The octarepeat region of prion protein, but not the TM1 domain, is important for the antioxidant effect of prion protein

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ABSTRACT

The cellular prion protein (PrP^c) plays a crucial role in the pathogenesis of prion diseases, but its physiological function is far from understood. Several candidate functions have been proposed including binding and internalization of metal ions, a superoxide dismutase-like activity, regulation of cellular antioxidant activities, and signal transduction. The transmembrane (TM1) region of PrP^c (residues 110–135) is particularly interesting because of its very high evolutionary conservation. We investigated a possible role of TM1 in the antioxidant defense, by assessing the impact of overexpressing wt-PrP or deletion mutants in N₂A mouse neuroblastoma cells on intracellular reactive oxygen species (ROS) levels. Under conditions of oxidative stress, intracellular ROS levels were significantly lowered in cells overexpressing either wild-type PrP^c (wt-PrP) or a deletion mutant affecting TM1 (Δ 8TM1-PrP), but, as expected, not in cultures overexpressing a deletion mutant lacking the octapeptide region (Δ octa-PrP). Overexpression of wt-PrP, Δ 8TM1-PrP, or Δ octa-PrP did not affect basal ROS levels. Interestingly, the mitochondrial membrane potential was significantly lowered in Δ octa-PrP-transfected cultures in the absence of oxidative stress. We conclude that the protective effect of PrP^c against oxidative stress involves the octarepeat region but not the TM1 domain nor the high-affinity copper binding site described for human residues His96/His111.

Keywords:

Antioxidant
Prion protein
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Introduction

Transmissible spongiform encephalopathies (TSEs), also termed prion diseases, are neurodegenerative lethal diseases occurring in various mammalian species [1,2]. TSEs are characterized by spongiform change, neuronal death, astrogliosis, and accumulation of a disease-associated, pathogenic isoform of prion protein (PrP), termed PrP^{Sc}, which arises from a normal cellular isoform, termed PrP^c, in a posttranslational process involving refolding [3]. PrP^c is encoded by the *PRNP* gene on human chromosome 20 and mouse chromosome 2 [4,5]. This gene is highly conserved during evolution [6–8]. The PrP^c protein contains a long flexible amino terminal tail (amino acids [aa] 23–128) followed by three α -helices and two β -sheets [9]. PrP^c contains an octarepeat region (amino acids 51–91) and a highly hydrophobic region also called transmembrane domain 1 (TM1) (aa

110–135). PrP^c can be glycosylated on Asn180 and Asn196 and comprises a disulfide bridge between Cys178 and Cys213. PrP^c is a glycosyl-phosphatidyl-inositol (GPI)-linked glycoprotein and is enriched in detergent-resistant membranes. The very high degree of conservation of the TM1 region is indicative of an essential contribution to the physiological function of PrP^c. This region extends from aa 110 through 135 and comprises an array of hydrophobic amino acids. It is also a highly flexible and unstructured portion of PrP^c as shown by NMR analysis of recombinant PrP [9].

Previously we have shown that expression of a deletion mutant of 8 aa in the TM1 domain (PrP Δ 114–121) is sufficient to inhibit PrP^{Sc} accumulation in prion-infected neuroblastoma cells in a *trans*-dominant fashion [10]. Recently, we generated transgenic mice expressing the same deletion mutant [11]. These mice (termed PrP Δ _{PHC} in Ref. [11]) did not show any obvious spontaneous phenotype. However, crossing PrP Δ _{PHC} mice on a *Prnp*^{0/0} background with transgenic mice carrying pathogenic PrP Δ 94–134 or PrP Δ 32–134 transgenes (“Shmerling mice”) led to enhanced pathogenicity of the PrP Δ 94–134 transgene whereas pathogenicity of PrP Δ 32–134 was diminished [11].

Apart from the above effects concerning prion propagation and modulation of the “Shmerling syndrome,” a variety of other putative functions have been proposed for PrP^c. These include immunoregulation [12,13], signal transduction [12,14–16], binding of copper and other metals and their sequestration [17–20], synaptic transmission

Abbreviations: $\Delta\Psi$, mitochondrial membrane potential; BSO, buthionine sulfoximine; DMEM, Dulbecco's modified Eagle's medium; FACS, fluorescence-activated cell sorting; FCS, fetal calf serum; GPI, glycosyl-phosphatidyl-inositol; H₂DCFDA, 2',7'-dichlorodihydrofluorescein diacetate; N₂A, mouse neuroblastoma cells; PBS, phosphate-buffered saline; PrP, prion protein; ROS, reactive oxygen species; RT, room temperature; SOD, superoxide dismutase; tBOOH, *tert*-butylhydroperoxide; TM, transmembrane; TMRE, tetramethylrhodamine ethyl ester perchlorate; TSE, transmissible spongiform encephalopathies.

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[21], induction of or protection against apoptosis [22–24], superoxide dismutase (SOD)-like activity [25–27], and regulation of cellular antioxidant activities [28,29]. The ability of PrP^c to bind copper via its octapeptide region and via two independent histidine residues (human residues His96 and His111 [corresponding to murine residues 95 and 110]) [30,31] might play an important role in PrP^c function(s). PrP^c could act as a sensor for oxidative stress [29,32–34], and trigger intracellular anti-stress signaling events. As to the proposed SOD-like activity of PrP^c [25,26,35], it should be noted that other groups either have been unable to confirm this [27] or have proposed some indirect mechanism [36]. Interestingly recent work on stroke models has associated PrP^c with a protective function [37–41], which may well be related with the above-noted antioxidant activity.

Intriguingly there exists a clear link between transition-metal binding and neurodegenerative diseases, including prion diseases [42]. In some paradigms transition metal binding seems to protect against oxidation, in others to enhance it by disturbing free radical homeostasis [43,44].

