SOD remain unchanged (see Table I). Interestingly, Western blot detection of CuZn-SOD in A74 cells indicates that the total level of this protein was unchanged in stimulated and unstimulated cells.
This may reflect an increase in CuZn-SOD activity of stimulated cells resulting from increased copper incorporation into SOD or from SOD-like activity of PrP-Copper complexes. Finally to detect if PrP expression decreases oxidative damage, lipid peroxidation was evaluated by measuring the formation of TBARS as a stress biomarker. The basal level of oxidative damage was significantly lower in stimulated cells as compared with unstimulated and control cells. These data indicate that PrP expression increases resistance to basal as well as induced oxidative stress by increasing cellular defenses.

DISCUSSION

In this study, we used a cellular model derived from a heterologous epithelial cell line (RK13) in which the expression of murine PrP<sup>C</sup> was regulatable in a dose-dependent manner by a doxycycline treatment. Actually most epithelial cell lines we have tested, unlike, RK13, do express PrP<sup>C</sup>.<sup>2</sup> The RK13 cells were chosen because they express no detectable levels of endogenous PrP. The risk that endogenous PrP could interfere with the function of transfected PrP is therefore reduced. This may be a reason why we succeeded in a previous study to infect RK13 cells transfected with ovine PrP<sup>C</sup> (18). It was then logical to generate a clone of cells overexpressing murine PrP<sup>C</sup>, which is used for cell biology and transmission studies. RK13 cells are the only available cell lines allowing a PrP expression from zero to high levels. In the present work we used radioactive copper (<sup>64</sup>Cu) to study the effect of PrP<sup>C</sup> expression on copper binding and uptake.

There is an increasing amount of data supporting a functional role for PrP<sup>C</sup> in copper metabolism. First the N-terminal half of PrP<sup>C</sup> contains five or six highly conserved octapeptide tandem motifs of the general form PHGGGWGQ, which are capable of binding copper ions with micromolar affinity (7, 8). Indeed, PrP<sup>C</sup> isolated from hamster brain can bind a copper affinity column (29). Second, copper content of membrane-enriched brain extract from PrP<sup>−/−</sup> mice is 10–15-fold lower than in wild type controls while no significant difference was observed for other metals (8). Third, neuronal CuZn-SOD from PrP<sup>−/−</sup> mice showed decreased activity linked to decreased copper incorporation by the enzyme (10, 30). Neurons cultured from PrP<sup>−/−</sup> mice were also more sensitive to oxidative stress, perhaps because of the alteration of CuZn-SOD (10). However, the significance of copper binding on PrP<sup>C</sup> functions or the role of PrP<sup>C</sup> copper metabolism has yet to be clarified. Several hypotheses have been proposed. Copper could have a role in the conformation of the protein (31). PrP<sup>C</sup> could have a role in copper transport across the cell membrane, and this could follow different processes. Copper needs to be mobilized from its extracellular ligands (albumin and histidine), and Cu(II) is reduced to Cu(I) by unknown metalloreductases at the membrane surface prior to its delivery across the plasma membrane by the high affinity transporter CTR1 (32). These processes are still unclear. It is also possible that copper may be transported by more than one transport system, at least in some tissues. The cooperative copper-binding mode of PrP<sup>C</sup> within the physiological concentration range suggests a role in copper transport (33). So, the contribution of PrP<sup>C</sup>, if any, in copper uptake

<sup>2</sup>F. Archer and H. Laude, unpublished data.
Prion Expression Increases PrP<sup>C</sup> Copper Binding

Fig. 6. PrP<sup>C</sup> expression increases resistance to copper but not to manganese toxicity. Cell lines were incubated with the indicated concentration of copper (A) or manganese (B) for 24 h, and viability was then measured as described under “Experimental Procedures.” Results are expressed as mean percentage ± S.D. of viable cells, assuming 100% viability for untreated A74 cells. *, p < 0.01; #, p < 0.005.

by cells could be a direct transport across the plasma membrane or a binding step allowing either the mobilization of copper from its ligand or its reduction prior to its effective transport by other systems.