The mitochondrial membrane potential ($\Delta\Psi$) arises from proton translocation from the mitochondrial matrix to the mitochondrial intermembrane space through the electron transport chain. $\Delta\Psi$ reflects the mitochondrial activity of the cell. The major mitochondrial activity is, of course, ATP production, but mitochondria can also generate ROS by occasional escape of electrons from the respiratory chain. Hyperpolarization of the inner mitochondrial membrane (i.e., increased $\Delta\Psi$) is known to facilitate ROS production, which in turn induces a decrease of $\Delta\Psi$ via the induction of uncoupling protein 2 (UCP2). Uncoupling proteins allow passage of protons through the inner mitochondrial membrane without ATP production, which leads to both deficiency in ATP formation and immediate lowering of mitochondrial production of ROS. The biological importance of this mechanism is highlighted by the fact that persistent overproduction of ROS will lead to calcium influx and cytochrome *c* release into the cytoplasm, thus triggering apoptosis [45,46]. Based on this knowledge it was interesting to study if the antioxidant effect of PrP^c is mediated by modulation of $\Delta\Psi$ and if TM1 plays a role in such an effect. It should be noted that previous publications from the literature have reported conflicting results as to the role of PrP^c on mitochondrial respiratory function [47–49] and therefore the situation was unclear.

The aim of the present study was to investigate a possible role of the highly conserved TM1 domain of PrP^c (i) in the cellular response to exogenous oxidative stress, (ii) in controlling endogenous ROS levels, and (iii) in modulation of mitochondrial membrane potential ($\Delta\Psi$). We chose N₂A mouse neuroblastoma cells as an experimental system, as these cells have extensively been used in research on PrP^c and on prions. We transfected N₂A cells with expression constructs either encoding wild-type (wt) mouse PrP^c or with the deletion mutant PrP Δ 114–121, carrying an 8-aa deletion in the TM1 region of PrP [10]. In some of the experiments an expression plasmid encoding a PrP version lacking the octapeptide region was also used.

In order to assess cellular ROS levels we used 2',7'-dichlorofluorescein diacetate (H₂DCFDA) as a probe for quantitative analysis by flow cytometry (FACS) and tetramethylrhodamine ethyl ester perchlorate (TMRE) in order to monitor changes in $\Delta\Psi$.

Experimental procedures

cDNA constructs

The expression plasmid pL15TK [50] (empty vector, used for mock transfections) was the basis for creating pCMV-wtPrP [10] (henceforth termed wt-PrP) and pCMV- Δ 114–121PrP [10] (henceforth termed Δ TM1-PrP). Plasmid pCDNA3.1 Δ 43–91 (henceforth termed Docta-PrP) was described before [51]. Plasmids pCR3-Thy1 and pEGFP were used as controls.

Cell culture

The murine neuroblastoma cell line N₂A and its subclones (H6, H12, D11, G9, and F1) were cultured as described [52]. The human cervical carcinoma cell line HeLa was cultured in DMEM (Gibco, Karlsruhe, Germany), 10% FCS (Biochrom, Berlin, Germany), 1% L-glutamine (Gibco), 1% penicillin/streptomycin (Gibco). A Casy counter Model TT (Schärfe System, Reutlingen, Germany) was used for automated determination of cell numbers and cell viability.

Transfection

N₂A cells were transiently transfected using JetPEI reagent (Qbiogene, Illkirch, France) according to the manufacturer's instructions. Briefly N₂A were seeded in 24-well plates. The next day, transfections were performed. One microgram of DNA dissolved in 50 μ l of 150 mM NaCl was combined with 2 μ l JetPEI reagent prediluted in 50 μ l of 150 mM NaCl. The mixture was incubated 30 min at RT and then added onto cells dropwise.

Endogenous ROS and reaction to oxidative stress monitoring by FACS analysis

The use of H₂DCFDA (Molecular Probes, Eugene, OR) as ROS staining and modified staining methods have been described by Sohn et al. [53] and Sauer et al. [54]. In order to set up the system, H₂O₂ (Merck, Darmstadt, Germany), *tert*-butylhydroperoxide (tBOOH) (Fluka, Munich, Germany), or the GSH-depleting compound buthionine sulfoximine (BSO) (Sigma, Munich, Germany) was applied, as indicated, to increase intracellular ROS levels. In subsequent experiments addressing the effect of transfected PrP versions, only H₂O₂ was used for the induction of oxidative stress. Briefly, 1 day posttransfection, medium was exchanged and cells were exposed or not to 100 μ M copper chloride hydrate (CuCl₂, Sigma) for 1 day. Then cells were washed with PBS 1 $\times$ and fresh medium was added. Cells were then treated with 15 μ M 2',7'-dichlorofluorescein diacetate (Molecular Probes) in the presence or in absence of 3 mM hydrogen peroxide (H₂O₂, Merck, Darmstadt, Germany) for 30 min. The cells were washed with PBS and trypsinized. Then 100 μ l FACS buffer was added and FACScan analysis was performed using a FACScan flow (Beckton Dickinson Biosciences, Mountain view, CA) equipped with Cell Quest version 3.3 software. Data analysis was performed using FlowJo 6.0 program (TreeStar, Asland, OR).

Mitochondrial membrane potential monitoring by FACS analysis

The use of TMRE (Sigma) as a probe for mitochondrial membrane potential has been described by Nicholls and Ward [55] and Scaduto et al. [56]. Briefly, 1 day posttransfection, medium was exchanged and cells were exposed or not to 100 μ M copper chloride hydrate (CuCl₂, Sigma) for 1 day. Then cells were washed with PBS 1 $\times$ and fresh medium was added. Cells were treated with 4 μ M TMRE for 15 min. Cells were washed with PBS, trypsinized, and resuspended in 100 μ l FACS buffer. Then FACScan analysis was performed.

Statistics and normalization

Each individual experiment was done at least in duplicates, and the mean values yielded the respective experimental data point of the day, to be used for further statistical analysis of the experiments performed independently on different days.

Crude data of transfected N₂A were normalized as follows: The mean values from all untreated mock samples were set to 100%. All other data were expressed as percentage compared to the mock value of the same day. Student's *t* test was performed on FACS results by using Analyse-It software (Analyse-it Software Ltd., Leeds, UK). *P* < 0.05 was considered statistically significant.

Results

In order to investigate the contribution of the TM1 domain of PrP^C to the physiological role(s) of this protein, N₂A cells were transfected with expression constructs for wt-PrP or Δ 8TM1-PrP. The empty vector plasmid served as a control. In the transfected cultures, intracellular ROS levels in response to oxidative stress; endogenous ROS levels; and mitochondrial membrane potential ($\Delta\Psi$) were assessed.

Endogenous ROS level after oxidant treatment of N₂A, N₂A subclones, and HeLa cells

H₂DCFDA, which permits monitoring of the ROS level, was used as a probe, and its fluorescent intensity was measured by flow cytometry. In order to set up the system, gating of live cells and staining of unstressed cells with H₂DCFDA was performed (Fig. 1A). In addition, ROS levels in untransfected cells were measured with or without prior addition of H₂O₂, *tert*-butylhydroperoxide, or the GSH-depleting

Fig. 1. Endogenous ROS and oxidative stress response to various compounds in N₂A cells. (A) FACS setting for measurements of ROS level in N₂A cells in response to oxidative stress. In order to assess endogenous reactive oxygen species H₂DCFDA was used (15 μM, 30 min) and the intensity of the resulting fluorescence was measured by FACS as described under Materials and methods; (a) gating of live cells; (b) cells without fluorescence probe, representing background; (c) cells stained with H₂DCFDA (FL-1) in the presence of H₂O₂; (d) Overlay of b and c. (B) Intracellular ROS levels were assessed in the presence of H₂O₂ (3 mM, 30 min), *tert*-butylhydroperoxide (tBOOH) or buthionine sulfoximine (BSO) added directly to the medium in the presence of H₂DCFDA. All experiments were done in duplicate. In each case a representative set of FACS data is shown; (a) addition of H₂O₂; (b) addition of tBOOH; (c) addition of BSO. Note that double peaks are present in all cases.

compound buthionine sulfoximine. In all three cases a similar shift to the right (i.e., increase of ROS) was observed (Fig. 1B), as expected, thus validating our readout system. All subsequent experiments were done with H_2O_2 .

Further analyses on randomly picked subclones of N_2A [56] as well as on HeLa cells (human cervical carcinoma) revealed that the double peak observed in Fig. 1B in the presence of H_2DCFDA and oxidants is due to some heterogeneity in the N_2A cell population. While HeLa cells showed a single peak (Fig. 2A), N_2A subclones (Figs. 2B–F) showed varying patterns. For the subsequent experiments N_2A mass cultures were used.

Fig. 2. Background and oxidative stress-induced DCF fluorescence in different cell populations. For each cells type a representative set of FACS data is shown. (A) HeLa; (B) N_2A subclone H12; (C) N_2A subclone D11; (D) N_2A subclone H6; (E) N_2A subclone F1; (F) N_2A subclone G9. Note the different patterns in the various cell populations.

Impact of overexpression of wt-PrP or $\Delta 8TM1$ -PrP on ROS levels in N_2A cells exposed to exogenous hydrogen peroxide

As we performed transient transfections assays, a green fluorescent protein (GFP) construct was used to assess transfection efficiency. Routinely, about 60% of cells were GFP positive (data not shown). In addition, Western blots revealed very similar amounts of the various overexpressed PrP versions in transfected cells (Fig. S1).

In order to compare the impact of $\Delta 8TM1$ -PrP with wt-PrP on intracellular ROS levels under conditions of oxidative stress, cells were transfected with the respective PrP constructs and analyzed for ROS levels 24 h later. ROS levels were measured after exposure of the cells to H_2O_2 added directly to the medium in the presence of H_2DCFDA . Our data showed that ROS levels were significantly lower both in wt-PrP and in $\Delta 8TM1$ -PrP-overexpressing cells compared to mock transfection (Figs. 3A and B), which indicates that both PrP versions exerted an antioxidant effect.

One possible explanation for such antioxidant effects could be a SOD-like activity of PrP or some change in upstream events controlling ROS levels. We therefore used a colorimetric test based on competition and xanthine/xanthine oxidase in N_2A cells overexpressing wt-PrP or $\Delta 8TM1$ -PrP. No difference, however, was observed among mock, wt-PrP, and $\Delta 8TM1$ -PrP-transfected cells in this system (data not shown), thus pointing to the relevance of other mechanisms.

Effect of copper treatment after oxidative stress in N_2A cells overexpressing wt-PrP, $\Delta 8TM1$ -Pr, or $\Delta octa$ -PrP

Because PrP^C can bind copper it was interesting to investigate the possible influence of copper on the oxidative stress response in PrP^C-overexpressing N_2A cells. Transfected N_2A cells were treated with copper (100 μM $CuCl_2$) for 1 day followed by washing, fluorescent staining with H_2DCFDA , H_2O_2 exposure, and FACS analysis. The experiments were repeated independently and the results are expressed as a percentage of DCF intensity for mock-transfected cells in the absence of copper treatment (Fig. 3C). The data show that, once again, the ROS level present in wt-PrP and $\Delta 8TM1$ -PrP-overexpressing cells is significantly reduced compared to mock-transfected cells ($P=0.003$ and $P=0.04$, respectively). By contrast, cells overexpressing $\Delta octa$ -PrP did not show any change in ROS levels under these conditions.

After copper treatment the ROS level in mock-transfected cells showed only a tendency toward increase ($P=0.058$). In wt-PrP and $\Delta 8TM1$ -PrP-overexpressing cells, however, copper treatment induced a significant increase of ROS ($P=0.046$ for wt-PrP compared to mock; $P=0.012$ for the comparison between wt-PrP with and without copper treatment; $P=0.021$ for the comparison between $\Delta 8TM1$ -PrP with and without copper treatment). By contrast, copper treatment had no significant effect on $\Delta octa$ -PrP-overexpressing cells.