PrP<sup>C</sup> is normally attached to the cell membrane via a phosphatidylinositol anchor (34). It has been shown that the enzyme PIPLC releases PrP<sup>C</sup> from the cells into media (35). Our ex vivo experiments confirm the copper binding activity of the PrP<sup>C</sup> protein, because we established a correlation between copper binding and PrP<sup>C</sup> expression. This finding was further confirmed when PrP<sup>C</sup> was cleaved with PIPLC prior to <sup>64</sup>Cu labeling. However, our work does not support that PrP<sup>C</sup> could be involved in the copper transport across the membrane, as suggested by studies reporting histidine-dependent uptake of <sup>67</sup>Cu proportional to PrP<sup>C</sup> expression in cerebellar cells derived from three lines of mice expressing various amounts of PrP<sup>C</sup> (36). However, Pauly and Harris (37) have reported that copper stimulates endocytosis of both mouse PrP and chicken PrP on the cell surface of N2a mouse neuroblastoma cells via clathrin-coated pits. They suggested that PrP<sup>C</sup> could serve as a recycling receptor for the uptake of copper ions from the extracellular milieu. Also, it has been shown that 100 µM copper resulted in the rapid endocytosis of biotinylated murin PrP<sup>ɛ</sup> expressed in human neuroblastoma SH-SY5Y cells (38). In these two studies the minimum concentration of CuSO<sub>4</sub> required to produce an observable increase in PrP<sup>C</sup> internalization was ~100 µM, which is 15-fold greater than the estimated K<sub>d</sub> for binding to synthetic PrP peptides and recombinant PrP (7, 9, 39). In our work we used very low levels of copper, which we believe renders our results much closer to physiological conditions. In any case when high concentrations of copper (100 µM) are used in our cultures the intracellular copper level increased up to 2-fold in both total and soluble fractions in stimulated cells (see Table II). So, only under high copper concentrations PrP<sup>C</sup> expression increases copper uptake in the cell. However, our results clearly demonstrated that at physiological concentrations of copper, murine PrP<sup>C</sup> binds copper at the outer side of the cell membrane but also indicates that PrP<sup>C</sup> does not function as a copper transporter from the extracellular medium to the cytoplasm. This supports the hypothesis that PrP<sup>C</sup> may rather be an extracellular copper sensor (33).

Murine PrP<sup>C</sup> may serve as a copper chelating or buffering agent in the outer side of the cell membrane, and this may serve to protect cells against toxicity of free copper ions or a copper and reactive oxygen species-dependent cleavage of PrP into the octapeptide repeat region. This process may be related to the function of the molecule in the response to oxidative stress and suggests that the binding of copper is important for its processing (40).

The link between copper and PrP<sup>C</sup> may explain the mechanism of neurodegeneration in prion diseases because copper and other transition metals play an important role in the neuropathology of neurodegenerative disorders such as Parkinson’s disease (PD), Alzheimer’s disease (AD), and Amyotrophic lateral sclerosis (ALS) (41). Copper is an important component of various redox enzymes because of its ability to readily adopt two ionic states Cu(I) and Cu(II). Free copper is also a toxic ion, as exemplified by its ability to inactivate proteins through tyrosine nitration, and both deficiency and excess lead to disorders such as Menkes syndrome or Wilson’s disease (42), illustrating its physiological importance and duality in the central nervous system. In the absence of copper chelating agent on the cell surface, free copper could react with peroxides such as hydrogen peroxide produced by superoxide dismutation or directly by many enzyme catabolites such as monooamine oxidase, urate oxidase, glucose oxidase, d-amino acid oxidase, and others to form the highly reactive hydroxyl radical (·OH), which can initiate lipid peroxidation as well as protein oxidation and cause apoptosis. It has been shown that in the brain, highest concentrations of PrP<sup>C</sup> are found at synapses, and copper binding by PrP<sup>C</sup> in the synaptic cleft has a significant influence on synaptic transmission (43). Changes in electrophysiological properties such as long-term potentiation (LTP), circadian rhythm between PrP<sup>C</sup>/<sup>C</sup> and wild-type mice could be related to a disturbed copper uptake in PrP<sup>C</sup>/<sup>C</sup> mice (43). Stimulated A74 cells undergo high resistance to copper but not to manganese or cadmium toxicity when compared with unstimulated or control cells. This specific protection against copper toxicity may be due to the chelating or buffering effect of murine PrP<sup>C</sup> on the cell surface. Previously PC12 cells selected for resistance to copper toxicity and oxidative stress showed high levels of PrP<sup>C</sup> (44). Primary cerebellar granule culture derived from PrP knockout mice were significantly more susceptible to H<sub>2</sub>O<sub>2</sub> toxicity than wild type; this toxicity was related to a significant decrease in glutathione reductase activity (45). Moreover, increased oxidative damage to proteins and lipids was observed in the brain lysates from Prnp<sup>−/−</sup> as compared with wild type mice of the same genetic background (46, 47). As oxidative stress has been frequently implicated in neurodegeneration it was very interesting to test the influence of PrP expression in stimulated A74 on antioxidant enzymes activities and resistance to oxidative stress. PrP<sup>C</sup> induction in stimulated cells increases significantly CuZn-SOD, catalase, glutathione reductase activities, and glutathione levels in cells. In addition stimulated cells were more resistant to oxidative stress caused by SIN-1. This active metabolite of the vasodilatatory drug molsidomine is frequently used as a model for the continuous release of different free radicals: O<sub>2</sub><sup>•−</sup>, NO<sup>•−</sup>, and/or NO<sub>3</sub><sup>−</sup>, and other potent oxidants such as ONOO<sup>−</sup> (48). The
The relationship between PrP<sup>C</sup> and oxidative stress arose from results showing an alteration in cellular response to stress with the decrease in PrP<sup>C</sup> expression or conversion to the infectious form. PrP<sup>C</sup>/H11002 mouse brains have reduced CuZn-SOD, and cerebellar cells derived from these mice were more sensitive to oxidative stress (10); increased levels of PrPC were linked to increased levels of CuZn-SOD activity because of an increase in copper incorporation (12). In our model, we believe that increased CuZn-SOD activity in stimulated cells is due to SOD-like activity of PrP-Cu complexes in the outer side of the cell membrane. Indeed, we detected no change in the protein levels of CuZn-SOD in stimulated and unstimulated cells (Fig. 8), and