Effect of copper treatment on endogenous ROS levels in N_2A cells overexpressing wt-PrP, $\Delta 8TM1$ -PrP, or $\Delta octa$ -PrP

In order to investigate the possible influence of overexpressing the above PrP versions on endogenous ROS in N_2A cells with or without copper treatment, the same experiments were performed in the absence of exogenous H_2O_2 . Here, no statistically significant differences could be observed (Fig. 4B).

Effect of copper treatment on mitochondrial membrane potential in N_2A cells overexpressing wt-PrP, $\Delta 8TM1$ -PrP, or $\Delta octa$ -PrP

We also investigated the influence of copper on $\Delta \Psi$ in N_2A cells overexpressing various PrP versions. Transfected N_2A cells were treated with copper for 1 day, followed by washing, fluorescent staining with TMRE, and FACS analysis. Our data revealed that copper treatment induced a significant decrease of $\Delta \Psi$ in mock, wt-PrP,

Fig. 3. DCF fluorescence on oxidative stress induction in N₂A cells overexpressing wt-PrP, Δ 8TM1-PrP, or Δ octa-PrP. (A) Cells were transfected and on the next day intracellular ROS levels were assessed after treatment of the cells with H₂O₂ (3 mM 30 min) added directly to the medium in the presence of H₂DCFDA (15 μM 30 min). Representative FACS data for mock-transfected and wt-PrP or Δ 8TM1-PrP-overexpressing cultures are shown. (B) Summary of 6 independent experiments on the oxidative stress response in transfected N₂A cultures. Each experiment was done in duplicate as described in A and their mean values were taken as the relevant data points from the respective experiment. Statistical significance was tested by using Student's *t* test; asterisk denotes $P < 0.05$. (C) Effect of copper treatment on cellular ROS after oxidative stress in N₂A cultures overexpressing various PrP versions. N₂A cells were plated on Day 0. On Day 1, cells were transfected with empty vector, wt-PrP, Δ 8TM1-PrP, or Δ octa-PrP, respectively. Medium was changed on Day 2, and 100 μM CuCl₂ was added or not in fresh medium. On Day 3, cells were washed with PBS, fresh medium containing H₂O₂ was added, and fluorescent staining with H₂DCFDA was performed. Results are expressed in percentage of DCF intensity for mock-transfected cells in absence of copper treatment. Note that wt-PrP and Δ 8TM1-PrP-overexpressing cultures once again show a significant decrease of ROS after H₂O₂ treatment, whereas Δ octa-PrP-overexpressing cells do not show any significant difference compared to mock-transfected cultures. Copper treatment preceding the H₂O₂ treatment induces a significant increase of the ROS level in wt-PrP and Δ 8TM1-PrP-overexpressing cultures but not in Δ octa-PrP-overexpressing cultures. Student's *t* test was used to determine statistically significant differences. * denotes significant difference ($P < 0.05$) to untreated mock cultures; @ denotes a near-significant difference between untreated and copper-treated mock cultures; # denotes significant difference between the absence and presence of copper treatment in wt-PrP-transfected cultures; § denotes significant difference between the absence and presence of copper treatment in Δ 8TM1-PrP-transfected cultures. The number of independent experiments is indicated in brackets.

Fig. 4. Lack of effect of overexpressing various PrP versions on intracellular ROS levels in N₂A cultures with or without copper treatment. (A) FACS setting for measurements of endogenous ROS level in N₂A cells; (a) gating of live cells; (b) cells without fluorescence probe, representing background; (c) cells stained with H₂DCFDA (FL-1); (d) overlay of b and c. (B) N₂A cells were plated on Day 0. On Day 1, cells were transfected with empty vector, wt-PrP, Δ 8TM1-PrP, or Δ octa-PrP, respectively. Medium was changed on Day 2, and 100 μ M CuCl₂ was added in fresh medium. On Day 3, cells were washed with PBS, fresh medium was added, and fluorescent staining with H₂DCFDA was performed in the absence of any oxidant treatment. FACS analysis was performed as described under Materials and methods. No significant difference is observable by Student's *t* test. The number of experiments is indicated in brackets.

Δ 8TM1-PrP, and Δ octa-PrP-transfected cells ($P=0.028$ for mock, $P=0.041$ for wt-PrP, $P=0.036$ for Δ 8TM1-PrP, and $P=0.027$ for Δ octa-PrP compared to mock without copper treatment; Fig. 5B). A possible explanation for this reduction of $\Delta\Psi$ could be copper toxicity. However, neither microscopic inspection nor automated viability testing (Fig. 6) revealed any difference between any of the transfected cultures in the presence or absence of copper.

Interestingly Δ octa-PrP-overexpressing cells showed a $\Delta\Psi$ pattern different from that of mock-transfected cells, in that Δ octa-PrP overexpression significantly decreased $\Delta\Psi$ even without copper treatment compared to mock-transfected cells ($P=0.029$).

In order to exclude any nonspecific effects due to overexpression of proteins carrying a GPI anchor, N₂A cells were transfected with a Thy-1 expression construct. Thy-1 is a GPI-anchored protein commonly used as a control for GPI-anchorage effects. Experiments to assess $\Delta\Psi$, endogenous ROS, and the reaction to oxidative stress experiments did not show any difference between mock and Thy-1-transfected cells (data not shown). Therefore the effects observed with wt-PrP, Δ 8TM1-PrP and Δ octa-PrP apparently cannot be interpreted as a nonspecific consequence of overexpressing GPI-anchored proteins.

Discussion

In the present work, we studied the effects a partial deletion of the highly conserved transmembrane-1 (TM1) domain of PrP^C on the

antioxidant effect of the protein. Our experimental strategy was to perform transient transfection assays in mouse N₂A neuroblastoma cells to study the effects of overexpression of the deletion mutant Δ 8TM1-PrP, in comparison with wild-type PrP^C, on intracellular ROS levels. We did this under both basal and oxidative stress conditions. Since PrP^C can bind copper ions and this transition metal plays important roles both in oxidative stress induction and in ROS detoxification, the experiments were performed with or without pretreatment with exogenously added copper, respectively. For the same reason we also included in some of the experiments Δ octa-PrP, a mutant with a deletion of the octarepeat region, where much of the copper binding occurs. Finally we monitored $\Delta\Psi$ in the transfected cultures in the presence or absence of copper.