**Table I**

| Parameter       | A74 0 dox | A74 500 dox | RK13 500 dox |
|-----------------|-----------|-------------|--------------|
| Total SOD       | 4.5 ± 0.65| 5.445 ± 0.55<sup>*</sup> | 4.2 ± 0.67   |
| CuZn-SOD        | 2.7 ± 0.25| 3.445 ± 0.46<sup>*</sup> | 2.42 ± 0.38  |
| Mn-SOD          | 1.8 ± 0.08| 2 ± 0.095    | 1.78 ± 0.085 |
| Catalase        | 7.45 ± 0.63| 8.75 ± 0.96<sup>3</sup> | 7.29 ± 0.42  |
| GPX             | 45.6 ± 3.4 | 49 ± 5.7    | 43 ± 5.9     |
| GSH             | 64.59 ± 6.29| 114.7 ± 12.16<sup>*</sup> | 70.45 ± 11.8 |
| GR              | 30.86 ± 2.7 | 50.58 ± 1.5<sup>*</sup> | 32.2 ± 3.8   |
| MDA             | 64.3 ± 10.5 | 40.7 ± 7.4<sup>*</sup> | 59.4 ± 5.8   |

**Fig. 7.** PrP<sup>C</sup> expression increases resistance to oxidative stress produced by SIN-1 but not to H<sub>2</sub>O<sub>2</sub> toxicity. Cell viability was evaluated by a modified MTT assay as described under "Experimental Procedures" in stimulated and unstimulated A74 and non-transfected RK13 cells after 24 h of exposure to 1 mM SIN-1 (A) or after 3 h of exposure to different concentrations of H<sub>2</sub>O<sub>2</sub> (B). Results are expressed as mean percentage ± S.D. of survival cell; survival was calculated as the percentage of the staining values of untreated cultures. * , p < 0.01; #, p < 0.005.
Prion Expression Increases PrP<sup>C</sup> Copper Binding

**TABLE II**

| Prion Expression Increases PrP<sup>C</sup> Copper Binding |
|--------------------------------------------------------|
| **Cells Whole cells Soluble fraction**                  |
| 0 Dox 8.3 ± 1.4                                         |
| 500 Dox 15.5 ± 2.9                                      |
| 0 Dox + 100 μM Cu 467.2 ± 18                           |
| 500 Dox + 100 μM Cu 1087 ± 28+                          |