We opted for using *transient* transfections rather than establishing stably transfected cells, which has a major advantage of avoiding the risk of artifacts based on clonal effects. On the other hand only a proportion of the cells in the transfected culture are reached, in our case about 60% as assessed with a GFP expression plasmid. As revealed by Western blots, the overall level of expression of the transfected PrP versions was comparable (Fig. S1). It is important to note that expression of the transfected gene only in about two-thirds of the cell population only will, of course, diminish the apparent biological effect of the transgene (i.e., inhibition of ROS formation) in the total population of cells analyzed by flow cytometry. This is reflected by the rather low amplitude of the statistically significant effects described in this paper.

Fig. 5. Effect of copper treatment on mitochondrial membrane potential ($\Delta\Psi$) in N₂A cultures overexpressing various PrP versions. (A) FACS setting for measurements of $\Delta\Psi$ in N₂A cells; (a) gating of live cells; (b) cells without fluorescence probe, representing background; (c) cells stained with TMRE (FL-2); (d) overlay of b and c. (B) N₂A cells were plated on Day 0. On Day 1, cells were transfected with empty vector, wt-PrP, Δ 8TM1-PrP, or Δ octa-PrP, respectively. Medium was changed on Day 2, and 100 μ M CuCl_2 was added in fresh medium. On Day 3, cells were washed with PBS, fresh medium was added, and fluorescent staining and FACS analysis were performed as described under Materials and methods. Note that in the absence of copper treatment Δ octa-PrP-overexpressing cultures show a significant decrease of $\Delta\Psi$. After copper treatment in all cases $\Delta\Psi$ decreased significantly. Asterisks represent statistically significant differences ($P < 0.05$) compared with untreated mock-transfected cultures. The number of independent experiments is indicated in brackets.

Our data showed a significant decrease of intracellular ROS levels after oxidative stress in cells overexpressing either wt-PrP or Δ 8TM1-PrP (Figs. 3A and B). While an antioxidant role of wt-PrP^c has already been described, our results provide new information for Δ 8TM1-PrP. By contrast, we observed no significant protective effect in Δ octa-PrP-overexpressing cultures, in perfect agreement with previously published data [57]. Interestingly a very recent publication described the antioxidant protection of human SH-SY5Y neuroblastoma cells against toxicity of the oxidant agent paraquat by overexpression of wt-PrP^c but not Δ octa-PrP [58]. The parameters measured were cell viability, loss of membrane integrity, and mitochondrial bioenergetics. While these authors used a different cellular system, a different oxidant agent and different biological readouts, these data and our own data shown here on Δ octa-PrP are fully compatible and complementary.

The protective effect of wt-PrP or Δ 8TM1-PrP overexpression was apparently lost after copper pretreatment followed by exposure to H_2O_2 . This may be due to some generally increased biological variation of our results obtained after copper treatment (Fig. 3C). A possible explanation for the latter observation is the fact that H_2O_2 exposure of cells preloaded with copper is likely to elicit the formation of large amounts of hydroxyl radicals through the interaction of H_2O_2 and copper, enabling Fenton chemistry. This notion is fully consistent with the increased DCF signal observed in all cases of copper pretreatment

combined with H_2O_2 challenge (Fig. 3C). Small differences in the uptake of one or both interaction partners, i.e., copper and/or H_2O_2 , or their intracellular metabolism may therefore well translate into the

Fig. 6. Viability after copper treatment of N₂A cultures overexpressing various PrP versions. The cell treatment protocol was identical to that used for FACS analysis except for the addition of the fluorescence probe. Instead, cells were analyzed in a Casy cell counter. Four independent experiments were performed. Statistical analysis by Student's *t* test revealed no significant difference among any of the conditions.

larger biological variation of intracellular ROS formation apparent in Fig. 3C.

A possible explanation for PrP's protective effect against oxidative stress (Figs. 3A and B) could be some SOD-like activity, as proposed by Brown and colleagues [25,26]. In our system, however, no enhanced SOD activity was detected in wt-PrP or Δ 8TM1-PrP-transfected N2A (data not shown), in agreement with data from the literature [27].

Interestingly, overexpression of wt-PrP, Δ 8TM1-PrP, or Δ octa-PrP did not decrease basal ROS levels (Fig. 4B). Furthermore, copper treatment had no impact on the basal ROS level in cultures overexpressing any of the three PrP versions (Fig. 4B). Viewed together with the antioxidant effect of PrP^C under conditions of oxidative stress this observation points to some role of the protein in the acute signaling of oxidative stress, rather than a constitutive antioxidant function.

Because of the close relationship between ROS formation and the mitochondrial respiratory chain [59], we compared $\Delta\Psi$ in mock-transfected cultures with that of PrP-wt, Δ 8TM1, or Δ octa-overexpressing cultures (Fig. 5B). Neither wt-PrP nor Δ 8TM1-PrP-overexpressing cultures showed any alteration in $\Delta\Psi$ compared to mock-transfected cultures. By contrast Δ octa-PrP-transfected cells showed a statistically significant decrease of $\Delta\Psi$. In all cases, $\Delta\Psi$ decreased significantly after copper treatment, which was apparently not due to copper-induced cytotoxicity (Fig. 6).

The decrease of $\Delta\Psi$ in Δ octa-PrP-transfected cultures is a novel and unexpected finding. The underlying mechanisms are unknown, and it will be interesting to follow these up in future work. For the interpretation of the present data, however, it is clear that this decrease of $\Delta\Psi$ did not translate into lower levels of basal (Fig. 4B) or oxidative stress-induced ROS (Fig. 3C), as one might have presumed.