**REFERENCES**

1. Parchi, P., and Gambetti, P. (1996) *Curr. Opin. Neurobiol.* 6, 286–289.
2. Prusiner, S. B. (1998) *Proc. Natl. Acad. Sci. U. S. A.* 95, 13363–13368.
3. Horiuchi, M., and Caughey, B. (1999) *Structure Fold Des.* 7, R231–R40.
4. Bueler, H., Aguzzi, A., Sailer, A., Greiner, R. A., Autenried, P., Aguet, M., and Prusiner, B. S. (1993) *Cell* 73, 1359–1347.
5. Kretzschmar, H. A., Storryn, L. E., Westaway, D., Stubbelie, H. W., Prusiner, S. B., and Deanmond, S. J. (1998) *J. Am. Pathol.* 152, 18–27.
6. Hornshaw, M. P., McDermott, J. R., and Candy, J. M. (1995) *Biochem. Biophys. Res. Commun.* 207, 621–629.
7. Brown, D. R., Qin, K., Herma, J. W., Madlagn, A., Munsom, J., Strone, R., Fraser, P. E., Kruck, T., van Bohlen, A., Schultz-Schaffner, W., Giese, A., Westaway, D., and Kretzschmar, H. (1997) *Nature* 389, 654–687.
8. Stockel, J., Saar, J., Wallace, A. C., Cohen, F. E., and Prusiner, S. B. (1998) *Biochemistry* 37, 7185–7193.
9. Brown, D. R., Schultz-Schaffner, W., Schmidt, B., and Kretzschmar, H. A. (1997) *Exp. Neurol.* 146, 104–112.
10. Waggoner, D. J., Drisaldi, B., Bartschka, T., Casarone, R. L., Prohaska, J. R., Gittin, J. D., and Harris, D. A. (2000) *J. Biol. Chem.* 275, 7455–7458.
11. Brown, D. R., and Besinger, A. (1998) *Biochem. J.* 334, 423–429.
12. Pattinson, I. H., and Jeabetti, J. N. (1971) *Nature* 230, 110–117.
13. Purdy, M. (2000) *Med. Hypotheses* 54, 278–206.
14. Milhavet, O., McMahan, H. E., Rachid, W., Nishida, N., Katamine, S., Mange, A., Arletto, M., Casanova, D., Rondell, J., Favier, A., and Lehmann, S. (2000) *Proc. Natl. Acad. Sci. U. S. A.* 97, 13937–13942.
15. Lee, D. W., Sothern, H. O., Lim, M. B., Le, Y. G., Kim, Y. S., Carpenter, R. I., and Favier, A. (1999) *Free Radic. Biol. Med.* 30, 499–507.
16. Choi, S. I., Ju, W. K., Choi, E. K., Kim, J., Lee, H. Z., Carpenter, R. I., Wisniewski, H. M., and Kim, Y. S. (1999) *Acta Neuropathol. (Berl)* 96, 279–286.
17. Vilette, D., Andreolletti, O., Archer, F., Madeleine, M. F., Vilotte, J. L., Lehm, M., and Laude, H. (2001) *Proc. Natl. Acad. Sci. U. S. A.* 98, 4055–4059.
18. Christofinis, G. J., and Beale, A. J. (1968) *J. Pathol. Bacteriol.* 95, 377–381.
19. Lehmann, S., and Harris, D. A. (1995) *J. Biol. Chem.* 270, 24589–24597.
20. Hansen, M. B., Nielsen, S. E., and Berg, K. (1989) *J. Immunol. Methods* 119, 203–210.
21. Richard, M. J., Portal, B., Meo, J., Coudray, C., Hadijan, A., and Favier, A. (1992) *Clin. Chem.* 38, 704–709.
22. Marklund, S., and Marklund, G. (1974) *Methods Enzymol.* 62, 70–72.
23. Marklund, S., and Marklund, G. (1974) *Methods Enzymol.* 62, 70–72.
24. Akerboom, T. P., and Sies, H. (1984) *Methods Enzymol.* 77, 373–382.
25. Gonzalez, W. A., Kremers, M., and Flohe, L. (1974) *Z. Klin. Chem. Klin. Biochem.* 12, 444–448.
26. Sponagel, R. J., Delides, A., and Goldberg, D. M. (1981) *Biochem. Med.* 26, 238–248.
27. Gossen, M., and Bujard, H. (1992) *Proc. Natl. Acad. Sci. U. S. A.* 89, 5547–5551.
28. Brown, D. R., Hafir, F., Glasssmith, L. L., Wong, B. S., Jones, I. M., Clive, C., and Haswell, S. J. (2000) *EMBO J.* 19, 1180–1186.
29. Pan, K. M., Stahl, N., and Prusiner, S. B. (1992) *Protein Sci.* 1, 1343–1352.
30. Brown, D. R., Beng, B. R., Hafir, F., Clive, C., Haswell, R. J., and Jones, I. M. (1999) *Biochemistry* 34, 1–5.
31. Badworth, J. B., Hill, A. R., Joiner, S., Jackson, G. S., Clarke, A. R., and Collinge, J. (1999) *Nat. Cell Biol.* 1, 55–59.
32. Lee, J., Pea, B. M., Nose, Y., and Thiele, D. E. (2002) *J. Biol. Chem.* 277, 4380–4387.
33. Kramer, M. L., Kratzen, H. D., Schmidt, B., Rom, A., Wind, O., Leimann, S., Horne, S., and Kretzschmar, H. (2001) *J. Biol. Chem.* 276, 2388–2390.
34. Stahl, N., Borchelt, D. R., and Prusiner, S. B. (1997) *Cell* 88, 229–240.
35. Stahl, N., Borchelt, D. R., and Prusiner, S. B. (1998) *Biochemistry* 29, 5405–5412.
36. Brown, D. R. (1999) *J. Neurosci. Res.* 58, 717–725.
37. Paul, A. C., and Harris, D. A. (1998) *J. Biol. Chem.* 273, 33107–33110.
38. Perera, W. S., and Hooper, N. M. (2001) *Curr. Biol.* 11, 519–523.
39. Hornshaw, M. P., McDermott, J. R., Candy, J. M., and Lakey, J. H. (1995) *Biochem. Biophys. Res. Commun.* 214, 995–999.
40. McMahan, H. E., Mange, A., Nishida, N., Creminion, C., Casanova, D., and Lehmann, S. (2001) *J. Biol. Chem.* 276, 2286–2291.
41. Sayre, L. M., Perry, G., and Smith, M. A. (1999) *Curr. Opin. Chem. Biol.* 3, 229–235.
42. Mercer, J. F. (2001) *Trends Mol. Med.* 7, 64–69.
43. Kretzschmar, H. A., Tings, T., Madlagn, A., Giese, A., and Herms, J. (2000) *Arch. Viral. Suppl.* 15, 239–249.
44. Brown, D. R., Schmidt, B., and Kretzschmar, H. A. (1997) *Int. J. Dev. Neurosci.* 15, 961–972.
45. White, A. R., Collins, S. J., Maher, F., Jobling, M. F., Stewart, L. R., Thyer, J. M., Beyreuther, K., Masters, C. L., and Cappai, R. (1999) Am. J. Pathol. 155, 1723–1730
46. Wong, B. S., Liu, T., Li, R., Pan, T., Petersen, R. B., Smith, M. A., Gambetti, P., Perry, G., Manson, J. C., Brown, D. R., and Sy, M. S. (2001) J. Neurochem. 76, 565–572
47. bio Klamt, F., Dal-Pizzol, F., Conte da Frota, M. L., Walz, R., Andrades, M. E., da Silva, E. G., Brentani, R. R., n Izquierdo, I., and Fonseca Moreira, J. C. (2001) Free Radic. Biol. Med. 30, 1137–1144
48. Gergel, D., Misik, V., Ondrias, K., and Cederbaum, A. I. (1995) J. Biol. Chem. 270, 20922–20929
49. Radi, R., Beckman, J. S., Bush, K. M., and Freeman, B. A. (1991) J. Biol. Chem. 266, 4244–4250
50. Radi, R., Beckman, J. S., Bush, K. M., and Freeman, B. A. (1991) Arch Biochem. Biophys. 288, 481–487
51. Beckman, J. S., Ischiropoulos, H., Zhu, L., van der Woerd, M., Smith, C., Chen, J., Harrison, J., Martin, J. C., and Tsai, M. (1992) Arch. Biochem. Biophys. 298, 438–445
52. Brito, C., Naviliat, M., Tiscornia, A. C., Vuillier, F., Gualco, G., Dighiero, G., Radi, R., and Cayota, A. M. (1999) J. Immunol. 162, 3356–3366
53. Mouillet-Richard, S., Ermonval, M., Chebassier, C., Laplanche, J. L., Lehmann, S., Lannay, J. M., and Kellermann, O. (2000) Science 289, 1925–1928
54. Wong, B. S., Brown, D. R., Pan, T., Whiteman, M., Liu, T., Bu, X., Li, R., Gambetti, P., Olesik, J., Rubenstein, R., and Sy, M. S. (2001) J. Neurochem. 79, 689–698

Prion Expression Increases PrP C Copper Binding
Expression of Prion Protein Increases Cellular Copper Binding and Antioxidant Enzyme Activities but Not Copper Delivery
Walid Rachidi, Didier Vilette, Pascale Guiraud, Marie Arlotto, Jacqueline Riondel, Hubert Laude, Sylvain Lehmann and Alain Favier

J. Biol. Chem. 2003, 278:9064-9072.
doi: 10.1074/jbc.M211830200 originally published online December 23, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M211830200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 54 references, 17 of which can be accessed free at http://www.jbc.org/content/278/11/9064.full.html#ref-list-1