In summary, the protective effect of PrP^C against oxidative stress involves the octarepeat region but not the highly conserved TM1 domain nor the high-affinity copper binding site at human residues His96/His111 (corresponding to murine residues 95/110), which are both present in the Δ octa-PrP mutant. While it is attractive to hypothesize that the primordial physiological function of PrP^C is in antioxidant defense, this view is not supported by our present data showing that the most highly conserved region of PrP^C is apparently not involved in this function. Therefore other known or suspected functions of PrP^C deserve increased scrutiny.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.freeradbiomed.2008.08.024.

References

- [1] Silveira, J. R.; Caughey, B.; Baron, G. S. Prion protein and the molecular features of transmissible spongiform encephalopathy agents. *Curr. Top. Microbiol. Immunol.* **284**:1–50; 2004.
- [2] McKintosh, E.; Tabrizi, S. J.; Collinge, J. Prion diseases. *J. Neurovirol.* **9**:183–193; 2003.
- [3] Budka, H. Neuropathology of prion diseases. *Br. Med. Bull.* **66**:121–130; 2003.
- [4] Basler, K.; Oesch, B.; Scott, M.; Westaway, D.; Walchli, M.; Groth, D. F.; McKinley, M. P.; Prusiner, S. B.; Weissmann, C. Scrapie and cellular PrP isoforms are encoded by the same chromosomal gene. *Cell* **46**:417–428; 1986.
- [5] Sparkes, R. S.; Simon, M.; Cohn, V. H.; Fournier, R. E.; Lem, J.; Klisak, I.; Heinzmann, C.; Blatt, C.; Lucero, M.; Mohandas, T., et al. Assignment of the human and mouse prion protein genes to homologous chromosomes. *Proc. Natl. Acad. Sci. USA* **83**:7358–7362; 1986.
- [6] Simonic, T.; Duga, S.; Strumbo, B.; Asselta, R.; Cecilian, F.; Ronchi, S. cDNA cloning of turtle prion protein. *FEBS Lett.* **469**:33–38; 2000.
- [7] Wopfner, F.; Weidenhofer, G.; Schneider, R.; von Brunn, A.; Gilch, S.; Schwarz, T. F.; Werner, T.; Schatzl, H. M. Analysis of 27 mammalian and 9 avian PrPs reveals high conservation of flexible regions of the prion protein. *J. Mol. Biol.* **289**:1163–1178; 1999.
- [8] Rivera-Milla, E.; Oidtmann, B.; Panagiotidis, C. H.; Baier, M.; Sklaviadis, T.; Hoffmann, R.; Zhou, Y.; Solis, G. P.; Stuermer, C. A.; Malaga-Trillo, E. Disparate evolution of prion protein domains and the distinct origin of Doppel- and prion-related loci revealed by fish-to-mammal comparisons. *FASEB J.* **20**:317–319; 2006.
- [9] Zahn, R.; Liu, A.; Luhrs, T.; Riek, R.; von Schroetter, C.; Lopez Garcia, F.; Billeter, M.; Calzolari, L.; Wider, G.; Wuthrich, K. NMR solution structure of the human prion protein. *Proc. Natl. Acad. Sci. USA* **97**:145–150; 2000.
- [10] Holscher, C.; Delius, H.; Bürkle, A. Overexpression of nonconvertible PrP^C delta114–121 in scrapie-infected mouse neuroblastoma cells leads to trans-dominant inhibition of wild-type PrP(Sc) accumulation. *J. Virol.* **72**:1153–1159; 1998.
- [11] Baumann, E.; Tolnay, M.; Brabeck, C.; Pahnke, J.; Klotz, U.; Niemann, H. H.; Heikenwalder, M.; Rulicke, T.; Bürkle, A.; Aguzzi, A. Lethal recessive myelin toxicity of prion protein lacking its central domain. *EMBO J.* **26**:538–547; 2007.
- [12] Krebs, B.; Dorner-Ciossek, C.; Schmalzbauer, R.; Vassallo, N.; Herms, J.; Kretzschmar, H. A. Prion protein induced signaling cascades in monocytes. *Biochem. Biophys. Res. Commun.* **340**:13–22; 2006.
- [13] de Almeida, C. J.; Chiarini, L. B.; da Silva, J. P.; e Silva, P. M.; Martins, M. A.; Linden, R. The cellular prion protein modulates phagocytosis and inflammatory response. *J. Leukoc. Biol.* **77**:238–246; 2005.
- [14] Mouillet-Richard, S.; Ermonval, M.; Chebassier, C.; Laplanche, J. L.; Lehmann, S.; Launay, J. M.; Kellermann, O. Signal transduction through prion protein. *Science* **289**:1925–1928; 2000.
- [15] Schneider, B.; Mutel, V.; Pietri, M.; Ermonval, M.; Mouillet-Richard, S.; Kellermann, O. NADPH oxidase and extracellular regulated kinases 1/2 are targets of prion protein signaling in neuronal and nonneuronal cells. *Proc. Natl. Acad. Sci. USA* **100**:13326–13331; 2003.
- [16] Spielhauer, C.; Schatzl, H. M. PrP^C directly interacts with proteins involved in signaling pathways. *J. Biol. Chem.* **276**:44604–44612; 2001.
- [17] Brown, D. R.; Qin, K.; Herms, J. W.; Madlung, A.; Manson, J.; Strome, R.; Fraser, P. E.; Kruck, T.; von Bohlen, A.; Schulz-Schaeffer, W.; Giese, A.; Westaway, D.; Kretzschmar, H. The cellular prion protein binds copper in vivo. *Nature* **390**:684–687; 1997.
- [18] Burns, C. S.; Aronoff-Spencer, E.; Legname, G.; Prusiner, S. B.; Antholine, W. E.; Gerfen, G. J.; Peisach, J.; Millhauser, G. L. Copper coordination in the full-length, recombinant prion protein. *Biochemistry* **42**:6794–6803; 2003.
- [19] Chattopadhyay, M.; Walter, E. D.; Newell, D. J.; Jackson, P. J.; Aronoff-Spencer, E.; Peisach, J.; Gerfen, G. J.; Bennett, B.; Antholine, W. E.; Millhauser, G. L. The octarepeat domain of the prion protein binds Cu(II) with three distinct coordination modes at pH 7.4. *J. Am. Chem. Soc.* **127**:12647–12656; 2005.
- [20] Millhauser, G. L. Copper binding in the prion protein. *Acc. Chem. Res.* **37**:79–85; 2004.
- [21] Collinge, J.; Whittington, M. A.; Sidle, K. C.; Smith, C. J.; Palmer, M. S.; Clarke, A. R.; Jefferys, J. G. Prion protein is necessary for normal synaptic function. *Nature* **370**:295–297; 1994.
- [22] Gains, M. J.; Roth, K. A.; LeBlanc, A. C. Prion protein protects against ethanol-induced Bax-mediated cell death in vivo. *Neuroreport* **17**:903–906; 2006.
- [23] Kristiansen, M.; Messenger, M. J.; Kohn, P. C.; Brandner, S.; Wadsworth, J. D.; Collinge, J.; Tabrizi, S. J. Disease-related prion protein forms aggregates in neuronal cells leading to caspase activation and apoptosis. *J. Biol. Chem.* **280**:38851–38861; 2005.
- [24] Roucou, X.; Giannopoulos, P. N.; Zhang, Y.; Jodoin, J.; Goodyer, C. G.; LeBlanc, A. Cellular prion protein inhibits proapoptotic Bax conformational change in human neurons and in breast carcinoma MCF-7 cells. *Cell Death Differ.* **12**:783–795; 2005.
- [25] Brown, D. R.; Besinger, A. Prion protein expression and superoxide dismutase activity. *Biochem. J.* **334** (Pt 2):423–429; 1998.
- [26] Brown, D. R.; Wong, B. S.; Hafiz, F.; Clive, C.; Haswell, S. J.; Jones, I. M. Normal prion protein has an activity like that of superoxide dismutase. *Biochem. J.* **344** (Pt 1):1–5; 1999.
- [27] Hutter, G.; Heppner, F. L.; Aguzzi, A. No superoxide dismutase activity of cellular prion protein in vivo. *Biol. Chem.* **384**:1279–1285; 2003.
- [28] Brown, D. R. Prion and prejudice: normal protein and the synapse. *Trends Neurosci.* **24**:85–90; 2001.
- [29] Rachidi, W.; Vilette, D.; Guiraud, P.; Arlotto, M.; Riondel, J.; Laude, H.; Lehmann, S.; Favier, A. Expression of prion protein increases cellular copper binding and antioxidant enzyme activities but not copper delivery. *J. Biol. Chem.* **278**:9064–9072; 2003.
- [30] Jones, C. E.; Abdelrahman, S. R.; Brown, D. R.; Viles, J. H. Preferential Cu²⁺ coordination by His96 and His111 induces beta-sheet formation in the unstructured amyloidogenic region of the prion protein. *J. Biol. Chem.* **279**:32018–32027; 2004.
- [31] Thompson, A. R.; Abdelrahman, S. R.; Daniels, M.; Brown, D. R. High affinity binding between copper and full-length prion protein identified by two different techniques. *J. Biol. Chem.* **280**:42750–42758; 2005.

- [32] Brown, D. R.; Schulz-Schaeffer, W. J.; Schmidt, B.; Kretschmar, H. A. Prion protein-deficient cells show altered response to oxidative stress due to decreased SOD-1 activity. *Exp. Neurol.* **146**:104–112; 1997.
- [33] Klamt, F.; Dal-Pizzol, F.; Conte da Frota, M. J.; Walz, R.; Andrades, M. E.; da Silva, E. G.; Brentani, R. R.; Izquierdo, I.; Fonseca Moreira, J. C. Imbalance of antioxidant defense in mice lacking cellular prion protein. *Free Radic. Biol. Med.* **30**:1137–1144; 2001.
- [34] White, A. R.; Collins, S. J.; Maher, F.; Jobling, M. F.; Stewart, L. R.; Thyer, J. M.; Beyreuther, K.; Masters, C. L.; Cappai, R. Prion protein-deficient neurons reveal lower glutathione reductase activity and increased susceptibility to hydrogen peroxide toxicity. *Am. J. Pathol.* **155**:1723–1730; 1999.
- [35] Brown, D. R.; Clive, C.; Haswell, S. J. Antioxidant activity related to copper binding of native prion protein. *J. Neurochem.* **76**:69–76; 2001.
- [36] Sakudo, A.; Lee, D. C.; Li, S.; Nakamura, T.; Matsumoto, Y.; Saeki, K.; Itoharu, S.; Ikuta, K.; Onodera, T. PrP cooperates with ST11 to regulate SOD activity in PrP-deficient neuronal cell line. *Biochem. Biophys. Res. Commun.* **328**:14–19; 2005.
- [37] Weise, J.; Crome, O.; Sandau, R.; Schulz-Schaeffer, W.; Bahr, M.; Zerr, I. Upregulation of cellular prion protein (PrP^c) after focal cerebral ischemia and influence of lesion severity. *Neurosci. Lett.* **372**:146–150; 2004.
- [38] Shyu, W. C.; Lin, S. Z.; Chiang, M. F.; Ding, D. C.; Li, K. W.; Chen, S. F.; Yang, H. I.; Li, H. Overexpression of PrP^c by adenovirus-mediated gene targeting reduces ischemic injury in a stroke rat model. *J. Neurosci.* **25**:8967–8977; 2005.
- [39] Spudich, A.; Frigg, R.; Kilic, E.; Kilic, U.; Oesch, B.; Raeber, A.; Bassetti, C. L.; Hermann, D. M. Aggravation of ischemic brain injury by prion protein deficiency: role of ERK-1/-2 and STAT-1. *Neurobiol. Dis.* **20**:442–449; 2005.
- [40] Weise, J.; Sandau, R.; Schwarting, S.; Crome, O.; Wrede, A.; Schulz-Schaeffer, W.; Zerr, I.; Bahr, M. Deletion of cellular prion protein results in reduced Akt activation, enhanced postischemic caspase-3 activation, and exacerbation of ischemic brain injury. *Stroke* **37**:1296–1300; 2006.
- [41] Mitsios, N.; Saka, M.; Krupinski, J.; Pennucci, R.; Sanfeliu, C.; Miguel Turu, M.; Gaffney, J.; Kumar, P.; Kumar, S.; Sullivan, M.; Slevin, M. Cellular prion protein is increased in the plasma and peri-infarcted brain tissue after acute stroke. *J. Neurosci. Res.* **85**:602–611; 2007.
- [42] Milhavel, O.; Lehmann, S. Oxidative stress and the prion protein in transmissible spongiform encephalopathies. *Brain Res. Brain Res. Rev.* **38**:328–339; 2002.
- [43] Simonian, N. A.; Coyle, J. T. Oxidative stress in neurodegenerative diseases. *Annu. Rev. Pharmacol. Toxicol.* **36**:83–106; 1996.
- [44] Sayre, L. M.; Perry, G.; Smith, M. A. Redox metals and neurodegenerative disease. *Curr. Opin. Chem. Biol.* **3**:220–225; 1999.
- [45] Smaili, S. S.; Hsu, Y. T.; Youle, R. J.; Russell, J. T. Mitochondria in Ca²⁺ signaling and apoptosis. *J. Bioenerg. Biomembr.* **32**:35–46; 2000.
- [46] Smaili, S. S.; Hsu, Y. T.; Carvalho, A. C.; Rosenstock, T. R.; Sharpe, J. C.; Youle, R. J. Mitochondria, calcium and pro-apoptotic proteins as mediators in cell death signaling. *Braz. J. Med. Biol. Res.* **36**:183–190; 2003.
- [47] Lobão-Soares, B.; Bianchin, M. M.; Linhares, M. N.; Carqueja, C. L.; Tasca, C. I.; Souza, M.; Marques Jr, W.; Brentani, R.; Martins, V. R.; Sakamoto, A. C.; Carlotti Jr, C. G.; Walz, R. Normal brain mitochondrial respiration in adult mice lacking cellular prion protein. *Neurosci. Lett.* **375**:203–206; 2005.
- [48] Nico, P. B.; Lobão-Soares, B.; Landemberger, M. C.; Marques Jr, W.; Tasca, C. I.; de Mello, C. F.; Walz, R.; Carlotti Jr, C. G.; Brentani, R. R.; Sakamoto, A. C.; Bianchin, M. M. Impaired exercise capacity, but unaltered mitochondrial respiration in skeletal or cardiac muscle of mice lacking cellular prion protein. *Neurosci. Lett.* **388**:21–26; 2005.
- [49] Paterson, A. W.; Curtis, J. C.; Macleod, N. K. Complex I specific increase in superoxide formation and respiration rate by PrP-null mouse brain mitochondria. *J. Neurochem.* **105**:177–191; 2008.
- [50] Küpper, J. H.; de Murcia, G.; Bürkle, A. Inhibition of poly(ADP-ribosyl)ation by overexpressing the poly(ADP-ribose) polymerase DNA-binding domain in mammalian cells. *J. Biol. Chem.* **265**:18721–18724; 1990.
- [51] Gilch, S.; Nunziante, M.; Ertmer, A.; Wopfner, F.; Laszlo, L.; Schatzl, H. M. Recognition of luminal prion protein aggregates by post-ER quality control mechanisms is mediated by the preoctarepeat region of PrP. *Traffic* **5**:300–313; 2004.
- [52] Zhang, Y.; Poirier, G. G.; Bürkle, A. In-situ analysis of cellular poly(ADP-ribose) production in scrapie-infected mouse neuroblastoma cells. *Histochem. J.* **34**:357–363; 2002.
- [53] Sohn, I. P.; Ahn, H. J.; Park, D. W.; Gye, M. C.; Jo do, H.; Kim, S. Y.; Min, C. K.; Kwon, H. C. Amelioration of mitochondrial dysfunction and apoptosis of two-cell mouse embryos after freezing and thawing by the high frequency liquid nitrogen infusion. *Mol. Cells* **13**:272–280; 2002.
- [54] Sauer, H.; Wefer, K.; Vetrugno, V.; Pocchiari, M.; Gissel, C.; Sachinidis, A.; Hescheler, J.; Wartenberg, M. Regulation of intrinsic prion protein by growth factors and TNF- α : the role of intracellular reactive oxygen species. *Free Radic. Biol. Med.* **35**:586–594; 2003.
- [55] Nicholls, D. G.; Ward, M. W. Mitochondrial membrane potential and neuronal glutamate excitotoxicity: mortality and millivolts. *Trends Neurosci.* **23**:166–174; 2000.
- [56] Scaduto, R. C., Jr.; Grotyohann, L. W. Measurement of mitochondrial membrane potential using fluorescent rhodamine derivatives. *Biophys. J.* **76**:469–477; 1999.
- [57] Watt, N. T.; Taylor, D. R.; Gillott, A.; Thomas, D. A.; Perera, W. S.; Hooper, N. M. Reactive oxygen species-mediated beta-cleavage of the prion protein in the cellular response to oxidative stress. *J. Biol. Chem.* **280**:35914–35921; 2005.
- [58] Dupiereux, I.; Falisse-Poirier, N.; Zorzi, W.; Watt, N. T.; Thellin, O.; Zorzi, D.; Pierard, O.; Hooper, N. M.; Heinen, E.; Elmoualij, B. Protective effect of prion protein via the N-terminal region in mediating a protective effect on paraquat-induced oxidative injury in neuronal cells. *J. Neurosci. Res.* **86**:653–659; 2008.
- [59] Kowaltowski, A. J.; Vercesi, A. E. Mitochondrial damage induced by conditions of oxidative stress. *Free Radic. Biol. Med.* **26**:463–471; 1999.